Ganoderma lucidum inhibits proliferation of human breast cancer cells by down-regulation of estrogen receptor and NF-κB signaling

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Abstract. *Ganoderma lucidum*, an oriental medical mushroom, has been used in Asia for the prevention and treatment of a variety of diseases, including cancer. We have previously demonstrated that *G. lucidum* inhibits growth and induces cell cycle arrest at G_0/G_1 phase through the inhibition of Akt/NF-κB signaling in estrogen-independent human breast cancer cells. However, the molecular mechanism(s) responsible for the inhibitory effects of *G. lucidum* on the proliferation of estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) breast cancer cells remain to be elucidated. Here, we show that *G. lucidum* inhibited the proliferation of breast cancer MCF-7 and MDA-MB-231 cells by the modulation of the estrogen receptor (ER) and NF-κB signaling. Thus, *G. lucidum* down-regulated the expression of ERα in MCF-7 cells but did not effect the expression of ERß in MCF-7 and MDA-MB-231 cells. In addition, *G. lucidum* inhibited estrogen-dependent as well as constitutive transactivation activity of ER through estrogen response element (ERE) in a reporter gene assay. *G. lucidum* decreased TNF-α-induced (MCF-7) as well as constitutive (MDA-MB-231) activity of NF-κB. The inhibition of ER and NF-κB pathways resulted in the down-regulation of expression of c-myc, finally suppressing proliferation of estrogen-dependent as well as estrogen-independent cancer cells. Collectively, these results suggest that *G. lucidum* inhibits proliferation of human breast cancer cells and contain biologically active compounds with specificity against estrogen receptor and NF-κB signaling, and implicate *G. lucidum* as a suitable herb for chemoprevention and chemotherapy of breast cancer.

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Introduction

Estrogens exert their biological effects such is the induction of cell proliferation through the interaction with estrogen receptor-alpha (ERα) and ER-beta (ERß) (1-3). The liganded ER forms homo-/hetero-dimers, translocate into nucleus and activate transcription of target genes through the binding to estrogen response elements (EREs) in their promoter region (2,4-6). The activation of ERα stimulates cell proliferation while ERß has an opposite effect (7-9). Therefore, selective estrogen receptor modulators (SERMs) are widely used for the treatment of breast cancer in ERα-positive breast tumors (10,11). However, breast cancer often progresses from the estrogen-dependent, nonmetastatic phenotype (ERα- and ERß-positive) to the estrogen-independent, metastatic phenotypes (ERα-negative and ERß-positive) (11).

Constitutively active transcription factor nuclear factor-κB (NF-κB) and increased expression of c-myc were found in breast cancer cells (12,13) and linked to their high proliferative potential (3,13,14). Since the expression of c-myc is regulated by 17ß-estradiol (E2) (15,16) and NF-κB (17), the levels of c-myc in estrogen-dependent and estrogen-independent breast cancer cells can be modulated through the inhibition of ER and NF-κB signaling pathways. We and others have demonstrated that the increased activity of NF-κB is associated with the invasive behavior and aggressive tumor growth of ERα-negative breast cancer cells, and found that the inhibition of NF-κB results in the suppression of invasiveness and growth of these cells (12,18).

The extracts from a medicinal mushroom *Ganoderma lucidum* (Reishi) have been widely used in Asian countries to treat various human diseases, including cancers. The anticancer properties of *G. lucidum* have been attributed to a variety bioactive compounds including polysaccharides and lanostane-based triterpenes (19,20). We have recently demonstrated that transcription factor NF-κB controls invasive behavior of highly metastatic breast cancer cells (18), and that *G. lucidum* inhibits invasiveness of breast cancer cells through the suppression of NF-κB, resulting in the down-regulation of the expression of urokinase-type plasminogen activator (uPA) and its receptor (uPAR) (21). We have also found that *G. lucidum* inhibits the growth of breast cancer cells through the cell cycle arrest at G_0/G_1, which was mediated by the suppression of Akt/NF-κB

signaling and the down-regulation of expression of cyclin D1 (22).

In the present study, we investigated the molecular mechanism(s) responsible for the inhibitory effects of *G. lucidum* on the proliferation of estrogen-dependent MCF-7 (ERα-, ERß-positive) and estrogen-independent MDA-MB-231 (ERα-negative, ERß-positive) human breast cancer cells. We demonstrated that *G. lucidum* down-regulates the expression of ERα, inhibits estrogen-inducible ER transactivation and inhibits TNF-α-stimulated activation of NF-κB in MCF-7 cells. *G. lucidum* also suppressed constitutive activity of ERE and NF-κB in MDA-MB-231 cells. The inhibition of inducible- as well as constitutive-estrogen receptor and NF-κB pathways resulted in the down-regulation of expression of c-myc, finally resulting in the suppression of proliferation of MCF-7 and MDA-MB-231 breast cancer cells.

Materials and methods

Materials. *G. lucidum* (Reishimax) was purchased from Pharmanex (Provo, UT). According to the manufacturer, this sample contains 13.5% polysaccharides and 6% triterpenes. Further analysis by LC-MS demonstrated the presence of ganoderic acids A, F and H, which can be used for the standardization of *G. lucidum* (Thyagarajan *et al*, unpublished data). *G. lucidum* was dissolved in sterile water at a concentration of 50 mg/ml and stored at 4˚C. 17ß-estradiol was purchased from Sigma (St. Louis, MO), tamoxifen was purchased from Calbiochem (La Jolla, CA).

Cell culture. The human breast cancer cell lines (MCF-7 and MDA-MB-231) were obtained from ATCC (Manassas, VA). MCF-7 and MDA-MB-231 cells were maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM) containing penicillin (50 U/ml), streptomycin (50 U/ml), and 10% fetal bovine serum (FBS). 293T/NF-κB-luc cell line was obtained from Panomics (Redwood City, CA). 293T/NF-κB-luc cells were maintained in DMEM containing penicillin (50 U/ml), streptomycin (50 U/ml), hygromycin (100 μg/ml) and 10% FBS. Media and supplements were from Invitrogen (Grand Island, NY). FBS and Charcoal/Dextran treated FBS (CDFBS) were obtained from Hyclone (Logan, UT). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD).

Cell proliferation assay. Cell proliferation was determined by the tetrazolium salt method (MTT method), according to the manufacturer's instructions (Promega, Madison, WI). Briefly, MCF-7 and MDA-MB-231 cells ($2.5x10^3$/well) were cultured in a 96-well plate and treated with *G. lucidum* (0-0.25 mg/ml) for 24 and 48 h. At the end of the incubation period, the cells were harvested and absorption was determined with an ELISA plate reader at 570 nm. Data points represent mean ± SD in one experiment repeated at least twice.

Real-time reverse transcription-PCR. Real-time RT-PCR was used to determine the expression levels of ERα and ERß mRNA in human breast cancer cells. The total RNA from breast cancer cells was isolated by RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed as previously described (23). The PCR primers and TaqMan probes for ERα, ERß, and ß-actin were synthesized from MWG (High Point, NC). PCR was performed by mixing 5 μl of RT product with 2X QuantiTect Probe PCR master mix (Qiagen), and 0.375 μl of primer/probe mixture in a total of 25 μl. Real-time quantitative PCR analysis was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The amplifications were performed in duplicate for each sample and the PCR conditions were as follows: initial incubation at 50˚C for 2 min, denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, and 60˚C for 1 min. We analyzed the relative quantitation of gene expression by the comparative C_T ($\Delta\Delta C_T$) method (24). Briefly, the threshold cycle (C_T) was obtained as the fractional cycle number at which the amount of amplified target reached a fix threshold. Data normalization was performed by subtracting C_T value of the ß-actin from that of the target gene. The $\Delta\Delta C_T$ was calculated as the difference of the normalized C_T value ΔC_T) of the treatment and control samples. $\Delta\Delta C_T = \Delta C_{T\ treatment} - \Delta C_{T\ control}$. The comparative expression level of target genes is equal to $2^{-\Delta\Delta CT}$.

DNA transfection and luciferase assay. MCF-7, MDA-MB-231 and 293T/NF-κB-luc cells ($2.5x10^5$/well) were seeded into 6-well plates in DMEM with 10% FBS prior to transfection. Transient transfections were performed with the Effectene reagent (Qiagen) according to the manufacturer's instructions. Briefly, the cells were transfected with ERE-II-luc (8) or NF-κB-luc (BD Biosciences Clontech, Palo Alto, CA) reporter constructs (1 μg) and ß-galactosidase expression vector pCH110 (1 μg) while 293T/NF-κB-luc cells were transfected with pSG5 or ERα (25) or ERß (26) expression vector constructs (1 μg) and ß-galactosidase expression vector pCH110 (1 μg). Twenty hours after transfection, cells were washed once with DPBS and the medium was replaced with DMEM with 2% CDFBS. Cells were treated with E2 (10 nM), tamoxifen (1 μM), and *G. lucidum* (0.5 mg/ml) for 20 h followed by the treatment with TNF-α (50 ng/ml) for an additional 4 h. ß-glactosidase activity was measured in cell lysates, as previously described (18). Normalized amounts (equal numbers ß-galactosidase units) of cell extracts were used in luciferase assay using Lmax luminometer (Molecular Devices, Sunnyvale, CA). Data points represent the mean ± SD of three independent transfection experiments.

Preparation of nuclear and whole cell extract. MCF-7 and MDA-MB-231 cells ($1x10^7$) were treated with *G. lucidum* (0-1.0 mg/ml) for 24 h. Whole cell extracts were prepared as described (22). Nuclear extracts were isolated from cells resuspended with 0.5 ml of ice-cold RSB lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1 mM DTT, and protease inhibitor cocktail Complete™), by dounce homogenization. The nuclei were collected by centrifugation and the nuclear pellet was resuspended in 3 Vol of Buffer C (20% glycerol, 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 100 μM Na_3VO_4 and

protease inhibitor cocktail Complete™), incubated for 30 min, and the final supernatant (nuclear extract) collected by centrifugation. The protein concentration in whole cell and nuclear extract was determined according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic mobility shift assay (EMSA). Oligonucleotide probes containing consensus sequences for ERE and NF-κB binding sites were purchased from Santa Cruz Biotechnology and Promega (Madison, WI), respectively. EMSA for NF-κB was performed as previously described (18). EMSA for ERE was carried out in binding buffer [5% glycerol, 20 mM HEPES pH 7.9, 100 mm KCl, 1 mM EDTA, 2.5 mM DTT, and 2.0 μg poly-dIdC] with 5 μg of nuclear protein on ice for 20 min. One μl (40,000 cpm) of [^{32}P]-labeled ERE probe was added to the reaction mixture and incubated for another 20 min at room temperature. The reaction mixture was then separated on a 4.5% PAGE gel in 0.25X Tris/borate/EDTA (TBE) buffer. The specificity was confirmed by competitive EMSA with cold ERE oligonucleotide or unrelated DNA (URL), and supershift analysis with anti-ERα or anti-ERß antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Western blot analysis. Equal amounts of proteins (20 μg/lane) were separated on 4-12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was incubated with the corresponding primary antibodies diluted 1:1000 in blocking solution, as follows: a mouse anti-ERα monoclonal antibody, a rabbit anti-ERß polyclonal antibody, a mouse anti-c-Myc monoclonal antibody, and a mouse anti-actin monoclonal antibody (Santa Cruz Biotechnology). Anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Buckinghamshire, UK) were used for detection and visualization by the ECL Western blotting detection system (Amersham Biosciences).

Densitometric analysis. Autoradiograms of the Western blots were scanned with hp scanjet 5470c scanner. The optical densities of ERα, ERß, c-myc and ß-actin proteins on the film were quantified and analyzed with the UN-SCAN-IT software (Silk Scientific, Orem, UT). The ratios of ERα/actin, ERß/actin, and c-myc/actin were calculated by standardizing the ratios of each control to the unit value.

Results

G. lucidum inhibits proliferation of non-invasive as well as invasive breast cancer cells. We have recently demonstrated that an extract from medicinal mushroom *G. lucidum* inhibits proliferation of highly invasive human breast cancer cells (22). In order to evaluate effects of *G. lucidum* on the growth of non-invasive, estrogen-dependent (MCF-7), and highly-invasive, estrogen-independent (MDA-MB-231) breast cancer cells, the cells were treated with *G. lucidum* (0-0.25 mg/ml) for 24 and 48 h. As seen in Fig. 1A, *G. lucidum* inhibits the growth of MDA-MB-231 cells in a dose- and-time dependent manner. However, *G. lucidum* demonstrates more profound effect on MCF-7 cells since the concentration of 31 μg/ml inhibited proliferation of MCF-7 cells by 47-61% within 24-48 h, whereas the same dose inhibited proliferation of

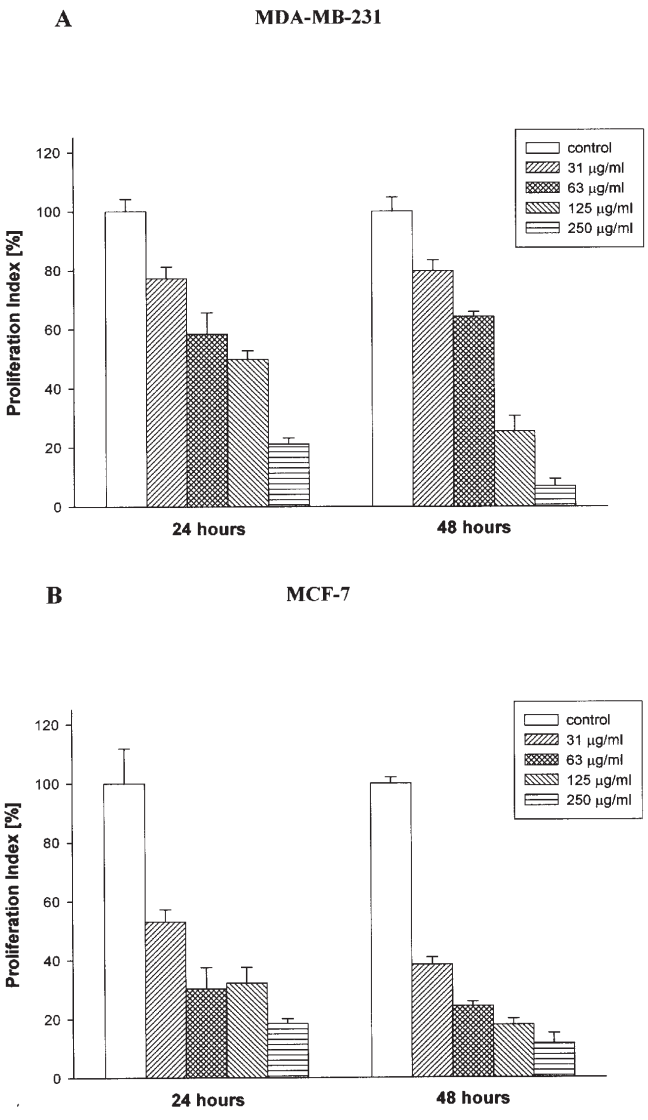

Figure 1. *G. lucidum* inhibits proliferation of breast cancer cells. (A) MDA-MB-231, (B) MCF-7 cells were treated with 0-250 μg/ml of *G. lucidum*. Proliferation was assessed after 24 and 48 h, as described in Materials and methods. Each bar represents the mean ± SD of three experiments.

MDA-MB-231 cells only by 23-20%, respectively (Fig. 1B). The inhibition of proliferation is not caused by the cytotoxicity of *G. lucidum* because, as we previously demonstrated, the viability of breast cancer cells is not affected by the concentration up to 1.0 mg/ml (22). These results suggest that estrogen-dependent (MCF-7) cells are more sensitive to *G. lucidum* than estrogen-independent (MDA-MB-231) cells.

Effect of G. lucidum on the expression of ER alpha and ER beta in breast cancer cells. To confirm the status of estrogen receptors in breast cancer cells, the whole cell lysates were prepared from MCF-7 and MDA-MB-231 cells and subjected to Western blot analysis with anti-ERα and anti-ERß antibody, respectively. As expected, MCF-7 estrogen-dependent breast cancer cells express ERα and ERß, whereas estrogen-independent MDA-MB-231 cells express only ERß (Fig. 2A). In order to determine whether the inhibition of cell growth is related to the status of estrogen receptors, we examined the

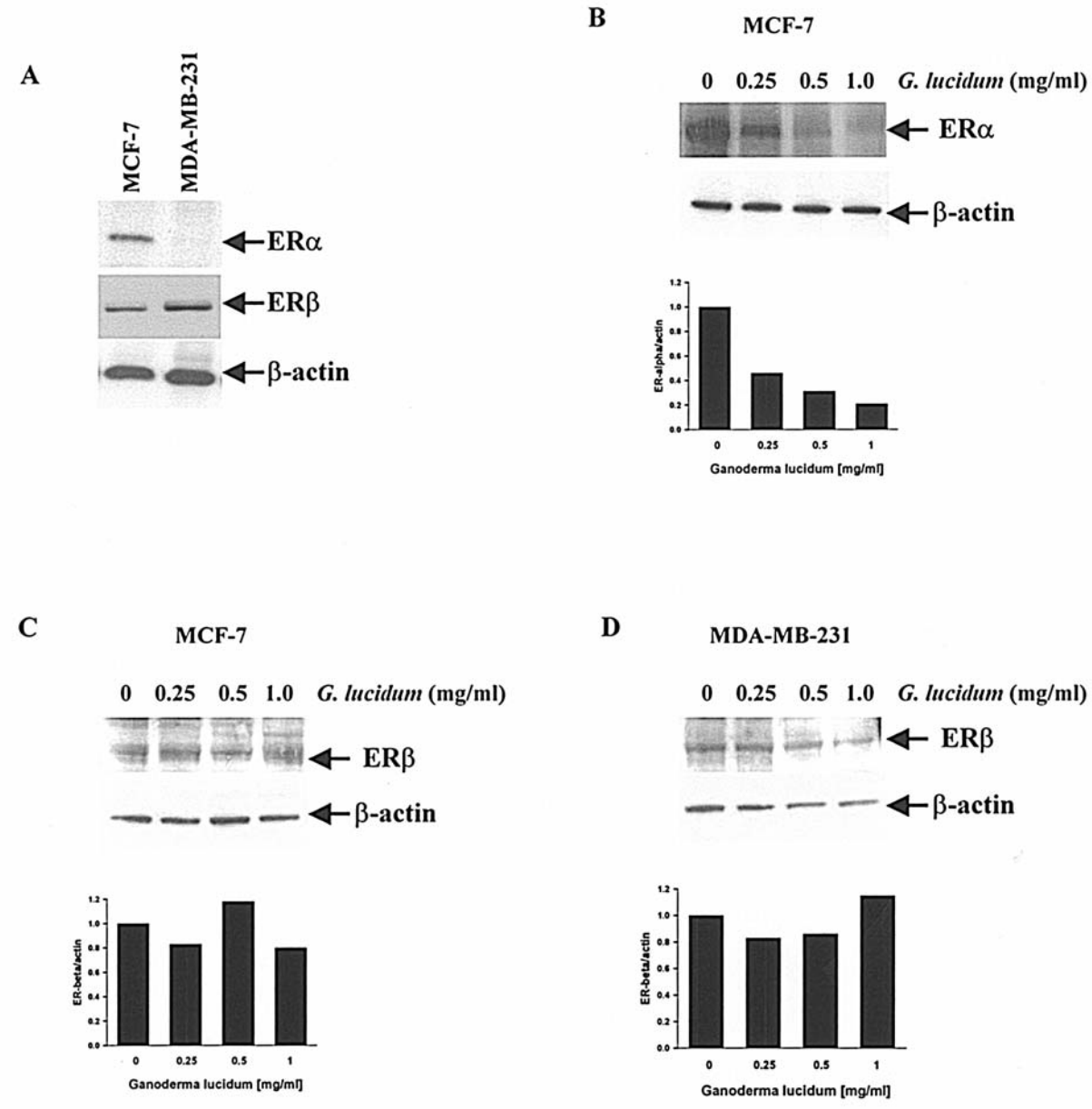

Figure 2. Effect of *G. lucidum* on the expression of ERα and ERß in breast cancer cells. (A) Whole cell extracts from MCF-7 and MDA-MB-231 cells were subjected to Western blot analysis with anti-ERα and anti-ERß antibodies. The equal protein loading was verified with anti-ß-actin antibody. The results are representative of three separate experiments. (B) MCF-7 cells were treated with *G. lucidum* (0-1.0 mg/ml) for 24 h and whole cell extracts were subjected to Western blot analysis with anti-ERα antibody. The equal protein loading was verified with anti-ß-actin antibody and the expression of ERα quantified by densitometry as described in Materials and methods. The results are representative of three separate experiments. (C) MCF-7 cells were treated with *G. lucidum* (0-1.0 mg/ml) for 24 h and whole cell extracts were subjected to Western blot analysis with anti-ERß antibody. The equal protein loading was verified with anti-ß-actin antibody and the expression of ERß quantified by densitometry as described in Materials and methods. The results are representative of three separate experiments. (D) MDA-MB-231 cells were treated with *G. lucidum* (0-1.0 mg/ml) for 24 h and whole cell extracts were subjected to Western blot analysis with anti-ERß antibody. The equal protein loading was verified with anti-ß-actin antibody and the expression of ERß quantified by densitometry as described in Materials and methods. The results are representative of three separate experiments. (E) MCF-7 cells were treated with vehicle (control), *G. lucidum* (GL, 0.5 mg/ml), Actinomycin D (ActD, 1 μg/ml), cycloheximide (CHX, 10 μg/ml), or the combination of GL with ActD or GL with CHX for 24 h, and whole cell extracts were subjected to Western blot analysis with anti-ERα antibody. The equal protein loading was verified with anti-ß-actin antibody. The results are representative of three separate experiments.

effects of *G. lucidum* on the expression of ERα and ERß in these cells. MCF-7 and MDA-MB-231 cells were treated with *G. lucidum* (0-1.0 mg/ml) for 24 h and the expression of ERα and ERß mRNA was evaluated by the real-time-PCR. The treatment with *G. lucidum* did not change mRNA of ERα or ERß in MCF-7 cells or ERß in MDA-MB-231 cells (not

shown). In contrast, *G. lucidum* markedly decreased the levels of ERα protein in MCF-7 cells in a dose-dependent manner (Fig. 2B), whereas the same treatment did not affect the expression of ERß (Fig. 2C). In addition, the expression of and ERß was also not affected by *G. lucidum* in MDA-MB-231 cells (Fig. 2D). To clarify the mechanism of regulation of ERα expression in breast cancer cells, MCF-7 cells were treated with *G. lucidum* (0.5 mg/ml), inhibitor of transcription Actinomycin D (Act D, 1 μg/ml), and inhibitor of translation cycloheximide (CHX, 10 μg/ml). As seen in Fig. 2E, *G. lucidum* as well as CHX or the combination of both markedly inhibited the expression of ERα, whereas Act D or the combination of ActD with *G. lucidum* did not markedly change the expression of ERα in MCF-7 cells. These results suggest that different mechanisms regulate expression of ERα and ERß, and that *G. lucidum* regulates expression of ERα at the translational level.

G. lucidum inhibits estrogen receptor mediated transcription activity in breast cancer cells. Natural estrogenic compounds mediate their transcriptional activity through the interaction with ERα and ERß (27). As we show above, *G. lucidum* inhibits proliferation of breast cancer cells expressing both ERα/ERß or only ERß. In order to determine whether the effects of *G. lucidum* are mediated through the inhibition of the transcriptional activation of estrogen receptor signaling, MCF-7 and MDA-MB-231 cells were transfected with reporter gene constructs ERE-luc, and treated with E2 (10 nM), TAM (1 μM), and *G. lucidum* (0.5 mg/ml) for 24 h, respectively. As seen in Fig. 3A, *G. lucidum* suppressed constitutive (control vs GL) as well as estrogen-induced (E2 vs E2 + GL) transcriptional activation in MCF-7 cells. Although *G. lucidum* and TAM inhibited the constitutive ERE activity in a similar manner, this effect was not additive or synergistic. Furthermore, *G. lucidum* inhibited also constitutive ERE activity (control vs GL) in MDA-MB-231 cells (Fig. 3B). As expected E2 does not stimulate ERE activity in MDA-MB-231 cells and also the effect of TAM was not so dramatic in these cells (Fig. 3B). To evaluate if the suppression of transcriptional activation through ERE is the result of the inhibition of the DNA-binding, we have performed gel shift analysis with nuclear extracts from MCF-7 and MDA-MB-231 cells with [^{32}]P-labeled ERE DNA-binding oligonucleotide probe. As seen in Fig. 3C, nuclear extracts from MCF-7 cells bind specifically to the ERE, and *G. lucidum* inhibited ERE-binding. In addition, *G. lucidum* also inhibited ERE-binding in MDA-MB-231 cells (not shown). In summary, *G. lucidum* inhibits ERE transactivation activity mediated through ERα and ERß in breast cancer cells.

G. lucidum inhibits constitutive as well as inducible NF-κB activity through estrogen receptor-independent as well as estrogen receptor-dependent pathways. The constitutive activation of NF-κB contributes to the progression of breast cancer to estrogen-independent growth and more aggressive phenotype of breast cancer cells (28,29). We have previously demonstrated that *G. lucidum* decreased constitutively active NF-κB resulting in the cell cycle arrest at G_0/G_1 and inhibition of proliferation of MDA-MB-231 cells (21,22). In order to evaluate the effect of *G. lucidum* on inducible NF-κB activation,

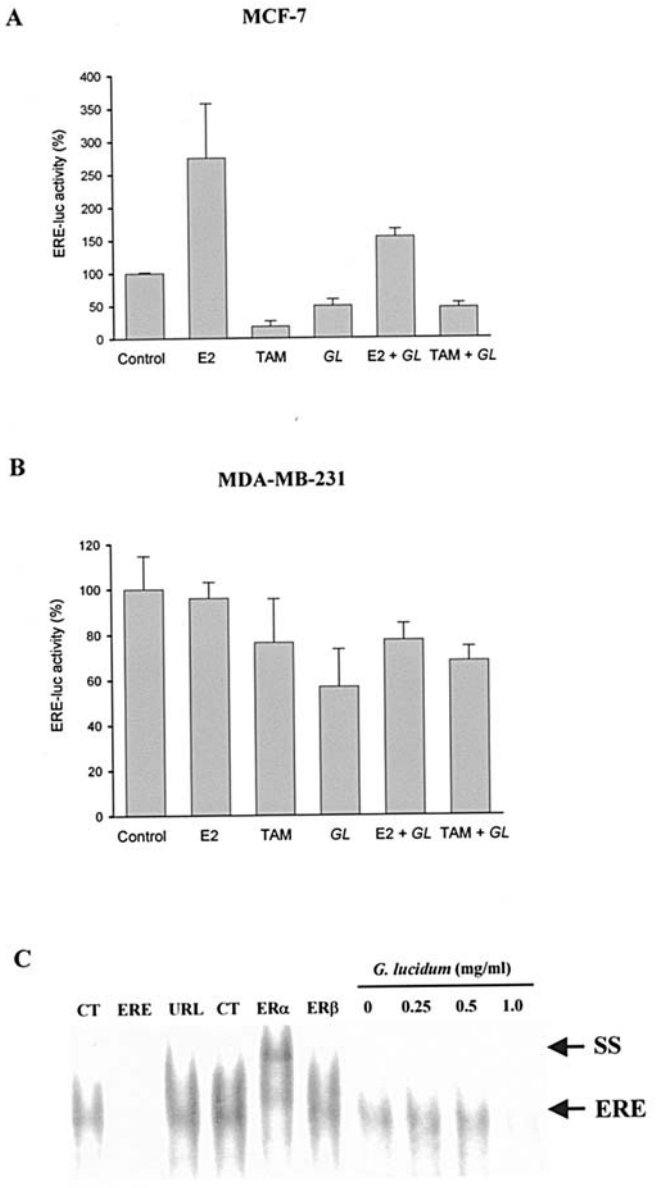

Figure 3. *G. lucidum* inhibits ERE activity in breast cancer cells. (A) MCF-7; (B) MDA-MB-231 cells were transfected with 1 μg ERE-luc reporter gene construct and 1 μg ß-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated with vehicle (control), E2 (10 nM), tamoxifen (TAM, 1 μM), *G. lucidum* (GL, 0.5 mg/ml), or the combination of E2 with GL or TAM with GL for 24 h. ERE activity was measured as described in Materials and methods. The results are expressed as the percentage of relative ERE activity. Each bar represents the mean ± SD of three experiments. (C) MCF-7 cells were treated with *G. lucidum* (0-1.0 mg/ml) for 24 h and nuclear extracts were subjected to EMSA with [^{32}P]-labeled ERE probe as described in Materials and methods. The specificity was confirmed by competitive EMSA with cold ERE oligonucleotide or unrelated DNA (URL), and supershift analysis with anti-ERα or anti-ERß antibody. The results are representative of three separate experiments.

MCF-7 cells were transfected with reporter gene constructs NF-κB-luc, and treated with TNF-α, E2, TAM and *G. lucidum* as described in Materials and methods. As expected, TNF-α stimulated NF-κB activity in MCF-7 cells, and this activity was inhibited by *G. lucidum* (Fig. 4A, control + TNF-α vs TNF-α + GL). In addition, E2 treatment further increased TNF-α stimulated NF-κB activity, which was also inhibited

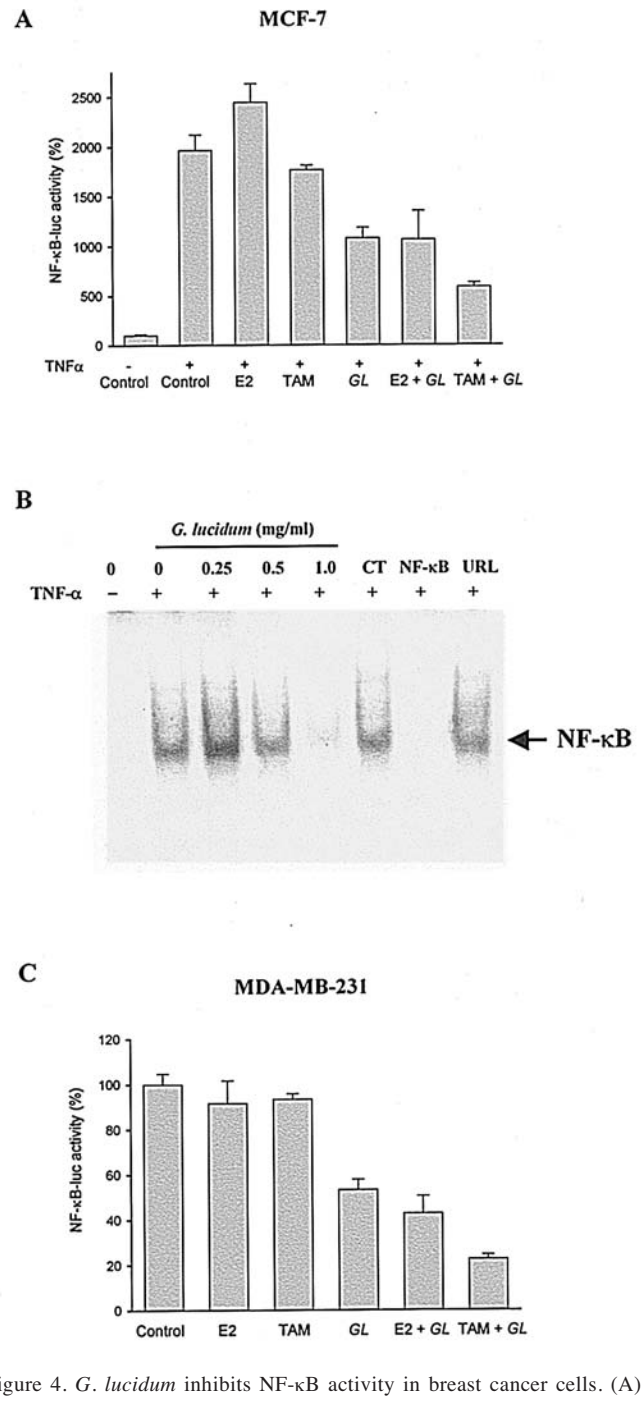

Figure 4. *G. lucidum* inhibits NF-κB activity in breast cancer cells. (A) MCF-7 cells were transfected with 1 μg NF-κB-luc reporter construct and 1 μg ß-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated with vehicle (control), E2 (10 nM), tamoxifen (TAM, 1 μM), *G. lucidum* (GL, 0.5 mg/ml), or the combination of E2 with GL or TAM with GL for 20 h, followed by a 4-h incubation with TNF-α (50 ng/ml). NF-κB activity was measured as described in Materials and methods. The results are expressed as the percentage of relative NF-κB activity. Each bar represents the mean ± SD of three experiments. (B) MCF-7 cells were treated with *G. lucidum* (0-1.0 mg/ml) for 20 h following by a 4-h incubation with TNF-α (50 ng/ml), and nuclear extracts were subjected to EMSA with [^{32}P]-labeled NF-κB probe as described in Materials and methods. The specificity was confirmed by competitive EMSA with cold NF-κB oligonucleotide or unrelated DNA (URL). The results are representative of three separate experiments. (C) MDA-MB-231 cells were transfected with 1 μg NF-κB-luc reporter construct and 1 μg ß-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated with vehicle (control), E2 (10 nM), tamoxifen (TAM, 1 μM), *G. lucidum* (GL, 0.5 mg/ml), or the combination of E2 with GL or TAM with GL for 24 h. NF-κB activity was measured as described in Materials and methods. The results are expressed as the percentage of relative NF-κB activity. Each bar represents the mean ± SD of three experiments.

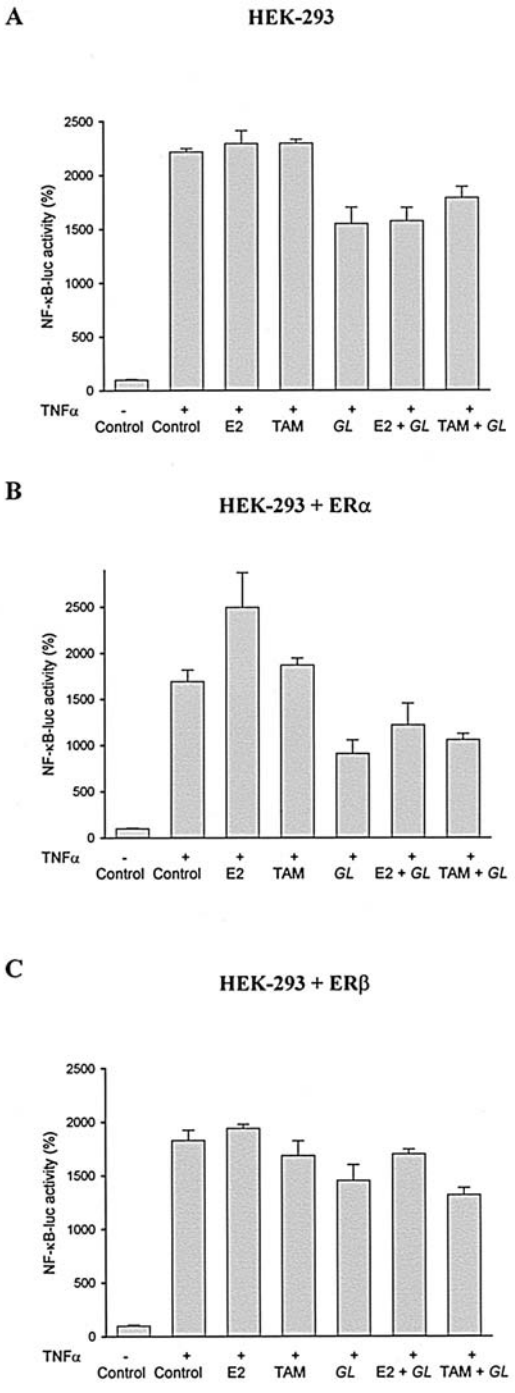

Figure 5. Effect of estrogen receptors on *G. lucidum* mediated inhibition of NF-κB. 293T/NF-κB-luc cells were transfected with: (A) 1 μg pSG5 empty expression vector; (B) 1 μg ERα expression vector; (C) 1 μg ERß expression vector and 1 μg ß-galactosidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated with E2 (10 nM), tamoxifen (TAM, 1 μM), *G. lucidum* (GL, 0.5 mg/ml) or the combination of E2 with GL or TAM with GL for 20 h, followed by a 4-h incubation with TNF-α (50 ng/ml). NF-κB activity was measured as described in Materials and methods. The results are expressed as the percentage of relative NF-κB activity. Each bar represents the mean ± SD of three experiments.

by the treatment with *G. lucidum* (Fig. 4A, TNF-α + E2 vs TNF-α + E2 + GL), and tamoxifen further enhanced the inhibitory effect of *G. lucidum* (Fig. 4A, TNF-α vs TNF-α + TAM + GL). In order to confirm that *G. lucidum* inhibits DNA-binding of NF-κB in TNF-α-stimulated MCF-7 cells, nuclear extracts were prepared and subjected to gel shift

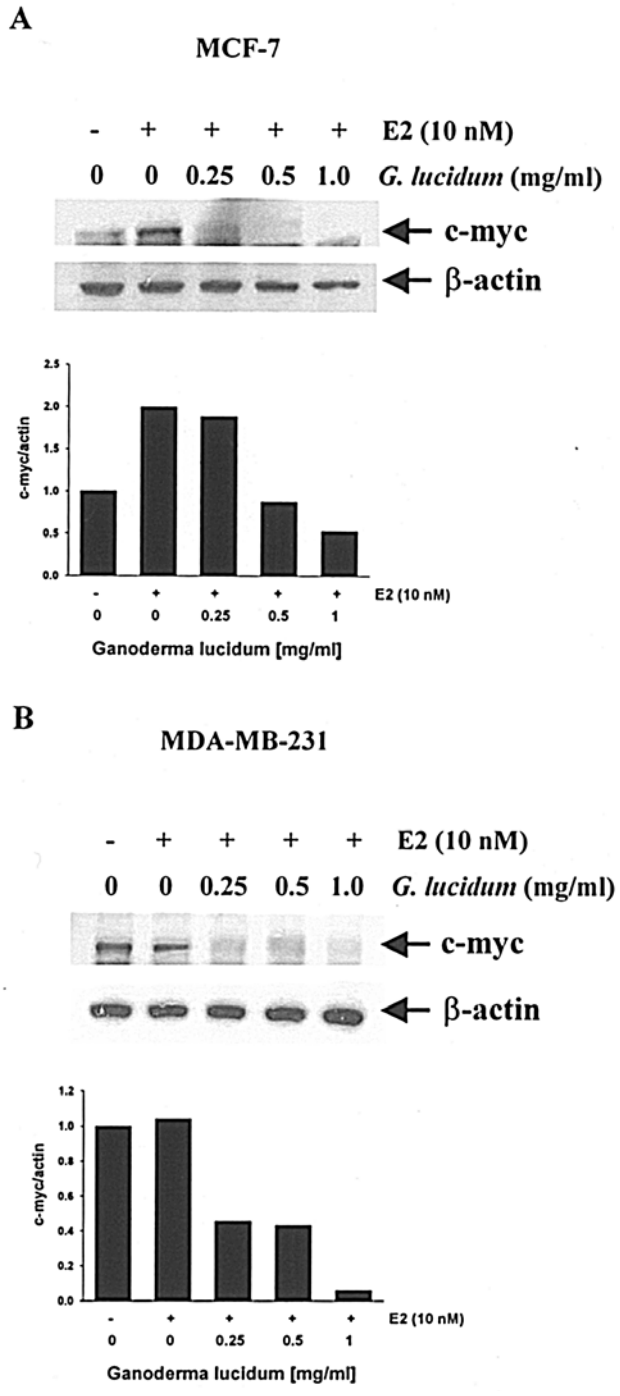

Figure 6. Effect of *G. lucidum* on the expression of c-myc in breast cancer cells. (A) MCF-7; (B) MDA-MB-231 cells were treated with E2 (10 nM) and *G. lucidum* (0-1.0 mg/ml) for 24 h. Whole cell extracts were subjected to Western blot analysis with anti-c-myc antibody. The equal protein loading was verified with anti-ß-actin antibody and the expression of c-myc quantified by densitometry as described in Materials and methods. The results are representative of three separate experiments.

analysis with [^{32}P]-labeled NF-κB oligonucleotide probe. As seen in Fig. 4B, *G. lucidum* inhibits DNA-binding of inducible NF-κB; the specificity of this inhibition was confirmed by competition and supershift assays (18). As we have previously reported, *G. lucidum* inhibits constitutively active NF-κB in MDA-MB-231 cells (21,22). However, this effect can be enhanced by the combination of *G. lucidum* with tamoxifen (Fig. 4C, control vs GL vs TAM + GL).

In order to elucidate the role of estrogen receptors in the inhibition of NF-κB mediated by *G. lucidum*, we employed HEK-293 containing NF-κB-luciferase reporter gene (293T/NF-κB-luc). These cells do not express endogenous ERα or ERß, and *G. lucidum* markedly inhibited TNF-α-induced NF-κB activity (Fig. 5A). Interestingly, E2 further stimulated TNF-α-induced NF-κB activity in 293T/NF-κB-luc cells transfected with ERα, and this NF-κB activity was also suppressed by *G. lucidum* (Fig. 5B). Moreover, TNF-α-induced NF-κB activity in 293T/NF-κB-luc cells transfected with ERß was also decreased by *G. lucidum* (Fig. 5C).

In summary, *G. lucidum* inhibits constitutive as well as inducible activation of NF-κB, and this inhibition can be mediated through ERα or ERß, or can be estrogen receptor independent.

G. lucidum down-regulates expression of c-myc in breast cancer cells. Increased expression of the oncogene c-myc has been detected in a majority of breast cancers (13). Because the expression of c-myc is controlled by estrogen and by NF-κB (15-17), we hypothesized that *G. lucidum* also modulates expression of c-myc in breast cancer cells. Although estradiol markedly induced expression of c-myc in MCF-7 cells, *G. lucidum* down-regulated expression of c-myc in a dose response manner (Fig. 6A). As expected, E2 did not stimulate c-myc expression in estrogen-independent MDA-MB-231 cells, and the constitutive c-myc expression was also down-regulated by *G. lucidum* in a dose-response manner (Fig. 6B). These results suggest that the inhibitory effects of *G. lucidum* on the growth of MCF-7 and MDA-MB-231 cells can be associated with the down-regulation of estrogen-dependent as well as estrogen-independent expression of c-myc.

Discussion

We have previously demonstrated that *G. lucidum* inhibits invasive behavior and proliferation of highly metastatic estrogen-independent breast cancer cells through the suppression of constitutively active NF-κB (21,22,30). Nevertheless, the effect of *G. lucidum* on the most pertinent signaling network involved in breast cancer - estrogen receptor signaling, remains to be elucidated. In the present study, we evaluated the effect of *G. lucidum* on the estrogen-dependent, poorly invasive breast cancer cells (MCF-7) and estrogen-independent, highly invasive breast cancer cells (MDA-MB-231). We showed that *G. lucidum* can mediate, in part, its anti-proliferative effect through the inhibition of ER and NF-κB signaling, resulting in the down-regulation of expression of c-myc in breast cancer cells (Fig. 7).

Estrogen receptor signaling is a complex process, which can be controlled at the level of expression of estrogen receptor or at the level of its function as a transcription factor regulating expression of estrogen-responsive genes. Here we show that *G. lucidum* down-regulated the expression of ERα in MCF-7 cells. This effect was specific for ERα because the expression of ERß in MCF-7 or MDA-MB-231 cells was not affected by the *G. lucidum* treatment. Since estrogen activities responsible for cell proliferation of breast cancer

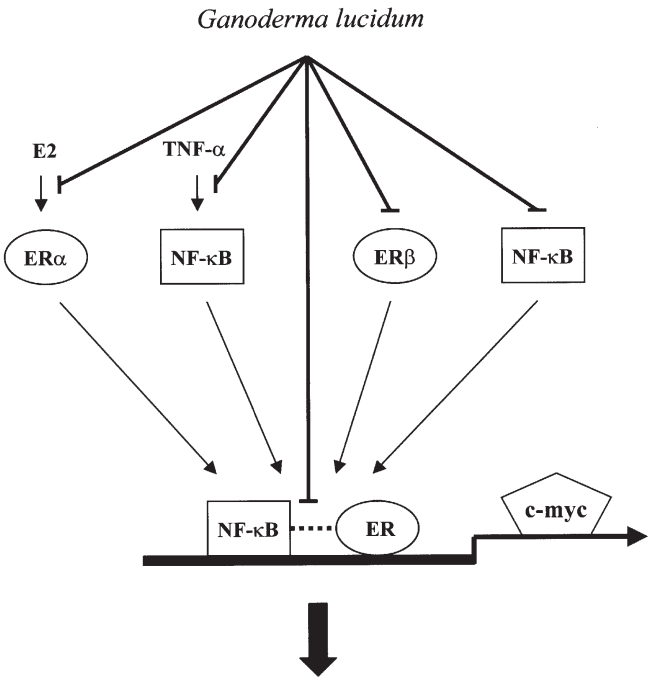

Figure 7. Proposed mechanisms for the inhibition of proliferation in estrogen-dependent and estrogen-independent breast cancer cells by *G. lucidum*. In estrogen-dependent MCF-7 cells, *G. lucidum* down-regulates the expression of ERα, inhibits E2-induced activation of ERα, and inhibits TNF-α-induced activation of NF-κB. In estrogen-independent MDA-MB-231 cells, *G. lucidum* inhibits constitutive trans-activation of ERß and NF-κB. *G. lucidum* also inhibits possible interaction between NF-κB and estrogen receptor, resulting in the down-regulation of expression of c-myc and inhibition of growth of human breast cancer cells.

cells are mediated through ERα (31), the down-regulation of ERα by *G. lucidum* resulted in the inhibition of proliferation of ERα-positive breast cancer cells. In contrast, *G. lucidum* did not down-regulate expression of ERß suggesting that *G. lucidum* inhibits proliferation of ERß-positive cells independently of ERß. Furthermore, *G. lucidum* suppressed growth of MDA-MB-231 through the inhibition of Akt/NF-κB signaling (22). Finally, the down-regulation of expression of ERα by ethanol extract of *G. lucidum* was also demonstrated in prostate cancer cells (32).

At the transcription level, *G. lucidum* suppressed estrogen signaling by the inhibition of transactivation of estrogen receptor using reporter gene construct linked to the estrogen response element (ERE). Therefore, *G. lucidum* inhibits constitutive as well as E2-induced ERE transactivation in estrogen-dependent MCF-7 cells, and constitutive ERE transactivation in estrogen-independent MDA-MB-231 cells. The suppression of transcriptional activity through ERα and ERß by *G. lucidum*, suggests that *G. lucidum* triterpenes could exert selective estrogen receptor modulator (SERM) activities. However, due to the complex composition of *G. lucidum* extract, containing polysaccharides and triterpenes, we were not able to detect specific binding to ER (not shown). Interestingly, structurally related triterpenes isolated from the plant *Ferula* modulated estrogenic activity also through the interaction with ERα and ERß (33). In addition, markedly lower doses of *G. lucidum* inhibited proliferation of MCF-7 (ERα-positive, ERß-positive) than of MDA-MB-231 (ERα-negative, ERß-positive) cells. This effect was mimicked by the typical SERM anti-estrogen tamoxifen, which inhibits ERα-negative breast cancer cells only at higher concentrations (34). Furthermore, inhibition of proliferation of breast cancer cells by *G. lucidum* is mediated by the cell cycle arrest at G_0/G_1 (22), which is a typical mechanism for anti-estrogens regulation of cell cycle (35).

We have previously demonstrated that *G. lucidum* inhibits constitutively active NF-κB in MDA-MB-231 cells (21). In the present study we show that *G. lucidum* also inhibits inducible activation of NF-κB independently of status of estrogen receptor in cells. Thus, *G. lucidum* inhibited NF-κB in cells which do not express ER (HEK-293), in cells expressing ERα (HEK-293 overexpressing ERα), in cells expressing ERß (MDA-MB-231 and HEK-293 overexpressing ERß) or in cells expressing ERα and ERß (MCF-7). However, estradiol and TNF-α-stimulated NF-κB activity was inhibited by *G. lucidum* only in cells expressing ERα, suggesting that *G. lucidum* modulates cross-talk between ER and NF-κB. Although the original model of the interaction between NF-κB and ERα suggested inhibition of NF-κB by E2 (36), here we demonstrate that E2 in fact, under certain conditions, may also induce NF-κB. Furthermore, the positive regulation of NF-κB- and ERα-responsive genes c-myc and cyclin D1, via the promoter association of IκB kinase (IKKα and ERα), resulted in the enhanced proliferation of breast cancer cells (37).

We demonstrate that *G. lucidum* down-regulated the E2-dependent as well as constitutive expression of c-myc in breast cancer cells. The inhibition of E2-stimulated c-myc expression in ERα-positive cells is consistent with our data demonstrating inhibition of E2-dependent trans-activation activity in MCF-7 cells by *G. lucidum*. Given that tamoxifen inhibits proliferation and c-myc expression in ERα-positive breast cancer cells (38-40) our data further suggest an anti-estrogenic effect of *G. lucidum*. However, the down-regulation of c-myc, which is constitutively active and is not activated by E2 in ERα-negative breast cancer cells, demonstrates the inhibitory effect of *G. lucidum* also on NF-κB pathway.

In conclusion, we present a possible mechanism by which *G. lucidum* inhibits proliferation of estrogen-dependent as well as estrogen-independent human breast cancer cells. The biological effects of *G. lucidum* on estrogen-dependent MCF-7 cells are mediated through the down-regulation of ERα expression, inhibition of estrogen-inducible ER trans-activation and inhibition of TNF-α-stimulated activation of NF-κB. The effects on estrogen-independent MDA-MB-231 cells are mediated through the suppression of constitutive activity of ERE and NF-κB. The inhibition of inducible- as well as constitutive ER and NF-κB pathways results in the down-regulation of expression of c-myc, finally resulting in the suppression of proliferation of estrogen-dependent and estrogen-independent human breast cancer cells. Further studies are in progress to identify biologically active compounds in *G. lucidum* with specificity against estrogen receptor and NF-κB signaling.

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