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ORIGINAL ARTICLE

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Estrogen-like activity of ethanol extract of *Ganoderma lucidum*

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Abstract The ethanol extract from the fruiting body of *Ganoderma lucidum* was tested for its estrogen-like activity by using the cell proliferation assay (MCF-7 cells, human breast cancer cells), as well as the estrogen receptor binding assay, and pS2 mRNA expression assay in MCF-7 cells in vitro and uterotrophic assay in vivo. The ethanol extract of *G. lucidum* showed significant positive effects on the proliferation of MCF-7 cells. This proliferation effect is related to the estrogenic activity of *G. lucidum*, because this proliferation activity was inhibited by the addition of the antiestrogenic compound ICI 182,780. The ability to bind to human estrogen receptors (hERs) α and β of the ethanol extract of *G. lucidum* was confirmed by using the coactivator-bacterial alkaline phosphatase system. ER-dependent cell responsibilities were investigated by examining the regulation of gene transcription for pS2 in MCF-7 cells. Our results demonstrated that the pS2 mRNA levels are significantly increased by the ethanol extract of *G. lucidum* via an estrogen-like manner. Additionally, young rats that received the ethanol extract of *G. lucidum* (200 mg/kg per day) for 3 days showed a significant increase (growth approximately twofold compared with the control group) in uterine weight after each treatment, which supports the estrogen-like activity of the ethanol extract of *G. lucidum* in vivo. It was concluded that the ethanol extract of *G. lucidum* showed estrogen-like activity, which may be useful in regulating hormone levels to treat related diseases such as osteoporosis if safety is fully guaranteed.

Key words *Ganoderma lucidum* · Estrogenic activity · Menopausal syndrome

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Introduction

Menopause brings about changes in the health of postmenopausal women that have a major impact on their lives. After menopause, osteoporosis associated with estrogen deficiency is the most common cause of age-related bone loss. Hormone replacement therapy (HRT) can resolve most postmenopausal problems, and the almost universal loss of skeletal mass in postmenopausal women can be prevented by estrogen replacement. However, compliance with HRT is poor because of the risks of breast and endometrial cancers associated with the long-term use of HRT.[1] Thus, a safe estrogenic ingredient originating from edible and medicinal natural products is needed.

Estrogens play an important role in bone maintenance, in the central nervous system, and in the cardiovascular system.[2] Estrogens carry out their action by binding to a high affinity nuclear receptor, the estrogen receptor (ER). Bound ER undergoes conformational change, interacts with chromatin, and modulates the transcription of target genes in estrogen-responsive tissues.[3] Many naturally occurring compounds, such as flavonoids, coumestan derivatives, and lignans, are nonsteroidal agents that have demonstrated weak estrogenic activity.[4] Recently, ginsenoside Rg1, a naturally derived triterpenoid isolated from *Panax notoginseng*, has been reported to have estrogen-like activity.[5]

The fruiting body of *Ganoderma lucidum* (Reishi or Ling-Zhi) is a well-known Chinese crude drug,[6] which has been used clinically in China, Japan, and Korea for hundreds of years as a tonic and sedative, as well as for the treatment of hypertension and chronic hepatitis.[7] It is also considered to promote longevity and help maintain vitality. Nowadays, this mushroom is used for leucopenia and is popular as a home remedy.[8] In a well-known book on Chinese herbal medicines written during the Ming dynasty, *P'en-Ts'ao Kang-Mu*, by Li Shin-Chen, the writer describes *G. lucidum* as being effective for menopausal troubles, which are known to be associated with estrogen deficiency. This information led us to further investigate the

medicinal properties of *G. lucidum*, especially its estrogenic activity.

The present study was designed to determine whether the ethanol extract of *G. lucidum* has estrogen-like activity. In this study, we evaluated the estrogen-like activity of the ethanol extract of the fruiting body of *G. lucidum*. To achieve this we measured the relative proliferative effects of MCF-7 cells and estrogen receptor (ERα, ERβ) binding assay, and pS2 mRNA expression of estrogen receptor (ER)-positive MCF-7 cells in vitro and uterotrophic assays in vivo to elucidate their ability to regulate the level of estrogen. Our findings indicated that the ethanol extract of *G. lucidum* does in fact protect against disorders caused by estrogen deficiency. Many beneficial effects of *G. lucidum* have been reported, but they may be mediated, at least in part, by the extract's estrogenic activity.

Materials and methods

The fruiting body of *Ganoderma lucidum* (strain BMC9049) was obtained from Bisoken (Oita, Japan). The chemicals used were 17β-estradiol (E2), Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), fetal bovine serum (FBS) (Gibco), activated charcoal, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO) (Wako, Osaka, Japan), glutamine (Nissui, Tokyo, Japan), penicillin, streptomycin, and trypsin (Invitrogen, Carlsbad, CA, USA).

Extract preparations of *Ganoderma lucidum*

The dried and chipped fruiting body of *G. lucidum* (15 kg) was extracted with 99% ethanol (126 l) at room temperature for 24 h by using a blender. The extracts were filtered through Advantec No. 2 filter papers, concentrated under vacuum, and then freeze-dried. The extracts (571.1 g) were used as ethanol extracts. Also, a portion of the residual *G. lucidum* after extraction with ethanol was reextracted with boiling water, and the aqueous extracts were freeze-dried and called water extracts.

Cell cultures

MCF-7 cells, which are estrogen-sensitive human breast cancer cells, were used in this study. The MCF-7 cells were kindly provided by Prof. K. Yamada (Kyushu University, Japan).

Charcoal treatment of serum

To remove the endogenous estrogens, 100 ml of FBS was treated twice with 5 g each of activated charcoal at 56°C for 30 min. Then the activated charcoal was removed by centrifugation at 10 000 *g* and 4°C for 20 min. This procedure was repeated twice, and the supernatant was filtered through a 0.22-µm cellulose acetate filter. The charcoal-treated FBS (cFBS) was then stored at –20°C until use.

Cell proliferation assay

The cells were maintained in a petri dish and incubated (37°C; 5% CO_2) until 70% confluence was reached. They were harvested weekly by 10-min exposure to 0.25% (w/v) trypsin, 1 mM EDTA in PBS and passaged at a 1:3 split ratio. The cells were subcultured into 24-well plates at a density of approximately 1×10^4 cells/well to determine the cell number. The cells were allowed to grow over 24 h in a humidified atmosphere of 5% CO_2 in air at 37°C until the cells adhered. To eliminate the effect of different factors in the growth media and the contribution of any estrogenic effect, the cells were placed under serum-free conditions for 24 h. The sample was dissolved in DMSO, and the final concentration of this solution in the culture medium of 0.1% did not affect the cell yields. The cells were detached using 0.25% (w/v) trypsin, 1 mM EDTA in PBS. The cell numbers were counted by a Bürker hemocytometer (Marienfeld, Marienfeld, Germany). The control condition also contained 0.1% DMSO. Three independent wells were used in the same 24-well plate to each sample, and repeated at least three times. Reproducible results were obtained. The average number of cells in the control was about 1×10^5 cells per well, so the results are expressed as the mean ± standard error. The mean of cell numbers from each experiment were normalized to the hormone-free control culture cells (100%) to correct for the difference in the initial plating density.

CoA-BAP method

This method was established by Kanayama et al.[9] The principle of in vitro assay for activation of ER induced by the ethanol extract of *G. lucidum* is based on protein–protein interactions on a microplate involving a GST-ER fusion protein and a BAP (bacterial alkaline phosphatase)-CoA (coactivator) fusion protein.

The assay was performed using the kit, TIF2-BAP-human ERα or ERβ (hERα or hERβ) (Microsystems, Kyoto, Japan) according to the manufacturer's instruction. Briefly, the experiment was performed as follows. All experiments were carried out on ice unless otherwise stated. A prepared microwell plate was rinsed with 120 µl of suspension buffer, followed by two further rinses with 150 µl of suspension buffer. Next, 100 µl of purified histidine-tagged hTIF2 NID-BAP protein (30 µg/ml) was added to the plate with an appropriate concentration of ethanol extract of *G. lucidum* or control solvent. After 1 h of incubation at 4°C, the plate was washed with 120 µl of wash buffer [50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 100 mM KCl, 0.1% Nondiet P-40]. Two further washes were then carried out with 150 µl of wash buffer. After removal of wash buffer, the enzyme reaction was started with the addition of 100 µl of a 10 mM NPP solution [100 mM Tris-HCl

(pH 8.0), 10 M *p*-nitrophenylphosphate (Nacalai Tesque, Kyoto)]. After incubation at 30°C for 3 h, the reaction was stopped with the addition of 25 µl of 0.5 N NaOH. Finally, the absorbance at 405 nm was measured with a microplate reader ELx 800 (Bio-Tec instruments). Alkaline phosphatase (AP) activity without GST-NR was also measured as background for this assay.

Real-time polymerase chain reaction analysis

Total RNA was extracted by use of Isogen (NipponGene, Toyama, Japan) from MCF-7 cells after being cultured for proliferation assay with or without ethanol extract of *G. lucidum*. Complementary DNA was synthesized in a final volume of 20 µl that included 1 µg of total RNA (4–5 µl of 0.2–0.3 µg/µl total RNA), 1 µM oligo-dT 18-mer primer, 10 units of RNase inhibitor, and 10 units of AMV reverse transcriptase (Takara, Japan) according to the manufacturer's instruction. After reverse transcription for 60 min at 45°C, the samples were heated for 5 min at 95°C to terminate the reaction. Real-time polymerase chain reaction (PCR) was performed in a final volume of 10 µl with a Line Gene (Bio Flux, Japan). The SYBR Premix Ex Taq kit (Takara, Japan) was used according to the manufacturer's instructions with a final concentration of 0.2 µM for each primer. PCR amplification was performed as follows: (1) an initial denaturation at 95°C for 1 min, (2) 45 cycles, with 1 cycle consisting of denaturation 95°C for 15 s, annealing at 60°C for 15 s, and elongation at 72°C for 30 s. Amplicon specificity was verified by melting-curve analysis conducted at 65° to 95°C with stepwise fluorescence acquisition and by 2% agarose gel electrophoresis staining with ethidium bromide. No fluorescence was detected from real-time PCR amplification without a template. Each primer sequence of pS2 (accession number: X52003) was 5′-TGG AGA ACA AGG TGA TCT GC-3′(forward) and 5′-ATC TGT GTT GTG AGC CGA AGG-3′(reverse), respectively. β-Actin was used as a reference gene (accession number: AY582799). Each primer sequence was 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ (forward) and 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′ (reverse), respectively. The ratio of gene specific expression was defined as relative expression to the actin expression.

Uterotrophic assays

The protocol for the uterotrophic assays was based on that described by Odum et al.[10] Female Fisher rats (21 days old; Charles river, Yokohama) were dosed by subcutaneous (sc) injection. Test agents were dissolved or homogeneously suspended in sesame oil (except where indicated otherwise). The dosing volume was 2 ml/kg body weight. Animals received three daily doses (20, 200 mg/kg per 2 ml oil) of the test sample and were killed by blood removal under anesthesia of pentobarbital 24 h after the final dose. Uteri were excised, trimmed free of fat, pierced, and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. The uterus was then weighed (wet weight). E2 was used as a positive control agent, and the study was accompanied by a vehicle (negative) control group.

Statistics

Results in vitro were expressed as mean ± standard error. Statistical significance was determined by Student's nonpaired *t*-test. Animal experiment results were expressed as mean ± standard error, statistical significance was determined by analysis of variance (ANOVA) and Bonferroni's multiple *t*-test.

Results

Effect of the extract of *Ganoderma lucidum* on the proliferation of MCF-7 cells

The MCF-7 cell proliferative assay is a well-established in vitro system characterized by its estrogen responsiveness through the expression of the estrogen receptor.[11] As shown in Fig. 1, E2 exhibited a strong proliferation-stimulating activity of about 145% in comparison with the control cells. The ethanol extract of *Ganoderma lucidum* exerted a proliferation-stimulation activity of more than 200% at 0.1 µg/ml. However, at a high concentration of more than 10 µg/ml, the ethanol extract lost the activity. The exact mechanism involved in the dose-dependent change in activ-

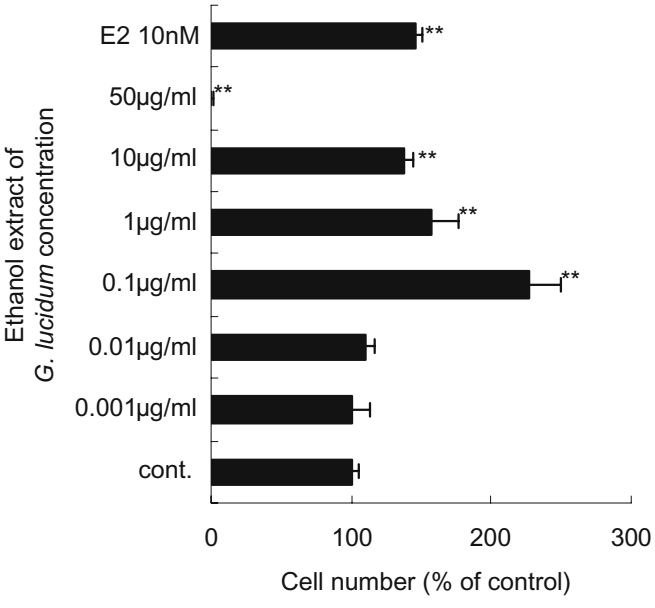

Fig. 1. Effect of ethanol extract of *Ganoderma lucidum* on the proliferation increase in MCF-7 cells. The average cell number of control was 1.03×10^5 cells/well. The average cell number of E2 was 1.47×10^5 cells/well. *Double asterisk* indicates a significant difference between control and ethanol extract of *G. lucidum*-treated cells as analyzed by Student's nonpaired *t*-test ($P < 0.01$)

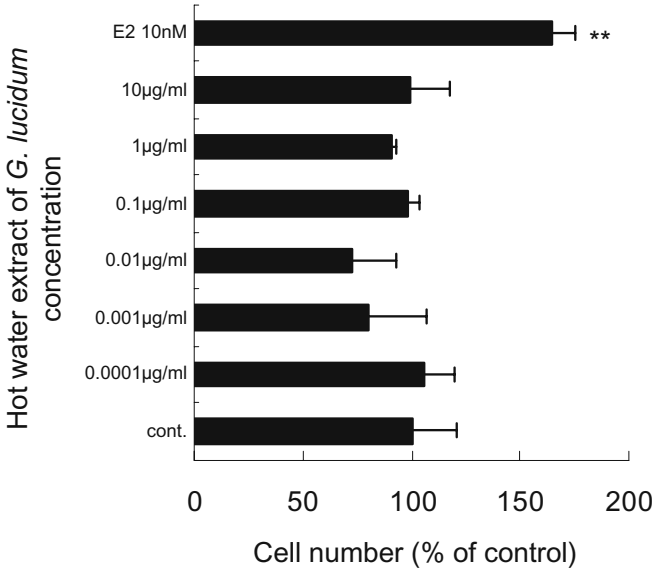

Fig. 2. Effect of hot water extracts of *G. lucidum* on the proliferation increase in MCF-7 cells. The average cell number of control was 1.08×10^5 cells/well. The average cell number of E2 was 1.66×10^5 cells/well. *Double asterisk* indicates a significant difference between control and *G. lucidum* hot water extracts or E2-treated cells as analyzed by Student's nonpaired *t*-test ($P < 0.01$)

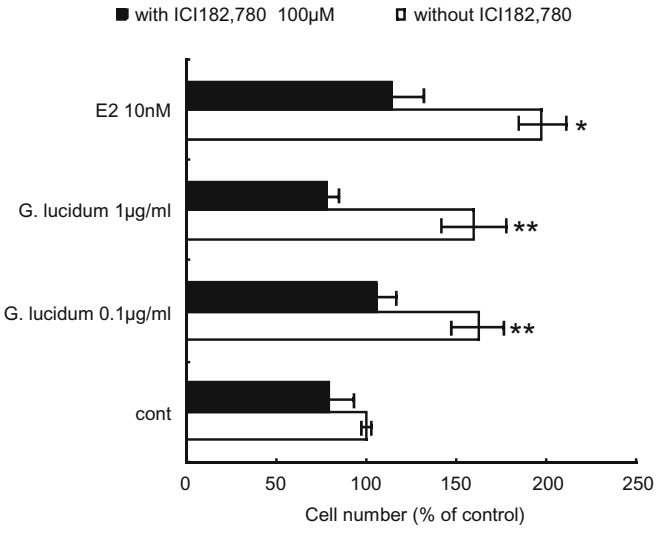

Fig. 3. Effect of ethanol extract of *G. lucidum* on the cell proliferation in MCF-7 cells. The average cell number of control was 1.05×10^5 cells/well. The average cell number of E2 was 1.96×10^5 cells/well. *Asterisks* indicate a significant difference between control and hot water extracts of *G. lucidum*-treated cells as analyzed by Student's nonpaired *t*-test (*$P < 0.05$, ** $P < 0.01$)

ity remains unclear. However, this phenomenon might be caused by the extract's cytotoxicity, at least in part.[12] Additionally, a recent report indicated that the *G. lucidum* extract can induce cell cycle arrest and apoptosis in MCF-7.[13] The concentration of ethanol extract for the maximal stimulation effect was 0.1 μg/ml (Fig. 1). In contrast, the water extract, which was prepared from *G. lucidum* after extraction with ethanol, did not show any activity (Fig. 2).

Ganoderma lucidum has been well known to produce polysaccharides[14] and oxygenated triterpenoids with a very broad spectrum of biological activities and pharmacological functions.[6] Triterpenoids show antiandrogenic activity, such as 5α-reductase inhibition,[15] and polysaccharides have been found to possess a potent antitumor effect, attributed to their immunomodulation property. As shown in Figs. 1 and 2, the ethanol extract of *G. lucidum* showed the proliferation increase in MCF-7, but hot water extracts did not. These results indicated that the active ingredients of *G. lucidum* are hydrophobic components, such as triterpenoids, and not polysaccharides. The steroid backbone of the triterpenoids in *G. lucidum* may make it a suitable candidate to interact and activate estrogen receptors.

ER-dependent action of the ethanol extract of *G. lucidum* on the proliferation of MCF-7 cells

To determine whether the proliferation-stimulating activity on MCF-7 cells is mediated via the ER, cells were incubated with the ethanol extract of *G. lucidum* in the presence or absence of the estrogen antagonist ICI 182,780. ICI 182,780 (100 μM) completely blocked the proliferation-stimulating activity on MCF-7 cells by the ethanol extract of *G. lucidum* and E2 (Fig. 3). The action of the ethanol extract of *G. lucidum* in MCF-7 can be specifically blocked by treating with an ER antagonist (ICI 182,780), providing further support for the role of activated ER in mediating its action in these cells.

Dietary phytoestrogens have been the focus of investigations for their potential beneficial effects on cancer prevention and the treatment of postmenopausal syndromes. Current lists of dietary phytoestrogens include isoflavones,[16] coumestans,[16] resveratrol,[17] and 8-prenylnaringenin.[18] The relative affinities of these compounds toward ER are in the microgram-per-milliliter range, similar to those of the ethanol extract of *G. lucidum*. More specifically, the effective concentration of the ethanol extract of *G. lucidum* in the stimulation of MCF-7 cell growth is in the microgram-per-milliliter range, as shown in Fig. 1.

ER binding assay against ethanol extract of *G. lucidum*

We examined the binding activity to hERs α and β of the ethanol extract of *G. lucidum* that stimulated the growth of MCF-7 cells by CoA-BAP system. The ethanol extract of *G. lucidum* bound quite poorly to both hERs compared with the E2 (Fig. 4). The ethanol extract of *G. lucidum* showed slight hER α and β-dependent AP activity induction.

Stimulation of endogenous estrogen responsive genes in MCF-7 cells

To evaluate the potential of the ethanol extract of *G. lucidum* as an activator of estrogen-responsive genes, we

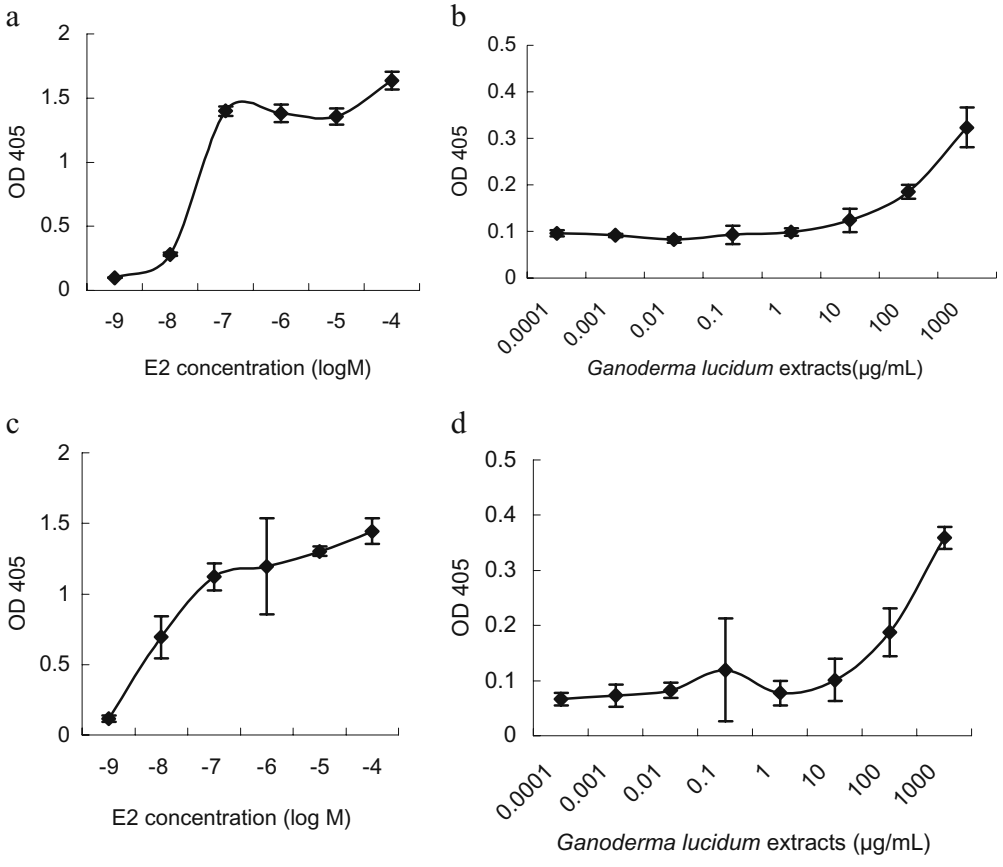

Fig. 4a–d. Estrogen receptor (ER)-binding assay by coactivator-bacterial alkaline phosphatase (CoA-BAP) system. ER-binding methods were used to determine **a** the binding affinity of E2 to ERα, **b** the binding affinity of ethanol extracts of *G. lucidum* to ERα, **c** the binding affinity of E2 to ERβ, and **d** the binding affinity of ethanol extracts of *G. lucidum* to ERβ

examined pS2 mRNA induction in MCF-7 cells after treatment with the ethanol extract of *G. lucidum*. Steady-state pS2 mRNA levels were measured by carrying out real-time PCR assays on total RNA prepared from MCF-7 cells treated with the samples. As an internal control, constitutively expressed human β-actin mRNA was used. As shown in pS2, the mRNA expression was induced after E2 treatment, as is well known. The ethanol extract of *G. lucidum* activated transcription of the pS2 gene (Fig. 5). These data confirm that the ethanol extract of *G. lucidum* can act as an ER agonist.

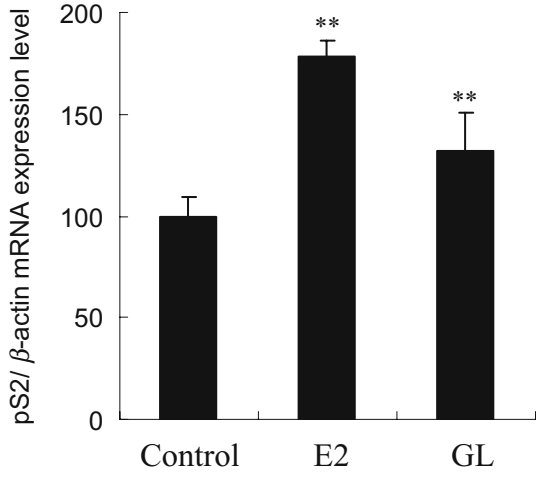

Fig. 5. pS2 expression in MCF-7-derived cells. Relative pS2 mRNA levels were determined using a real-time polymerase chain reaction assay. Regulation of pS2 mRNA expression by 10 nM E2 and 0.1 μg/ml ethanol extracts of *G. lucidum* (*GL*). Each value is the mean ± standard deviation of three determinations. *Double asterisk* indicates significant difference between control and ethanol extracts of *G. lucidum* or E2-treated cells as analyzed by Student's nonpaired *t*-test ($P < 0.01$)

Immature uterus tests

The uterine effects were also studied by treating prepubertal 3-week-old female rats injected for 3 days with increasing doses of ethanol extract of *G. lucidum* and by comparing the effects with those of E2. Figure 6 shows that the ethanol extract of *G. lucidum* had a slight intrinsic agonistic activity. The effect of E2 is more than 5000 times as strong as that of the ethanol extract of *G. lucidum.*

Discussion

In our study, we found that the ethanol extract of *Ganoderma lucidum* had estrogenic activity on MCF-7 cells. Coadministration of ICI 182,780 virtually blocked the proliferation-stimulatory effects induced by the ethanol extract of *G. lucidum*. This complete reversal indicated that the ethanol extract of *G. lucidum* exerted its effect through estrogen receptors in MCF-7 cells.

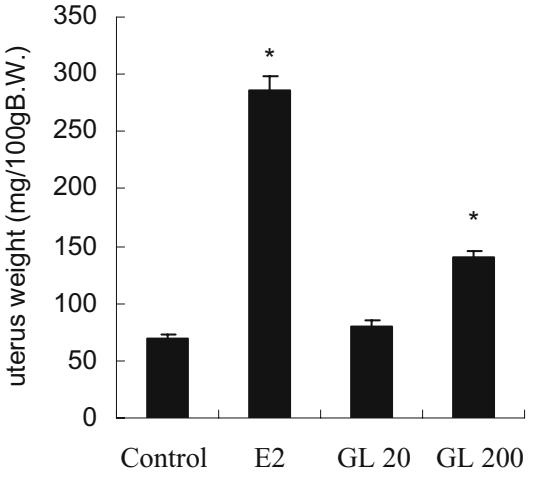

Fig. 6. The effect of E2 (0.04 mg/kg) and ethanol extracts of *G. lucidum* (*GL*) (20 or 200 mg/kg) on uterus wet weight in immature rats. Controls received sesame oil only. Data represent group means ± standard error (control and GL groups, $n = 3$; E2, $n = 4$). *Asterisk* indicates significant difference between control and ethanol extracts of *G. lucidum* or E2-treated animals as analyzed by analysis of variance and Bonferroni's multiple *t*-test ($P < 0.05$)

It is reported that the proliferation rate of MCF-7 cells is modulated markedly by the conditions under which the cells are grown. The MCF-7cells respond almost immediately to the lack of estrogens with a decreased proliferation rate. The reduced rate of proliferation in the absence of estrogens is maintained by MCF-7 cells for at least 1 month. For 5 or 6 months, E2 was not able to further stimulate the proliferation of these long-term withdrawn cells, and the antiestrogen was still inhibitory to cell proliferation.[19] The same phenomenon was observed in our experiment (Figs. 1–3) and is the reason for the different proliferation of E2 in each experiment.

From our result, the ethanol extract of *G. lucidum* showed very weak binding activity to hERα and hERβ (Fig. 4). Recently, membrane estrogen binding sites (mER) were found in breast cancer cell lines MCF-7.[20] In paraffin tissue sections of breast cancer patients, estrogen membrane receptor was identified in cancerous tissue. In breast cancer cells, the fact that estrogen effects were reported in the absence of classical steroid receptors suggested the existence of a distinct membrane site. The ethanol extracts of *G. lucidum* may act on this membrane estrogen binding site rather than binding to the ERα and ERβ. Our results demonstrate that the pS2 mRNA levels are significantly increased by the ethanol extract of *G. lucidum* via an estrogen-like manner. Additionally, young rats that received the ethanol extract of *G. lucidum* (200 mg/kg per day) for 3 days showed a significant increase (growth of approximately twofold compared with the control group) in uterine weight after each treatment, which supports the estrogen-like activity of the ethanol extract of *G. lucidum* in vivo. It was concluded that the ethanol extract of *G. lucidum* showed estrogen-like activity, which may be useful in regulating the hormone levels to treat related diseases (osteoporosis, etc.) if safety is fully guaranteed.

When activated by E2, ERα plays an important role in the stimulation of cancer cell proliferation and prevention of apoptosis. The biological actions of E2 are mediated both by genomic transcriptional effects in the nucleus and by nongenomic actions via ERα acting outside the nuclear compartment. Depending on the cell type and context, the nongenomic effects of E2 can lead to rapid activation of many signaling molecules, such as (1) IGF-I receptor (IGF-IR) and epidermal growth factor receptor (EGFR), (2) $p21^{ras}$ and Raf-1, (3) MAPK and Akt, (4) protein kinase C, (5) release of nitric oxide and stimulation of prolactin secretion, and (6) alteration of calcium and Maxi-K channels. Both genomic and nongenomic actions of E2 play pivotal roles in E2-induced cancer cell proliferation and survival.

Genistein inhibits hormone-dependent breast cancer cells.[21] The important action of genistein, which may contribute to its chemopreventative properties, is inhibition of the EGFR tyrosine kinase.[22] The known effects of tamoxifen on the IGF system in breast cancer cells include: inhibition of IGF-I stimulated growth, modulation of IGFBP expression, reduced secretion of autocrine IGF, down-regulation of plasma levels of IGF-I in breast cancer patients, and decreased levels of IGF-I binding sites.[23] However, at concentrations less than 10 μmol/l, MCF-7 cell growth is stimulated by genistein.[24–26] This situation may be analogous to tamoxifen as noted by Bouker and Hilakivi-Clarke[27] in that in male rat anterior pituitary cells, low (100 nmol/l) concentrations of tamoxifen increased prolactin secretion, but, at higher concentrations, estrogen-stimulated prolactin secretion was completely inhibited by tamoxifen.[28]

In our study, the ethanol extracts of *G. lucidum* showed both stimulation and inhibition effect on proliferation of MCF-7 cell. The components of *G. lucidum* may have the same effects as genistein and tamoxifen. These components exerted estrogen-like effects at low concentrations that may be considered physiologic concentrations, but at high concentrations exerted other effects, like inhibition of the activity of one or more cellular molecules that control cell signaling, growth, and death.

Safety is a primary consideration in the use of natural products, such as those from *G. lucidum* with estrogenic activity, and especially those in food and supplemental products, which may be utilized in unregulated quantities on a regular basis. In this study, we showed that the extract of *G. lucidum* has a proliferation effect on breast cancer cells (MCF-7) under limited experimental conditions, more specifically in cFBS, in which the hydrophobic steroidal hormone was removed by treatment with charcoal. This result might be linked to carcinogenicity. The potential application of *G. lucidum* in the treatment of estrogen-dependent symptoms, such as those found in postmenopausal women, requires further detailed study on its potential risk of contributing to the development of breast and endometrial cancers. However, the risk may be negligible, because many reports about the anticancer effect of *G. lucidum* have been published.[29] Furthermore, it has been reported that the ethanol extract of *G. lucidum* is able to induce cell cycle arrest at the G1 phase and induce apoptosis in MCF-7 cells with FBS, not cFBS,[13] and *G. lucidum*

has been demonstrated to possess antitumor activities,[30] immunomodulatory activities,[31] neuroactive effects,[32] and antiandrogen activity.[33] In addition, *G. lucidum* extract was described to have no effect on genotoxic chromosomal breakage and no cytotoxic effects in a mouse test.[34] The fruiting body of *G. lucidum* has proven its safety through many years of human medicinal use and consumption. Recent studies also showed that the alcohol extract or the triterpene fraction of *G. lucidum* possessed an antitumor effect, which seems to be related to the cytotoxic activity against tumor cells directly. Preliminary study indicated that the antiangiogenic effect may be involved in the antitumor activity of *G. lucidum*.[35] Although many beneficial effects of *G. lucidum* have been reported as described above, most of the mechanisms remain unclear. However, they might be mediated, at least in part, by the extract's estrogenic activity.

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References

1. Persson I, Weiderpass E, Bergkvist L, Bergstrom R, Schairer C (1999) Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. Cancer Causes Control 10:253–260
2. Sudhir K, Komesaroff PA (1999) Clinical review 110: cardiovascular actions of estrogens in men. J Clin Endocrinol Metab 84: 3411–3415
3. Cassidy A (1999) Potential tissue selectivity of dietary phytoestrogens and estrogens. Curr Opin Lipidol 10:47–52
4. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
5. Chan RY, Chen WF, Dong A, Guo D, Wong MS (2002) Estrogen-like activity of ginsenoside Rg1 derived from *Panax notoginseng*. J Clin Endocrinol Metab 87:3691–3695
6. Wasser SP, Weis AL (1999) Therapeutic effects of substances occurring in higher basidiomycetes mushrooms: a modern perspective. Crit Rev Immunol 19:65–96
7. Shiao MS (2003) Natural products of the medicinal fungus *Ganoderma lucidum*: occurrence, biological activities, and pharmacological functions. Chem Rec 3:172–180
8. Lieu CW, Lee SS, Wang SY (1992) The effect of *Ganoderma lucidum* on induction of differentiation in leukemic U937 cells. Anticancer Res 12:1211–1215
9. Kanayama T, Mamiya S, Nishihara T, Nishikawa J (2003) Basis of a high-throughput method for nuclear receptor ligands. J Biochem 133:791–797
10. Odum J, Lefevre PA, Tittensor S, Paton D, Routledge EJ, Beresford NA, Sumpter JP, Ashby J (1997) The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. Regul Toxicol Pharmacol 25:175–188
11. Leclercq G, Heuson JC (1979) Physiological and pharmacological effects of estrogens in breast cancer. Biochem Biophys Acta 560:427–455
12. Wu TS, Shi LS, Kuo SC (2001) Cytotoxicity of *Ganoderma lucidum* triterpenes. J Nat Prod 64:1121–1122
13. Hu H, Ahn NS, Yang X, Lee YS, Kang KS (2002) *Ganoderma lucidum* extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. Int J Cancer 102:250–253
14. Hsu HY, Hua KF, Lin CC, Lin CH, Hsu J, Wong CH (2004) Extract of Reishi polysaccharides induces cytokine expression via TLR4-modulated protein kinase signaling pathways. J Immunol 173:5989–5999
15. Soule HD, Vazguez J, Long A, Albert S, Brennan M (1973) A human cell line from a pleural effusion derived from a breast carcinoma. J Nat Cancer Inst 51:1409–1416
16. Liu J, Fujita R, Shimizu K, Konishi F, Noda K, Kumamoto S, Kaneko S, Suimi Y, Kondo R (2005) The effect of strain, growth stage and cultivating condition of *Ganoderma lucidum* on 5α–reductase inhibition. J Wood Sci 51:189–192
17. Tham DM, Gardner CD, Haskell WL (1998) Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. J Clin Endocrinol Metab 83:2223–2235
18. Brooks SC, Locke ER, Soule HD (1973) Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 248:6251–6253
19. Katzenellenbogen BS, Rendra KL, Norman MJ, Berthois Y (1987) Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence. Cancer Res 47:4355–4360
20. Berthois Y, Pourreau-Schneider N, Gandilhon P, Mittre H, Tubiana N, Martin PM (1986) Estradiol membrane binding sites on human breast cancer cell lines. J Steroid Biochem 25:963–972
21. Constantinou A, Huberman E (1995) Genistein as an inducer of tumor cell differentiation: possible mechanisms of action. Proc Soc Exp Biol Med 208:109–115
22. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262:5592–5595
23. Guvakova MA, Surmacz E (1997) Tamoxifen interferes with the insulin-like growth factor I receptor (IGF-IR) signaling pathway in breast cancer cells. Cancer Res 57:2606–2610
24. Zava DT, Duwe G (1997) Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. Nutr Cancer 27:31–40
25. Miodini P, Fioravanti L, Di Fronzo G, Cappelletti V (1999) The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. Br J Cancer 80:1150–1155
26. Fioravanti L, Cappelletti V, Miodini P, Ronchi E, Brivio M, Di Fronzo G (1998) Genistein in the control of breast cancer cell growth: insights into the mechanism of action in vitro. Cancer Lett 130:143–152
27. Bouker KB, Hilakivi-Clarke L (2000) Genistein: does it prevent or promote breast cancer? Environ Health Perspect 108:701–708
28. Martinez-Campos A, Amara JF, Dannies PS (1986) Antiestrogens are partial estrogen agonists for prolactin production in primary pituitary cultures. Mol Cell Endocrinol 48:127–133
29. Lin SB, Li CH, Lee SS, Kan LS (2003) Triterpene-enriched extracts from *Ganoderma lucidum* inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. Life Sci 72:2381–2390
30. Wang SY, Hsu ML, Hsu HC, Tzeng CH, Lee SS, Shiao MS, Ho CK (1997) The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. Int J Canc 70:699–705
31. Bao XF, Wang XS, Dong Q, Fang JN, Li XY (2002) Structural features of immunologically active polysaccharides from *Ganoderma lucidum*. Phytochem 59:175–181
32. Cheung WM, Hui WS, Chu PW, Chiu SW, Ip NY (2000) Ganoderma extract activates MAP kinases and induces the neuronal differentiation of rat pheochromocytoma PC12 cells. FEBS Lett 486:291–296
33. Fujita R, Liu J, Shimizu K, Konishi F, Noda K, Kumamoto S, Kurashiki K, Kondo R (2005) Anti-androgenic activities of *Ganoderma lucidum*. J Ethnopharmacol 102:107–112
34. Chiu SW, Wang ZM, Leung TM, Moore D (2000) Nutritional value of Ganoderma extract and assessment of its genotoxicity and antigenotoxicity using comet assays of mouse lymphocytes. Food Chem Toxicol 38:173–178
35. Lin ZB, Zhang HN (2004) Anti-tumor and immunoregulatory activities of *Ganoderma lucidum* and its possible mechanisms. Acta Pharmacol Sin 25:1387–1395