Simvastatin blocks TGF-β1-induced epithelial-mesenchymal transition in human prostate cancer cells

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Abstract. In recent years, the use of statins has been reported to be associated with a reduced risk of prostate cancer (PCa), particularly metastatic PCa. The mechanisms underlying these epidemiological observations are poorly understood. Epithelial-mesenchymal transition (EMT) is a critical initial step and a hallmark for cancer metastasis. In the present study, the relationship between simvastatin and EMT in PCa and the mechanism involved was investigated. It was demonstrated that simvastatin inhibited the EMT as assessed by reduced expression of N-cadherin and vimentin, and increased E-cadherin in TGF-β1 treated DU145 PCa cells. Furthermore, simvastatin inhibited TGF-β1-induced migration and invasion of DU145 cells. The TGF-β1/Smad pathway and non-Smad pathway were investigated in simvastatin-treated DU145 cells. Simvastatin had no effect on TGF-β1-induced phosphorylation of Smad2 and Smad3. In the non-Smad pathway, simvastatin reduced TGF-β1-induced p38 MAPK phosphorylation, but had no effect on TGF-β1-induced Erk1/2 phosphorylation. Simvastatin attenuated TGF-β1-induced EMT, cell migration and invasion in DU145 cells. These effects may have been mediated by the inhibition of p38 MAPK phosphorylation, not through the canonical Smad pathway. Therefore simvastatin may be a promising therapeutic agent for treating PCa.

Introduction

Prostate cancer (PCa) is the most common malignant cancer in men and the second leading cause of cancer in men in the western world (1). Patients usually respond initially to androgen deprivation therapy but eventually relapse and metastasize because of the development of castration resistant prostate cancer (CRPC).

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Statins are commonly prescribed cholesterol-lowering drug that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. In recent years, statins use has been reported to be associated with a reduced risk of PCa, particularly reducing the reduced risk of advanced or metastatic PCa (2). A previous study reviewing the clinical data of 1351 cases after radical prostatectomy showed less aggressive histological features in men taking statins (3). The mechanisms for these epidemiological observations are partially understood. *In vitro* experiments have demonstrated that simvastatin induces apoptosis in CRPC cells by inhibiting nuclear factor-κB pathway (4). It was reported that mevastatin and simvastatin can downregulate androgen receptor (AR) expression and prostate specific antigen (PSA) secretion, thus exhibiting an anti-proliferation effect on PCa cells (5). Brown *et al* (6) found that statins, including atorvastatin, mevastatin, simvastatin and rosuvastatin, reduced the migration and colony formation of PC-3 cells co-cultured with bone marrow stroma cells. Because of the high prevalence of PCa and lack of potent chemoprevention strategies, the application of statins on the prevention and treatment of PCa is promising, and would be beneficial to public health.

Epithelial-mesenchymal transition (EMT) is a highly conserved process that allows polarized, immobile epithelial cells to trans-differentiate to those with motile mesenchymal phenotypes. Accumulating evidence indicates that EMT is a critical initial step and a hallmark of cancer cell migration, invasion and metastasis (7). Transforming growth factor-β1 (TGF-β1) is widely recognized as an inducer and a chief regulator of EMT in a variety of cell types, including cancer cells (8). It was reported that overproduction of TGF-β1 was associated with high grade, metastasis and poor clinical outcome in PCa, and TGF-β1 may promote tumor progression by stimulating metastasis (9). At present, to the best of our knowledge, no data has been reported to demonstrate the relationship between simvastatin and EMT in PCa. The present study is the first to investigate the effects of simvastatin on the TGF-β1-induced EMT in PCa cells. DU145 PCa cells were treated with simvastatin, at non-apoptotic concentrations, and the expression levels of the epithelial markers E-cadherin and mesenchymal markers such as N-cadherin and vimentin were assessed. Wound-healing and transwell assays were used to investigate the effects of simvastatin on the motility and invasion of PCa cancer cells. Further studies investigated the effects of simvastatin on TGF-β1-induced EMT by assessing

the p38 Mitogen-Activated Protein Kinases (p38 MAPK) pathway, non-canonical Smad signaling. The present study aimed to determine whether simvastatin has potential as an anti-metastatic agent for PCa.

Materials and methods

Cell culture and reagents. Human DU145 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (ThermoFisher Scientific, Inc., Schuylerville, NY, USA), and 100 units/ml penicillin and 0.1 mg/ml streptomycin (Thermofisher Scientific, Inc.) at 37˚C in a humidified 5% CO_2 incubator. Simvastatin was obtained from Sigma-Aldrich (Santa Clara, CA, USA) and dissolved at a concentration of 50 μM in dimethyl sulfoxide (DMSO) (stored in aliquots at -20˚C). The maximum final concentration of DMSO was <0.1% for each treatment, and was also used as a control. Recombinant human TGF-β1 was purchased from PeproTech (Rocky Hill, CT, USA) and used at a final concentration of 5 ng/ml. The cells were incubated in serum-free medium overnight, and then treated with TGF-β1 for 48 h to induce EMT.

Cell viability assay. DU145 cells ($3x10^3$ cells/well) were seeded in 96-well plates and incubated overnight. After serum starvation for 24 h, the cells were treated with 5 ng/ml TGF-β1 and various concentrations of simvastatin for 48 h. Cell viability was measured using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Japan) according to the manufacturer's instructions. The absorbance was measured using a VARIOSCAN FLASH (ThermoFisher Scientific, Inc.).

Western blot analysis. DU145 cells were pre-treated with simvastatin (0, 5 or 10 μM) for 2 h followed by incubation with or without 5 ng/ml TGF-β1 for another 48 h. Total protein extracts were prepared using a Protein Extraction kit (KeyGEN BioTECH, Beijing, China). Protein samples were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, UK). Blots were blocked for 1 h with 5% nonfat milk in Tris-buffered saline/Tween (TBST, 0.05% Tween-20 in TBS), and then probed overnight at 4˚C with primary antibodies (Smad2/3 Antibody Sampler Kit; cat. no. 12747; dilution, 1:1,000). All primary antibodies in this study were from Cell Signaling Technologies, Inc., (Beverly, MA, USA). After incubation with HRP-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. 7074; dilution, 1:2,000; Cell Signaling Technologies Inc.) for 1 h at room temperature, the membranes were then developed using a chemiluminescence kit (Millipore) with G: BOX Chemi XL1. GENESys (Syngene, Cambridge, UK) and analyzed by Image J software 1.48 (imagej.nih.gov/).

Wound healing assay. DU145 cells were seeded at a density of $2x10^5$ cells/ml in 6-well plates and cultured to confluence. After serum starvation for 24 h, cell monolayers were scratched with a sterilized 200 ml pipette tip and washed 3 times with phosphate-buffered saline (PBS). Cells were further treated with 5 μM or 10 μM simvastatin and 5 ng/ml TGF-β1. The ability of cells to migrate into the cleared section was observed and images were captured using the IX73 inverted microscope system for advanced live cell imaging (Olympus Corporation, Tokyo, Japan) at 48 h.

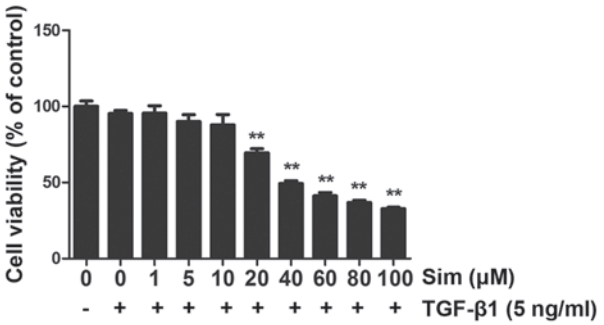

Figure 1. The effect of simvastatin on the viability of DU145 cells in the presence of TGF-β1. Cells were treated with 5 ng/ml TGF-β1 alone or in combination with indicated concentrations of simvastatin for 48 h. Cell viability was measured by CCK-8 assay. **P<0.01 vs. the TGF-β1 alone group.

Transwell invasion assay. After serum starvation for 24 h, DU145 cells were pre-treated with 5 ng/ml TGF-β1 and 5 μM or 10 μM simvastatin for 48 h. The filter was coated with 15 μl of matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The pretreated cells were trypsinized and resuspended in serum-free medium and seeded at a density of $1x10^4$ cells/well onto the top chamber. Next, 500 μl of culture medium containing 10% FBS was added to the bottom chamber. After incubation for 36 h, non-invading cells were carefully removed with a cotton swab. The filter membrane was fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 15 min and stained with 0.1% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) for 15 min. The migrated cells were counted in 5 randomly selected fields at 200x magnification using the IX73 inverted microscope (Olympus Corporation).

Statistical analysis. The mean values from the experiments were pooled for statistical analysis. One-way analysis of variance followed by a least significant difference test or Student's *t*-test was performed to determine the differences between groups. All statistical analysis was performed by the SPSS statistics 17.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Simvastatin blocks TGF-β1-induced EMT in DU145 cells. To investigate simvastatin on TGF-β1-induced EMT in DU145 cells, cell viability assay was performed to determine the cytotoxic effects of simvastatin. Figure 1 shows that simvastatin had a dose dependent cytotoxic effect on DU145 cells. The calculated IC_{50} value was 50.90 mM at 48 h treatment. After 48 h treatment, no significant differences were observed on cell viability at concentrations up to 10 μM in the presence of TGF-β1, so 5 and 10 μM simvastatin were used in the following experiments.

TGF-β1 has been widely recognized as an inducer of EMT in a variety of types of epithelial cell (8). In the present study, TGF-β1 stimulation was used to induce the occurrence

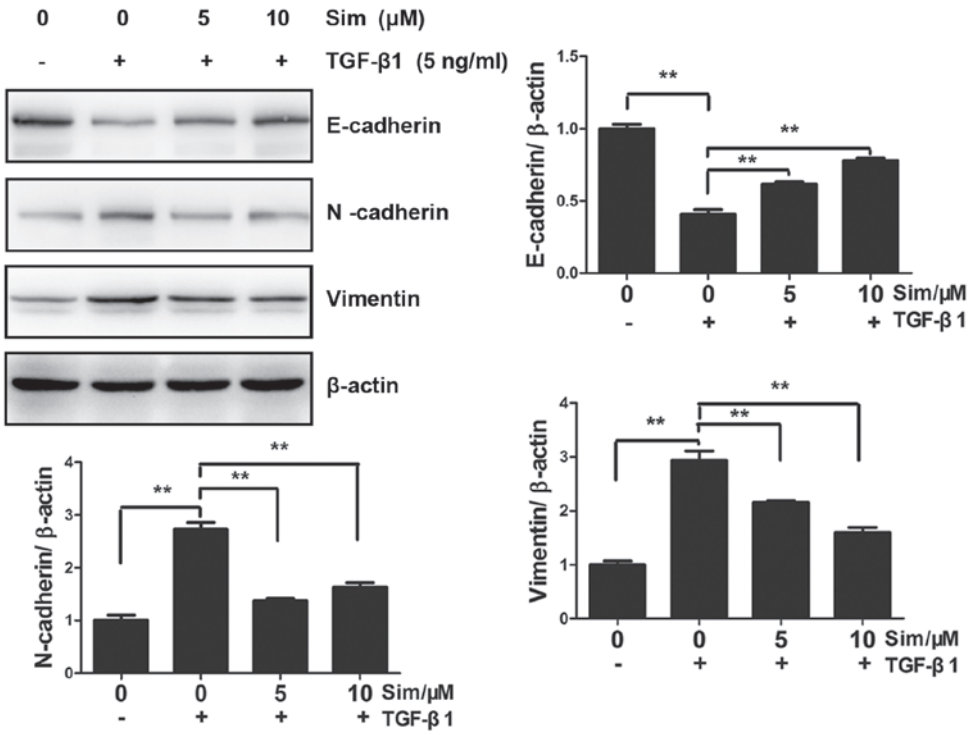

Figure 2. Simvastatin inhibits TGF-β1 induced-EMT. DU145 cells were incubated with indicated concentrations of simvastatin for 2 h, followed by treatment with TGF-β1 for 48 h. Relative expression of E-cadherin, N-candherin and vimentin were analyzed by Western blot analysis. β-actin was used as an internal loading control. The quantitative ratios are shown as relative optical densities of bands that are normalized to the expression of β-actin. Data shown are representative of 3 independent experiments (**P<0.01 vs. the TGF-β1 alone group).

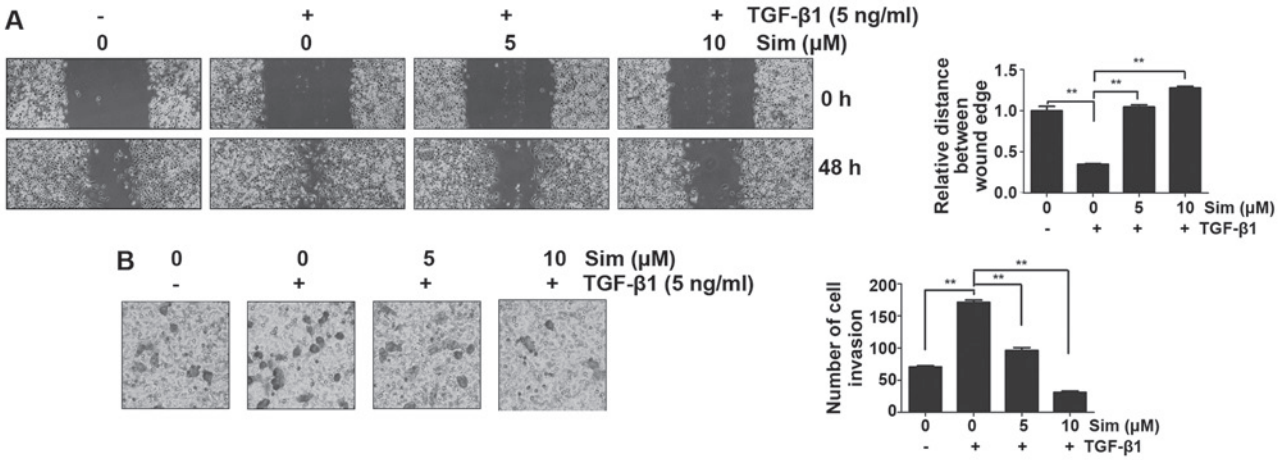

Figure 3. Simvastatin inhibits TGF-β1-induced migration and invasion. (A) The effect of simvastatin on the TGF-β1-induced migration of DU145 cells was evaluated using would-healing assay. Values of relative distance between wound edges represent mean ± standard deviation of triplicate samples (**P<0.01). (B) The effect of simvastatin on the TGF-β1-induced invasion of DU145 cells was evaluated using a matrigel invasion assay. Migrated cells were stained with crystal violet and counted for quantitative analysis. Data shown are representative of 3 independent experiments (**P<0.01 vs. the TGF-β1 alone group).

of EMT in DU145 cells, to identify whether TGF-β1-induced EMT is suppressed by simvastatin. The effect of simvastatin on TGF-β1-induced EMT was evaluated by checking the well-established markers using western blotting. As shown in Fig. 2, DU145 cells treated with 5 ng/ml TGF-β1 for 48 h led to significant reduction in the epithelial phenotype marker E-cadherin and an increase in the mesenchymal phenotype markers N-cadherin and Vimentin. However, when combined with simvastatin (5 or 10 μM), expression of E-cadherin was significantly increased, and the expression levels of N-cadherin and vimentin were significantly reduced compared with the TGF-β1 alone group (P<0.01). These findings indicate that simvastatin inhibited the effects of TGF-β1-induced EMT in DU145 cells.

Simvastatin inhibits TGF-β1-induced migration and invasion. To explore the potential role of simvastatin in prostate cancer invasion and metastasis by inhibiting TGF-β1-induced EMT, the effects of simvastatin on cellular migration and invasion were evaluated using a wound-healing assay and

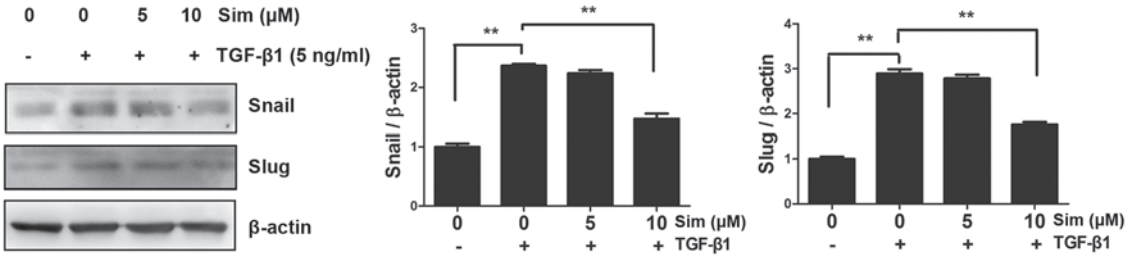

Figure 4. Simvastatin inhibits TGF-β1-induced EMT- transcriptional factors. DU145 cells were incubated with indicated concentrations of simvastatin for 2 h, followed by treatment with TGF-β1 for 48 h. Relative expression of snail and slug were analyzed by Western blot analysis. β-actin was used as an internal loading control. Data shown are representative of 3 independent experiments (**P<0.01 vs. the TGF-β1 alone group).

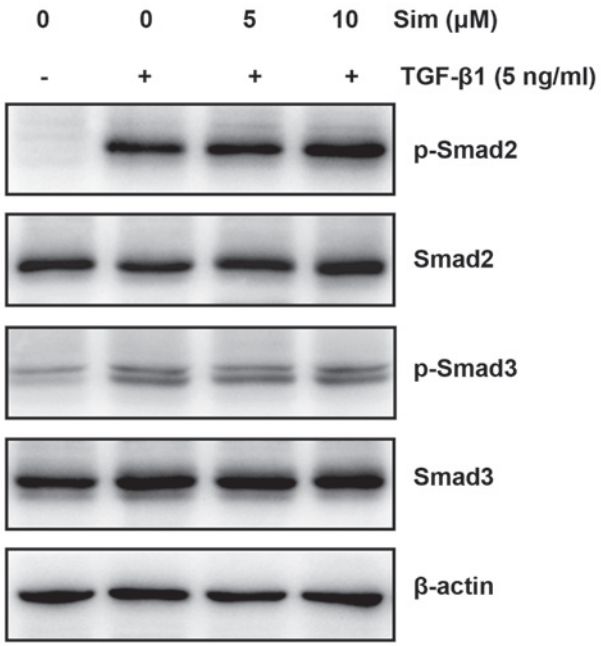

Figure 5. Simvastatin has no effect on TGF-β1/Smad signaling. DU145 cells were incubated with indicated concentrations of simvastatin for 2 h, followed by treatment with TGF-β1 for 48 h. Relative expression of p-Smad2, Smad2, p-Smad3 and Smad3 were analyzed by Western blot analysis. β-actin was used as an internal loading control. Data shown are representative of 3 independent experiments.

a matrigel invasion assay *in vitro*, respectively. The wound healing assay revealed that TGF-β1 treatment stimulated DU145 cells to migrate and close the wound (P<0.01 versus no TGF-β1 control), while co-incubation with simvastatin (5 or 10 μM) distinctly reversed TGF-β1-mediated migration (Fig. 3A; P<0.01 versus TGF-β1 alone). The invasion assay demonstrated that TGF-β1 treatment markedly enhanced the ability of DU145 cells to cross the basement membrane matrix (P<0.01 versus no TGF-β1 control). In the matrigel invasion assay, simvastatin (5 or 10 μM) significantly inhibited the TGF-β1-induced invasion of DU145 cells across the gelatin-coated membrane (Fig. 3B; P<0.01 versus TGF-β1 alone). These results indicate that simvastatin is an effective inhibitor of cell migration and invasion in TGF-β1-induced DU145 cells.

Simvastatin inhibits TGF-β1-induced EMT-transcriptional factors. To determine whether simvastatin inhibits Snail and Slug induction via TGF-β1, the expression levels of Snail and Slug were measured using Western blotting. TGF-β1 treatment for 48 h led to significant increase in the expression of Snail and Slug (P<0.01). However, Snail and Slug were significantly inhibited by 10 μM simvastatin compared with TGF-β1 alone group cells (P<0.01) (Fig. 4). Simvastatin reduced TGF-β1-induced Snail and Slug levels at the concentration of 10 μM. These results show that TGF-β1-induced expression of EMT inducting transcription factors, Snail and Slug, were inhibited by 10 μM simvastatin.

Simvastatin has no effect on TGF-β1/Smads signaling. Because simvastatin could inhibit the TGF-β1-induced EMT in DU145 cells, the effects of simvastatin on Smad2 and Smad3, two important mediators of the TGF-β/Smads signaling, were studied to investigate whether simvastatin could modulate the canonical TGF-β/Smads pathway. The levels of phosphorylated and total Smad2, as well as phosphorylated and total Smad3, were detected using western blotting. As is shown in Fig. 5, TGF-β1 triggers phosphorylation of Smad2 and Smad3, while simvastatin has no effects on TGF-β1-induced phosphorylation of Smad2 and Smad3, even at 10 μM. These data indicated that the inhibition of TGF-β1-induced EMT by simvastatin is not through the TGF-β/Smad signaling pathway.

Simvastatin attenuates TGF-β1-induced p38 phosphorylation in DU145 cells. Besides canonical Smads signaling, TGF-β1 can mediate the EMT process independently of Smads. The mitogen-activated protein kinase (MAPK) signaling pathway is induced by TGF-β1, and can modulate the outcome of TGF-β1-induced responses (10). The above results indicated that simvastatin had no effect on TGF-β1/Smad signaling, so the influence of simvastatin on MAPK signaling, including p38 MAPK and Erk1/2, was next investigtaed. As shown in Fig. 6, TGF-β1 induced the phosphorylation of p38 MAPK and Erk1/2 protein in DU145 cells, and this TGF-β1-induced p38 MAPK phosphorylation was strongly inhibited by the addition of 5 or 10 μM simvastatin, whereas no major difference in Erk1/2 expression was demonstrated (Fig. 6). These data indicated that simvastatin may inhibit TGFb1-induced EMT through the p38 MAPK pathway, not through TGF-β1/Smad signaling.

Discussion

EMT comprises a conversion in cell phenotype and serves crucial roles in the development of multiple organs and in tissue repair. It also adversely contributes to organ fibrosis and carcinoma invasion and metastasis (7). In epithelial cancers, EMT

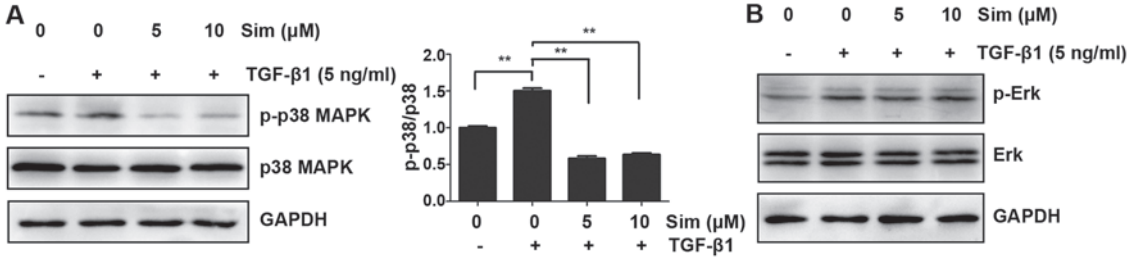

Figure 6. Simvastatin attenuates TGF-β1-induced p38 MAPK activation. DU145 cells were incubated with indicated concentrations of simvastatin for 2 h, followed by treatment with TGF-β1 for 48 h. Relative expression of (A) p-p38 and p38 MAPK and (B) p-ERK and Erk were analyzed by Western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. Data shown are representative of 3 independent experiments (**P<0.01 vs. the TGF-β1 alone group).

is characterized by a switch in cell membrane cadherins (from E-cadherin to N-cadherin) and a shift from apical-basal to front-back end polarity. During EMT, tumor cells lose cell-cell adhesion and acquire motility and invasion. These tumor cells that have gone through EMT are thought to be responsible for seeding distant dissemination from primary tumors (11). EMT is orchestrated by a set of pleiotropically acting transcription factors, such as Snail (12), Slug (13), Zeb1 (14) and Twist (15), which can directly repress mediators of epithelial adhesion, including the hallmark of EMT: E-cadherin.

TGF-β signaling has been implicated as major inducer of EMT (16). High production and activity of TGF-β1 are associated with highly aggressive cancer and poorly prognostic patients (17). In tumor cell lines, TGF-β1 signaling has been reported to induce EMT via up-regulating EMT-TFs, such as Snail and Slug, thereby allowing cancer cells to become more motile and invasive (18,19). In mouse tumor models, activation of the TGF-β1 pathway has been shown to induce EMT and promote cells to spread to distant organs in mice (20,21).

Statins, which can inhibit HMG-CoA reductase, are frequently prescribed medications for treating high cholesterol. In addition to their cholesterol-lowing effect, epidemiological evidence suggests that statins may be associated with a lower risk of aggressive prostate cancer (2). Therefore, it was hypothesized that statins may have anti-metastasis effect in PCa. Recently, statins has been shown to attenuate TGF-β1-induced EMT in EMT-related diseases, such as lung fibrosis (22), renal fibrosis (23), atherosclerotic renal artery stenosis (24), and postoperative complications associated with EMT of lens epithelial cells (25). These findings suggested that statins may be a promising therapeutic strategy to treat EMT-related disorders, including cancer metastasis. The present study focused on exploring the effect of simvastatin on TGF-β1-induced EMT in prostate cancer cells. It was demonstrated that simvastatin inhibited TGF-β1-induced EMT in DU145 cells at a concentration of 5 and 10 μM, and suppressed cell migration and invasion, although at these concentrations it exerted no influence on cell proliferation. These data indicate that simvastatin could be an effective antagonizer on metastasis.

A previous study reported that stimulation of TGF-β1 could induce EMT in prostate cancer (26). In the present study, DU145 cells treated with 5 ng/ml TGF-β1 for 48 h exhibited reduced levels of E-cadherin and increased expression of N-cadherin and vimentin. Thus, an EMT model in DU145 cells to study the therapeutic effect of simvastatin on this pathological process was successfully established. Simvastatin pretreatment prior to TGF-β1 stimulation in DU145 cells inhibited TGF-β1-induced EMT characteristics, the loss of E-cadherin and the increase of N-cadherin and vimentin. Also, simvastatin inhibited the TGF-β1-induced migration and invasion of DU145 cells (Fig. 3).

EMT programs are orchestrated by a set of pleiotropically transcription factors, including Snail and Slug, which suppress expression of epithelial markers, induce expression of mesenchymal markers, and promote the dissociation of cell adhesion, thereby allowing the migration of cancer cells. After treatment for 48 h, TGF-β1 induced Snail and slug expression in DU145 cells (Fig. 4). The Fig. 4 also showed that expression of TGF-β1-induced upregulation of Snail and Slug, were inhibited by 10 μM simvastatin. Both Snail and Slug belong to the super-family of zinc finger transcription factors, which can bind proximal promoter sequences of E-cadherin, namely E-box elements, thus repressing its transcription (27). So the present study next sought to elucidate the upstream regulatory mechanisms underlying the alteration in Snail and Slug expression.

TGF-β1 induces EMT via two specific pathways, the canonical Smad signaling pathway and a non-Smad signaling pathway (28). Binding of TGF-β1 to heterotetrameric complexes of type I (TβRI) and type II TGF-β receptors (TβRII) leads to the phosphorylation/activation of the TβRI. With the assistance of adapter proteins, TβRI binds and phosphorylates the Smad2 and/or Smad3. The phosphorylated Smad2 or Smad3 will then associate with Smad4, translocate into the nucleus, and activate target gene expression through interaction with other transcription factors, such as snail and slug (29). Yang *et al* (22) reported that simvastatin attenuated TGF-β1 induced EMT in human alveolar epithelial cells associated with modulation of TGF-β1-Smad2/3 pathway. Other studies have also reported simvastatin inhibited TGF-β1 induced Smad2/3 phosphorylation during intestinal fibroblast (30) and myofibroblast differentiation in nasal polyp-derived fibroblasts (31), in which EMT also plays an important role. The present study focused on the influence of simvastatin on TGF-β1 mediated Smad2/3 signaling and found that simvastatin did not affect TGF-β1 induced phosphorylation of Smad2 and Smad3. Notably, the results suggested that simvastatin abrogated TGF-β1-induced EMT and cell migration and invasion in DU145 cells not through canonical TGF-β1-Smads signaling.

TGF-β1 can also transduct independently of Smads. TGF-β1 induces the MAPK signaling pathway and can modulate the outcome of TGF-β1-induced responses (10). In particular, p38 MAPK has been shown to mediate Smad-independent TGF-β1 responses (32). To further investigate the underlying mechanism of simvastatin inhibition on TGF-β1 induced EMT, p38 and Erk1/2 MAPK signaling were investigated. It was demonstrated that TGF-β1 induced phosphorylation of both p38 and Erk1/2 (Fig. 6) after 48 h of incubation. The activation of p38 was significantly reduced in the presence of 5 and 10 μM simvastatin, whereas Erk1/2 phosphorylation was unaffected by simvastatin, even at 10 μM. These data suggested that p38 MAPK signaling involved in action of simvastatin inhibiting the TGF-β1 induced EMT in DU 145 cells. A similar mechanism of statins attenuating EMT via inhibition of p38 MAPK activation was also reported in human tendon fibroblast cells (33). These data clearly demonstrate that the specific mechanism involved in the effect of statins on TGF-β1 induced EMT depends on the cancer cell type.

Overproduction of TGF-β1 is associated with poor clinical outcome in prostate cancer, and TGF-β1 may promote tumor progression by stimulating metastasis (9). Previous studies have demonstrated that statins reduced advanced PCa risk but not overall PCa risk (2,34,35). The present study is the first to show the inhibitory effect of simvastatin on TGF-β1 induced EMT and in TGF-β1-induced DU145 cells. This indicates that simvastatin attenuated TGF-β1 induced EMT associated with modulation of P38 MAPK signaling, not through the canonical Smad-dependent pathway. These data could to some degree explain why statins exhibit a protective effect in PCa and reduce advanced PCa risk.

In summary, the present study demonstrated that simvastatin was able to suppress PCa cell migration and invasion *in vitro* by inhibiting TGF-β1 induced EMT. These effects may have been mediated by the inhibition of p38 MAPK phosphorylation. These findings provide a novel insight into the actions of simvastatin as an inhibitor of EMT and cancer metastasis in PCa.

Acknowledgements

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