

Ginsenoside Rp1 Inhibits Proliferation and Migration of Human Lung Cancer Cells

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Abstract

Ginsenoside Rp1 (G-Rp1) is a novel ginseng saponin derivative with anti-tumor activity. However, the biochemical and molecular mechanisms of G-Rp1 on anti-tumor activity are not fully understood. In the present study, we report that G-Rp1 inhibits lung cancer cell proliferation, migration and adhesion in p53 wild-type A549 and p53-deficient H1299 cells. Anti-proliferative activity of G-Rp1 in lung cancer cells is mediated by enhanced nuclear localization of cyclin-dependent kinase inhibitors including p27^{Kip1} and p21^{WAF1/Cip1}, and subsequent inhibition of pRb phosphorylation. We also show that these anti-tumor activities of G-Rp1 in both A549 and H1299 cells appear to be mediated by suppression of mitogenic signaling pathways such as ERK, Akt and p70^{S6K}. Taken together, these findings suggest further development and evaluation of G-Rp1 for the treatment of lung cancers with mutated p53 as well as wild-type p53.

Key Words: Ginsenoside Rp1, Cell proliferation, Cell migration, p53, Lung cancer

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), has long been used as traditional medicine for thousands of years in Korea, China, and Japan. It is well appreciated that ginseng is one of the most extensively used herbal medicines around the world. Ginsenosides, the main active ingredients of ginseng, mediate multiple pharmacological activities such as antioxidant, anti-inflammatory, immunostimulatory, anti-diabetic and anti-tumorigenic activities (Attele *et al.*, 1999; Sala *et al.*, 2002; Lee *et al.*, 2007; Qi *et al.*, 2011), however, cellular and molecular mechanisms of ginsenoside activities are not fully understood. Ginsenosides are triterpenoid saponins that have a common four-ring hydrophobic steroid-like structure with sugar moieties. More than 30 different types of ginsenosides have been identified from ginseng (Baek *et al.*, 1996; Tachikawa *et al.*, 1999). Based on different carbohydrate moieties at the C-3, and C-6 positions, ginsenosides are mainly classified into three groups: protopanaxadiols, protopanaxatriols, and the oleanolic acid derivatives (Tachikawa *et al.*, 1999; Qi *et al.*, 2011). The binding of sugar derivatives to the side chains (C-3, C-6, or C-20 position) in ginsenosides mediates the diverse biological and pharmacological effects

on the regulation of central nervous system, cardiovascular system, endocrine secretion, lipid metabolism, and stress-related diseases (Choi *et al.*, 2001; Shibata, 2001; Leung *et al.*, 2006; Yue *et al.*, 2006). In addition, many studies have reported that ginsenosides have anti-tumor activities *in vitro* and *in vivo* (Yun, 2001; Yun, 2003; Wang *et al.*, 2009; Park *et al.*, 2011). Among the ginsenosides, Rg1 functions to induce angiogenic responses related to cell migration, adhesion and proliferation (Leung *et al.*, 2006; Shi *et al.*, 2009), however, Rg3 has anti-angiogenic and anti-tumor activities (Yue *et al.*, 2006). In addition, Rg5 shows more potent anti-tumor and chemopreventive activities than Rg3 (Park *et al.*, 2002), but its chemical instability limits the development of Rg5 for cancer therapeutics (Han *et al.*, 1982).

A novel ginsenoside derivative, ginsenoside Rp1(G-Rp1) [3-O-β-D-Glucopyranosyl(1→2)-β-D-glucopyranosyl dammarane-3β, 12β-diol] has recently been prepared on a large scale from crude ginsenosides (Kumar *et al.*, 2006; Park *et al.*, 2008). G-Rp1 is more stable, and shows stronger *in vitro* and *in vivo* anti-tumor activity than Rg3 (Kumar *et al.*, 2006; Park *et al.*, 2008; Kim and Cho, 2009; Kumar *et al.*, 2009). Although significant advances have been made in understanding the function of G-Rp1 and in improvement of stability and solubil-

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pISSN: 1976-9148 eISSN: 2005-4483

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Received Jun 29, 2011 Revised Aug 9, 2011 Accepted Aug 9, 2011

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ity, the diverse biological effects and molecular mechanisms of G-Rp1 on cellular behaviors such as cell proliferation, migration, and adhesion still remain unexplored. In the present study, we evaluated the efficacy and molecular mechanisms of G-Rp1 on proliferation, migration and adhesion in p53 wild-type A549 and p53-deficient H1299 cells.

MATERIALS AND METHODS

Cell culture conditions

Human lung carcinoma cells (A549, H1299) from American Tissue Culture Collection (Manassas, VA, USA) were grown in 10% fetal bovine serum-Dulbecco's Modified Eagle's Medium (FBS-DMEM) (HyClone Laboratories, Logan, UT, USA).

Reagents

G-Rp1 of 97% purity was prepared using established protocols (Kumar *et al.*, 2006; Park *et al.*, 2008). The stock solution (10 mM) of G-Rp1 was dissolved in 100% dimethyl sulfoxide. The following antibodies were purchased from commercial sources: anti-phospho-ERK (T202/Y204), anti-phospho-Akt (Ser473), anti-phospho-p70^{S6K} (T421/S424), anti-phospho-pRb (S780, S807/811), and anti-Lamin A/C (Cell Signaling, Beverly, MA, USA) antibodies; anti-ERK, anti-Akt, anti-p70^{S6K}, anti-p27^{Kip1}, anti-p21^{WAF1/Cip1}, anti-actin antibodies, and mouse and rabbit IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell growth assay

Subconfluent A549 and H1299 cells, plated on 6-well plates (1×10^5 cells/well), were grown in basal DMEM without serum for 48 h to synchronize cells in G₁/G₀ phase of cell cycle, pretreated with G-Rp1 at different concentrations (1-20 μ M) for 30 min, and further incubated with 10% FBS-DMEM for 24 h. Following culture for 24 h, the cell numbers were quantified using trypan blue exclusion method. The results from triplicate determinations (mean \pm standard deviation) are presented as the numbers of cells per culture.

Migration assay

Cell migration was quantified in the *in vitro* wound-healing assay as described previously (Cho *et al.*, 2011). After cells were plated on 48-well plates, grown to confluence, and a single wound was created in the center of the cell monolayer by the gentle removal of the attached cells with a sterile plastic pipette tip. Cells were pretreated with or without G-Rp1 (10, 20 μ M) for 30 min, followed by serum stimulation for 32 h (A549) and 20 h (H1299), respectively. Cells were fixed with methanol, and then stained with 0.04% GIEMSA staining solution (Sigma-Aldrich, St. Louis, MO, USA). The migration of the cells into the wound was observed with still images taken at the indicated time point.

Adhesion assay

Subconfluent cells were detached with trypsin and allowed to recover in 10% FBS-DMEM for 1 h at 37°C with gentle rocking. After recovery, the cells were collected by low-speed centrifugation and resuspended in serum-free DMEM. The cell suspension were pretreated with or without G-Rp1 for 30 min, and followed by serum treatment. The cells were plated on 96-well plates (1×10^4 cells/well), and further incubated for 2 h

at 37°C. Following incubation unattached cells were removed by washing the wells three times with ice-cold phosphate-buffered saline (PBS). Attached cells were fixed with methanol, and then stained with 0.04% GIEMSA staining solution. The cells were photographed and counted. The results (mean \pm standard deviation) are presented as the numbers of adherent cells.

Western blot analysis

Subconfluent cells in 100 mm dishes (BD biosciences, Bedford, MA, USA) were serum-starved for 48 h in DMEM and replaced with fresh media, followed by treatments for different time points, as indicated, at 37°C. Cells were rinsed twice with ice-cold PBS and lysed by incubation in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 100 μ g/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 0.5 μ g/ml leupeptin, 80 mM β -glycerophosphate, 25 mM NaF and 1 mM sodium orthovanadate for 30 min at 4°C. Cell lysates were clarified at 13,000 \times g for 20 min at 4°C, and the supernatants were subjected to Western blot as described previously (Seo *et al.*, 2003; Seo *et al.*, 2008).

Subcellular fractionation

Following treatments as indicated, cells were rinsed twice with ice-cold PBS, and cytoplasmic and nuclear extracts were prepared using Nuclear/Cytosol Fractionation Kit (BioVision Inc., Mountain View, CA, USA), according to the manufacturer's instructions (Seo *et al.*, 2006).

Zymogram analysis

Activities of MMP-2 were measured by zymography (Alper *et al.*, 2001). Aliquots of conditioned medium were diluted in sample buffer, applied to 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich Co.) as a substrate. After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 1 h to remove SDS and allow re-naturalization of MMPs, and further incubated in developing buffer containing 50 mM Tris (pH 7.5), 10 mM CaCl₂, and 150 mM NaCl for 20 h at 37°C. The gels were stained with 0.5% Coomassie brilliant blue R-250 in 30% methanol-10% acetic acid) for 2 h and followed by destaining with 30% methanol-10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of the Coomassie blue-stained gelatin.

Statistical analysis

Statistical analysis was performed using Student's *t* test, and was based on at least three different experiments. The results were considered to be statistically significant when $p < 0.05$.

RESULTS

G-Rp1 inhibition of cell proliferation is mediated by induction of Cdk inhibitor nuclear localization and pRb hypophosphorylation

Previous studies have reported that ginsenoside Rp1 (G-Rp1) possesses the potential chemopreventive and anti-metastatic activities (Kumar *et al.*, 2006; Park *et al.*, 2008; Kumar *et al.*, 2009). These studies suggest that G-Rp1 can alter the signaling pathways and sequentially regulate the ex-

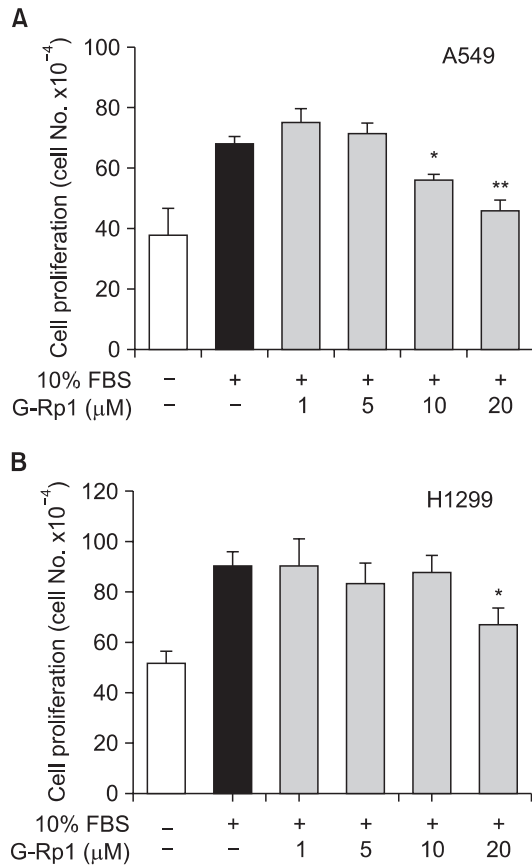


Fig. 1. G-Rp1 inhibits cell proliferation in A549 and H1299 cells. Quiescent A549 (A) and H1299 (B) cells were pretreated with or without G-Rp1 at different concentrations ranging from 1 to 20 μM for 30 min, followed by 10% serum stimulation for 24 h. Values represent the mean ± S.D. of three independent experiments. Statistical significance is indicated (**p*<0.05, ***p*<0.01, compared with 10% serum-treated cells).

pression and/or activation of cell mitogenesis-related proteins. However, little is known about functional relationship between G-Rp1-mediated inhibition of cell mitogenesis and tumor suppressor protein expression. We first examined the ability of G-Rp1 to regulate cell proliferation in p53 wild-type A549 and p53-deficient H1299 cells. G-Rp1 pretreatment significantly suppressed cell proliferation in a dose-dependent manner (Fig. 1). H1299 cells were less sensitive to G-Rp1 (10, 20 μM) treatment, as compared with A549 cells, raising the possibility that G-Rp1 might regulate cell proliferation by p53-dependent mechanism. However, our initial experiments demonstrate that G-Rp1 markedly inhibits p53-deficient SKOV-3 ovarian cancer cell proliferation to levels similar to that observed in A549 and HepG2 cells which express wild-type p53 (Hong and Seo, Data not shown). This observation demonstrates that G-Rp1 potently inhibits cell proliferation of diverse cancer cell lines and this anti-proliferative effect and potency appear to be dependent on the specific cell or tissue types, independent of p53 expression levels. Based on these findings, we next analyzed the phosphorylation status of retinoblastoma protein (pRb). During cell cycle progression, pRb is sequentially phosphorylated by cyclin-dependent kinase (cdk)/cyclin complexes, resulting in transition from G₁ to S phase of the

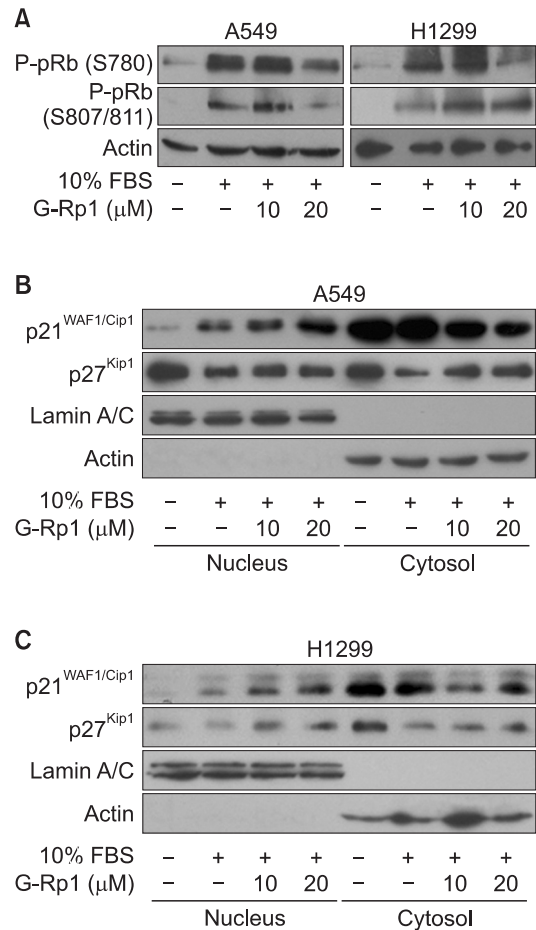


Fig. 2. G-Rp1 suppression of cell proliferation is mediated by nuclear localization of p27^{Kip1} and p21^{WAF1/Cip1}, and inhibition of pRb phosphorylation. (A) Quiescent cells were pretreated with or without G-Rp1 (10, 20 μM) for 30 min, followed by 10% serum stimulation for 24 h. Cell lysates were Western-blotted with anti-phospho-pRb, anti-pRb, or anti-actin antibodies. Quiescent A549 (B) and H1299 (C) cells were treated with G-Rp1 as in (A), and subjected to subcellular fractionation into nuclear and cytosolic extracts as described in Materials and Methods section. The fractions were Western-blotted with anti-p21^{WAF1/Cip1}, anti-p27^{Kip1}, anti-Lamin A/C, or anti-actin antibodies. Lamin A/C served as a marker for the nucleus and actin for the cytosol. Results shown are representative of three independent experiments.

cell cycle (Harbour *et al.*, 1999; Malumbres and Barbacid, 2001). Specifically, we examined the phosphorylation of pRb on residues Ser 780 and Ser 807/811, sites specific for cdk4 and cdk2 phosphorylation, respectively. As shown in Fig. 2A, G-Rp1 pretreatment dose-dependently blocked pRb phosphorylation at both sites in A549 cells. In contrast, G-Rp1 inhibited pRb phosphorylation at Ser 780 residue only in H1299 cells, but did marginally induce the phosphorylation of pRb at Ser 807/811 residue (Fig. 2A). This finding may partially explain the marginal effect of G-Rp1 on regulating H1299 cell proliferation. Collectively, G-Rp1-mediated inhibition of pRb phosphorylation is consistent with anti-proliferative activity of G-Rp1 in lung cancer cells, possibly through inhibition of cdk activities.

It has been reported that cell cycle progression specifically

requires activation of cdks through formation with cyclins, their cognate regulatory subunits (Malumbres and Barbacid, 2001). The kinase activity of these complexes is regulated by the Cip/Kip family of cdk inhibitors such as p27^{Kip1} and p21^{WAF1/Cip1} as well as INK-4 family that selectively inhibit cdk4/6-cyclin D complexes (Sherr and Roberts, 1999; Hanahan and Weinberg, 2011). Enhanced expression of the cdk inhibitors attenuates proliferative responses, whereas reduced levels of these inhibitors are associated with increased cell proliferation. The regulation of cdk inhibitor levels is complex, involving both *de novo* protein synthesis and/or proteasome-dependent protein degradation. Furthermore, cdk inhibitors must be present in the cell nucleus to inhibit the G₁ to S phase transition of the cell cycle. Thus, we next analyzed the expression and localization of cdk inhibitors by Western blot analysis of nuclear and cytosolic extracts with or without G-Rp1 treatment. As shown in Fig. 2B and 2C, mitogenic stimulation for 24 h markedly reduced the cytosolic levels of p27^{Kip1} to ~30% levels observed in unstimulated cells, consistent with previous findings that the cellular levels of p27^{Kip1} is mainly controlled by proteasome-dependent degradation before S-phase entry (Sherr and Roberts, 1999; Hanahan and Weinberg, 2011). Moreover, mitogenic stimulation resulted in a concomitant decrease in nuclear localization of p27^{Kip1} as previously reported (Seo *et al.*, 2006). G-Rp1 pretreatment significantly prevented the decrease in cytosolic levels and nuclear localization of p27^{Kip1} in response to mitogenic stimuli (Fig. 2B, C).

In contrast to p27^{Kip1}, mitogenic stimulation did not alter the cytosolic levels of another cdk inhibitor p21^{WAF1/Cip1} as compared with unstimulated cells, but marginally increased p21^{WAF1/Cip1} nuclear localization. G-Rp1 pretreatment further enhanced the nuclear localization of p21^{WAF1/Cip1} in a dose-dependent fashion. Collectively, these findings suggest that G-Rp1 may inhibit the phosphorylation of pRb through the regulation of expression and nuclear localization of cdk inhibitors, resulting in suppression of lung cancer cell proliferation.

G-Rp1 inhibits cell migration and adhesion in lung cancer cells

Cell migration and adhesion, which are controlled by coordinated events through the interactions with extracellular matrix molecules as well as intercellular components, has been known to play the pivotal roles in angiogenesis, tumor invasion and metastasis (Stetler-Stevenson *et al.*, 1993; Christofori, 2006; Bourbouliad and Stetler-Stevenson, 2010). We first examined the ability of G-Rp1 to regulate lung cancer cell migration. In contrast to cell proliferation, G-Rp1 pretreatment at higher concentration (20 μM) resulted in a 30% and 60% reduction in A549 and H1299 cell migration, respectively (Fig. 3A, B). Clearly, H1299 cells were more sensitive to G-Rp1 than A549 cells. Expression of MMPs, especially MMP-2 and MMP-9, can promote migration and invasion by selective proteolysis of extracellular matrix components (Stetler-Stevenson *et al.*, 1993; Bourbouliad and Stetler-Stevenson, 2010). Based on G-Rp1-mediated inhibition of cell migration, we next analyzed the activities of MMP-2 and MMP-9 in both cell lines (Fig. 3C). The conditioned media from A549 cell cultures had high levels of MMP-2 activity relative to those of MMP-9. G-Rp1 pretreatment showed little or no change of MMP-2 activity, and slightly reduced MMP-9 activity in A549 cells (Fig. 3C, left panel). However, the activities of MMPs in H1299 cells were not clearly detectable and altered by G-Rp1 treatment

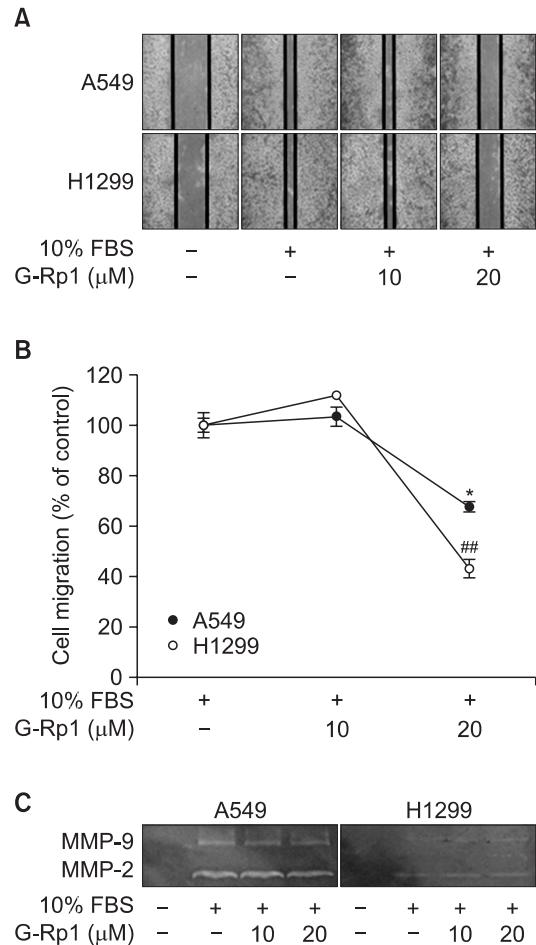


Fig. 3. G-Rp1 inhibits cell migration in A549 and H1299 cells. (A) Cells were pretreated with G-Rp1 (10, 20 μM) for 30 min, followed by 10% serum stimulation for 32 h (A549 cells) and 20 h (H1299 cells), respectively. (B) Migration into the wounded area was quantified by measuring the migration distance of cells from wound edge. Results from six independent experiments (mean ± S.D.) are represented as the percentage of maximally induced migration with 10% serum. Statistical significance is indicated (**p*<0.05, compared with 10% serum-treated A549 cells; ##*p*<0.01, compared with 10% serum-treated H1299 cells). (C) Cells were treated with G-Rp1 as in (A). Gelatin zymogram analysis was carried out by use of conditioned media from A549 and H1299 cells. Zymogram gel loading was normalized to total protein concentration. Results are representative of three independent experiments.

(Fig. 3C, right panel). These observations suggest that G-Rp1 inhibition of cell migration may not require the regulation of MMP activity, and are similar to previous findings that G-Rp1 does not have MMP-2 inhibitory activity (Park *et al.*, 2008). We next examined the effect of G-Rp1 on cell adhesion. As shown in Fig. 4, cells readily adhered to tissue culture plastic in response to mitogenic stimuli, as compared with unstimulated control cells (Fig. 4). G-Rp1 pretreatment markedly inhibited cell attachment to the levels observed in unstimulated controls. Collectively, these observations demonstrate that G-Rp1 effectively regulates cellular behaviors such as cell proliferation, migration, and adhesion in both p53 wild-type and p53-deficient lung cancer cell lines.

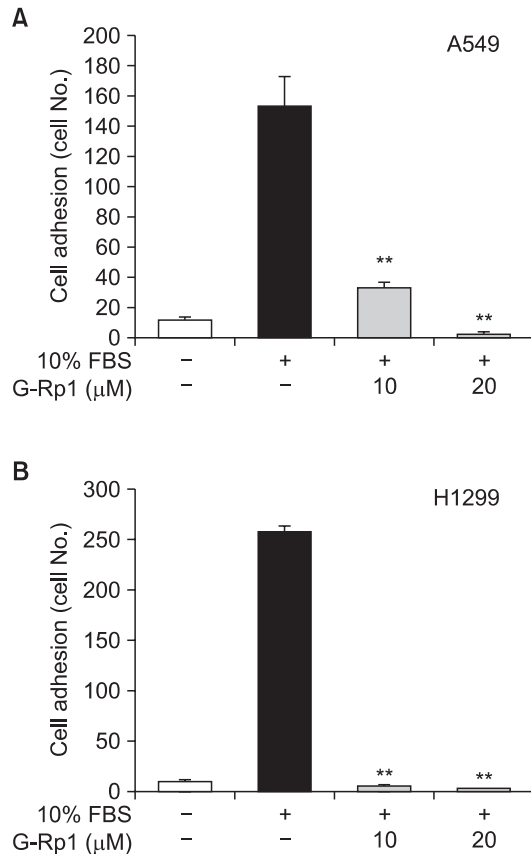


Fig. 4. G-Rp1 inhibits cell adhesion in A549 and H1299 cells. The cell suspension was pretreated with or without G-Rp1 (10, 20 μ M) for 30 min, followed by 10% serum stimulation. A549 (A) and H1299 (B) cells were plated and further incubated for 2 h. The number of attached cells was determined by counting 10 random fields of cells (100X field). Cell adhesion results from six independent experiments (mean \pm S.D.) are represented as the numbers of attached cells. Statistical significance is indicated (** p <0.01, compared with 10% serum-treated cells).

G-Rp1 inhibition of cell proliferation and migration is mediated through down-regulation of mitogenic signaling pathways

To further investigate the molecular mechanism by which G-Rp1 inhibits cell proliferation, migration and adhesion in lung cancer cells (Fig. 1, 3 and 4), we examined the changes in activation of extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt, and mammalian target of rapamycin (mTOR)/p70^{S6K} which are central to the coordinated control of cellular fates such as cell proliferation, migration, adhesion, survival and apoptosis (Lemmon and Schlessinger, 2010; Fenton and Gout, 2011; Hanahan and Weinberg, 2011). As expected, mitogenic stimulation for 15 min markedly increased the phosphorylation of ERK, Akt and p70^{S6K} as compared with unstimulated controls (Fig. 5A). G-Rp1 pretreatment significantly inhibited the phosphorylation of ERK, Akt and p70^{S6K} in a dose-dependent manner. However, G-Rp1-mediated inhibition of Akt phosphorylation appeared to be a little weaker than that of ERK and p70^{S6K} in both cell lines. These inhibitory effects of G-Rp1 on ERK, Akt and p70^{S6K} phosphorylation were found to be independent of

p53 expression levels, consistent with our previous findings (Fig. 1, 3, 4). Finally, pretreatment of cells with PD98059, an inhibitor of ERK pathway, LY294002, an inhibitor of PI3K/Akt pathway, or rapamycin, an inhibitor of mTOR/p70^{S6K} pathway, mimicked the suppressive effects on G-Rp1 on cell proliferation and migration in both cell lines. Co-treatment with G-Rp1 did slightly but not significantly enhance the ability of these pharmacological inhibitors to regulate cell proliferation (Fig. 5B, C) and migration (Fig. 5D, E), indicating that G-Rp1 and these pharmacological inhibitors may share similar roles and mechanisms of action in regulating cellular processes. Taken together, these findings demonstrate that anti-tumor activities of G-Rp1 might be attributed to the inactivation of ERK, Akt and p70^{S6K}-dependent signaling pathways.

DISCUSSION

Ginseng, which has been used for thousands of years in treating a variety of diseases, has many bioactive constituents including ginsenosides, phytosterols, peptides, fatty acids, polysaccharides, polyacetylenes, vitamins, and minerals (Attele *et al.*, 1999; Qi *et al.*, 2011). The most active constituent of ginseng is a saponin glycoside known as ginsenosides. Among the ginsenosides, Rh2 has anti-proliferative and apoptotic activities against various cancer cell lines including lung, breast and liver cancers (Lee *et al.*, 1996; Jin *et al.*, 2000; Cheng *et al.*, 2005; Choi *et al.*, 2009). In addition, Rg3 has recently been used as a new anti-tumor drug in China, and reported to exhibit anti-angiogenic and anti-tumor activities such as the regulation of tumor invasion and metastasis (Yue *et al.*, 2006). Ginsenoside Rp1, a semi-synthesized Rg3 derivative, is known to be more stable and less toxic, and has stronger anti-tumor activity than Rg3 (Kumar *et al.*, 2006; Park *et al.*, 2008; Kim and Cho, 2009; Kumar *et al.*, 2009). However, no detailed mechanisms of G-Rp1 responsible for regulation of tumor growth and progression have been clearly reported to date.

Lung cancer is the most common cause of cancer-related deaths worldwide, due to lack of potent therapeutic targets and strategies against highly aggressive and drug resistant phenotypes. The p53 gene is the most frequently mutated tumor suppressor gene in lung cancers, and loss of p53 function is closely associated with the treatment failure and poor survival (Fuster *et al.*, 2007). In the present study, pharmacological effects and molecular mechanisms of G-Rp1 on cell proliferation, migration, and adhesion were investigated using lung cancer cell lines such as p53 wild-type A549 and p53-deficient H1299 cells. G-Rp1 treatment markedly inhibited the proliferation of lung cancer cells in a dose-dependent manner, and this anti-proliferative activity of G-Rp1 was mediated through enhanced nuclear localization of cdk inhibitors and inhibition of pRb phosphorylation, independently of the levels of p53 protein expression (Fig. 1, 2). Tumor invasion, an important step in the sequential process of metastasis, requires tumor cell adhesion, migration and enzymatic degradation of the extracellular matrix and basement membranes (Bourboulia and Stetler-Stevenson, 2010). G-Rp1 treatment reduced cell migration with little or no change of MMP activities (Fig. 3), similar to previous reports (Park *et al.*, 2008). These findings are clearly distinct from other ginsenoside derivatives which repress MMP activities (Yue *et al.*, 2006; Park *et al.*, 2011),

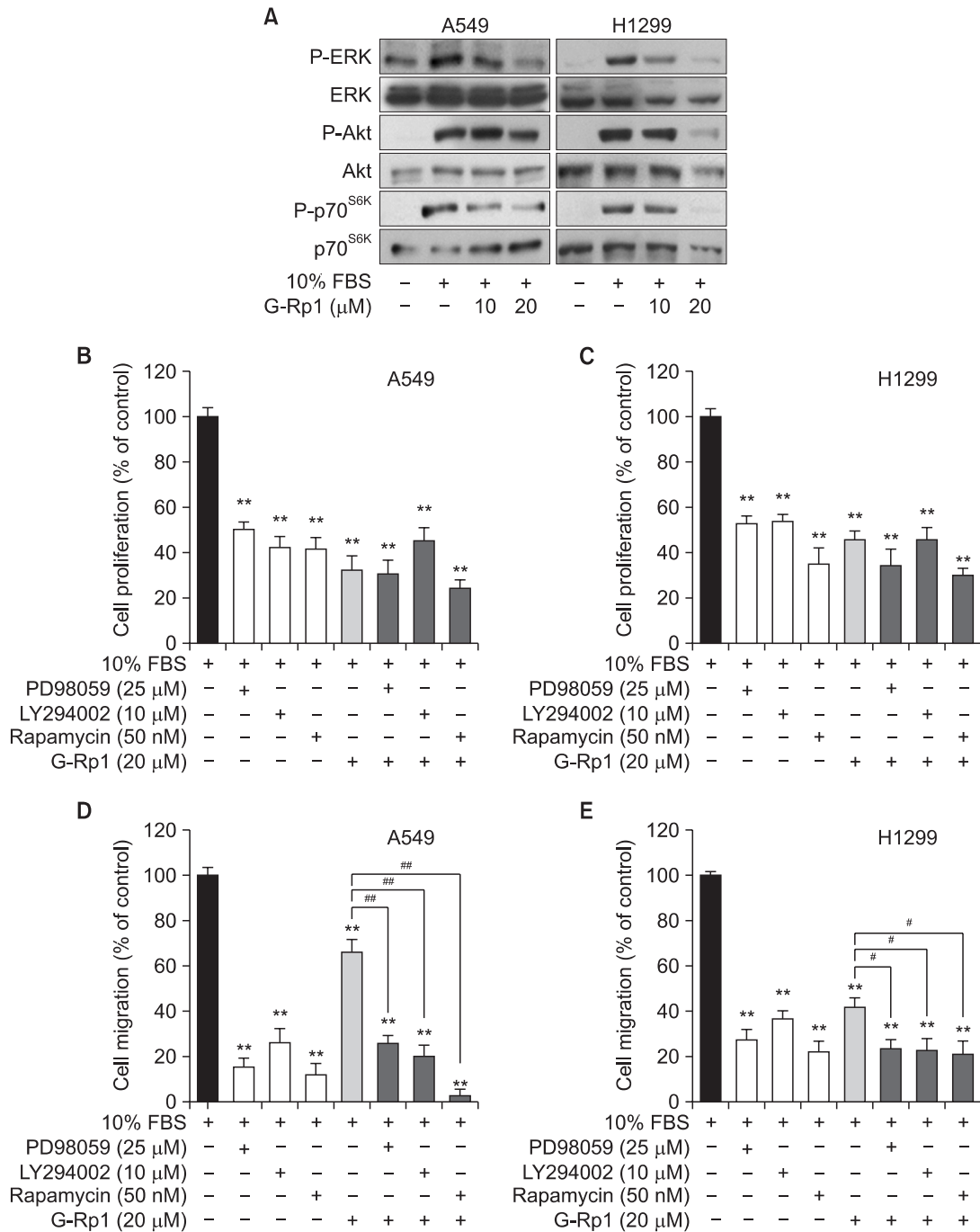


Fig. 5. G-Rp1 inhibition of cell mitogenic responses is mediated by down-regulation of signaling pathways. (A) Quiescent cells were pre-treated with or without G-Rp1 (10, 20 μM) for 30 min, followed by 10% serum stimulation for 15 min. Cell lysates were Western-blotted with anti-phospho-ERK, anti-ERK, anti-phospho-Akt, anti-Akt, anti-phospho-p70^{S6K}, or anti-p70^{S6K} antibodies. Results shown are representative of at least three independent experiments. Quiescent A549 (B and D) and H1299 (C and E) cells were pretreated with G-Rp1 (20 μM) for 30 min in the presence or absence of PD98059 (25 μM), LY294002 (10 μM) or rapamycin (50 nM), followed by 10% serum stimulation for cell proliferation and migration experiments as previously described. Results from at least four independent experiments (mean ± S.D.) are represented as the percentage of maximally induced proliferation or migration with 10% serum. Statistical significance is indicated (***p*<0.01, compared with 10% serum-treated cells; #*p*<0.05, ##*p*<0.01, compared with 10% serum plus G-Rp1-treated cells).

suggesting the existence of alternative mechanism to regulate cell migration and invasion, independently of regulation of MMP activity. In addition, G-Rp1 dramatically inhibited cell adhesion (Fig. 4), however, G-Rp1 anti-adhesive activity did not appear to affect the ability of G-Rp1 to inhibit cell proliferation and migration (Fig. 1, 3A and 3B). Finally, G-Rp1 treatment inhibited ERK, Akt, and p70^{S6K}-dependent signaling pathways (Fig. 5A), resulting in suppression of cell proliferation and migration, as evidenced by using pharmacological inhibitors such as PD98059, LY294002 and rapamycin (Fig. 5B-E).

In conclusion, we demonstrate for the first time that G-Rp1 mediates anti-tumor activities of lung cancer cells through inhibition of ERK, Akt and p70^{S6K}-dependent signaling pathways and these modulating effects are independent of p53 expression. Our findings provide important insights into the roles and pharmacological efficacy of G-Rp1 in regulation of cell fates, and support the preclinical and clinical development of G-Rp1 as a potent anti-tumor agent for the treatment of cancers with mutated p53 as well as wild-type p53.

ACKNOWLEDGMENTS

This work was supported by Basic Science Research Program (KRF-2008-313-C00555) (2010-0021913) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science Technology (MEST), 2008 Research Grant from Kangwon National University and by the second stage of the Brain Korea 21 program.

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