

# Hepatitis B virus X protein enhances liver cancer cell migration by regulating calmodulin-associated actin polymerization

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**Hepatitis B virus (HBV) infection is a major cause of hepatocellular carcinoma (HCC), which is a highly aggressive cancer. HBV X protein (HBx), one of four HBV gene products, plays pivotal roles in the development and metastasis of HCC. It has been reported that HBx induces liver cancer cell migration and reorganizes actin cytoskeleton, however the molecular basis for actin cytoskeleton reorganization remains obscure. In this study, we for the first time report that HBx promotes actin polymerization and liver cancer cell migration by regulating calcium modulated protein, calmodulin (CaM). HBx physically interacts with CaM to control the level of phosphorylated cofilin, an actin depolymerizing factor. Mechanistically, HBx interacts with CaM, liberates Hsp90 from its inhibitory partner CaM, and increases the activity of Hsp90, thus activating LIMK1/cofilin pathway. Interestingly, the interaction between HBx and CaM is calcium-dependent and requires the CaM binding motif on HBx. These results indicate that HBx modulates CaM which plays a regulatory role in Hsp90/LIMK1/cofilin pathway of actin reorganization, suggesting a new mechanism of HBV-induced HCC metastasis specifically derived by HBx. [BMB Reports 2021; 54(12): 614-619]**

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers with high mortality rates worldwide (1). HCC is a very aggressive tumor with poor prognosis, because of frequent extrahepatic and intrahepatic metastasis (2). Especially vascular invasion to portal vein is a special feature of intrahepatic metastasis of HCC (3). Therefore, elucidation of the mechanisms underlying HCC metastasis would affect the survival rate and prognosis of HCC patients.

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Chronic infection of hepatitis B virus (HBV) is closely associated with the initiation and development of HCC (4). Approximately up to 53% of HCC cases in the world are related to HBV (5). There are various possible mechanisms whereby HBV may cause HCC, however the exact mechanism remains unclear.

Among four HBV proteins, a small promiscuous protein, HBx is required for the establishment of viral infection and the development of HCC (6). HBx performs a variety of biological functions including transcriptional activation of various viral and cellular promoters, interaction with p53, interference with host DNA repair, and modulation of cell proliferation and apoptosis (7). In addition, HBx affects cellular adhesion, epithelial-mesenchymal transition, and cytoskeletal rearrangement. Intriguingly, HBx promotes invasiveness and metastasis of HCC by inducing secretion of HMGB1 and upregulation of MMPs (8, 9). These features show that HBx is closely associated with the regulation of HCC metastasis.

Actin cytoskeleton rearrangement is required for cell morphogenesis, polarization, and motility which are critical cellular processes in metastasis (10). Actin cytoskeletal dynamics is controlled by the polymerization rate of globular actin monomers to filamentous actin (F-actin) (11). One of the regulatory cascades for actin polymerization is the cofilin-LIM kinase (LIMK) axis. LIMK phosphorylates and inactivates an actin depolymerizing factor cofilin, leading to F-actin formation (12). Although LIMK is phosphorylated and inactivated by the member of Rho-GTPase effectors such as Rho-associated protein kinase (ROCK) (13), it is also controlled by heat shock protein 90 (Hsp90), a molecular chaperone. Hsp90 associates with LIMK1 and induces its homodimerization to increase the activity and stability of LIMK1 (14). Indeed, we found that the post-translational modification of Hsp90, especially deacetylation of Hsp90 by SIRT2, plays important roles in the regulation of LIMK1/cofilin pathway and actin polymerization (15).

We previously performed a yeast two-hybrid screening and found several cellular proteins interacting with HBx (16). Among them, calmodulin (CaM) stood out as a candidate factor connecting HBx to actin polymerization. CaM is a highly conserved calcium binding protein that relays the calcium signal (17). The effect of the calcium ion on actin organization has been well studied in the immune synapse between T cells and antigen-presenting cells (18). The elevated calcium ion concentra-

tions negatively affect on actin cytoskeleton organization. It has been reported that CaM modulates actin polymerization by inhibiting the interaction between actin and actin-binding proteins, such as caldesmon and Hsp90 (19, 20). However, the signaling network in actin cytoskeleton dynamics associated with CaM-Hsp90 has not been elucidated yet.

Here, we first report that HBx physically binds to CaM and interferes with the interaction between CaM and Hsp90. Subsequently HBx releases Hsp90 from CaM, activates LIMK1, and increases the level of phosphorylated cofilin, which enhances actin polymerization and cell migration. Through this study, it is evident that the specific interaction between HBx and CaM plays a crucial role in actin rearrangement and liver cancer cell migration associated with HCC metastasis.

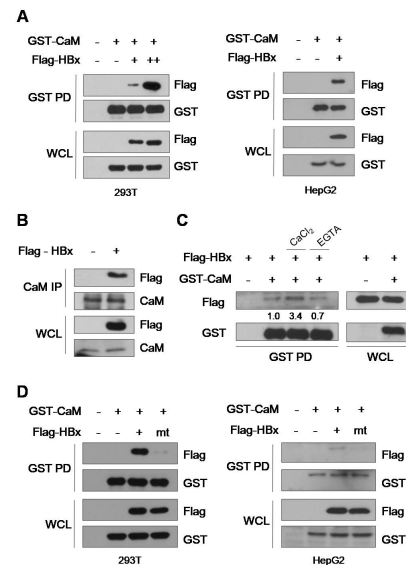
## RESULTS

### HBx binds to calmodulin in liver cancer cells

HBx is known to affect actin cytoskeleton reorganization and cell migration in liver cancer cells, however the relevant molecular events have been poorly studied. To dissect how HBx regulates actin organization and cell motility, we attempted to find HBx-binding cellular proteins associated with actin dynamics. In previous research, to identify cellular proteins interacting with HBx, yeast two-hybrid screening analysis was carried out using the full length of HBx as bait (16). Among putative HBx-interacting cellular proteins, the most promising candidate was calcium modulating protein, calmodulin (CaM), which is very closely related to actin cytoskeletal rearrangement.

We tested the physical interaction between HBx and CaM by GST pull-down assay and co-immunoprecipitation (IP) assay in 293T cells and human hepatoma HepG2 cells respectively, and confirmed the physical binding of these proteins (Fig. 1A). The physical interaction of HBx and endogenous CaM was further reconfirmed in HepG2 cells by co-IP assay (Fig. 1B). Since most CaM binding proteins have calcium dependency, we checked whether the interaction between HBx and CaM is calcium-dependent or not. In the presence of calcium ion, the interaction between HBx and CaM was increased, while in the absence of calcium ion, the interaction was reduced in HepG2 cells, showing calcium-dependent interaction between HBx and CaM (Fig. 1C).

To dissect the CaM-binding site in HBx, we analyzed 'calmodulin target database (21)' and predicted a putative CaM-binding motif that might be located between amino acids 123 and 142 in HBx. We introduced the point mutation into HBx by substituting lysine residue (aa 130) with alanine (K130A). This residue is adjacent to the first hydrophobic region (aa 131-aa 134) of two anchoring hydrophobic domains, which are considered to be CaM-binding motifs (22). Very interestingly, the HBx mutant (K130A) lost the binding affinity to CaM significantly compared with wild type HBx (Fig. 1D).

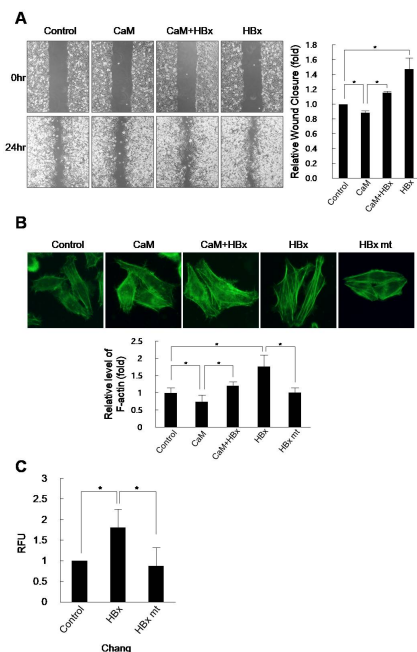


**Fig. 1.** HBx binds to CaM in liver cancer cells. (A) 293T and HepG2 cells were cotransfected with plasmids expressing GST-CaM and Flag-HBx. Cell lysates were incubated with glutathione-sepharose beads, and analyzed by Western blotting. (B) HepG2 cells were transfected with plasmid expressing Flag-HBx. Cell lysates were immunoprecipitated with anti-CaM antibody, and analyzed by Western blotting. (C) The extracts of cotransfected HepG2 cells were incubated with glutathione-sepharose beads in the presence of 1 mM CaCl<sub>2</sub> or 1 mM EGTA, and analyzed by Western blotting. (D) 293T and HepG2 cells were cotransfected with plasmids expressing GST-CaM, Flag-HBx, and Flag-HBx mutant. Cell lysates were pulled-down with glutathione-sepharose beads and analyzed by Western blotting.

### HBx represses the inhibitory effects of CaM on cell migration and actin polymerization

Since CaM inhibits actin polymerization, the interaction between oncogenic HBx and CaM might be involved in actin cytoskeleton rearrangement and liver cancer cell migration associated with HCC metastasis. To check the very possibility, we tested the effects of HBx and CaM on cellular motility in HepG2 cells. CaM decreased hepatoma cell migration, whereas HBx increased the migration conversely (Fig. 2A). Since cell migration is closely related with actin fiber dynamics, we analyzed actin polymer rearrangement in the presence of CaM and HBx by phalloidin staining assay. Interestingly, HBx elevated actin polymerization to lead stress fiber formation, which was reduced by CaM (Fig. 2B). In addition, the HBx mutant devoid of CaM-binding affinity apparently did not increase F-actin formation suggesting that HBx elevates actin polymerization by regulating CaM.

To further confirm that HBx enhances cell motility through CaM, we did quantitative analysis of cell motility by chemotaxis cell migration assay using DMEM containing 10% FBS as a chemoattractant. Chang liver cells expressing mutant HBx lost cell mobility significantly compared with cells expressing wild



**Fig. 2.** HBx represses the inhibitory effects of CaM on cell migration and actin polymerization. (A) HepG2 cells were transfected with plasmids expressing GST-CaM and Flag-HBx. The cell monolayer was scratched with a sterile micropipette tip and photographed using a phase-contrast microscopy (Wound closure % =  $[(A_0 - A_{24}) / A_0] \times 100$ ;  $A_t$  is the area of wound measured at  $t$  hours after scratch, \* $P < 0.05$ ). (B) HepG2 cells expressing GST-CaM, Flag-HBx, and Flag-HBx mutant were fixed and stained with phalloidin for F-actin staining. The level of actin fiber were quantified using Image-J (\* $P < 0.05$ ). (C) Chang liver cells were transfected with plasmids expressing Flag-HBx and Flag-HBx mutant. Cell migration rate was determined using a QCM Chemotaxis Cell migration kit and quantified as a relative fluorescence unit (\* $P < 0.05$ ).

type HBx (Fig. 2C). Taken together, these results suggest that HBx augments F-actin rearrangement associated with cell migration through its interaction with CaM.

### HBx increases the level of phosphorylated cofilin reduced by CaM in liver cancer cells

To find out how HBx affects actin polymerization and cell migration by interacting with CaM, we checked the phosphorylation level of cofilin, which is pivotal for actin dynamics. When HBx expression was knocked-down by HBx shRNA in HepG2.2.15 cells which produce all viral proteins along with HBV particle, the level of phosphorylated cofilin (p-cofilin) was dramatically reduced, suggesting that p-cofilin is modulated by HBx in HBV-replicating cells which mimic pathophysiological conditions (Fig. 3A). Since HBx inhibits the regulatory effect of CaM on F-actin rearrangement associated with cell migration, we compared the effects of HBx and mutant HBx devoid of CaM-binding affinity on the level of p-cofilin in HepG2 cells. As expected,

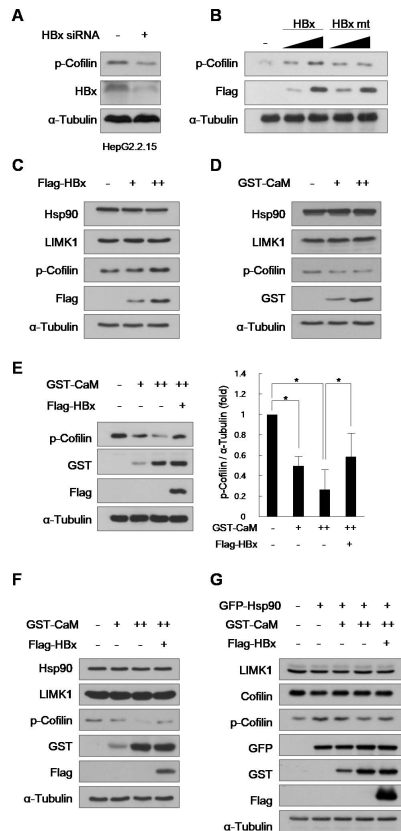
HBx elevated the level of p-cofilin in a dose-dependent manner, whereas the HBx mutant did not (Fig. 3B), suggesting that the binding ability of HBx to CaM is critical for HBx-induced actin polymerization derived by p-cofilin.

Since LIMK1 is closely regulated by Hsp90 to control the phosphorylation of cofilin (14), we tested whether HBx affects the levels of Hsp90 and LIMK1 in HepG2 cells. HBx increased the p-cofilin level in a dose-dependent manner without affecting the levels of Hsp90 and LIMK1 (Fig. 3C), whereas CaM decreased the p-cofilin level without changing the levels of Hsp90 and LIMK1 (Fig. 3D). Furthermore, the expression of HBx certainly restored the p-cofilin level which was repressed by CaM (Fig. 3E), without affecting the expression levels of Hsp90 and LIMK1 (Fig. 3F). In addition, HBx consistently restored the inhibitory activity of CaM on the level of p-cofilin, which was increased by Hsp90 in hepatoma cells, without affecting the level of cofilin (Fig. 3G). It is evident that HBx increases actin polymerization by elevating the level of p-cofilin, but not cofilin, suggesting that LIMK1 activity is important for HBx-induced actin polymerization and cell motility.

### HBx interferes with the interaction between CaM and Hsp90 to facilitate the interaction between Hsp90 and LIMK1

Hsp90 forms a constitutive dimer, which binds to CaM in a calcium-dependent manner (23). Therefore, it is possible that HBx may interact with CaM to disturb the physical binding between CaM and Hsp90. Actually, the interaction between Hsp90 and CaM was significantly inhibited by HBx, but not by the HBx mutant devoid of CaM binding affinity in HepG2 cells (Fig. 4A), suggesting that HBx hinders the interaction between CaM and Hsp90 by competitive binding to CaM and consequently releases Hsp90 from CaM under physiological calcium concentration.

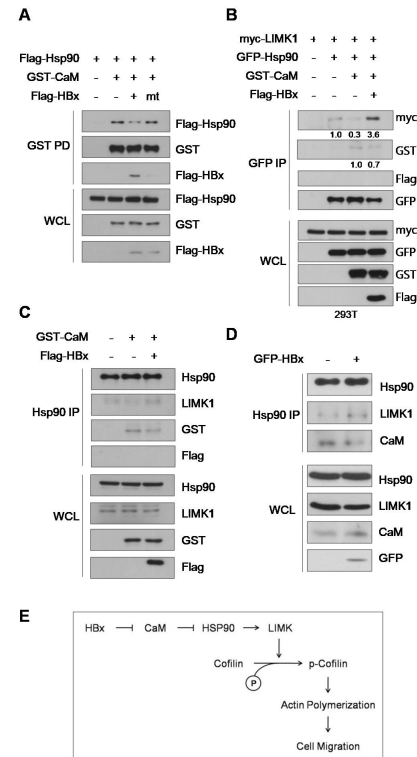
Since Hsp90 associates with LIMK1 to increase the activity and stability of LIMK1 (14), we analyzed the effects of CaM and HBx on the interaction between Hsp90 and LIMK1. While CaM reduced the interaction between Hsp90 and LIMK1, HBx inhibited the interaction between CaM and Hsp90, and subsequently elevated the interaction between Hsp90 and LIMK1 which was reduced by CaM (Fig. 4B). We reconfirmed the same effects of CaM and HBx on the interaction between endogenous Hsp90 and LIMK1 in HepG2 cells (Fig. 4C). In addition, we further confirmed the effect of HBx on the endogenous interaction of Hsp90 with CaM and LIMK1 (Fig. 4D). It is clear that HBx interferes with the interaction between CaM and Hsp90 by competitive binding to CaM, and subsequently increases the interaction between Hsp90 and LIMK1 which elevates p-cofilin. In addition, the HBx mutant devoid of CaM binding affinity does not have these effects at all, suggesting that the interaction of HBx with CaM is a key step for cofilin signaling associated with Hsp90 and LIMK1. Taken together, a bona fide interaction of HBx and CaM is fundamental for the HBx-mediated actin polymerization pathway consisting of Hsp90/LIMK1/p-cofilin.



**Fig. 3.** HBx elevates the level of p-cofilin which is repressed by CaM in liver cancer cells. (A) HepG2.2.15 cells were transfected with plasmid expressing HBx shRNA. At 48 h after transfection, cell extracts were prepared and analyzed by Western blotting. (B) HepG2 cells were transfected with increasing amounts of plasmids expressing Flag-HBx and Flag-HBx mutant. Whole cell extracts were analyzed by Western blotting. (C) HepG2 cells were transfected with various amounts of Flag-HBx plasmid and analyzed by Western blotting using indicated antibodies. (D) HepG2 cells transfected with GST-CaM plasmid were analyzed by Western blotting using indicated antibodies. (E) HepG2 cells were cotransfected with plasmids expressing GST-CaM and Flag-HBx, and analyzed by Western blotting. The relative levels of p-cofilin were quantified by Image-J (\*P < 0.05). (F) HepG2 cells expressing GST-CaM and Flag-HBx were analyzed by Western blotting to check the protein levels of cofilin-signaling pathway. (G) HepG2 cells were transfected with various amounts of plasmids expressing GFP-Hsp90, GST-CaM, and Flag-HBx. Cell extracts were analyzed by Western blotting.

## DISCUSSION

Recently, it has been shown that the  $Ca^{2+}$ /CaM complex regulates the activity of several small GTPases which play an essential role in cytoskeleton remodeling and cell migration (24). The  $Ca^{2+}$ /CaM complex also activates several CaM dependent kinases which play crucial roles in the regulation of cell motility (25). However, the role of the  $Ca^{2+}$ /CaM complex in actin localization and polymerization is poorly understood.



**Fig. 4.** HBx inhibits the interaction between CaM and Hsp90, and elevates the interaction between Hsp90 and LIMK1. (A) Transfected cell lysates were incubated with glutathione-sepharose beads, pulled-down, and analyzed by Western blotting. (B) 293T cells were cotransfected with plasmids expressing Myc-LIMK1, GFP-Hsp90, GST-CaM, and Flag-HBx. Cell lysates were immunoprecipitated with anti-GFP antibody and analyzed by Western blotting. (C) HepG2 cells were transfected with plasmids expressing GST-CaM and Flag-HBx. The cell extracts were immunoprecipitated with anti-Hsp90 antibody and analyzed by Western blotting. (D) The extracts of HepG2 cells transfected with GFP-HBx plasmid were immunoprecipitated and analyzed by Western blotting. (E) Proposed model of actin polymerization induced by HBx interacting with CaM.

Here, we first report that HBx binds to CaM in a calcium ion-dependent manner suggesting that HBx is closely related to the calcium signaling pathway. Intracellular calcium is a ubiquitous signal that controls a variety of cellular processes including cell cycle progression, tumorigenesis, and actin polymerization (26, 27). Therefore, through CaM, HBx may also contribute to the enhancement of actin polymerization induced by intracellular calcium ions.

Interestingly, we found that CaM decreased the level of p-cofilin, while HBx increased it conversely. Cofilin plays an essential role in regulating actin filament dynamics and its actin-depolymerizing activity is inhibited by phosphorylation at Ser-3 by LIMKs (12). Actually LIMK1/cofilin pathway is involved in the metastasis of various cancer cells including ovarian cancer cells (28). In addition, since SSH1L, a slingshot (SSH)

family, specifically dephosphorylates p-cofilin, and SSH1L is regulated by calcineurin which is a CaM-dependent protein phosphatase (29), we can not rule out the possibility that calcineurin and SSH1L may be associated with the regulation of cofilin phosphorylation induced by HBx through CaM.

Hsp90 is a molecular chaperone that is one of the most abundant proteins expressed in cells. Hsp90 interacts with many client proteins involved in actin dynamics and metastasis of cancer cells (30). Hsp90 interacts with HER-2, also known as ErbB-2, and activates signal transduction leading to cytoskeletal rearrangement and invasion of human breast cancer cells (31). Moreover, it has been demonstrated that LIMK1/2 has a short amino acid sequence similar to that of the ErbB-2 kinase domain, which interacts with Hsp90. Actually Hsp90 interacts with LIMK1 and promotes the stability and activity of LIMK1 (14). Previously, we reported that Hsp90 and LIMK1 closely interact with each other and cooperatively affect the phosphorylation of cofilin, cytoskeletal rearrangement, and cell migration in human colorectal carcinoma cells (15).

Our data clearly indicate that HBx interacts with CaM, releases Hsp90 from CaM, and activates LIMK1, which increases the phosphorylation of cofilin, a regulatory factor of actin cytoskeleton reorganization (Fig. 4E). It has been reported that HBx up-regulates Hsp90 $\alpha$  expression by c-Myc mediated transcriptional activation (32), however, our data show that HBx does not affect the level of Hsp90. It is clear that HBx may activate Hsp90 in various ways, including transcription regulation and post-translational interactions. Evidently HBx activates Hsp90 by blocking CaM, since CaM represses the dimerization of Hsp90. Moreover, to further explore the metastatic potential of HBx-CaM interaction *in vivo*, we injected B16F10 mouse melanoma cells expressing either HBx or HBx mutant devoid of CaM binding affinity intravenously into nude mice. Interestingly, we observed that HBx induced lung metastasis significantly compared with HBx mutant (data not shown).

Taken together, HBx regulates the migration of liver cells by interacting with CaM, a regulatory protein of actin polymerization, via Hsp90 and LIMK along with p-cofilin suggesting new mechanism of HBx on liver cancer cell migration associated with calcium signaling factor CaM.

## MATERIALS AND METHODS

### Cell culture and transfection

HepG2 cells, HepG2.2.15 cells, and Chang liver cells, 293T cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics at 37°C in 5% CO<sub>2</sub>. Transfections were performed using PEI (Sigma-Aldrich) and Fugene HD transfection reagent (Roche).

### Plasmids

Flag-HBx was previously described (16) and Myc-LIMK1 was provided by Dr. K. Itoh (33). Calmodulin 3 full gene was sub-cloned into the mammalian expression vector, pEBG to express

GST-CaM. The point-mutant plasmid of Flag-HBx was constructed by replacing three nucleotides AAG (nt 388–390) encoding lysine with GCC encoding alanine in HBx open reading frame.

### Pull-down assay and co-immunoprecipitation

Transfected cell lysates were prepared in lysis buffer and incubated with glutathione-sepharose beads (Incospharm) for overnight at 4°C. The beads were collected by centrifugation and rinsed several times with lysis buffer. The pellets were resuspended in sample buffer, boiled for 10 min, and analyzed by Western blotting.

For Co-IP assay, cell lysates were incubated with indicated antibodies overnight and mixed with protein A/G agarose beads (Incospharm) for 2 h at 4°C. The beads were harvested, dissolved, and analyzed.

### Western blot analysis

Cell lysates were separated on SDS-PAGE and transferred onto a PVDF membrane (Millipore). The membranes were blocked with 5% skim milk in PBSt (PBS containing 0.2% Tween 20) for 1 h, and then incubated with primary antibodies overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG for 1 h at room temperature. The proteins were detected with ECL reagent (Millipore).

### Wound healing assay

Cells were seeded on 6-well plates coated with 20  $\mu$ g/ml type I collagen. At 24 h after transfection, the cell monolayer was scratched with a sterile micropipette tip, and photographed at 0 h and 24 h after scratch using a phase-contrast microscopy. The area of wound was measured and quantified using Image-J program (NIH).

### Cell migration assay

Cell migration rates were determined using a QCM chemotaxis cell migration assay kit (Chemicon). Cells in serum-free DMEM were added to the upper chambers which were placed on the lower chamber containing DMEM and 10% FBS. After 24 h, migratory cells on polycarbonate membrane at the bottom of upper chamber were dissociated and stained with CyQuant GR dye. Fluorescence intensities were detected using a fluorometer at 480/520 nm.

### Actin staining assay

Cells were seeded on sterile glass cover slip, which was coated with poly-L-lysine. At 24 h after transfection, cells were fixed with 4% paraformaldehyde for 10 min and washed three times with cold PBS. Cells were permeabilized with cold acetone for 30 sec, washed with cold PBS, and then incubated with alexa-fluor 488 phalloidin (Invitrogen) for 1 h. Cover slip glass was mounted with anti-fade mounting reagent (Invitrogen), and fluorescence was examined using a fluorescence microscope.

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## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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