A supercritical-CO₂ extract of *Ganoderma lucidum* spores inhibits cholangiocarcinoma cell migration by reversing the epithelial–mesenchymal transition

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ABSTRACT

Background: *Ganoderma lucidum* (*G. lucidum*) is an oriental medical mushroom that has been widely used in Asian countries for centuries to prevent and treat different diseases, including cancer.

Hypothesis/Purpose: The objective of this study was to investigate the effect of A supercritical-CO₂ extract of *G. lucidum* spores on the transforming growth factor beta 1 (TGF- β 1)-induced epithelial–mesenchymal transition (EMT) of cholangiocarcinoma cells.

Study design: This was an in vitro study with human cholangiocarcinoma TFK-1 cells treated with varying concentrations of *G. lucidum*.

Methods: A supercritical-CO₂ extract of *G. lucidum* spores (GLE) was obtained from completely sporoderm-broken germinating *G. lucidum* spores by supercritical fluid carbon dioxide (SCF-CO₂) extraction. GLE pre-incubated with human cholangiocarcinoma TFK-1 cells prior to TGF- β 1 treatment (2 ng/ml) for 48 h. Changes in EMT markers were analyzed by western blotting and immunofluorescence. The formation of F-actin stress fibers was assessed via immunostaining with phalloidin and examined using confocal microscopy. Additionally, the effect of the GLE on TGF- β 1-induced migration was investigated by a Boyden chamber assay.

Results: TGF- β 1-induced reduction in E-cadherin expression was associated with a loss of epithelial morphology and cell–cell contact. Concomitant increases in N-cadherin and fibronectin were evident in predominantly elongated fibroblast-like cells. The GLE suppressed the TGF- β 1-induced morphological changes and the changes in cadherin expression, and also inhibited the formation of F-actin stress fibers, which are a hallmark of

EMT. The GLE also inhibited TGF- β 1-induced migration of TFK-1 cells.

Conclusion: Our findings provide new evidence that GLE suppress cholangiocarcinoma migration in vitro through inhibition of TGF- β 1-induced EMT. The GLE may be clinically applied in the prevention and/or treatment of cancer metastasis.

Keywords:

Supercritical-CO₂ extract; *Ganoderma lucidum*; Epithelial–mesenchymal transition; Cholangiocarcinoma TFK-1; Migration.

Abbreviations:

G. lucidum, *Ganoderma lucidum*; GLE, supercritical-CO₂ extract of *G. lucidum* spores; CAA, cholangiocarcinoma; TGF- β 1, transforming growth factor beta 1; EMT, epithelial–mesenchymal transition;

Introduction

Cholangiocarcinoma (CCA) is the second most common primary malignancy of the liver (Carriaga and Henson, 1995) and the incidence and mortality rate has been steadily growing worldwide (von Hahn et al., 2011). Despite advances in surgical and medical therapy, treatment options for CCA remain unsatisfactory (Blechacz and Gores, 2008). The primary reason for the poor prognosis is metastasis, which precludes curative surgical resection. Therefore, screening suitable compounds targeting tumor metastasis is an effective way to treat this highly invasive cancer.

Cancer metastasis refers to the spread of cancer cells from the primary neoplasm and the growth of secondary tumors at sites distant from the primary tumor. Metastasis occurs through a complex multistep process consisting of invasion into the circulation from a primary tumor, immigration to distant organs, adhesion to endothelial cells and infiltration into the tissue. The process of epithelial–mesenchymal transition (EMT) is characterized by the epithelial cells converting into the elongated, motile and invasive mesenchymal phenotype (Thiery et al., 2009), which enhances cellular motility. It is considered an early event of metastasis and a critical step in the dissemination of tumor cells (Yilmaz and Christofori, 2010). Increasing evidence suggests that EMT is important during the progression and recurrence of CCA (Ryu et al., 2012). Therefore, the inhibition of EMT could be therapeutically important for the inhibition of invasion and metastasis in CCA.

Ganoderma lucidum (*G. lucidum*) has been used as a dietary therapeutic in traditional Chinese medicine for several millennia. The fruit bodies, cultured mycelia and the spores of *G. lucidum* have been reported to be effective in the treatment of various types of diseases,

including cancer (Chen and Zhong, 2011; Liu and Zhong, 2011; Seto et al., 2009). Although the fruiting body of *G. lucidum* has been used as a traditional herbal medicine since ancient times, the spores only came to be utilized in the late 20th century. It is known that the anticancer effects of *G. lucidum* may be derived from the triterpenoids, polysaccharides or immunomodulatory protein components (Zhou et al., 2007). A supercritical-CO₂ extract that we have obtained from *G. lucidum* spores (GLE) has high bioactive triterpenoid contents. More recently, we have reported GLE exert antitumor activities by direct tumoricidal effects or indirectly by activating monocytes/macrophages (Zhang et al., 2009).

However, to date, the ability of GLE to antagonize transforming growth factor beta 1 (TGF- β 1)-mediated changes associated with EMT in CCA cells has not been explored. In the present study, we evaluated the effect of GLE on the TGF- β 1-induced EMT of CCA cells. Our findings suggest that GLE inhibits TGF- β 1-induced EMT development, thus suppressing EMT-activated CCA cell migration.

Materials and methods

Reagents

Fetal bovine serum (FBS) was obtained from Hyclone, Thermo Fisher Scientific, Victoria, Australia, and Roswell Park Memorial Institute 1640 medium (RPMI 1640) was from Hyclone, Logan, UT, USA. TGF- β 1 was from R&D Systems, Minneapolis, MN, USA, and 4'-6-diamidino-2-phenylindole (DAPI) and Alexa Fluor® 555 phalloidin were from Sigma-Aldrich, Seelze. Mouse monoclonal antibodies (mAb) against E-cadherin (610181), N-cadherin (610920) and fibronectin (610077) were from BD Pharmingen, San Diego, CA,

USA.

Plant and spores

G. lucidum was cultivated at a base in a high alpine forested area (1000 m above sea level) in Fujian Province, Southeast China, which was established by the Academy of Food and Health Engineering at Sun Yat-Sen University. The fungus was *G. lucidum* (Curtis: Fr.) Karst. (Polyporaceae), and voucher specimen of the sample was deposited in the Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences. The spores of *G. lucidum* were collected and activated by placing the germination-induced spores in a well-ventilated culture box kept at constant temperature and humidity (relative humidity, 60% to 98%; temperature, 16 °C to 43 °C; activation period, 10 minutes to 24 hours) to obtain the germination-activated spores. The germination rate of the spores was more than 95%. The sporoderm of germinating spores was then broken and the broken rate could reach 99.8%.

Sample preparation and determination of ganoderic acids content

The germination-activated, sporoderm-broken spores were placed in supercritical fluid carbon dioxide (SCF-CO₂) extracting apparatus, the supercritical conditions included 5 M to 60 M Pa of pressure; 32 °C to 85 °C of temperature; and 5 kg/h to 80 kg/h of flow capacity rate. The total extraction time was between 0.5 to 6 hour. 37g extract was obtained from 100 g of completely sporoderm-broken germinating ganoderma spores (extraction yield = 37 g / 100 g). The extract mainly consisted of triglycerides and *G. lucidum* triterpenoids (Liu et al., 2007; Yuan et al., 2006).

Ganoderic acids, the major bioactive triterpenoids components from *G. lucidum*, could be considered as the “marker compounds” for the chemical evaluation. Thus, the contents of

the ganoderic acids in the GLE were determined following the previous method (Gao et al., 2004). Briefly, GLE was dissolved in MeOH to prepare stock solution of 0.5 g/ml and filtered through a 0.45 μ M membrane. Then, 20 μ l of the supernatant diluted to appropriate concentration was injected into the HPLC System (Binary HPLC Pump 1525, Refractive Index Detector 2414, Photodiode Array Detector 2996; Waters, Milford, MA, USA) which included a Symmetrys C18 column (4.6 mm $\times$ 250 mm, 5 μ m; Waters) and a Guard column (3.9 mm $\times$ 20 mm $\times$ 5 mm; Waters). The mobile phase was made by mixing solvent A (0.1 % phosphoric acid aqueous solution) and solvent B (acetonitrile) using the following gradient program: 0 min, 20% B; 5 min, 30% B; 10 min, 36% B; 60 min, 40% B; 61 min, 20% B; 65 min, 20% B. The flow rate was set at 1.0 ml/min, and the detecting wavelength was set at 254 nm. The operating temperature was maintained at 30 °C. Chromatographic peak was identified by comparing the retention times and spectra against known standards. The method was validated for parameters such as linearity, precision, and accuracy following the previous method (Gao et al., 2004).

Cell culture and treatment

The human CCA TFK-1 cell line was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Cells were maintained in RPMI 1640 medium with 10% FBS and 1% penicillin–streptomycin at 37 °C in 5% CO₂ with constant humidity. The cells were provided with fresh medium every 2–3 days.

TFK-1 cells were pretreated with GLE at various concentrations (GLE at the concentrations less 400 μ g/ml has no effect on the TGF- β 1-induced EMT, data not shown) for 8 h, then incubated with 2 ng/ml TGF- β 1 for 48 h. The GLE was dissolved in dimethyl

144 sulfoxide (DMSO) so that the final concentration of DMSO was less than 0.1% (v/v).

145 ***Cell viability assay***

146 TFK-1 cells were seeded at 2×10^4 cells/well in 96-well plates, and then cultured with
147 GLE, TGF- β or medium alone for the indicated time. The viability of tumor cells was
148 determined by Alamar Blue assay (AbD Serotec, Oxford, UK). The cell viability was
149 calculated according to the following formula: Cell viability (%) = $(1 - \text{absorbance of}$
150 $\text{experimental group/absorbance of control group}) \times 100\%$. Values are represented as means
151 $\pm$ SEM of three independent experiments performed in triplicate.

152 ***Western blotting***

153 Proteins were extracted as previously described and separated by 10% sodium dodecyl
154 sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotted with an mAb
155 against E-cadherin, N-cadherin, fibronectin or actin (used as an internal control), and
156 visualized with an ECL Kit (Pierce, Rockford, IL, USA).

157 ***Immunofluorescence***

158 For immunofluorescence analysis, TFK-1 cells cultured on coverslips were fixed with
159 ice-cold methanol and then stained with mouse anti-human E-cadherin, N-cadherin or
160 Fibronectin, followed by incubation with donkey anti-mouse Alexa Fluor® 555 and nuclear
161 counterstaining with DAPI. Alexa Fluor® 555 phalloidin was used to stain F-actin.
162 Fluorescent images were examined using a Leica TCS SP5 confocal microscope.

163 ***Transwell assays***

164 The migration of tumor cells was analyzed in a 24-well Boyden chamber with an 8 mm
165 pore size polycarbonate membrane (Corning Glass Works, Corning, NY, USA). TFK-1 cells

were treated with GLE at various concentrations for 8 h and then harvested. Cells (5×10^4) were suspended in 100 μ l serum-free RPMI 1640 and added to the upper chamber, while the lower compartment was filled with 600 μ l RPMI 1640 containing 10% FBS as a chemo-attractant to encourage cell migration. The media in the upper and lower compartments contained TGF- β 1 or the GLE at the same concentration. After incubation at 37 °C for 24 h, non-migrated cells remaining on the upper surface of the membrane were removed with cotton swabs. The migrated tumor cells on the lower surface of the membrane were fixed with methanol, stained with crystal violet and then counted in five random fields per well under a light microscope at a magnification of 200 $\times$. Representative images and the number of migrated cells per field are shown. Data represent the mean $\pm$ SEM of three independent experiments.

Statistical analysis

Results are expressed as the mean $\pm$ SEM of three independent experiments. Statistical differences between means were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. $p < 0.05$ was considered significant.

Results

Content of ganoderic acids in GLE

The contents of the ganoderic acids in the GLE were determined by the HPLC. The method was proved to be valid according to the results of linearity, precision, and accuracy (data not shown). The compounds of peaks in the HPLC chromatogram of GLE were identified. The contents of 10 ganoderic acids in the GLE were quantified with ganoderic acid

A $816 \pm 7.67 \mu\text{g/g}$, ganoderic acid B $301 \pm 3.28 \mu\text{g/g}$, ganoderic acid C2 $134 \pm 1.62 \mu\text{g/g}$,
ganoderic acid C6 $18 \pm 0.27 \mu\text{g/g}$, ganoderic acid D $548 \pm 7.18 \mu\text{g/g}$, ganoderic acid F $282 \pm$
 $3.16 \mu\text{g/g}$, ganoderic acid G $702 \pm 7.58 \mu\text{g/g}$, lucidenic acid A $300 \pm 3.99 \mu\text{g/g}$, lucidenic acid
B $72 \pm 0.95 \mu\text{g/g}$ and lucidenic acid N $161 \pm 2.21 \mu\text{g/g}$, respectively. The HPLC
chromatograms of **GLE** are shown in Fig. 1.

GLE inhibited TGF- β 1-induced EMT morphological changes

TGF- β 1 modulates cell morphology changes and EMTs in many cell types. In the
presence of 2 ng/ml TGF- β 1, the morphology of TFK-1 cells changed from the classic
cobblestone appearance of epithelial cells to predominantly elongated fibroblast-like cells. To
determine the regulatory effect of the GLE during TGF- β 1-induced EMT, we analyzed the
morphological changes in TGF- β 1-treated cells pre-incubated with the GLE. Treatment with
TGF- β 1 induced prominent morphological changes in TFK-1 cells, including elongated and
spindle-like shapes. Pretreatment with the GLE suppressed the spindle-shaped morphological
change induced by TGF- β 1 in a dose-dependent manner. Most of the cells treated with
800 $\mu\text{g/ml}$ GLE maintained their oval shape, which is typical of epithelial cell morphology.
Meanwhile, 400 $\mu\text{g/ml}$ GLE restored the epithelial morphology of the cells to a certain extent
(Fig. 2).

We then evaluated the effect of the GLE on the viability of TFK-1 cells. Cells were
exposed to incremental concentrations of the GLE for 48 h and the growth of the cells was
determined by Alamar Blue assay. The results showed that GLE at the indicated
concentrations did not affect the viability of TFK-1 cells (Fig. 3). Taken together, these
findings showed that the GLE inhibited the effects of TGF- β 1.

GLE regulated EMT marker expression during TGF- β 1-induced EMT

Reduced levels of the epithelial marker (E-cadherin) and increased levels of the mesenchymal markers (N-cadherin and fibronectin) are important characteristics of EMT. We determined the effect of the GLE on the expression of EMT markers by western blotting. As shown in Fig. 4A, under basal conditions TFK-1 cells expressed relatively higher levels of E-cadherin than N-cadherin and Fibronectin, consistent with their epithelial phenotype. Decreased E-cadherin and increased N-cadherin and fibronectin levels were observed in the TFK-1 cells treated with TGF- β 1. In contrast, GLE treatment blocked the expression of mesenchymal markers (N-cadherin and fibronectin) and restored the expression of E-cadherin.

To verify these findings, we performed immunofluorescence staining to investigate the effects of GLE on the distribution of EMT markers in TFK-1 cells. As shown in Fig. 4B, E-cadherin expression was lost and mesenchymal markers (N-cadherin and fibronectin) were induced in TGF- β 1-stimulated TFK-1 cells. In contrast, pretreatment with 800 μ g/ml GLE effectively prevented these expressional variations in TGF- β 1-treated TFK-1 cells (Fig. 4B). Taken together, these findings showed that the GLE inhibited the effects of TGF- β 1-induced EMT in TFK-1 cells.

GLE affected F-actin formation and distribution induced by TGF- β 1 stimulation

The actin cytoskeleton has been implicated in cellular motility and chemotaxis during EMT. Formation of F-actin stress fibers is a characteristic development in cells undergoing EMT (Buckley et al., 2012; Zhang et al., 2009). Hence, we investigated the F-actin content and its distribution in cells upon stimulation with TGF- β 1 in the presence of GLE. As shown

in Fig. 5, TFK-1 cells showed an increased number of stress fibers during TGF- β 1 stimulation. The stress fibers were mainly distributed in the cytoplasm and near the nucleus. However, GLE treatment reduced the TGF- β 1-induced formation of stress fibers and actin relocated under the cellular membrane.

GLE prevented migration via inhibition of TGF- β 1-induced EMT

Since the GLE was able to inhibit the TGF- β 1-induced EMT of TFK-1 cells, we further dissected the effects of the GLE on migration using a transwell migration assay in vitro. Treatment with TGF- β 1 resulted in an increased number of migrated cells on the lower face of the chambers. Pretreatment with the GLE showed impaired migration through the filters and the action of TGF- β 1 appeared completely antagonized. Quantization of these results further revealed that the GLE inhibited the migration capacity induced by TGF- β 1 in TFK-1 cells (Figs. 6A, B).

Discussion

The extracts of *G. lucidum* are known for their anti-cancer activities and are thought to act through a variety of mechanisms, including the inhibition of proliferation, cell cycle arrest, the induction of apoptosis (Wang et al., 2012; Zhang et al., 2009) and autophagy (Thyagarajan et al., 2010). Although several reports have suggested the involvement of the extracts of *G. lucidum* in the inhibition of tumor metastasis (Weng et al., 2008; Weng et al., 2009), its role in EMT has not been examined. In this study, we demonstrated that GLE inhibit TGF- β 1-induced morphological changes in TFK-1 cells. Furthermore, GLE prevented and reversed the changes in the protein levels of E-cadherin and N-cadherin following

treatment with TGF- β 1, and decreased intracellular F-actin fibril expression and cell migration in TFK-1 cells. Our data demonstrated that GLE suppress TGF- β 1-induced EMT, which suggested that GLE may have therapeutic implications in TGF- β 1-induced EMT, which is involved in phenomena such as invasion and metastasis.

Cancer invasiveness and metastasis requires tightly adherent epithelial cells to convert to a more motile phenotype expressing several mesenchymal features, which is referred to as EMT (Yilmaz and Christofori, 2010). During this process, E-cadherin, a hallmark of the epithelial phenotype, is downregulated, while neural cadherin (N-cadherin), normally expressed in fibroblasts, is upregulated (Thiery et al., 2009). CCA is characterized by a poor prognosis and strong invasiveness. The evidence from clinical studies has demonstrated that CCA patients with decreasing E-cadherin and increasing N-cadherin expression have significantly lower survival rates than patients with increasing E-cadherin and decreasing N-cadherin expression (Ryu et al., 2012). Accordingly, the inhibition of EMT may improve the poor prognosis for patients with CCA.

In the present study, we confirmed that an E/N-cadherin switch occurred in CCA cells following TGF- β 1-induced EMT, whereby exposure of TFK-1 cells to TGF- β 1 resulted in the loss of the epithelial marker E-cadherin, and increased N-cadherin and fibronectin, resulting in transformation of the myofibroblastic morphology (Sato et al., 2010), as well as stress fiber reorganization by F-actin. When TGF- β 1-induced EMT was inhibited by the GLE in TFK-1 cells, E-cadherin expression was rescued and the mesenchymal phenotype was strongly inhibited. Our results confirmed that TGF- β 1-induced EMT may activate cell migration. When the TGF- β 1-stimulated TFK-1 cells were treated with the GLE, the GLE inhibited

EMT migration functions and characteristics. It should be noted that tumors undergoing EMT acquire stem cell properties (Mani et al., 2008), which contribute to resistance to chemotherapy (Yang et al., 2006). Thus, we intend to explore the effect and mechanism of GLE on resistance to chemotherapy in our future work.

Triterpenoid bioactive compounds extracted from *G. lucidum* have been shown to have anti-invasive or anti-metastatic activities and are thought to affect various invasive behaviors in various cell lines and animal models (Chen and Zhong, 2011; Weng and Yen, 2010; Wu et al., 2013). The anti-metastasis effects of ganoderic acids, which are major triterpenic acids found in *G. lucidum*, were associated with several molecular mechanisms. Ganoderic acids A and H inhibited invasive behavior of MDA-MB-231 cells through down-regulation of CDK4 and suppression of uPA secretion (Jiang et al., 2008). Ganoderic acid Me suppressed tumor invasion by down-regulating MMP-2/9 gene expression in Lewis Lung Carcinoma cells (Chen et al., 2008). Ganoderic acid T inhibited tumor invasion of HCT-116 colon cancer cell through down-regulated expression of MMP-9, iNOS, and uPA (Chen et al., 2010). Lucidenic acid-rich extract had potential anti-invasive activity on PMA-induced HepG2 cells by suppressing MMP-9 activity (Weng et al., 2009). The ethanol extract of the *G. lucidum* fruiting body was also shown to have strong anti-angiogenic activity (Stanley et al., 2005; Thyagarajan et al., 2006). Here, we found that GLE inhibited the TGF- β 1-induced migration of TFK-1 CCA cancer cells through suppression of EMT, suggesting a novel mechanism of action for the anti-metastatic effects of this mushroom.

Our previous studies in animal models have shown that the supercritical-CO₂ extract of completely sporoderm-broken germinating ganoderma spores are able to suppress tumor

growth in vivo (Liu et al., 2002). Furthermore, the supercritical-CO₂ extract induced potent antitumor immune responses in normal human monocytes, macrophages, and immunosuppressive tumor-associated macrophages (Zhang et al., 2009), which indicated that the supercritical-CO₂ extract of completely sporoderm-broken germinating ganoderma spores showed profound immunomodulatory effects on the hosts' immune systems. The present study showed that the supercritical-CO₂ extract from spore of *Ganoderma lucidum* could exert anti-metastatic activities by inhibition of TGF- β 1-induced EMT. From these results, it seems likely that supercritical-CO₂ extract of ganoderma spores may exert antitumor activity through multiple mechanisms of action. Thus, the supercritical-CO₂ extract of ganoderma spores may have the potential to serve as novel anticancer agents.

Conclusions

Our findings provide new evidence that GLE suppress CCA migration in vitro through inhibition of TGF- β 1-induced EMT. With further study, GLE may be clinically applied in the prevention and/or treatment of cancer metastasis.

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References

- Blechacz, B., Gores, G. J., 2008. Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. *Hepatology* 48, 308-321.
- Buckley, S. T., Medina, C., Davies, A. M., Ehrhardt, C., 2012. Cytoskeletal re-arrangement in TGF-beta1-induced alveolar epithelial-mesenchymal transition studied by atomic force microscopy and high-content analysis. *Nanomedicine : nanotechnology, biology, and medicine* 8, 355-364.
- Carriaga, M. T., Henson, D. E., 1995. Liver, gallbladder, extrahepatic bile ducts, and pancreas. *Cancer* 75, 171-190.
- Chen, N. H., Liu, J. W., Zhong, J. J., 2008. Ganoderic acid Me inhibits tumor invasion through down-regulating matrix metalloproteinases 2/9 gene expression. *J Pharmacol Sci* 108, 212-216.
- Chen, N. H., Liu, J. W., Zhong, J. J., 2010. Ganoderic acid T inhibits tumor invasion in vitro and in vivo through inhibition of MMP expression. *Pharmacol Rep* 62, 150-163.
- Chen, N. H., Zhong, J. J., 2011. p53 is important for the anti-invasion of ganoderic acid T in human carcinoma cells. *Phytomedicine* 18, 719-725.
- Gao, J. J., Nakamura, N., Min, B. S., Hirakawa, A., Zuo, F., Hattori, M., 2004. Quantitative determination of bitter principles in specimens of *Ganoderma lucidum* using high-performance liquid chromatography and its application to the evaluation of ganoderma products. *Chem Pharm Bull (Tokyo)* 52, 688-695.
- Jiang, J., Grieb, B., Thyagarajan, A., Sliva, D., 2008. Ganoderic acids suppress growth and invasive behavior of breast cancer cells by modulating AP-1 and NF-kappaB signaling. *Int J Mol Med* 21, 577-584.
- Liu, R. M., Zhong, J. J., 2011. Ganoderic acid Mf and S induce mitochondria mediated apoptosis in human cervical carcinoma HeLa cells. *Phytomedicine* 18, 349-355.
- Liu, X., Xu, S. P., Wang, J. H., Yuan, J. P., Guo, L. X., Li, X., Huang, X. N., 2007. Characterization of ganoderma spore lipid by stable carbon isotope analysis: implications for authentication. *Analytical and bioanalytical chemistry* 388, 723-731.
- Liu, X., Yuan, J. P., Chung, C. K., Chen, X. J., 2002. Antitumor activity of the

sporoderm-broken germinating spores of *Ganoderma lucidum*. *Cancer Lett* 182, 155-161.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Brisken, C., Yang, J., Weinberg, R. A., 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133, 704-715.

Ryu, H. S., Chung, J. H., Lee, K., Shin, E., Jing, J., Choe, G., Kim, H., Xu, X., Lee, H. E., Kim, D. G., Lee, H., Jang, J. J., 2012. Overexpression of epithelial-mesenchymal transition-related markers according to cell dedifferentiation: clinical implications as an independent predictor of poor prognosis in cholangiocarcinoma. *Hum Pathol* 43, 2360-2370.

Sato, Y., Harada, K., Itatsu, K., Ikeda, H., Kakuda, Y., Shimomura, S., Shan Ren, X., Yoneda, N., Sasaki, M., Nakanuma, Y., 2010. Epithelial-mesenchymal transition induced by transforming growth factor- β 1/Snail activation aggravates invasive growth of cholangiocarcinoma. *Am J Pathol* 177, 141-152.

Seto, S. W., Lam, T. Y., Tam, H. L., Au, A. L., Chan, S. W., Wu, J. H., Yu, P. H., Leung, G. P., Ngai, S. M., Yeung, J. H., Leung, P. S., Lee, S. M., Kwan, Y. W., 2009. Novel hypoglycemic effects of *Ganoderma lucidum* water-extract in obese/diabetic (+db/+db) mice. *Phytomedicine* 16, 426-436.

Stanley, G., Harvey, K., Slivova, V., Jiang, J., Sliva, D., 2005. *Ganoderma lucidum* suppresses angiogenesis through the inhibition of secretion of VEGF and TGF- β 1 from prostate cancer cells. *Biochem Biophys Res Commun* 330, 46-52.

Thiery, J. P., Acloque, H., Huang, R. Y., Nieto, M. A., 2009. Epithelial-mesenchymal transitions in development and disease. *Cell* 139, 871-890.

Thyagarajan, A., Jedinak, A., Nguyen, H., Terry, C., Baldrige, L. A., Jiang, J., Sliva, D., 2010. Triterpenes from *Ganoderma Lucidum* induce autophagy in colon cancer through the inhibition of p38 mitogen-activated kinase (p38 MAPK). *Nutr Cancer* 62, 630-640.

Thyagarajan, A., Jiang, J., Hopf, A., Adamec, J., Sliva, D., 2006. Inhibition of oxidative stress-induced invasiveness of cancer cells by *Ganoderma lucidum* is mediated through the suppression of interleukin-8 secretion. *Int J Mol Med* 18, 657-664.

von Hahn, T., Ciesek, S., Wegener, G., Plentz, R. R., Weismuller, T. J., Wedemeyer, H., Manns, M. P., Greten, T. F., Malek, N. P., 2011. Epidemiological trends in incidence and

380 mortality of hepatobiliary cancers in Germany. *Scand J Gastroenterol* 46, 1092-1098.

381 Wang, J. H., Zhou, Y. J., Zhang, M., Kan, L., He, P., 2012. Active lipids of *Ganoderma*
382 *lucidum* spores-induced apoptosis in human leukemia THP-1 cells via MAPK and PI3K
383 pathways. *J Ethnopharmacol* 139, 582-589.

384 Weng, C. J., Chau, C. F., Hsieh, Y. S., Yang, S. F., Yen, G. C., 2008. Lucidenic acid inhibits
385 PMA-induced invasion of human hepatoma cells through inactivating MAPK/ERK signal
386 transduction pathway and reducing binding activities of NF-kappaB and AP-1.
387 *Carcinogenesis* 29, 147-156.

388 Weng, C. J., Chau, C. F., Yen, G. C., Liao, J. W., Chen, D. H., Chen, K. D., 2009. Inhibitory
389 effects of *ganoderma lucidum* on tumorigenesis and metastasis of human hepatoma cells in
390 cells and animal models. *J Agric Food Chem* 57, 5049-5057.

391 Weng, C. J., Yen, G. C., 2010. The in vitro and in vivo experimental evidences disclose the
392 chemopreventive effects of *Ganoderma lucidum* on cancer invasion and metastasis. *Clin Exp*
393 *Metastasis* 27, 361-369.

394 Wu, G. S., Song, Y. L., Yin, Z. Q., Guo, J. J., Wang, S. P., Zhao, W. W., Chen, X. P., Zhang,
395 Q. W., Lu, J. J., Wang, Y. T., 2013. Ganoderiol A-enriched extract suppresses migration and
396 adhesion of MDA-MB-231 cells by inhibiting FAK-SRC-paxillin cascade pathway. *PLoS*
397 *One* 8, e76620.

398 Yang, A. D., Fan, F., Camp, E. R., van Buren, G., Liu, W., Somcio, R., Gray, M. J., Cheng,
399 H., Hoff, P. M., Ellis, L. M., 2006. Chronic oxaliplatin resistance induces
400 epithelial-to-mesenchymal transition in colorectal cancer cell lines. *Clin Cancer Res* 12,
401 4147-4153.

402 Yilmaz, M., Christofori, G., 2010. Mechanisms of motility in metastasizing cells. *Mol Cancer*
403 *Res* 8, 629-642.

404 Yuan, J. P., Wang, J. H., Liu, X., Kuang, H. C., Huang, X. N., 2006. Determination of
405 ergosterol in *ganoderma* spore lipid from the germinating spores of *Ganoderma lucidum* by
406 high-performance liquid chromatography. *J Agric Food Chem* 54, 6172-6176.

407 Zhang, J. P., Zheng, L., Wang, J. H., Magnusson, K. E., Liu, X., 2009. Lipid extract from
408 completely sporoderm-broken germinating *Ganoderma sinensis* spores elicits potent
409 antitumor immune responses in human macrophages. *Phytother Res* 23, 844-850.

410 Zhou, X., Lin, J., Yin, Y., Zhao, J., Sun, X., Tang, K., 2007. Ganodermataceae: natural
411 products and their related pharmacological functions. Am J Chin Med 35, 559-574.

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Figure legends

Fig. 1. The HPLC chromatographic profile of ganoderic acids in GLE. (1) ganoderic acid C2; (2) lucidenic acid N; (3) ganoderic acid C6; (4) ganoderic acid G; (5) ganoderic acid B; (6) ganoderic acid A; (7) lucidenic acid B; (8) lucidenic acid A; (9) ganoderic acid D; (10) ganoderic acid F.

Fig. 2. Effect of GLE on TGF- β 1-induced morphological changes in TFK-1 cells. TFK-1 cells were pretreated with the indicated concentrations of the GLE for 8 h and then stimulated with 2 ng/ml TGF- β 1 for 48 h. Treatment with TGF- β 1 alone induced cell elongation and increased scattering, while the GLE inhibited the activation of these processes in a dose-dependent manner. Magnification 100 $\times$.

Fig. 3. Cell viability after pre-exposure (8 h) to various concentrations of the GLE in combination with TGF- β 1 for 48 h. The effects of the GLE on cell viability were determined by Alamar Blue assay. TFK-1 cells stimulated with 2 ng/ml TGF- β 1 were treated with GLE at concentrations of 0 (DMSO only as a control), 400 and 800 μ g/ml. Changes in cell viability were determined. Values are represented as means $\pm$ SEM of three independent experiments performed in triplicate.

Fig. 4. Effect of the GLE on the expression of EMT markers in TGF- β 1-treated TFK-1 cells. TFK-1 cells were incubated with the indicated concentration of the GLE for 8 h followed by treatment with TGF- β 1 for 48 h. (A) Western blotting for EMT marker expression in TFK-1 cells. (B) The cells were immunostained with anti-E-cadherin, anti-N-cadherin and anti-fibronectin antibodies, and nuclei were counterstained with DAPI. Fluorescence imaging was performed on a confocal microscope.

436 **Fig. 5.** Effect of the GLE on the formation of F-actin stress fibers in TFK-1 cells. Cells were
437 labeled with Alexa Fluor® phalloidin (red) and their nuclei counterstained with DAPI (blue).
438 Imaging and analysis was performed using a confocal microscope.

439 **Fig. 6.** Inhibitory effect of GLE on TGF- β 1-induced TFK-1 cell migration. TFK-1 cells were
440 incubated with the GLE for 8 h, Migration was determined using a Transwell assay as
441 described in methods. Migrated cells were stained with crystal violet and counted for
442 quantitative analysis. Representative images (A) and statistical analysis (B). The average
443 number of migrated cells per field is shown. The original magnification was 200 $\times$. Data
444 represent the mean $\pm$ SEM of three independent experiments. *** $p < 0.01$ versus TGF- β 1
445 alone.

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Figure 6

A

B