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Honokiol inhibits hepatoma carcinoma cell migration through downregulated Cyclophilin B expression

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ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth common types of cancer with poor prognosis in the world. Honokiol (HNK), a natural biphenyl compound derived from the magnolia plant, has been reported to exert anticancer effects, but its mechanism has not been elucidated exactly. In the present study, HNK treatment significantly suppressed the migration ability of HepG2 and Hep3B human hepatocellular carcinoma. The treatment reduced the expression levels of the genes associated with cell migration, such as S100A4, MMP-2, MMP-9 and Vimentin. Interestingly, treatment with HNK significantly reduced the expression level of Cyclophilin B (CypB) which stimulates cancer cell migration. However, overexpressed CypB abolished HNK-mediated suppression of cell migration, and reversed the apoptotic effects of HNK. Altogether, we concluded that the suppression of migration activities by HNK was through down-regulated CypB in HCC. These finding suggest that HNK may be a promising candidate for HCC treatment via regulation of CypB.

1. Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide. The regions with the highest rates of liver cancer are found in Asia and Africa [1]. Recently, numerous active treatments for HCC have been widely applied in clinics, such as hepatic resection, chemotherapy, immunotherapy, and targeted therapy. However, 5-year survival rate remains undesirable [2]. Therefore, it is necessary to develop new therapeutic agents for the treatment of HCC and to study its mechanism.

Cyclophilin B (CypB) helps the protein fold appropriately through peptidyl-prolyl cis/*trans*-isomerase activity. CypB is mainly localized in the endoplasmic reticulum (ER) and attenuates ER stress through its PPIase activity [3]. Previous studies have shown that CypB is involved in multiple functions, including cell proliferation, hepatitis C virus replication and intracellular signal transduction system [4,5]. Recently, it has been reported that inhibition of CypB effectively impedes cancer cell migration via modulating the STAT3 pathway, suggesting an important role of CypB in cancer cell migration [6].

Honokiol (HNK) is a natural compound derived from the bark of the plant *Magnolia officinalis*. HNK have many biological effects, including anti-inflammatory, reactive oxygen species, antioxidant, and anticancer properties [7–10]. Also, HNK inhibits HepG2 cell migration by repressing the process of epithelial-mesenchymal transition (EMT) [11]. However, the mechanism of the anti-migration activity of HNK is still completely unclear.

In this present study, we presented the novel mechanism by which HNK treatment inhibits cancer cell migration. Our results provided insights into the mechanism of anti-HCC through the regulation of CypB expression.

2. Material and methods

2.1. Cell culture and reagents

The human HepG2 cell line was maintained in DMEM medium containing 10% fetal bovine serum (HyClone, UT, USA) and

Abbreviations: CypB, Cyclophilin B; HNK, Honokiol; HCC, Hepatocellular carcinoma; PARP, Poly (ADP-ribose) polymerase; Bax, Bcl-2-associated X protein; S100A4, S100 Calcium-binding protein A4; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9.

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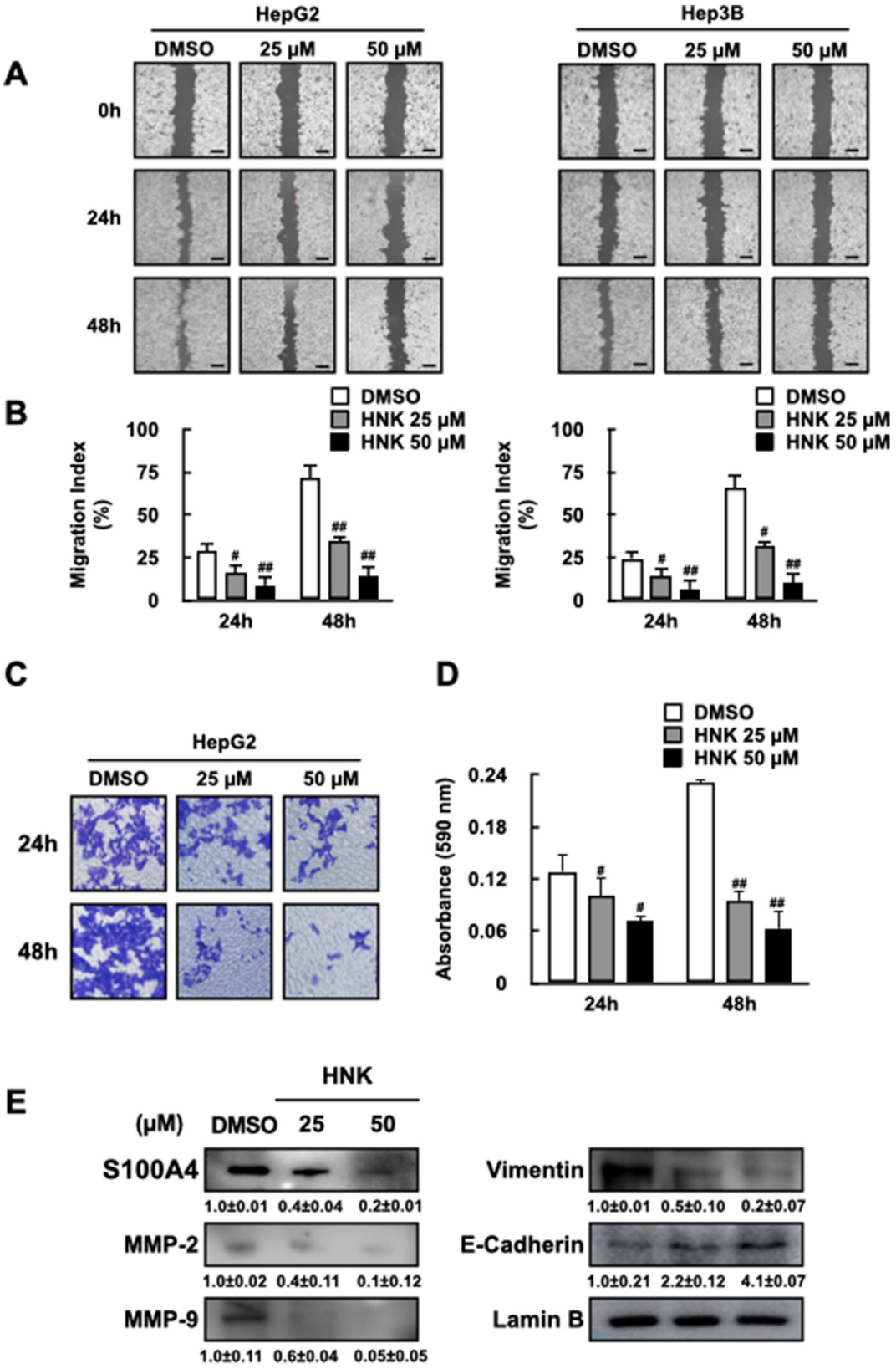

Fig. 1. HNK reduces cancer cell migration and invasion in HepG2 cells.
(A) Wound healing or scratch assay was performed to assess the effects of HNK on cancer cell migration using HepG2, and Hep3B cells. Cells were incubated with or without HNK (25 or 50 μM) for 24 h and 48 h. HNK treatment inhibits migration of cells, compared to control (DMSO-treated) cells. Control (0 h) panel indicates the original space between the cell layers immediate after making a scratch or wound. The dark space between the cell layers indicates the space largely unoccupied by hepatoma cancer cells. The representative photomicrographs are shown from three independent experiments. Scale bar, 2 mm
(B) Quantification of the wound healing assay with HNK (25 or 50 μM) treatment for 24 h and 48 h. The unoccupied empty space by the cells between the cell layers was measured

using CellSens software, OLYMPUS under microscope, and the data are presented as an empty space in terms of μm ±SD for each cell line. Significant inhibition in cell migration versus DMSO-treated controls. $^{\#}P < 0.01$, $^{\#\#}P < 0.05$ versus DMSO-treated controls.
(C) Cells incubated with or without HNK (25 or 50 μM) for 24 h or 48 h were plated onto the upper part of the Matrigel invasion chamber. After incubation, invasive cells that had migrated to the lower part of the membrane were stained with crystal violet and observed using a microscope.
(D) Quantification of the transwell invasion assay with HNK (25 or 50 μM) treatment for 24 h and 48 h. Absorbance at 590 nm showed quantitative Matrigel invasion data. Significant inhibition in cell invasion versus DMSO-treated controls. $^{\#}P < 0.01$, $^{\#\#}P < 0.05$ versus DMSO-treated controls.
(E) After treatment with HNK (25 or 50 μM) for 48 h, the protein expressions of S100A4, MMP-2, MMP-9, Vimentin and E-Cadherin were determined by Western blot analysis, Lamin B was used as a loading control. The control was treated with DMSO. Data are expressed as mean ± SD of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

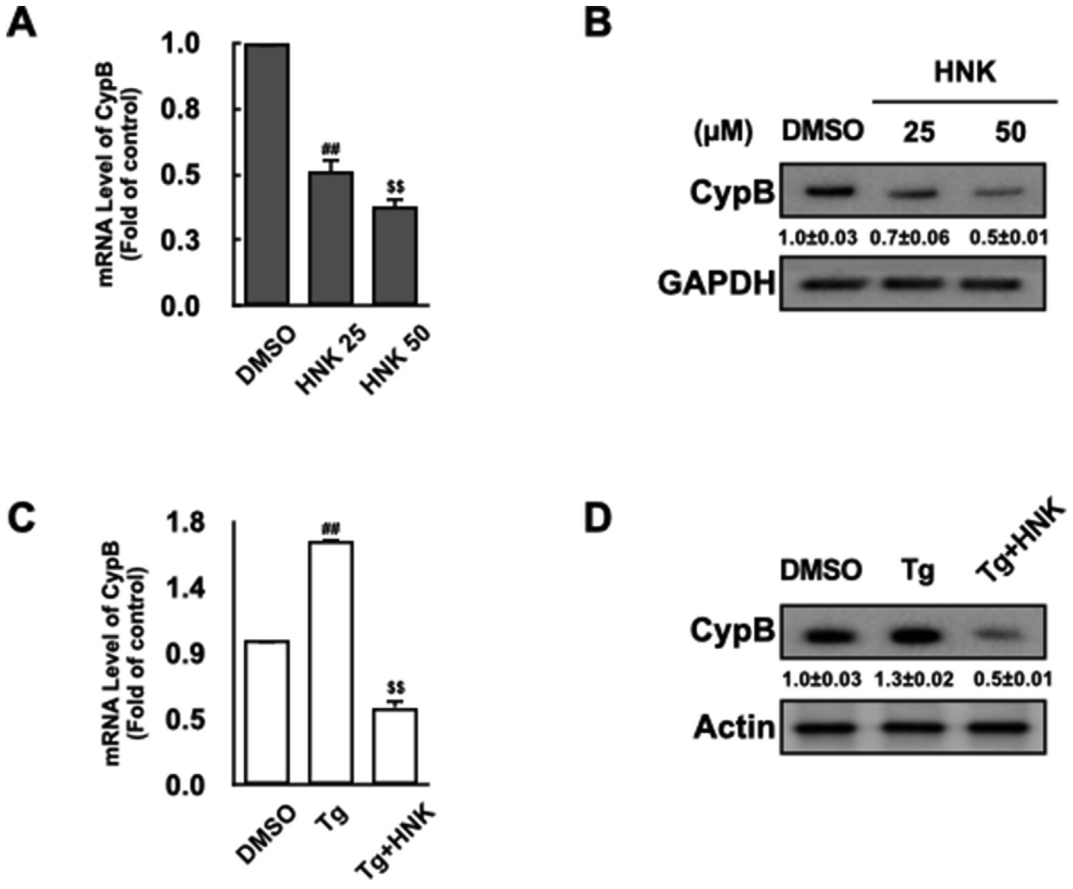

Fig. 2. CypB expression was reduced by HNK treatment in HepG2 cells.
After treatment of HepG2 cells with HNK at the indicated doses for 48 h, (A) the mRNA expression level and (B) the protein expression level of CypB were analyzed by qRT-PCR and Western blotting, respectively. After treatment of HepG2 cells with both 5 μM thapsigargin and 50 μM HNK or 5 μM thapsigargin only for 48 h, (C) the mRNA expression level and (D) the protein expression level of CypB were analyzed by qRT-PCR and Western blotting, respectively. GAPDH or Actin was used as a loading control. The control was treated with DMSO. $^{\#\#}P < 0.05$, $^{\$\$}P < 0.05$ versus DMSO-treated controls. Data are expressed as mean ± SD of three independent experiments.

Penicillin (Corning, NY, USA) in a 37 °C incubator with 5% CO_2. HNK was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10mM and stored at -20°C. Plasmids and siRNAs were transfected using X-tremeGENE™ (Invitrogen, CA, USA) and VIROMER® BLUE (lipocalyx, Germany) according to manufacturers' instructions. The following antibodies were used: S100A4, CypB, HA, Vimentin, MMP-2, MMP-9, Pro-caspase-3, Pro-PARP (Cell signaling, MA, USA), Actin, Bax (Santa Cruz, TX, USA).

2.2. Plasmid constructs

Cyclophilin B was HA-tagged at its 5′ end and subcloned into a vector (HA tag; 5′- TACCCATACGACGTCCCAGACTACGCT-3′).

2.3. Real-time PCR analysis

Total cellular RNA was extracted from cells using the TRIzol reagent (Invitrogen, CA, USA). A cDNA was synthesized from 0.2 μg total RNA using M-MLV reverse transcriptase (Fermentas, MD, USA). The specific primers for Real-time PCR included the following: CypB forward 5′-GCACAGGAGGAAAGAGCATC-3′ and reverse 5′-AGCCAGGCTGTCTTGACTGT-3'; and GAPDH, forward 5′-CAAGGTCATCCATGACAACTTTG-3′ and reverse 5′-GTCCAC-CACCCTGTTGCTGTAG-3'. Real-time PCR was performed using an ABI prism 7500 Sequence Detection System (Applied biosystems, CA, USA) with SYBR-Green PCR Master Mix (Applied Biosystems, CA, USA). The PCR reaction was carried out for 40 thermal cycles. Expression of the target gene was analyzed by an absolute quantification method and normalized using GAPDH levels. Results were

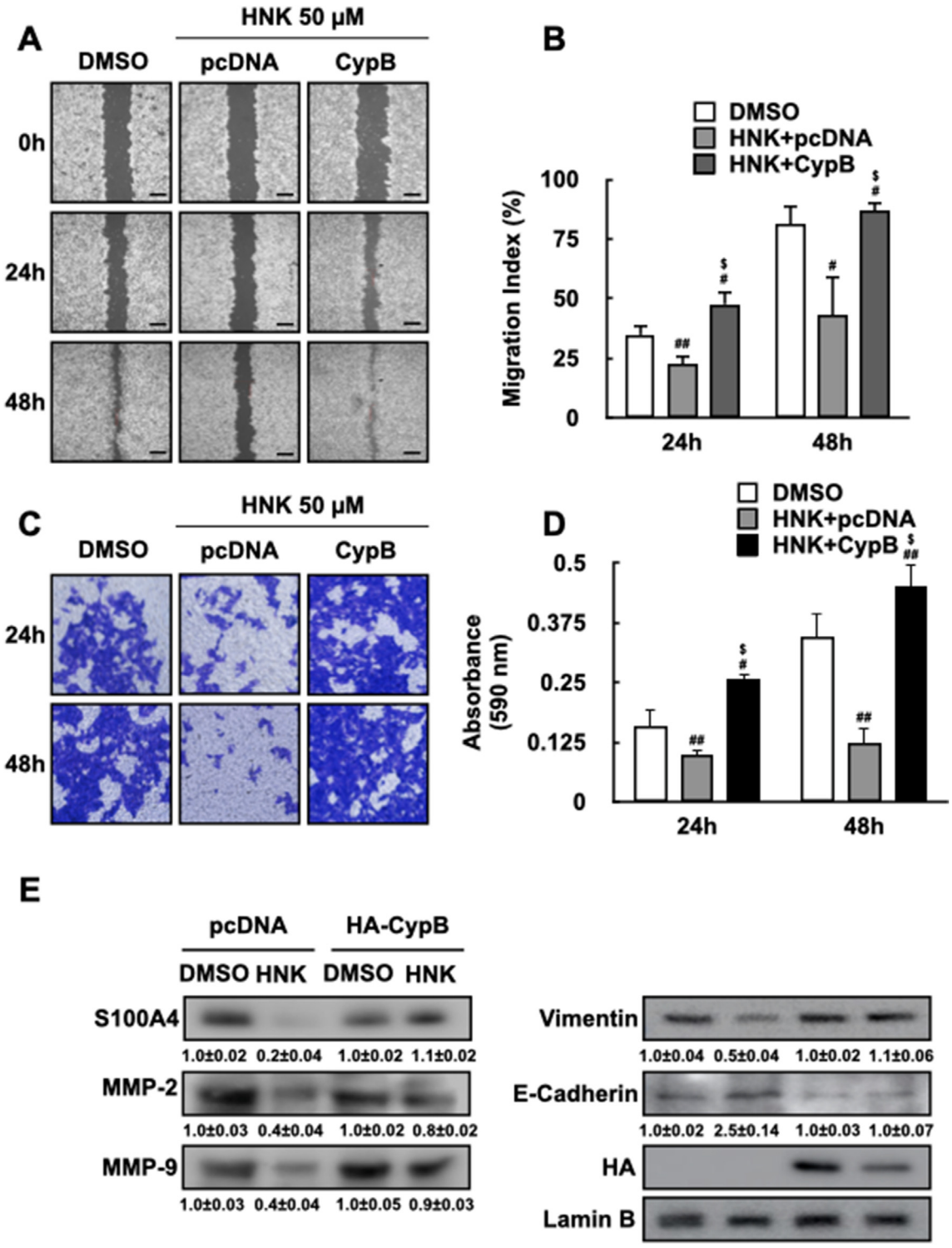

Fig. 3. Honokiol inhibits cell migration via CypB regulation.

(A) Wound healing or scratch assay was performed to assess the effects of CypB on HNK-mediated inhibition of cancer cell migration in HepG2 cells. HepG2 cells were transiently transfected with pcDNA or HA-CypB,(CypB), followed by treatment with 50 μM HNK for 24 h, or 48 h. Control (0 h) panel indicates the original space between the cell layers immediate after making a scratch or wound. The dark space between the cell layers indicates the space largely unoccupied by hepatoma cancer cells. The representative photomicrographs are shown from three independent experiments. Scale bar, 2 mm

(B) Quantification of the wound healing assay with 50 μM HNK treatment for 24 h or 48 h, after transfection with pcDNA or HA-CypB,(CypB), The unoccupied empty space by the cells between the cell layers was measured using CellSens software, OLYMPUS under microscope, and the data are presented as an empty space in terms of μm ±SD for each cell line. Significant inhibition in cell migration versus DMSO-treated controls. $^{\#}P < 0.01$, $^{\#\#}P < 0.05$ versus pcDNA-transfected cells treated with DMSO; $^{\$}P < 0.05$ versus pcDNA-transfected cells treated with HNK.

(C) Transwell invasion assay was performed to assess the effects of CypB on HNK-mediated inhibition of cancer cell invasion in HepG2 cells. HepG2 cells were transiently transfected with pcDNA or HA-CypB,

(CypB), followed by treatment with 50 μM HNK for 24 h, or 48 h. Cells incubated with or without HNK (25 or 50 μM) for 24 h or 48 h were plated onto the upper part of the Matrigel

calculated by using the follow equation: R (ration) = $2^{-[\Delta CTsample - \Delta CTcontrol]}$.

2.4. Western blot analysis

Cells were lysed in Laemmli lysis buffer (2% SDS, 10% Glycerol, 0.002% bromphenole blue, 0.0625 M Tris-Cl, pH 6.8, 5% 2-mercaptoethanol and 1% protease inhibitor, 1% phosphatease inhibitor). Protein concentrations of the total lysates were determined by the BCA (Bicinchoninic Acid) protein assay. Twenty micrograms of protein from the total lysates were subjected to SDS-PAGE, transferred to BioTrace™ NT nitrocellulose membrane (Pall corporation, FL, USA), and incubated with primary antibodies in blocking solution (5% BSA). Proteins were visualized using the ECL-plus detection system (Santa Cruz, TX, USA). The immunoreactive protein band was detected and band density was quantified by densitometry using an Amersham Imager 600 (GE Healthcare Life Sciences, Little Chalfont, UK).

2.5. Wound healing assay

Cell were seeded at a density of 5×10^5 cells/well into 6-well dishes and treated with DMSO, 25 or 50 μM HNK. After 24 h, a wound scratch was made a 200 μl pipette tip on cell and images were captured after 24 h, or 48 h to estimate the area occupied by migratory cells. Cells were maintained at 37 °C. The migration capacity was calculated using [Δ area/area (0 h)] x100%.

2.6. Transwell invasion assay

After treatment with different concentrations of honokiol, 1×10^5 HepG2 cells were diluted in 500 μl serum-free DMEM medium and inoculated in the upper Transwell chamber coated with growth factor-reduced Matrigel(Corning, AZ, USA). DMEM medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 24 h or 48 h, cells on upper surface of the membrane were removed using a cotton swab, and invaded cells were fixed with 4% formaldehyde for 10 min at room temperature followed by 0.25% crystal violet staining (Sigma-Aldrich; Merck KGaA, MO, USA) for another 10 min at room temperature. Crystal violet staining was dissolved in 33% acetic acid and optical density was detected at 590 nm.

2.7. Cell viability assay

Cell viability was assayed using Chromo-CK™ cell viability assay kit (Chromogen, Korea). HepG2 cells were seeded in a 24-well plate at a density of 2.5×10^4 cells/well for 24 h and were cultured overnight for cell adhesion, then treat different concentrations HNK (0–100 μM) for 24 h or 48 h. At the end of treatment, 50 μl MTS solution was added to each well and incubated 37 °C for 30min. Absorbance was measured at wavelength 450 nm with SYNERGY HTX multi-mode reader (BioTek, VT, USA). Cell viability is calculated as the percentages change of the absorbance of treated cells divided by the absorbance of DMSO cells.

2.8. Statistical analysis

Results were expressed as mean ± SD. Error bars represent the mean ± SD of three independent experiments performed in triplicate. The difference between two mean values was analyzed using Student's *t*-test.

3. Results

3.1. Honokiol inhibits cancer cell migration

To evaluate the effects of HNK on cancer cell migration, we first performed the wound healing assay in human hepatocellular carcinoma (HepG2, and Hep3B) cell line. The cells were treated with different concentrations of HNK (0, 25, and 50 μM/ml) for 24 h or 48 h. The width of the wound in the treated cells showed a lower propensity for closure compared to that of the vehicle control, DMSO-treated. Treatment with 25 μM and 50 μM of HNK for 48 h, reduced the cell migration of HepG2 or Hep3B by 50% and up to 80%, respectively, compared with the vehicle control, DMSO (Fig. 1A and B). In addition, invasion capacity of HepG2 cells was measured using Transwell assays. The cells invading into the lower chambers were significantly decreased upon treatment with HNK for 24 h or 48 h, compared with the vehicle-treated control group (Fig. 1C and D). To investigate the mechanism of inhibition of HNK in HepG2 cell migration, the expression levels of the genes associated with cell migration, such as S100A4, MMP-2, MMP-9, Vimentin and E-Cadherin were determined by Western blotting assays. The results demonstrated that the expression of S100A4, MMP-2, MMP-9 and Vimentin was significantly downregulated by HNK treatment, while E-cadherin was upregulated (Fig. 1E). Our data suggest that treatment with HNK suppresses cancer cell migration in human hepatocellular carcinoma.

3.2. CypB expression level is reduced by Honokiol treatment

Next, we investigated in detail the molecular mechanism by which HNK reduces cell migration. Previous studies have reported that CypB stimulates cancer cell migration [6]. Therefore, to investigate the effects of HNK on CypB expression, the mRNA and protein expression levels of CypB were measured after treatment with HNK. Interestingly, treatment with HNK significantly reduced the mRNA and protein expression levels of CypB (Fig. 2A and B).

Thapsigargin, an inducer of CypB, is known to increase the mRNA and protein levels of CypB [12]. As expected, the induced CypB expression level by thapsigargin was abolished after co-treatment with HNK (Fig. 2C and D). These results suggest that HNK treatment reduces expression of CypB.

3.3. Honokiol inhibits cell migration via CypB regulation

To further study the effects of CypB overexpression on the inhibitory effect of HNK on cell migration, HepG2 cells overexpressing CypB were treated with HNK (50 μM) for 24 h or 48 h. As expected, HNK treatment reduced cell migration, compare to the vehicle control, DMSO. However, overexpressed CypB abrogated HNK-mediated suppression of cell migration (Fig. 3A and B). Also,

invasion chamber. After incubation, invasive cells that had migrated to the lower part of the membrane were stained with crystal violet and observed using a microscope. (D) Quantification of transwell invasion assay with 50 μM HNK treatment for 24 h or 48 h, after transfection with pcDNA or HA-CypB,(CypB), Absorbance at 590 nm showed quantitative Matrigel invasion data. $^{\#}P < 0.01$, $^{\#\#}P < 0.05$ versus pcDNA-transfected cells treated with DMSO; $^{\$}P < 0.05$ versus pcDNA-transfected cells treated with HNK. (E) After transfection with pcDNA or HA-CypB HepG2 cells were treated with 50 μM HNK for 48 h the protein expressions of S100A4, MMP-2, MMP-9, Vimentin and E-Cadherin were determined by Western blot analysis. Overexpression of HA-CypB was verified by immunoblotting with anti-HA antibody. Lamin B was used as a loading control. The control was treated with DMSO. Data are expressed as mean ± SD of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

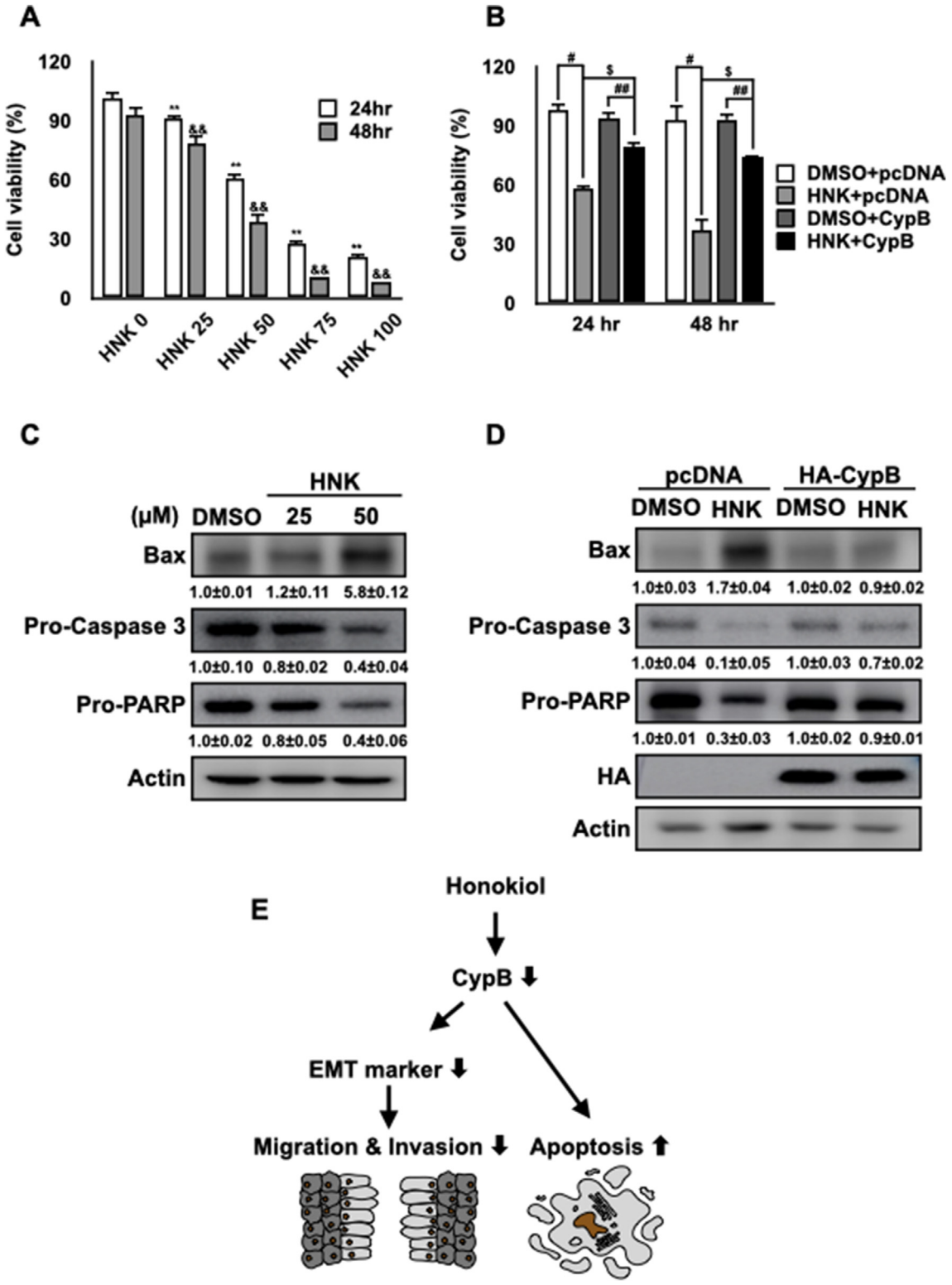

Fig. 4. Overexpressed CypB reversed the apoptotic effects of Honokiol.
(A) HepG2 cells treated with varying concentrations of HNK (0, 25 μM, 50 μM, 75 μM, 100 μM) for 24 h or 48 h. Cell viability was measured by MTS assay. $^{**}P < 0.05$ versus cells treated with DMSO for 24 h; $^{\&\&}P < 0.05$ versus cells treated with DMSO for 48 h.
(B) After transfection with pcDNA or HA-CypB, HepG2 cells were treated with 50 μM HNK for 24 h or 48 h. Cell viability were measured by MTS assay. $^{\#}P < 0.01$ versus pcDNA-transfected cells treated with DMSO; $^{\#\#}P < 0.05$ versus HA-CypB-transfected cells treated with DMSO; $^{\$}P < 0.05$ versus pcDNA-transfected cells treated with 50 μM HNK.
(C) HepG2 cells were treated with HNK (25 μM or 50 μM) and the cell lysates were subjected to Western blot analysis. The control was treated with DMSO. (D) HepG2 cells were transiently transfected with pcDNA or HA-CypB, followed by treatment with 50 μM HNK. The cell lysates were subjected to Western blot analysis. Overexpression of HA-CypB was verified by immunoblotting with anti-HA antibody. Actin was used as a loading control. Data are expressed as mean ± SD of three independent experiments.
(E) A schematic diagram shows that HNK inhibits CypB expression leading to upregulated apoptosis and reduced cancer cell migration.

the reduced invasion ability by honokiol was restored by CypB overexpression (Fig. 3C and D). To confirm our results, the expression levels of the genes associated with cell migration, such as S100A4, MMP-2, MMP-9, Vimentin and E-Cadherin were determined by Western blotting assays. Fig. 3C showed that HNK treatment failed to inhibit the expressions of S100A4, MMP-2, MMP-9, and Vimentin in CypB-overexpressed cells, compared to DMSO-treated control Also. CypB overexpression suppressed HNK-induced E-Cadherin (Fig. 3E). These data suggest that HNK affects cancer cell migration by targeting CypB signaling pathway.

3.4. Overexpressed CypB reverses the apoptotic effects of Honokiol

We also investigated the effects of HNK treatment on cancer cell death. MTS assays were performed with HepG2 cells treated with HNK for 24 h or 48 h. HNK increased cell death in time- and dose-dependent manners (Fig. 4A).

Next, we studied the effects of CypB on HNK-mediated cell death. Overexpression of CypB protected HepG2 cells from HNK-mediated cell death at both time points (Fig. 4B). In order to confirm our data, the expression levels of cell death marker molecules including Bax, pro-PARP and Pro-caspase were analyzed by Western blotting. HNK treatment up-regulated Bax expression and down-regulated the expressions of pro-PARP and pro-Caspase3 (Fig. 4C). As expected, overexpressed CypB reversed the apoptotic effects of HNK in HepG2 cells (Fig. 4D).

Taken together, these results suggest that HNK inhibits CypB expression, resulting in upregulated apoptosis and reduced cancer cell migration (Fig. 4E).

4. Discussion

Honokiol is of great interest due to its various pharmacological properties. HNK inhibits the production of numerous cytokines and adhesion proteins associated with tumor promotion, progression, metastasis and cell viability. Moreover, the benefits of HNK overcoming chemoresistance in human myeloma cells have been demonstrated [13]. In addition, the combination of HNK and various other chemotherapeutic agents significantly enhances the efficacy of breast, colon and ovarian cancers treatment [9,13–16]. And also, HNK reduced cancer cell metastasis through migration regulation [17]. Therefore, it has considerable potential to serve as a novel agent for cancer prevention and primary treatment. However, the mechanism by which HNK works in cancer cells is still unclear. Here, we explored the role of CypB in HNK-induced cell migration and cell death in HepG2 human hepatoma cells.

Even though the exact inhibitory mechanism of HNK in cancer cell migration remains to be elucidated, previous studies have suggested that HNK affects melanoma and breast cancer cell growth by targeting AMPK signaling [18]. HNK-induced AMPK activation may induce apoptosis and cell cycle arrest in various types of cancer, including breast, colon and oral cancer [19]. On the other hand, Since MMPs, especially MMP-2 and MMP-9 are important in metastatic processes, it has been postulated that HNK down-regulates the expressions of MMP2 and MMP9. Recently, HNK has been shown to inhibit MMP9 expression through post-translational regulation [20]. Consistently, we showed that HNK treatment reduces the expression levels of MMP2 and MMP9. Also Reactive Oxygen Species(ROS) and ERK pathway have been considered to play central roles in HNK-induced anti-cancer activity [21]. However, we showed for the first time that HNK regulates CypB expression to affect cancer cell migration. HNK treatment reduced both mRNA and protein levels of CypB (Fig. 2). Overexpressed CypB abrogated HNK-mediated suppression of cell migration (Fig. 3). Therefore, we concluded that CypB regulation is an essential mechanism for regulation of HNK-mediated migration. It has been reported that CypB is overexpressed in breast, liver, colon, and pancreatic cancer and plays an crucial role in the malignant progression of tumors [4]. Consistent with our data, CypB-silencing or down-regulated CypB results in the inhibition of proliferation, migration and invasion [22]. Moreover, Previously we have shown that CypB protects hepatoma cell from oxidative stress induced apoptosis via ERK activation [4]. Further studies may be needed to define the exact roles of the ERK pathway and CypB in treatment with HNK. Like CypB, oncogenic transcription factor FOXM1 is one of the most overexpressed oncoproteins in human cancer. It has been reported found that HNK inhibits FOXM1 protein expression and that HNK inhibitory effect on FOXM1 is a result of binding of HNK to FOXM1 [23]. Likewise, it might be possible for HNK to physically interact with CypB. Therefore, in the future, it will be interesting to study the molecular mechanism by which HNK down-regulate CypB expression.

In summary, HNK was able to suppress cell migration in the HepG2 cell line, which was mediated by down-regulated CypB. In addition, HNK also inhibited proliferation in HepG2 human hepatoma cell line through reduced expression of CypB. These data suggest that CypB expression is important for the anti-cancer activity of HNK. Also, our findings provide more evidence to support HNK as a potential therapeutic candidate in the treatment of metastatic cancers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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