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Diosgenin Suppresses Hepatocyte Growth Factor (HGF)-Induced Epithelial−Mesenchymal Transition by Down-regulation of Mdm2 and Vimentin

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ABSTRACT: Substantial activation of the hepatocyte growth factor (HGF)/c-Met pathway leads to cancer cell scattering and invasion and has been observed in several types of cancers, including prostate and colorectal cancers. The phosphorylation cascade downstream of HGF, particularly PI3K/Akt signaling, regulates epithelial-to-mesenchymal transition (EMT). How this signaling governs EMT and whether specific kinases respond to particular EMT effectors remain unclear. This study found specific increases in Mdm2 and vimentin rather than the coregulation of an array of EMT marker proteins in response to HGF-induced EMT in DU145 prostate cancer cells. Importantly, it was further found that diosgenin abrogated HGF-induced DU145 cell scattering and invasion. Moreover, diosgenin effectively inhibited the HGF-induced increases in Mdm2 and vimentin by down-regulating phosphorylated Akt and mTOR. In summary, the results suggest that diosgenin may be a potential compound for use in prostate cancer therapy to target the major HGF-induced EMT pathway.

KEYWORDS: diosgenin, epithelial−mesenchymal transition, c-Met, Akt, Mdm2

INTRODUCTION

The epithelial-to-mesenchymal transition (EMT) is a complex process occurring during cancer development and progression.[1,2] The crucial cellular changes have been identified, including cytoskeletal remodeling by a polarized phenotype, the disassembly of cell−cell junctions by relocalization of matrix adhesion, and changes in transcriptional regulation to increase cell mobility and invasion.[3,4] In addition to cancer progression and metastasis, EMT also contributes to the emergence of cancer stem cells.[5−7]

A number of molecules have served as epithelial or mesenchymal markers since the early 1980s.[3] Down-regulation of E-cadherin and up-regulation of vimentin are often associated with EMT. Recently, the tumor suppressor p53 was found to play an important role in the maintenance of epithelial characteristics. Wang et al. demonstrated that wild-type p53 induces Mdm2-mediated degradation of Slug, a transcriptional repressor of E-cadherin, and thus controls invasion in non-small-cell lung cancer.[8] However, down-regulation of E-cadherin by mutant p53 is independent of cell invasion in colon carcinoma cell lines.[9]

Activation of TGF-β or hepatocyte growth factor (HGF) signaling is believed to be a key event underlying EMT in various cancers.[7,10−12] A significant correlation between the overexpression of c-Met, a transmembrane receptor for HGF, and the histological grade of patients has been demonstrated, suggesting that HGF/c-Met is a strong prognostic factor.[10−16] In addition, several experiments performed in cellular models have demonstrated that overexpression of HGF or c-Met results in the constitutive activation of signaling pathways leading to invasion, metastasis, proliferation, and morphogenesis.[10,12,17,18] These results are consistent with observations in nude mouse models.[19−21] Indeed, substantial activation of the phosphorylation cascade by HGF has been observed in several types of cancer, including prostate cancer and colorectal cancer.[10] (−)Epigallocatechin-3-gallate (EGCG) and luteolin have been identified as inhibitors of HGF-induced activation of tumorigenesis by blocking c-Met phosphorylation and the downstream phosphorylation cascade, including suppressing the phosphorylation of Akt and mTOR.[22−24]

Given that current cancer therapy incurs a high cost and requires repeated treatments, significant emphasis has recently been placed on prevention by natural products. Phytochemicals with cancer chemopreventive activities, such as curcumin, EGCG, resveratrol, and diallyl sulfide, have been widely studied.[25] Among these phytochemicals, diosgenin, a steroid saponin found in the seed of *Trigonella foenum graecum* and the roots of *Dioscorea villosa*, has been shown to be cancer chemopreventive in an azoxymethane (AOM)-induced rodent colon cancer model.[25] Furthermore, diosgenin has demonstrated disruption of Ca^{2+} homeostasis,

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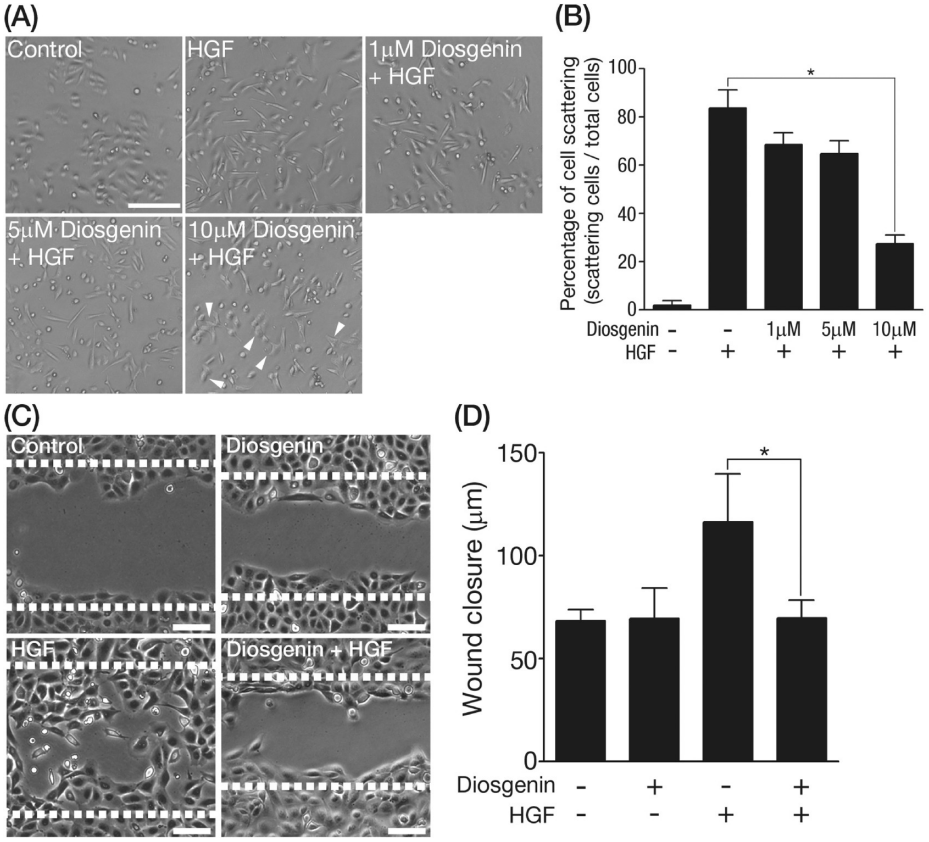

Figure 1. Diosgenin inhibits HGF-induced cell scattering and migration. (A) Du145 cells were pretreated with 100% ethanol or various concentrations of diosgenin for 1 h. Cell scattering was blocked by 10 μM diosgenin, as indicated by the arrowhead. (B) Statistical analysis of the cell scattering. When treated with 10 μM diosgenin, scattering was dramatically inhibited ($n = 3$; *, $p < 0.05$). (C) For the wound healing assay, confluent DU145 cells were pretreated with 100% ethanol or with 10 μM diosgenin for 8 h. Cells were then scratched with pipet tips and washed to remove the debris. Fresh medium containing 0.5% serum with 100% ethanol or 10 μM diosgenin was added. Cells were then incubated with 33 ng/mL HGF or PBS for 18 h. The dotted lines indicate the cell edges at the T_0 point. Representative pictures are shown. Scale bar = 50 μm. In the control, cells were treated with 100% ethanol and PBS. (D) HGF-induced cell motility was determined by measuring the closure of the wound in panel C. Data are plotted as means with SD. Diosgenin significantly inhibited HGF-induced cell motility ($n = 3$; *, $p < 0.05$).

inhibition of NF-κB, reduction of Akt and mTOR phosphorylation, and modulation of caspase-3 activity.[26,27] In addition, the diosgenin glycoside dioscin binds to the chemokine receptor CXCR3 and is involved in inflammation.[28] Because diosgenin can inhibit the phosphorylation of Akt and mTOR, we further examined its potential role in HGF/c-Met mediated cancer progression.[27] In this study, we present evidence that diosgenin inhibits HGF/c-Met signaling by blocking the downstream Akt and mTOR phosphorylation cascade and effectively abrogating the HGF/c-Met-induced cell scattering and invasion of DU145 prostate cancer cells. Interestingly, we also found the specific activation of Mdm2 and vimentin, rather than other EMT marker proteins, in HGF/c-Met-induced EMT in DU145 prostate cancer cells and dissected the specific signaling pathway upstream of Mdm2 and vimentin in prostate cancer cells.

MATERIALS AND METHODS

Cell Culture. DU145 prostate cancer cells were maintained in MEM (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen) and 1% penicillin−streptomycin (Invitrogen). Cells were maintained in a 37 °C incubator with 5% CO_2.

Reagents and Antibodies. Diosgenin, LY294002, and rapamycin were purchased from Sigma Chemical Co. (St. Louis, MO). Diosgenin was dissolved in 100% ethanol, whereas LY294002 and rapamycin were dissolved in DMSO. HGF was purchased from Invitrogen and dissolved in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4 at pH 7.4) with 0.1% BSA. Primary antibodies against phospho-c-Met (Y1234/35), phospho-Erk (T202/Y204), phospho-Akt (S473), phospho-mTOR (S2448), c-Met, Erk, Akt, mTOR, E-cadherin, p53, and α-tubulin were purchased from Cell Signaling Technology (Beverly, MA). Primary antibody against vimentin was purchased from Abcam Inc. (Cambridge, MA). Primary anti-Mdm2 antibody was purchased from BD Biosciences (Los Angeles, CA). The secondary antibodies, HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG, were obtained from Millipore (Billerica, MA).

Scattering Assay. DU145 cells (2×10^4) were seeded in each well of a 24-well plate and incubated overnight in a 37 °C incubator with 5% CO_2. DU145 cells were pretreated with diosgenin for 1 h at the indicated concentrations. HGF was added to each well at the final concentration of 33 ng/mL. Cells were then incubated at 37 °C for 18 h. Representative photographs were taken at 200× magnification using a Nikon TE2000-U inverted microscope.

Wound Healing Assay. The wound healing assay was performed as described previously.[29] To generate confluent cell monolayers, 4.5×10^5 DU145 cells per well of a 6-well plate were cultured overnight in

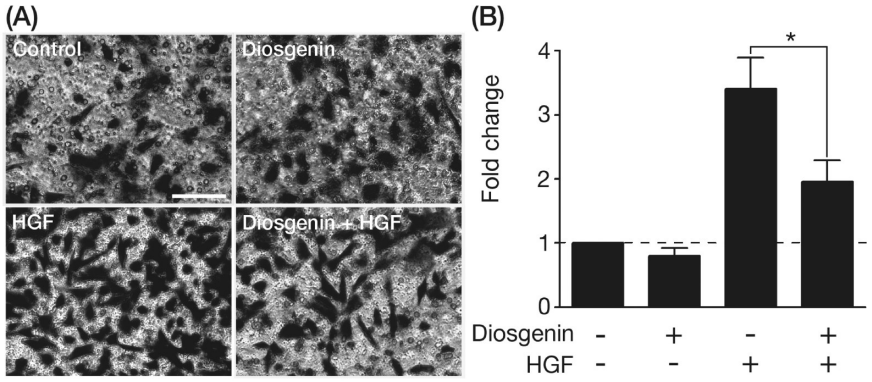

Figure 2. Diosgenin inhibits HGF-induced invasion in DU145 cells: (A) representative pictures (scale bar = 50 μm); (B) statistics of invading cells. After Matrigel coating, 2×10^4 cells were cultured in MEM containing 0.1% serum $\pm$ 10 μM diosgenin in 24-well transwell chambers. Serum-containing MEM (500 μL) $\pm$ 33 ng/mL HGF was plated in the lower chamber. The cells were incubated for 22 h. Invading cells were then fixed, stained with Toluidine Blue, and counted in five random fields. The experiment was repeated three times. *, $p < 0.05$. The diosgenin treatment reduced HGF-induced DU145 invasion.

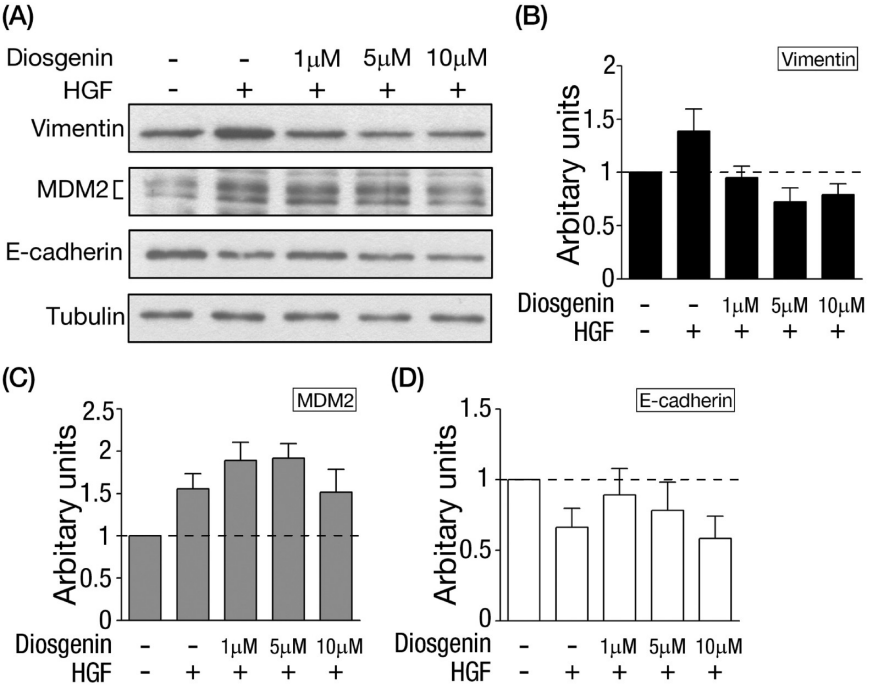

Figure 3. Diosgenin inhibits HGF-induced EMT of DU145 cells. (A) DU145 cells were pretreated with 1, 5, or 10 μM diosgenin for 1 h. The increased expression of the mesenchymal marker vimentin, which was induced by HGF, was down-regulated by diosgenin. The quantification of vimentin (B), MDM2 (C), and E-cadherin (D) is normalized to actin and shown in the lower panel. The value of the control cells was set to 1 (n = 3).

serum-containing MEM in a 37 °C incubator with 5% CO_2. The monolayers were pretreated with 10 μM diosgenin for 8 h before being scratched. The monolayers were scratched with a plastic tip and washed by PBS to remove cell debris. Next, 0.5% serum-containing MEM, 10 μM diosgenin, and 33 ng/mL HGF were added to each well, and the scratched monolayers were incubated in a 37 °C incubator with 5% CO_2 for 18 h. Wound closure was measured in 10 random fields at 200× magnification using Image-Pro Express software and a Nikon TE2000-U inverted microscope. Data from three independent experiments were analyzed by t test using GraphPad Prism 5 software. The distance between groups was considered to be statistically significant when $p < 0.05$.

In Vitro Invasion Assay. The Matrigel invasion assay was performed in 24-well Transwell culture plates. Briefly, 25 μL of BD Matrigel Basement Membrane Matrix (BD Biosciences) was resolved at 4 °C overnight and coated on the transwell insert membrane. The inserts were then incubated at 37 °C for 30 min to gel. After Matrigel coating, 2×10^4 DU145 cells in 100 μL of MEM with 0.1% serum and 10 μM diosgenin were added to the top chamber. Serum-containing MEM (500 μL) and 33 ng/mL HGF were plated in the bottom chamber. The cells were incubated in a 37 °C incubator with 5% CO_2 for 22 h. After incubation, the medium was aspirated and the noninvading cells were scrubbed with a wet cotton swab. The cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 15 min. Fixed cells were washed three times with PBS and stained with 0.5% Toluidine Blue O (Sigma-Aldrich, St. Louis, MO) in 2% Na_2CO_3 for 10 min. Excess stain was removed by three washes with distilled water. The invading cells were counted in five random fields at 400× magnification.

Three independent experiments were conducted, and the data were analyzed with a *t* test using GraphPad Prism 5 software. The distance between groups was considered to be statistically significant when $p < 0.05$.

Western Blot Analysis. DU145 cells (1.3×10^5) were seeded in each well of a 6-well plate and incubated overnight in a 37 °C incubator with 5% CO_2. For HGF-induced epithelial−mesenchymal transition studies, cells were were pretreated with diosgenin, LY294002, or rapamycin for 1 h with indicated concentrations. HGF was then added to a final concentration of 33 ng/mL, and cells were incubated at 37 °C for 18 h. To examine diosgenin's inhibition of HGF-induced Met signaling, cells were pretreated with or without 10 μM diosgenin for the indicated times and treated with 33 ng/mL HGF for 20 min. Cells were washed three times with PBS and lysed in gold lysis buffer (10% glycerol, 1% Triton X-100, 137 mM NaCl, 10 mM NaF, 1 mM EGTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris-HCl, pH 7.9, 100 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1% SDS, 10 μg/mL aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/mL leupeptin). Protein content was determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories). A total of 50 μg of protein was separated by SDS-PAGE and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing nonfat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS. The filter paper was incubated with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:2500 dilution; Millipore, Billerica, MA).

RESULTS

Diosgenin Blocks the HGF-Induced Motility of Prostate Cancer Cells. The activation of HGF/c-Met signaling caused significant polarization of cell shape in DU145 cells (Figure 1A). To examine whether diosgenin blocks HGF-induced polarization of DU145 cells, we pretreated cells with diosgenin for 8 h at the indicated concentrations, followed by HGF stimulation, and observed the cell morphology and motility. As shown in Figure 1A, diosgenin did not induce cell death under the conditions used in these experiments. The cell number, which is resistant to phenotypic change, slightly increased upon treatment with 10 μM diosgenin (Figure 1A,B, arrowheads). We further examined whether diosgenin also affected HGF-induced cell migration. Confluent DU145 cells were pretreated with 10 μM diosgenin for 8 h and scratched with pipet tips, washed to remove the debris, and treated with fresh medium containing 0.5% serum with 10 μM diosgenin. Cells were then incubated with 33 ng/mL HGF for 18 h. HGF-induced cell migration was determined by measuring the wound closure. Dotted lines indicate the edges at the t_0 time point (Figure 1C). There was no effect on cells treated with diosgenin alone (Figure 1C). Compared to cells treated with PBS (Figure 1C), HGF significantly induced cell migration (Figure 1C). Notably, the HGF-induced cell migration was abrogated in the presence of diosgenin (Figure 1C). The cell motility data obtained from Figure 1C are plotted as means with SD (Figure 1D).

Diosgenin Blocks HGF-Induced Invasion. A modified invasion assay was performed to further determine whether diosgenin was also capable of blocking HGF-induced invasion. DU145 cells were plated on top of Matrigel-coated 24-well transwell chambers. Cells were cultured in 0.1% serum-containing MEM, 33 ng/mL HGF, and 10 μM diosgenin for 22 h. Invading cells were then fixed, stained with Toluidine Blue, and counted in five random fields. Consistent with the results presented in Figure 1, treatment with diosgenin reduced DU145 invasion induced by HGF (Figure 2). The HGF treatment increased the number of invading cells by 3.5-fold compared to untreated controls or to cells treated with diosgenin alone. Treatment of cells with 10 μM diosgenin significantly decreased the amount of invasion induced by 2-fold compared to the control set.

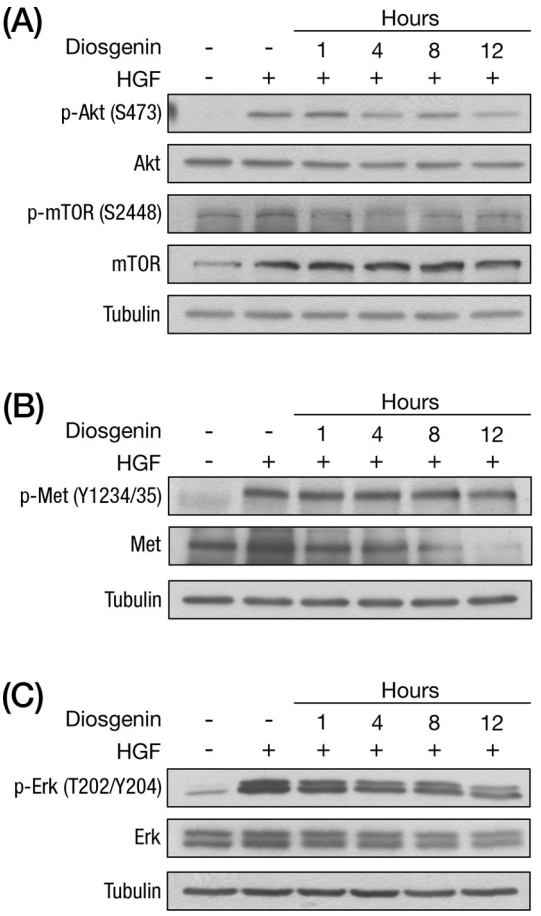

Figure 4. Diosgenin blocks HGF-induced Met signaling by down-regulating total Met and the phosphorylation of Akt and mTOR (S2448): (A) pretreatment with diosgenin blocked the phosphorylation of Akt and mTOR; (B) diosgenin down-regulated the expression of total c-Met; (C) diosgenin slightly decreased phospho- and total ERK after long-term pretreatment. DU145 cells were pretreated with 100% ethanol or 10 μM diosgenin for 1, 4, 8, or 12 h before HGF treatment for 20 min. Stimulation with HGF induced phosphorylation of Met (Y1234/35), ERK (T202/Y204), Akt (S473), and mTOR (S2448). HGF treatment also increased the amount of total mTOR.

Diosgenin Suppresses HGF-Induced EMT by Down-regulating Mdm2 and Vimentin, Instead of Up-regulating E-Cadherin, in DU145 Cells. To further clarify whether inhibiting HGF-induced scattering, migration, and invasion in diosgenin-treated DU145 cells resulted from dysregulation of EMT-related proteins, we examined the expression of the EMT marker proteins vimentin and E-cadherin and their upstream regulator Mdm2. As shown in Figure 3A, the expression levels of vimentin and Mdm2 were increased, whereas the expression of E-cadherin was decreased when cells were activated by HGF. Pretreatment with diosgenin for 1 h significantly suppressed the HGF-induced expression of vimentin and Mdm2 (Figure 3A). However,

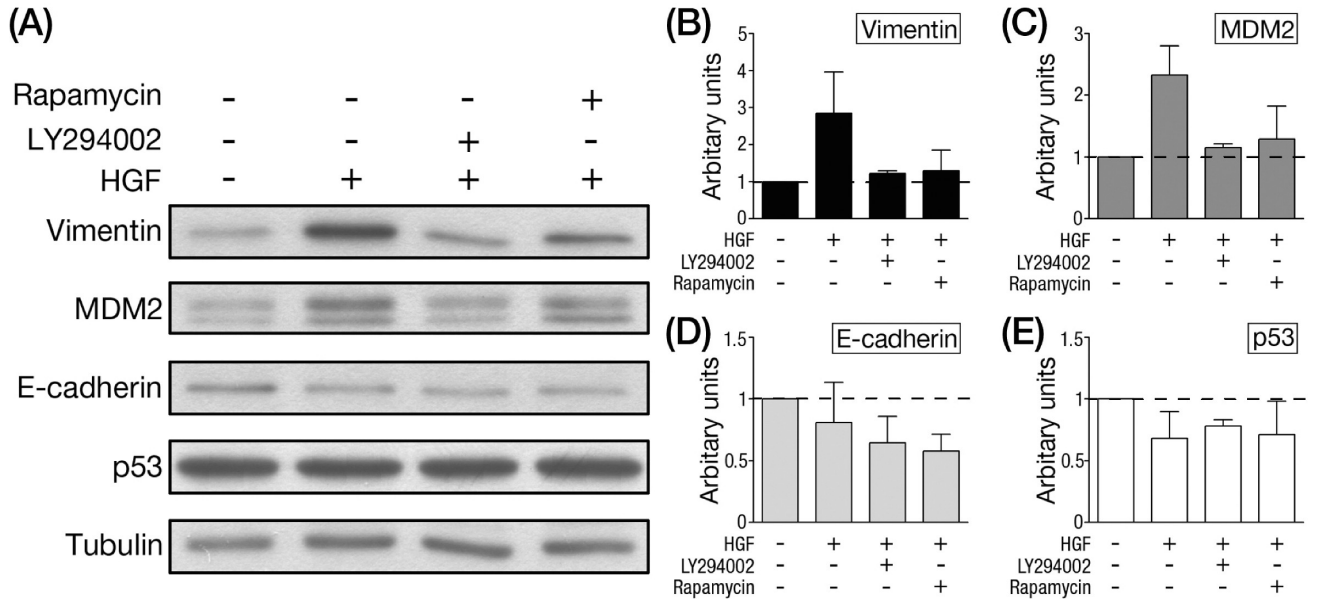

Figure 5. LY294002 and rapamycin inhibit HGF-induced EMT in DU145 cells. (A) Pretreatment with 10 μM LY294002 and 100 nM rapamycin for 1 h reduced the expression of vimentin and MDM2. (B−E) The quantified levels of vimentin, MDM2, p53, and E-cadherin were normalized to tubulin, and the value from the control cells was set to 1 (n = 3).

pretreatment with diosgenin did not affect the HGF-induced down-regulation of E-cadherin (Figure 3A). The quantification of vimentin, Mdm2, and E-cadherin is normalized to tubulin and shown in Figure 3B−D.

Diosgenin Inhibits HGF-Induced Phosphorylation of Akt and mTOR in DU145 Cells. Diosgenin can block the phosphorylation of Akt and mTOR in various cancer cell lines.[25,26] These two kinases can be activated by HGF stimulation. To assess whether diosgenin modulated the upstream signaling events induced by HGF/c-Met, DU145 cells were pretreated with 10 μM diosgenin for the indicated durations and stimulated with or without 33 ng/mL HGF. The protein lysates from individual treatment conditions were harvested and subjected to Western blot analysis, which revealed that 10 μM diosgenin effectively blocked HGF-induced phosphorylation of Akt and mTOR (Figure 4A). Given that diosgenin is a fatty acid synthase (FASN) inhibitor, we also tested whether it could inhibit c-Met phosphorylation as luteolin does.[23,27] With long-term pretreatment, diosgenin down-regulated the expression of total c-Met but did not significantly inhibit the phosphorylation of c-Met (Figure 4B). The RAF-MEK-ERK pathway is required for HGF-induced EMT.[11,12,30] We thus additionally examined whether diosgenin could inhibit the activation of ERK. Diosgenin slightly reduced the phosphorylation of ERK and total ERK under long-term pretreatment (Figure 4C). In summary, diosgenin blocks HGF-induced signaling via the specific repression of activation of Akt and mTOR (Figure 4).

Selective Inhibition of Akt or mTOR Phosphorylation Inhibits the HGF-Induced Increases in Mdm2 and Vimentin in DU145 Cells. To confirm whether the phosphorylation of AKT or mTOR is the major pathway leading to increased vimentin in cells activated by HGF, DU145 cells were pretreated with 10 μM LY294002 or 100 nM rapamycin for 1 h and then treated with HGF for 18 h. Similar to the results presented in Figure 3, pretreatment with LY294002 or rapamycin significantly reduced HGF-induced vimentin and Mdm2 expression but did not affect E-cadherin (Figure 5A). DU145 cells contain two p53 mutant alleles, p53P223L and p53 V274F.[31] The cooperative expression of both mutant p53 proteins in DU145 results in resistance to Fas-mediated apoptosis.[32] Given that p53 is a regulator of EMT,[8] we also investigated its role in the EMT of DU145 cells. Unlike other p53 mutants that down-regulate E-cadherin expression,[8,9] the p53 mutant proteins in DU145 did not affect the expression of E-cadherin. In addition, the expression of mutant p53 was not considerably affected when DU145 cells were pretreated with LY294002 or rapamycin, suggesting that HGF-induced EMT might be independent of mutant p53 activity (Figure 5A). The quantification of E-cadherin, vimentin, Mdm2, and p53 levels was normalized to that of tubulin, and the value of the control cells was set to 1 (Figure 5B−D).

DISCUSSION

Underlying tumorigenesis, various signaling pathways involved in proliferation, motility, migration, and invasion are activated, and the levels of EMT marker proteins show a positive correlation with progression in patients.[33,34] Robust activation of the HGF/c-Met pathway causes scattering, migration, and invasion of cells in several types of cancer.[10−16] Herein, we characterized and dissected a specific cascade in prostate cancer DU145 cells. HGF stimulated the phosphorylation of c-Met, Akt, ERK, and mTOR, followed by an increase in the levels of Mdm2 and vimentin (Figures 1−3). The suppression of Akt and mTOR phosphorylation significantly reduced the HGF-induced increases in Mdm2, vimentin, cell migration, and cell invasion (Figure 5). These results suggest that a specific signaling axis controls HGF-induced EMT, in which Mdm2 and vimentin are key effectors in DU145 cells. Significantly, we further found that diosgenin inhibited HGF-induced EMT by down-regulating phosphorylated Akt and mTOR. These findings suggest diosgenin is a potential natural therapeutic compound for prostate cancer.

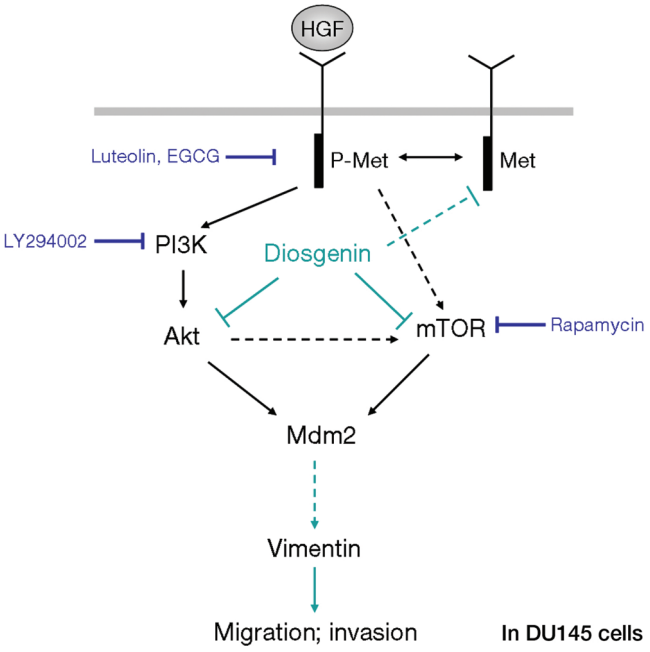

Figure 6. Working model for the mechanism of inhibition of HGF-induced EMT by diosgenin in DU145 cells.

The function of diosgenin in HGF-induced EMT of DU145 cells is summarized in Figure 6, illustrating the following working model: PI3K/Akt/Mdm2/vimentin is a central signaling pathway of HGF-induced EMT in DU145 cells and is suppressed by diosgenin. The PI3K/Akt pathway is indispensable for HGF-induced EMT.[10−12] At this moment, we can not distinguish whether diosgenin targets Akt directly. Isolation of diosgenin-binding cellular proteins and RNAi knock-down of the individual binding protein one-by-one may help to clarify this question. Recently, Zhu et al. showed that Akt1 regulates the motility and invasion of soft-tissue sarcoma by phosphorylating vimentin at Ser39.[35] Additionally, EMT-induced vimentin expression positively correlates with Axl, a receptor tyrosine kinase responsible for migration, in clinical samples and breast cancer cell lines.[36] These observations draw attention to EMT-related proteins, such as vimentin, as therapeutic targets.

Moreover, in contrast to other known compounds, such as EGCG or the flavonoid luteolin, which can inhibit HGF-induced migration and invasion, diosgenin down-regulated c-Met but did not affect phosphorylated c-Met. There are at least two possible mechanisms of diosgenin's function. One possibility is that diosgenin suppresses protein translation via mTOR. In support of this is our finding that diosgenin specifically down-regulated total c-Met protein, but not phosphorylated c-Met. The other possibility is that diosgenin specifically targets unphosphorylated c-Met via activation of proteins involved in a c-Met internalization mechanism, such as Cb1. Cb1 has been suggested to compete with other proteins with SH2 domains, such as PI3K.[37] Although the detailed mechanism remains to be characterized, down-regulation of total c-Met by diosgenin would, in turn, contribute to reduced HGF/EMT signaling and inhibit tumorigenesis.

Mani et al. demonstrated that EMT induced by TGF-β treatment or ectopic expression of E-cadherin repressors, such as Twist or Snail, can also confer stem cell-like properties to nontumorigenic, immortalized human mammary epithelial cells, providing the first link of EMT and "stemness".[5] Thus, inhibition of EMT (targeting EMT-associated factors) could be a strategy not only for reducing cancer metastasis but also for targeting drug resistance caused by cancer stem cells.[7] Indeed, Gupta et al. have reported that salinomycin significantly reduces the proportion of epithelial cancer stem cells and enhances epithelial gene expression, although the detailed mechanism remains elusive.[38] In addition, HGF can costimulate Wnt and Akt activity to modulate the features of colon cancer stem cells in a paracrine fashion, suggesting that HGF/c-Met signaling could be a therapeutic target.[39] In prostate cancer cells, inhibition of EMT by blocking the PI3K/Akt pathway or by miR-200 re-expression eliminates the "stemness" induced by sphere-forming conditions or overexpression of PDGF-D, respectively.[40,41] The potential application of diosgenin to cancer stem cells, particularly prostate cancer, is further supported by the findings reported here.

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