Abstract. Silibinin, a naturally occurring flavanone isolated from milk thistle extract, has been shown to possess strong anticancer efficacy against both androgen-dependent and androgen-independent prostate cancer, wherein it inhibits not only cell growth, but also cell invasion and metastasis. Inhibitory effects of silibinin on prostate cancer invasion, motility and migration were previously observed in the highly bone metastatic ARCaPM cell line; however, mechanisms of such efficacy are not completely elucidated. The epithelial-to-mesenchymal transition (EMT) is a crucial step in the progression of prostate cancer, reversal or inhibition of EMT by drugs thus provides a new approach to prostate cancer therapy. In the present study, we found that silibinin treatment resulted in the up-regulation of cytokeratin-18 and down-regulation of vimentin and MMP2, which was consistent with morphologic reversal of EMT phenotype leading to epithelial. Moreover, we found that silibinin could inhibit the nuclear factor κB (NF-κB) p50 translocation via the up-regulation of IκB protein, and possibly subsequently down-regulated the expression of two major EMT regulators, ZEB1 and SLUG transcription factors. Overall these findings demonstrate silibinin was able to reverse EMT to suppress the invasive property of metastatic prostate cancer cells at the transcriptional level.

Introduction

Bone metastatic disease is the primary cause of death for most prostate cancer patients (1). Recently, accumulating evidence suggests that epithelial-to-mesenchymal transition (EMT), an essential process during embryogenesis, plays a crucial role in the initial step of cancer metastasis. During EMT, cells lose epithelial polarity and acquire a spindle-shaped, highly motile fibroblastoid phenotype. These transitions endow cancer cells with more invasive and metastatic properties (2-4). Therefore, attenuation or reversal of EMT allows the development of novel drug-based strategies for the prevention and therapy of prostate cancer metastasis (5,6).

Silibinin, the active constituent of silymarin isolated from the dried fruits of milk thistle (Silybum marianum) plant, possesses pleiotropic anticancer capabilities in different cancer cells, including prostate cancer (7-10). Silibinin possesses strong anticancer efficacy against both androgen-dependent and androgen-independent prostate cancer, wherein it inhibits not only cell growth, but also cell invasion and metastasis (11-14). Singh et al have reported that silibinin treatment of TRAMP mice bearing prostate tumor inhibited tumor growth, progression, local invasion, and distant metastasis involving suppression of tumor angiogenesis and EMT (14). However, the regulational mechanisms by which silibinin suppresses EMT of prostate cancer as an anti-metastatic agent, are still largely unknown.

EMT is a dynamic process and triggered by many factors, such as Wnt, transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and insulin-like growth factor (IGF) (15-17). These signaling pathways leading to EMT are regulated through the modulation of multiple transcription factors. The nuclear factor κB (NF-κB) family of transcription factors has been identified as a central mediator of EMT, and plays pivotal roles in both promoting and maintaining an invasive phenotype (18,19). In a combined
in vitro/in vivo model of mammary carcinogenesis, both Ras- and TGF-β-dependent effects on EMT-specific genes are mediated via NF-κB activity (20). EMT is also directly regulated by a family of zinc finger master regulatory proteins, including SNAIL (SNAIL1 and SLUG), ZEB (ZEB1 and ZEB2) and TWIST1 (21-24). Many studies have emphasized the major roles of signaling pathways leading to the transcriptional repression of epithelial phenotype by these factors, but different repressors may participate in different types of tumors (25). Overall, given the vital roles of NF-κB and zinc finger proteins in the regulation of EMT, NF-κB and these zinc finger proteins have been identified as the potential targets in prevention and treatment of metastatic carcinomas.

Utilizing the unique highly bone metastatic ARCaP M cell model with a spindle-shaped mesenchymal phenotype, we have previously demonstrated that silibinin could inhibit the invasion, motility and migration of prostate cancer cells in vitro (26). The ARCaP M cells are derived from ARCaP E cells which undergo EMT by exposing to soluble factors such as TGF-B1 plus EGF, IGF-1, β2-microglobulin or a bone microenvironment. Comparison to the epithelia-like ARCaP E cells, ARCaP M cells express mesenchymal biomarkers, and gain increased incidence of bone metastasis (27-29). Therefore, silibinin that could inhibit the invasive potential of ARCaP M cells appears to a promising therapeutic agent for treatment of prostate cancer bone metastasis. Herein, we further investigated whether this anti-metastatic efficacy of silibinin on ARCaP M cells may be associated with reversal of EMT, and elucidated the possible roles of NF-κB and the specific zinc finger proteins in the regulation of EMT in ARCaP M cells.

Materials and methods

Cell culture and silibinin treatment. Human prostate cancer cell lines ARCaP M, ARCaP E, LNCaP and DU145 were generously donated by Dr Leland W.K. Chung (Cedars-Sinai Medical Center, Los Angeles, CA, USA) and were cultured in RPMI-1640 medium (Gibco, San Diego, CA, USA) supplemented with 10% (v/v) fetal bovine serum and 100 U/ml penicillin-100 μg/ml streptomycin at 37˚C with 5% CO2 in a humidified incubator. Silibinin was obtained from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). For silibinin treatment, stock solution (0.05 M in DMSO) was added into culture medium to achieve appropriate concentration (100 μmol/l) as described (26), and then incubated with cells for indicated time periods (24-72 h), whereas DMSO solution without silibinin was used as control.

Preparation of total, cytosolic and nuclear cellular extracts. Following the desired treatments, total cellular extracts were prepared as previously described (26). Briefly, cells were scraped from the plates in ice-cold RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate] containing protease inhibitors, 1% cocktail and 1 mM PMSF (Sigma) for 10 min, cell lysate was then subjected to a centrifugation of 14,000 rpm for 10 min at 4˚C. The resulting supernatant was collected as total cellular extract.

Preparation of cytosolic and nuclear extracts was carried out as described previously with modifications as follows (30). Briefly, cells were scraped from the plates in ice-cold buffer A [10 mmol/l HEPES (pH 7.9), 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.2% NP40, 1 mmol/l DTT, 0.1 mmol/l PMSF and 1% cocktail] and left on ice for 10 min. After vigorous vortex for 10 sec, homogenates were centrifuged at 14,000 rpm for 10 min. The resulting supernatant was collected as cytosolic extract. The pellet was then resuspended in buffer B [20 mmol/l HEPES (pH 7.4), 420 mmol/l NaCl, 0.1 mmol/l EDTA, 1.5 mmol/l MgCl2, 25% glycerol, 1 mmol/l DTT, 0.1 mmol/l PMSF and 1% cocktail], vigorously rocked at 4˚C for 20 min, and centrifuged for 3 min at 14,000 rpm. The resulting supernatant was collected as nuclear extract. All the samples were stored at -80˚C, and their concentrations were determined by Bradford assay.

Western blotting. For immunoblotting, 20-50 μg of protein lysate per sample was denatured in 5X sample buffer and subjected to SDS-PAGE on 10 or 12% Tris-glycine gels. The separated proteins were transferred onto nitrocellulose membranes followed by blocking with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST, pH 7.6) for 1 h at room temperature. Membranes were incubated with the desired primary antibody overnight at 4˚C. Antibodies for Western blotting were: mouse anti-cytokeratin18 (sc-6259, Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 1:1000 dilutions, mouse anti-vimentin (sc-6260, Santa Cruz Biotechnology) with 1:2000 dilutions, rabbit anti-IκB-α (bs-0412R, Bioss, Beijing, China) with 1:1000 dilutions, rabbit anti-IκB (sc-371, Santa Cruz Biotechnology) with 1:500 dilutions, rabbit anti-p50 (sc-114, Santa Cruz Biotechnology) with 1:500 dilutions, and Histone H1 antibodies. The mean density for each band was determined using Glyko BandScan software (Glyko, Novato, CA, USA).

Immunofluorescence staining. ARCaP M cells were grown on glass coverslips and treated according to the followed experimental procedures for 48 h. The cells were washed with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were then incubated in 10% normal goat serum and PBS containing 0.25% Triton X-100 for 30 min at room temperature to block nonspecific protein-protein interactions and permeabilize the cell membranes. After washing, the cells were then incubated with primary antibody in 1:100 dilutions in PBS containing 10% bovine serum for overnight at 4˚C. After thoroughly washing the primary antibody, the cells were stained with FITC conjugated anti-Rabbit IgG in 1:100 dilutions for 30 min at room temperature. The coverslips were examined and photomicrographs were taken under Olympus IX-50 fluorescence inverted microscope (Olympus, Tokyo, Japan).
Semiquantitative RT-PCR. Total RNA was extracted and purified from ARCaPM cells treated with 100 μmol/l silibinin for different time periods using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantitated by absorbance at 260 nm. cDNA synthesis was performed using RevertAid™ kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. All PCR analyses were subsequently performed using the gene-specific primers, and the primer sequences are listed as follows (Table I): F, forward primer; R, reverse primer. Cycling conditions utilized were: 94˚C, 5 min, 30-35 cycles of 94˚C, 30 sec; 58˚C, 30 sec; 72˚C, 45 sec. Reactions were finished with 72˚C, 7 min extension. Each measurement was performed in triplicate. PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The mean density for each band was also quantitated using Glyko BandScan software.

Transwell cell invasion assay. Cell invasion was tested using a transwell chamber (Corning, NY, USA) with an 8-μm

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID</th>
<th>Primer sequence</th>
<th>Position</th>
<th>Product (bp)</th>
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<td>NM_20613</td>
<td>F: 5'-GCCTTCAACTGCAAATACTGC-3'</td>
<td>527-775</td>
<td>249</td>
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<td></td>
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<tr>
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<td>178-361</td>
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<td>R: 5'-TGGAATTGAGCAGCGGTAGTC-3'</td>
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</tr>
<tr>
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<td>770-1024</td>
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<td>448-765</td>
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<td></td>
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Figure 1. ARCaPM cells represent a spindle-shaped mesenchymal phenotype compared with two other prostate cancer cells. (A) Western blot analysis showed the expression of markers of epithelial and mesenchymal phenotypes in the ARCaPM, ARCaPE, and LNCaP cells. (B) Representative morphology of ARCaPM cells and the original ARCaPE cells is shown. (C) Transwell cell invasion assay was used to quantify the relative invasive properties of ARCaPM, ARCaPE, and LNCaP cells for 48 h. *p<0.05 versus ARCaPM cells.
pore size polycarbonate membrane filter coated with 50 μl of 2 mg/ml Matrigel (Sigma). Cells were suspended in 100 μl of serum-free medium with 5x10^4 cells/well and added into the upper chamber for 48 h. RPMI-1640 medium (1 ml) containing 20% FBS was added to the lower chamber as a chemoattractant. For silibinin treatment, ARCaPM cells treated with 100 μmol/l silibinin for different time periods were harvested and seeded with 5x10^3 cells/well. Cells invading through Matrigel were fixed by 4% paraformaldehyde and stained with Giemsa, and then counted under the microscope at x100 magnification in three random fields.

**Statistical analysis.** All assays were repeated in triplicate in three independent experiments. All data were expressed as a means ± SEM. Analysis of variance (ANOVA) for multiple comparisons among each group was used as noted. In all
cases, \( p < 0.05 \) was considered significant. Statistical analyses were performed using the SPSS software (Chicago, IL, USA).

**Results**

**ARCaP_M cells exhibit a spindle-shaped mesenchymal phenotype with more invasive property**. To confirm that the ARCaP_M cells underwent EMT as reported previously, we compared the expression of markers of epithelial and mesenchymal phenotype in ARCaP_M and ARCaPE cells. We found that the ARCaP_M cells expressed relatively reduced levels of cytokeratin-18 (epithelial marker) and elevated levels of vimentin and MMP2 (mesenchymal markers) compared with the original ARCaPE cells (Fig. 1A), and the ARCaP_M cells expressed active cleaved MMP2 (63 kDa) while the ARCaPE cells did not. These results were consistent with their morphology and relative invasive activities (Fig. 1B and C). The ARCaP_M cells displayed elongated fibroblastoid morphology and more invasive potential. In contrast, the ARCaPE cells showed a rounded shape, typical of an epithelial cobblestone appearance, and they grew in clusters. Herein, LNCaP cells, another type of prostate cancer cell with epithelial phenotype, were also determined as control (31). The expression of cytokeratin-18 and lower invasive property were shown in LNCaP cells; however, vimentin and MMP2 were rarely expressed (Fig. 1A and C).

**Silibinin inhibits the invasion of ARCaP_M cells via reversal of EMT, and regulates the EMT phenotype in DU-145 cells**. We have previously shown that silibinin inhibited the invasion, motility and migration of ARCaP_M cells. Herein, we further investigated whether silibinin could reverse the EMT phenotype of ARCaP_M cells. After the treatment of ARCaP_M cells with 100 \( \mu \text{mol/l} \) silibinin for 24, 48 and 72 h, up-regulation of cytokeratin-18 and down-regulation of vimentin and MMP2 (both the pro-MMP2 and active cleaved MMP2) in a time-dependent manner were observed (Fig. 1A), which was consistent with the inhibition of their invasive property of ARCaP_M cells (Fig. 2B). Importantly, we also observed that the morphology of ARCaP_M cells after silibinin treatment was partially changed from elongated fibroblastoid to epithelial cobblestone-like appearance, and the cells appeared to grow in close contact with each other (Fig. 1C). These results showed that silibinin could inhibit the invasion, motility and

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**Figure 3.** Up-regulation of the expression of I\( \kappa B \) protein and suppression of the nuclear translocation of NF-\( \kappa B \) p50 subunit are shown in ARCaP_M cells after silibinin treatment. (A) Western blot analysis was used to quantify the change of I\( \kappa B \) protein, and the quantitative analyses are done below the gel data. (B) Immunofluorescent staining analysis showed that I\( \kappa B \) protein levels were up-regulated after silibinin treatment (x100, magnification). (C) The cytosolic and nuclear p50 protein expression was detected using Western blot analysis when GAPDH and Histone H1 served as cytosolic and nuclear loading controls, respectively. The quantitative analyses are shown in the right panel.
Therefore, we assessed whether the NF-κB treatment. We found that the IκB protein was altered or not in the reversal of EMT in ARCaP M cells after silibinin treatment. We also noted that silibinin could inhibit the nuclear translocation of NF-κB protein (p50 subunit of NF-κB) in ARCaP_M cells when GAPDH and Histone H1 served as a cytosolic and nuclear loading control, respectively. These findings suggest up-regulation of IκBα proteins and subsequent inhibition of NF-κB proteins nuclear translocation may play important roles in silibinin-induced suppression of invasion in our experimental system.

Silibinin inhibits the expression of ZEB1 and SLUG transcription factors in ARCaP_M cells. Transcriptional repression is the major determinant in EMT during cancer progression, and activation of SNAIL, ZEB and TWIST transcription factors results in the induction and maintenance of the EMT phenotype. Since the inhibition of NF-κB signaling pathway was observed, we further investigated whether silibinin could down-regulate the expression of other transcription factors, which in turn resulted in the reversal of EMT characteristics of ARCaP_M cells by RT-PCR. We found that silibinin inhibited the expression of ZEB1 and SLUG transcription factors in ARCaP_M cells (Fig. 4), the two key mediators of EMT in prostate cancer ARCaP model system (data not shown). However, other factors including SNAIL1, ZEB2 and TWIST1 had no significant changes in ARCaP M cells after the same concentrations of silibinin treatment. These findings suggest that silibinin could target the specific transcription factors which determine the EMT status of ARCaP_M cells.

Discussion

ARCaP cells, isolated from the ascites fluid of a prostate cancer patient with bone metastasis, express a host of human prostate cancer biomarkers, and have high propensity for rapid and predictable bone and soft tissue metastases through orthotopic, intracardic and intraosseous injections (28). The ARCaP_M cells obtain increased cell migratory, invasive and metastatic potential to soft tissue and bone after EMT from ARCaP_E cells on exposure to soluble factors or host bone microenvironment, and this ARCaP EMT model has been widely used for understanding the biology and targeting therapy of the lethal progression of human prostate cancer to bone (27-29). We have previously shown that silibinin could inhibit the invasion, motility and migration of ARCaP_M cells in a dose- and time-dependent manner, and suppresses the expression of vimentin protein and MMP2 mRNA simultaneously (26). In this study, we have further used this model to investigate the potential regulational mechanisms of the anti-metastatic activities of silibinin on prostate cancer.

In agreement with the previous study showing that dietary silibinin may modulate MMPs, E-cadherin and vimentin via down-regulation of SNAIL1 to inhibit EMT and consequently suppress invasion and metastasis of prostate tumor in TRAMP mice (14), we also found that silibinin could reverse the mesenchymal phenotype by modulating the expression of cytokeratin-18, vimentin and MMP2 proteins in metastatic ARCaP_M or DU145 cells in vitro. Consistently, the morphological changes of ARCaP_M cells from fibroblastoid to

![Figure 4. Silibinin inhibits the expression of ZEB1 and SLUG mRNA in ARCaP_M cells. RT-PCR reaction showed the down-regulation of ZEB1 and SLUG expression in ARCaP_M cells, while SNAIL1, ZEB2 and TWIST1 had no significant changes after silibinin treatment. Quantitative analyses are shown below the gel data, and data represent the means ± SEM of three independent experiments.](image)
cobblestone-like after silibinin treatment were also observed. The process of EMT, involving the loss of E-cadherin, cytokeratin for impairing the epithelial-cell-cell junction, gain the expression of vimentin, α-smooth muscle actin for reorganization of cytoskeleton to be motile, production of metalloproteinases for degradation and remodeling of the extracellular matrix (ECM) endows cancer cells with more invasive and metastatic properties (32). Therefore, up-regulation of cytokeratin-18 and down-regulation of vimentin and MMP2 impaired the invasive property of ARCaPM cells. These findings demonstrated that silibinin could also target the EMT process and be useful for the intervention of prostate cancer diagnosed at the advanced stages.

The NF-κB family of transcription factors, serving as the master regulators of EMT, plays pivotal roles in both promoting and maintaining an invasive phenotype (18). Inhibition of NF-κB in Ras-transformed epithelial cells (EpRas cells) could lead to a 10-fold reduction in metastases to the lungs following tail vein injection into nude mice (20). Therefore, NF-κB has been identified as a key target in the prevention and therapy of progressive carcinomas (19). In this study, we showed that silibinin inhibited the nuclear translocation of NF-κB p50 subunits by increasing the IκBα levels in ARCaPM cells. It is known that IκBα binds to the p50/p65 heterodimer core, the most dominant proteins in the NF-κB family, and it maintains the heterodimers in the cytoplasm and prevents the activation of NF-κB target gene (33). Our findings suggest silibinin may inhibit the phosphorylation and degradation of IκBα, then blocks the constitutive activation of NF-κB in ARCaPM cells, and subsequently regulates the expression of the target genes cytokeratin-18, vimentin and MMP2. Consistently, similar inhibitory effects of silibinin on the constitutive activation of NF-κB have been documented in advanced prostate carcinoma DU145 cells (34).

Since many zinc finger transcription factors involved in the regulation of EMT were reported to be regulated by NF-κB, we next investigated the potential effects of silibinin on the expression of zinc-finger transcription factors in ARCaPM cells. Silibinin inhibited the expression of ZEB1 and SLUG transcription factors while SNAIL1, ZEB2 and TWIST1 were not affected. Different transcription factors, including SNAIL1, SLUG, ZEB1, ZEB2 and TWIST1, may participate in EMT in different types of tumors (25). Our previous studies have shown ZEB1 and SLUG are the two key determinants of EMT status in ARCaPM cells (data not shown), and Graham et al have demonstrated that ZEB1 is an important mediator of ARCaPM cell migration and invasion, and serves as a critical regulator of EMT to promote the metastasis of prostate cancer (35). Therefore, targeting ZEB1 and SLUG by silibinin may be associated with the reversal of EMT and subsequently suppression of the cell invasion of ARCaPM cells. In addition, accumulating evidence indicates that these master regulatory transcription factors are regulated either directly or indirectly by NF-κB. NF-κB was reported to directly induce ZEB1 expression and promoter activity (36), and indirectly regulated the expression of SLUG via the dioxin-activated aryl hydrocarbon receptor (AhR) (37). Thus, we supposed that repression of NF-κB signaling followed by ZEB1 and SLUG down-regulation might be the molecular mechanisms responsive for the regulation of EMT phenotype by silibinin at the transcriptional level, which consequently contribute to the inhibition of ARCaPM cell invasion.

In summary, these findings suggested that the anti-metastatic efficacy of silibinin on prostate cancer cells was associated with reversal of EMT at the transcriptional level. However, the exact roles of NF-κB through directly regulating ZEB1 and SLUG in ARCaPM cells require further investigation.

Acknowledgements

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