ORIGINAL RESEARCH

# Modulation of MnSOD and FoxMI Is Involved in Invasion and EMT Suppression by Isovitexin in Hepatocellular Carcinoma Cells

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MnSOD) ind. xM1 expression, Background: Manganese superoxide dismutase subsequently contributing to migration in second cap r cells. Isovitexin (ISOV) was ecreasing temness in hepatocellular recently found to downregulate MnSOD ar FoxM carcinoma (HCC) stem-like cells (HCS). The current study aimed to determine whether inhibition of migration, invasion a EM in HCSLCs y ISOV results from MnSOD/ FoxM1 signaling blockade and subsequent Twice Slug, ZEB1 and MMP-2 downregulation. Materials and Methods: Y examined the mig. ory and invasive capabilities and EMT phenotype in HCC cells are their HCSL<sup>(4)</sup>, respectively, by wound-healing assay, transwell invasion assay and Western lot after treament with non-cytotoxic concentrations of ISOV, and explored the mechanish by which SOV affects migration, invasion and EMT by MnSOD or Fox kdown and or overexpression in HCSLCs or HCC cells. ISOV not only downregulated MnSOD and FoxM1 but **Results:** The rest sho ...

also supervised the violatory and invasive capabilities and reversed the EMT phenotype in HCSLCs, which was reflected by elevated E-cadherin protein amounts, and reduced headhering dwistl, Sig, ZEB1 and MMP-2 protein levels. The suppressive effects of ISO pointer migratory and invasive capabilities and EMT phenotype could be potentiated by MnOD or FoxM1 knockdown in HCSLCs, and attenuated by MnSOD or FoxM1 overexpression reversed MnSOD knockwn combined with ISOV suppression on the migratory and invasive capabilities and EMT phenotype in HCSLCs, while having little effects on MnSOD expression.

**Conclusion:** Collectively, the above findings demonstrated that ISOV suppresses migration, invasion and EMT in HCSLCs by blocking MnSOD/FoxM1 signaling subsequently inhibiting the expression of EMT-related transcription factors and MMP-2.

**Keywords:** hepatocellular carcinoma, cancer stem cell, isovitexin, epithelial-mesenchymal transition, MnSOD, FoxM1, Twist1, MMP-2

#### Introduction

Hepatocellular carcinoma (HCC) ranks fifth among malignancies in terms of incidence, and represents the third cause of malignant tumor-related death around the world; its low survival rate is due to a lack of efficient therapeutics.<sup>1,2</sup> Although the cytologic pathogenesis of HCC is not completely elucidated, a small cell subset with stem cell characteristics, namely cancer stem cell-like cells (CSLCs) can initiate the tumor and promote cancer progression, recurrence and acquisition of resistance to chemotherapy.<sup>3,4</sup> To date, it has been confirmed that epithelial-mesenchymal transition (EMT), an

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Forkhead box M1 (FoxM1) is a carcinogenic transcription factor that is abnormally upregulated in various cancers, including HCC.<sup>7,8</sup> Suppression of FoxM1 has inhibitory effects on tumor progression and metastasis.<sup>9,10</sup> Increased expression of FoxM1 was observed in HCC tissues, in association with poor prognosis of patients with HCC.<sup>11–14</sup> FoxM1 silencing in mouse hepatocytes inhibits cell proliferation and reduces the formation of diethyl-nitrosamineinduced hepatoma.<sup>15</sup> Our laboratory and others demonstrated that elevated FoxM1 by manganese superoxide dismutase (MnSOD) promotes migration and invasion.<sup>16,17</sup> Whether and how FoxM1 upregulated by MnSOD induces the migratory and invasive capabilities and EMT phenotype in HCC stem-like cells (HCSLCs), thereby stimulating tumor progression, remains unknown.

It has been reported that MnSOD induces FoxM1 expression and promotes aggressiveness in lung cancer.<sup>17</sup> Our recent study demonstrated that MnSOD is overexpressed in lung CSLCs from H460 cells, and confers carcinogenesis and lung CSLC properties through activation of the FoxM1 transcriptional factor.<sup>16</sup> Because FoxM contributes to migration, invasion and EMT in various cancers,<sup>7,8,14</sup> we initially aimed to evaluate wether FoxM1 upregulation by MnSOD overexpression leads to migration, invasion and EMT in HCSLCs.

Isovitexin (ISOV, apigenin-6-Conucoside) was been shown to possess extensive biological activities.<sup>15</sup> Clatural flavone C-glycosides occur indifferent earle or medicinal plants.<sup>19,20</sup> It is well known that ISOV and vitexin exert antitumor effects on LCC by togeting cell apoptosis and autophagy through regulation of aport sis-related proteins such as Bax are Bere as we as the autophagy-associated protein LC2 in.<sup>21–24</sup> W recently confirmed that ISOV inhibits stemness in Fre Sters.<sup>25</sup> However, whether ISOV suppresses migration, invasio and EMT in HCSLCs remains unknown.

This work demonstrated enhanced migratory and invasive capabilities and induced EMT in HCSLCs compared with HCC cells. We firstly showed that ISOV suppressed migration and invasion, and reversed the EMT phenotype by downregulating MnSOD, FoxM1, Twist1, Slug, ZEB1 and MMP-2 in HCSLCs. These findings suggest MnSOD, and FoxM1 and its target proteins Twist1, Slug, ZEB1 and MMP-2 may promote migration, invasion and EMT, and ISOV might constitute a novel candidate for treating human HCC via suppression of migration and invasion as well as EMT inversion in HCSLCs.

## Materials and Methods Cell and Sphere Culture

MHCC97H and Sk-Hep-1 HCC cells as well as L-02 liver embryonic cells obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) were grown in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Invitrogen) under a humid environment with 5%  $CO_2$  at 37°C.

To obtain HCSLCs,MHCC971 or Sk-Hep-ucells were cultured in cancer stem cell condium (CCC-M) as described previously.<sup>25</sup> The second operation spheres were considered HCSL s.<sup>25</sup> For any interaction, in primary sphere culture centions of 1.2V (Sigma-Aldrich St.; final concentration of 1.10 and 2000A, respectively) in fresh CSC-M for 72h; then the second-generation spheres were culture without ISOV.

### Wound-Healing Assay

MHCC. Mode  $(2 \times 10^5)$  or Sk-Hep-1 cells  $(2 \times 10^5)$ , or recentive HCSLCs  $(2 \times 10^5)$  incubated with or without FOV were cultured in DMEM (Invitrogen) with 10% FBS (Invitrogen) until 90% confluence. Then, a wound as created by scratching across the center of the well with a sterile 100 µL pipette tip. Next, the cells were washed and photographed in the same high-power field for analysis at 0 and 24h, respectively. MHCC97H cells, Sk-Hep-1 cells or respective HCSLCs treated with vehicle (0.1% DMSO) were used to standardize the number of migrated cells.

#### Invasion Assay

Cell invasion assay was performed in transwell systems with 8  $\mu$ m pore membrane coating matrigel (Corning Incorporated, NY, USA). Briefly, MHCC97H cells (2×10<sup>5</sup>) or Sk-Hep-1 cells (2×10<sup>5</sup>), or respective HCSLC (2×10<sup>5</sup>) incubated with or without ISOV in CSC-M without growth factors were plated in upper chambers (Corning Incorporated). CSC-M containing growth factors served as a chemoattractant in the bottom wells for 24h. Then, the invading cells were fixed with methanol, dyed with Wright's stain, and mounted with Vectashield mounting medium (Vectorshield, Vector Laboratories). An optical microscope (Olympus DP72, Hamburg, Germany) was used for imaging (×200). Experiments were independently performed in triplicate.

#### Immunoblot

Immunoblot was carried out as described previously.<sup>16</sup> Antibodies including anti-MnSOD, anti-FoxM1, anti-E-cadherin, anti-N-cadherin, anti-Twist, anti-MMP-2, anti-Slug, anti-ZEB1 (Cell Signaling Technology, Inc. USA) and anti- $\beta$ -actin (Sigma, MA, USA) were used as primary antibodies. HRP-conjugated antibodies (Beyotime Institute; China) were used as secondary antibodies. Immunoreactive protein bands were detected using the enhanced chemiluminescence (ECL) reagent (Millipore; USA).

### Adenoviruses and Infection

MHCC97H cells  $(2 \times 10^5)$  or HCSLC cells  $(2 \times 10^5)$  were infected with various adenoviruses harboring the indicated shRNA or cDNA at a multiplicity of infection (MOI) of 100, as described previously.<sup>16</sup>

### Statistical Analysis

Statistical analyses were performed with the SPSS 20.0 software (IBM, Armonk, NY, USA) and SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA). Data are new  $\pm$ standard deviation (SD). Unpaired two-tailed Student's *t*-test was used to compare various group control. One-way ANOVA followed by post-hot fukey stest was performed for pairwise comparison an eng group 20.05 was considered statistically significant.

### Results

## ISOV Inhibits In asion and Reverses EMT Phenotype in HCSLCs from MHCC97H Cells

performed image of to analyze MnSOD and We initial rale and found that they were higher in FoxM: rotein HCSLCs pared with MHCC97H cells (Figure 1A). migration and invasion rates were substan-Meanwhile, C tially elevated in ACSLCs compared with MHCC97H cells (Figure 1B and C). In addition, increased N-cadherin, Twist1, Slug, ZEB1 and MMP-2 amounts, and decreased E-cadherin levels, were found in HCSLCs compared with MHCC97H cells (Figures 1D and E; Supplementary Figure 1). Our results revealed that HCSLCs had higher migratory and invasive capabilities and enhanced EMT phenotype, which may be associated with increased MnSOD, FoxM1 and its target molecules Twist1, slug, ZEB1 and MMP-2 protein amounts.

ISOV and vitexin were shown to possess antitumor activities in hepatoma via cell apoptosis and autophagy.21-24 Therefore, we employed the CCK-8 kit to measure cell viability in liver embryonic L-02 cells, MHCC97H cells and the corresponding HCSLCs treated with ISOV. The results showed that ISOV remarkably suppressed MHCC97H cells and the corresponding HCSLCs compared with L02 cells (Figure 2A). IC<sub>50</sub> was about 80 µM for MHCC97H cells and 40 µM for the corresponding HCSLCs (Figure 2A). We next examined the effects of ISOV on protein (MnSOD and FoxM1) levels, cell for tions (migration and invasion) and EMT phenotype clated physical (E-cadherin, N-cadherin, Twist1, Slug, 221 and MM-2) levels in HCSLCs after treatment with no sytotoxic concentrations (5.0, 10 and 20  $\mu$ M of ISOV. The vertex revealed that ISOV significantly wnr alated MnSOD and FoxM1 in HCSLCs (Fig. e 2B). Addition , substantial reduction of cell migro, and invasi HCSLCs was found after incubation with ISOV for 24h (Figure 2C and D). nore, ISOV ignificantly altered the levels of the Fv MT-related proteins Twist1, Slug, ZEB1 and MMP-2, in dose-dependent manner (Figures 2E and F; Supplementary ure 2).Ou lata showed that ISOV could inhibit cell migration and reverse EMT phenotype, which may be d to downregulation of MnSOD, FoxM1 and its downstream targets Twist1, Slug, ZEB1 and MMP-2.

# Changes of MnSOD Expression Affect Cell Invasion and the EMT Phenotype in HCSLCs and MHCC97H Cells

We next demonstrated that MnSOD shRNA significantly downregulated MnSOD and FoxM1 in HCSLCs relative to vector control or non-transduced cells (Figure 3A). Substantial reductions of cell migration and invasion in HCSLCs after MnSOD silencing were found in comparison with vector control- or non-transduced cells (Figure 3B and C). In addition, decreased N-cadherin, Twist1, Slug, ZEB1 and MMP-2 levels as well as increased E-cadherin expression were observed in comparison with vector control or nontransduced MHCC97H cells (Figures 3D and E; Supplementary Figure 3A). Conversely, MnSOD overexpression significantly increased MnSOD and FoxM1 expression amounts in MHCC97H cells relative to vector control or nontransduced cells (Figure 3F). Substantially elevated cell migration and invasion in MHCC97H cells overexpressing MnSOD were found compared with vector control or non-transduced cells (Figure 3G and H). Meanwhile, increased N-cadherin, Twist1, Slug, ZEB1 and MMP-2 expression levels as well as decreased E-cadherin amounts were observed in MHCC97H cells overexpressing MnSOD relative to vector control or non-transduced cells (Figures 3I and J; <u>Supplementary Figure 3B</u>). Collectively, the above findings indicate that promotion of cell migration, invasion and EMT in HCSLCs may require elevated MnSOD expression.

# Changes of MnSOD Expression Affect ISOV-Associated Inhibition of Migration and EMT in HCSLCs and MHCC97H Cells

For determining the relationship between MnSOD regulation and ISOV-associated repression of cell migration, invasion and EMT, MnSOD-knockdown HCSLCs and MHCC97H cells overexpressing MnSOD were incubated with or without ISOV. Silencing of MnSOD in combination with ISOV treatment more pronouncedly reduced MnSOD and FoxM1 levels compared with MnSOD silencing or ISOV alone (Figure 4A). Meanwhile, a starker reduction of cell migration and invasion was found after MnSOD knockdown combined with ISOV

treatment in comparison with MnSOD knockdown or ISOV alone in HCSLCs (Figure 4B and C). In addition, more pronouncedly decreased N-cadherin, Twist1, Slug, ZEB1 and MMP-2 expression levels and increased E-cadherin expression were observed after MnSOD knockdown plus ISOV administration relative to MnSOD knockdown or ISOV treatment alone in HCSLCs (Figures 4D and E; Supplementary Figure 4A). Conversely, the suppressive effects of ISOV on MnSOD and FoxM1 expression as well as migration, invasion and EMT were nearly abrogated by MnSOD overexpression in MHCC97H cells (Figures 4F-J; Supple tary Figure 4B). Collectively, the above findings indicate that the suppressive effects of ISOV on cell migration, 1 invasion a 1 EMT in HCSLCs may require MnSO<sup>r</sup> downic dation.

Changes of Foxe L Expression Affect Invasive Cartoility and EM in HCSLCs and MHCC9 H Cells To analyze the link between FoxM1 and cell migration, invasion and EMT, FOXM1 aRNA and FoxM1 cDNA were



Figure I Comparison of invasion and EMT between HCSLCs and MHCC97H cells. MnSOD and FoxMI protein levels (A), cell migratory and invasive capacities (B and C), and E-cadherin, N-cadherin, TwistI and MMP-2 protein amounts (D and E) in HCSLCs and MHCC97H Cells are shown (n=3; unpaired two-tailed Student's t-test, \*P<0.05 vs MHCC97H cells).





transduced into HCSLCs and AIR 97H cells, pectively. FOXM1 knockdown reparkably deck red FoxM1 expresaffecting MnSOD pression (Figure sion amounts, without 5A). Substantial de pases of ell migration and invasion were FoxM1\_\_\_\_encingrelative to vectorobserved in HCSLC se or non-trav <u>\_]]</u>\_ Figure 5B and C). In addi-Juce ontro M1 kno down decreased N-cadherin, Twist1, Slug, tion. F MP-2 protein amounts, and upregulated ZEB1 an E-cadherin in **WCSLCs** upon FoxM1 silencing compared with vector context or non-transduced HCSLCs (Figures 5D and E; Supplementary Figure 5A). Conversely, FoxM1 overexpression significantly elevated FoxM1 expression amounts, without affecting MnSOD expression (Figure 5F). Significantly increased cell migration and invasion in MHCC97H cells overexpressing FoxM1 were observed compared with vector control or non-transduced cells (Figure 5G and H). In addition, FoxM1 overexpression also elevated N-cadherin, Twist1, Slug, ZEB1 and MMP-2 expression

levels, and downregulated E-cadherin compared with vector control or non-transduced MHCC97H cells (Figures 5I and J; <u>Supplementary Figure 5B</u>). Taken together, the above findings indicate that promotion of cell migration, invasion and EMT in HCSLCs require elevated FoxM1, and its downstream targets Twist1, Slug, ZEB1 and MMP-2 might be controlled by MnSOD.

### Changes of FoxMI Expression Affect ISOV-Associated Inhibition of Invasion and EMT in HCSLCs or MHCC97H Cells

To determine whether ISOV inhibition of cell migration, invasion and EMT require FoxM1 repression, FoxM1 shRNA and FoxM1 cDNA were transduced into HCSLCs and MHCC97H cells, respectively. These cells were then treated with ISOV. We found that FoxM1 silencing significantly enhanced the suppressive effects of ISOV on FoxM1



Figure 3 Effects of MnSOD silencing and overexpression on migration, invasion and EMT. Ad-GFP and Ad-shMnSOD were transduced into HCSLCs, respectively. MnSOD and FoxM1 protein levels (**A**), cell migratory and invasive capacities (**B** and **C**), and E-cadherin, N-cadherin, Twist1 and MMP-2 protein amounts (**D** and **E**) in untreated-HCSLCs (Mock), harboring Ad-GFP or Ad-shMnSOD (n=3; \*P<0.05 vs Mock; #P<0.05 vs Ad-GFP; one-way ANOVA) are shown. MHCC97H cells were transduced with Ad-GFP or Ad-MnSOD and FoxM1 protein levels (**F**), cell migratory and invasive capacities (**G** and **H**), and E-cadherin, N-cadherin, N-cadherin, Twist1 and MMP-2 protein amounts (**I** and **J**) in MHCC97H cells transfected with Ad-GFP or Ad-MnSOD (n=3; \*P<0.05 vs Mock; #P<0.05 vs Mock; #P<0.05 vs Ad-GFP; one-way ANOVA) are shown.

expression, while ISOV-associated MnSOD downregulation was not altered after FoxM1 knockdown (Figure 6A). A more pronounced inhibition of cell migration and invasion was found after FoxM1 knockdown combined with ISOV relative to FoxM1 silencing or ISOV alone in HCSLCs (Figure 6B and C). In addition, FoxM1 knockdown also



Figure 4 Effects of ISOV combined with MnSOD silencing and overexpression on migration, invasion and the EMT phenotype. MnSOD and FoxM1 protein levels (A), cell migratory and invasive capacities (B and C), and E-cadherin, N-cadherin, Twist1 and MMP-2 protein amounts (D and E) in HCSLCs transfected with Ad-GFP or Ad-shMnSOD, and incubated with or without ISOV(10  $\mu$ M) (n=3; \*P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP plus10.0  $\mu$ M ISOV; one-way ANOVA) are shown. MHCC97H cells were transfected with Ad-GFP or Ad-MnSOD, and incubated with ISOV (10  $\mu$ M). MnSOD and FoxM1 protein levels (F), cell migratory and invasive capacities (G and H), and E-cadherin, N-cadherin, Twist1 and MMP-2 protein amounts (I and J) in MHCC97H cells transfected with Ad-GFP or Ad-MnSOD, incubated with or without ISOV(10  $\mu$ M) (n=3; \*P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP plus 10.0  $\mu$ M ISOV; <sup>8</sup>P<0.05 vs Ad-MnSOD alone; one-way ANOVA) are shown.



Figure 5 Effects of Four 1 silencing and overexpression on migration, invasion and the EMT phenotype. MnSOD and FoxMI protein levels (**A**), cell migratory and invasive capacities (**B** and **C**), and readherin, N-cadherin, TwistI and MMP-2 protein amounts (**D** and **E**) in HCSLCs transfected with Ad-GFP or Ad-shFoxMI (n=3; \*P<0.05 vs Mock; "P<0.05 vs Ad-GFP;one ray ANOVA) are shown. MnSOD and FoxMI protein levels (**F**), cell migratory and invasive capacities (**G** and **H**), and E-cadherin, N-cadherin, TwistI and MMP-2 protein levels (**F**), cell migratory and invasive capacities (**G** and **H**), and E-cadherin, N-cadherin, TwistI and MMP-2 protein levels (**F**) or Ad-FoxMI (n=3; \*P<0.05 vs Mock; "P<0.05 vs Ad-GFP; one-way ANOVA) are shown. MnSOD and FoxMI protein levels (**F**) or Ad-FoxMI (n=3; \*P<0.05 vs Mock; "P<0.05 vs Ad-GFP; one-way ANOVA) are shown.

potentiated the downregulation of N-cadherin, Twist1,Slug, ZEB1 and MMP-2 as well as E-cadherin upregulation (Figures 6D and E; <u>Supplementary Figure 6A</u>) by ISOV. Conversely, repression of FoxM1 expression, cell migration, invasion and EMT phenotype induced by ISOV was nearly

abrogated by overexpressing FoxM1 in MHCC97H cells (Figures 6F–6J; <u>Supplementary Figure 6B</u>). It should be noted that altered FoxM1 expression did not affect ISOV-associated suppression of MnSOD expression. Taken together, the above findings indicate that the suppressive



**Figure 6** Effects of ISOV combined with FoxM1 silencing and overexpression on migration, invasion and the EMT phenotype. MnSOD and FoxM1 protein levels (**A**), cell migratory and invasive capacities (**B** and **C**), and E-cadherin, N-cadherin, Twist1 and MMP-2 protein amounts (**D** and **E**) in HCSLCs transfected with Ad-GFP or Ad-shFoxM1, and incubated with or without ISOV (10  $\mu$ M) (n=3; \*P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP plus 10.0  $\mu$ M ISOV; <sup>%</sup>P<0.05 vs.Ad-shFoxM1 alone;one-way ANOVA) are shown. Ad-GFP and Ad-FoxM1 were transduced into MHCC97H cells incubated with or without ISOV (10  $\mu$ M), and E-cadherin, N-cadherin, Twist1 and MMP-2 protein amounts (**I** and **J**) in MHCC97H cells transfected with Ad-GFP or Ad-incubated with or without ISOV (10  $\mu$ M) (n=3; \*P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP plus 10.0  $\mu$ M ISOV; <sup>%</sup>P<0.05 vs Ad-GFP or Ad-FoxM1, and incubated with or without ISOV (10  $\mu$ M) (n=3; \*P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP plus 10.0  $\mu$ M ISOV; <sup>%</sup>P<0.05 vs Ad-GFP or Ad-FoxM1, and incubated with or without ISOV (10  $\mu$ M) (n=3; \*P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP plus 10.0  $\mu$ M ISOV; <sup>%</sup>P<0.05 vs Ad-GFP or Ad-FoxM1, and incubated with or without ISOV (10  $\mu$ M) (n=3; \*P<0.05 vs Ad-GFP; <sup>#</sup>P<0.05 vs Ad-GFP plus 10.0  $\mu$ M ISOV; <sup>%</sup>P<0.05 vs Ad-FoxM1 alone; one-way ANOVA).

effects of ISOV on cell migration, invasion and EMT in HCSLCs may require FoxM1 repression by MnSOD downregulation.

# ISOV Inhibits Invasion and Reverses the EMT Phenotype in HCSLCs from SK-Hep-1 Cells

To determine whether ISOV represses cell migration, invasion and EMT by a mechanism similar to that observed in other HCC cell lines, spheres derived from the SK-Hep-1 cell line (SK-SC) were obtained. We then used the CCK-8 kit to measure cell viability in liver embryonic L-02 cells, SK-Hep-1 cells and SK-SC treated with ISOV. We found ISOV remarkably suppressed cell viability in SK-Hep-1 cells and SK-SC compared with L02 cells (Figure 7A). ISOV also significantly reduced the amounts of MnSOD and FoxM1 in SK-SC (Figure 7B). In addition, substantial reductions of cell migration and invasion in SK-SC were found after incubation with non-cytotoxic concentrations (10 and 20  $\mu$ M) of ISOV for 24h (Figure 7C and D). Furthermore, ISOV significantly enhanced E-cadherin, attenuated N-cadherin, Twist1, Slug, ZEB1 and MMP-2 expressions in SK-SC (Figures 7E and F; Supplementary Figure 7). These results suggest that ISOV inhibited cell migration and invasion, and reversed the EMT phenotype by downregulating MnSOD, FoxM1, Twist1, Slug, ZEB1 and MMP-2, which was not limited to a specific type of cells.

### Discussion

In the current study, we firstly rified the elevated FoxM1 expression by Mr OD overex could proessic and E 7 in HCCs, indicating mote migration, invasi and met casis of HCC is, at that the initiation, ) gre. least partly, sed by M SOD with the underlying g FoxM, upregulation. This is mechanism nvol



Figure 7 ISOV inhibits migratory and invasive capability and EMT in HCSLCs from SK-Hep-I cells (SK-SC). Effects of ISOV (20, 40, 80 and 160  $\mu$ M) on cell viability (**A**) in L-02 cells, SK-Hep-I cells and SK-SC (n=3; \*P<0.05 vs vehicle; <sup>#</sup>P<0.05 vs 80  $\mu$ M ISOV; one-way ANOVA). Effects of ISOV (10, 20  $\mu$ M) on MnSOD and FoxMI protein levels (**B**), cell migratory and invasive capacities (**C** and **D**), and E-cadherin, N-cadherin, Twist I and MMP-2 protein amounts (**E** and **F**) in SK-SC (n=3; \*P<0.05 vs vehicle; <sup>#</sup>P<0.05 vs 10.0  $\mu$ M ISOV; one-way ANOVA).

because EMT is a key step in the initiation, progression and metastasis of cancers.<sup>6,26–29</sup> Importantly, we firstly demonstrated that the mechanisms underlying ISOV's inhibitory effects on migratory and invasive capabilities as well as the EMT in HCSLCs occurred through downregulation of MnSOD, FoxM1, Twist1, Slug, ZEB1 and MMP-2. These findings suggest that HCSLCs present elevated migratory and invasive capabilities and induced EMT, which could significantly help in designing novel therapeutic agents targeting HCSLCs in HCC.<sup>30</sup>

MnSOD is considered an enzyme localized in the mitochondria; its overexpression impacts migration and invasion as well as the metabolic shift towards aerobic glycolysis in cancers.<sup>17,30</sup> Our team and others have reported abnormal MnSOD is related to self-renewal in CSLCs.<sup>17,29,31–34</sup> In the current study, we revealed that MnSOD may elicit migration and invasion as well as the EMT phenotype in HCSLCs.

It was shown that overexpression of MnSOD upregulates the cancerogenic transcriptional factor FoxM1 by releasing E2F1, thereby promoting the migration and invasion of lung cancer cells.<sup>17</sup> Studies also showed that FoxM1 upregulated by MnSOD overexpressionis associated with stemness in CSLCs.<sup>17,29,31-34</sup> Consister these results, the present study found that change of MnSOD expression markedly affected FoxM1-express whereas FoxM1 level change did not alter MnSC expre sion. In addition, overexpression of xM1 arged the inhibitory effects of MnSOD sile ring figratory and invasive abilities and EM in MHC 97H cells. Accordingly, FoxM1 appears to be downstream effector of MnSOD, with Mp D/FoxM1 paling promoting migration, invasion and EMT in HCSLCs.

EMT was initian identified as a process in embryogenesis. Subsequent study confirment that EMT mediates the e of SLC or relevant enhances cell maintenar migrate x and *jectory* capabilities in many cancers.<sup>35,36</sup> The basis / fix-loop-helix transcription factor Twist1 represses E-caperin expression to endow the EMT phenotype in different vancers.<sup>27,37</sup> Slug, also known as SNAIL2, is a transcription factor that regulates the EMT process in cancer progression.<sup>38</sup> Slug can induce cancer cell metastasis and invasion by suppressing the epithelial phenotype and initiating EMT via binding to E-box DNA sequences found within the proximal promoter region of the E-cadherin gene.<sup>39,40</sup> ZEB1 is considered a transcriptional repressor of epithelial genes involved in EMT.<sup>41</sup> In the current study, we demonstrated that FoxM1 expression elevated

by MnSOD overexpression raised Twist1, Slug and ZEB1 expression levels. Qian et al showed that Twist1 overexpression results in gastric cancer cell proliferation through FoxM1 upregulation.<sup>42</sup> As FoxM1 upregulation may occur downstream of MnSOD as demonstrated above, the mutual activation of FoxM1 and Twist1, Slug and ZEB1 deserves further investigation.

FoxM1 has been reported to enhance MMP2-mediated invasion in human non-small cell lung cancer.<sup>43,44</sup> MnSOD or FoxM1 overexpression elevated MMP2 levels, accompanied by increased invasion. The above reponses were attenuated by MnSOD or FoxM1 shRM2, indicate a MMP2 may be a downstream effector of the hesSOD/FoxM1 axis.

Our previous study stand the extracts arboring ISOV inhibit CSLC characteristics in lun, or cer.<sup>19</sup> ISOV and vitexin act as chemical isomers, exhibit strong cytotoxic activities against ve ous call, cells, in adding HCC cells, in vitro raft tumor the nouse model in vivo.<sup>21–25,45</sup> and in a x However, no investigation on reversal of the EMT phenotype by has been ported so far. In the current study, we early provided evidence that ISOV represses migratory capbility and refersed the EMT phenotype in HCSLCs by sking Mn DD/FoxM1 signaling, subsequently downregu-1, Slug, ZEB1 and MMP-2 in vitro. Nevertheless, lating development of ISOV as a potential hepatoma chemopreventive and adjuvant chemotherapeutic agent warrants further investigation.

#### Conclusions

Collectively, our study demonstrated that repression of migration, invasion and EMT in HCSLCs by ISOV is due to MnSOD/FoxM1 signaling blockade, subsequently downregulating Twist1, Slug, ZEB1 and MMP-2. These findings suggest that ISOV may serve as a novel candidate for the treatment of HCC patients.

#### **Abbreviations**

ANOVA, analysis of variance; CSC-M, CSC-conditioned medium; CSLCs, cancer stem-like cells; EMT, epithelialmesenchymal transition; FoxM1,Forkhead box M1; HCC, hepatocellular carcinoma; HCSLCs, hepatocellular carcinoma stem-like cells; ISOV, isovitexin; MnSOD, manganese superoxide dismutase; SD, standard deviation.

### **Data Sharing Statement**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Ethics Approval and Informed Consent

The procedures were approved by the Ethics Committee of the Hunan Normal University.

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### **Author Contributions**

QYB and CXC performed and analyzed the experiments, wrote the paper. LLH, CXZ, YQ, LX, CYH, XC and ZC carried out the data collection, and data analysis. CJG and RKQ conceived and coordinated the study, designed, data analysis, revised the paper. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

### Disclosure

The authors declare that they have no conflicts of interest

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