Marmesin-mediated suppression of VEGF/VEGFR and integrin β1 expression: Its implication in non-small cell lung cancer cell responses and tumor angiogenesis

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Received May 19, 2016; Accepted June 29, 2016

DOI: 10.3892/or.2016.5245

Abstract. In the present study, we investigated the effects and molecular mechanism of marmesin, a natural coumarin compound isolated from *Broussonetia kazinoki*, on non-small cell lung cancer (NSCLC) cell responses and tumor angiogenesis. Marmesin abrogated mitogen-stimulated proliferation and invasion in both p53 wild-type A549 and p53-deficient H1299 NSCLC cells. These antitumor activities of marmesin were mediated by the inactivation of mitogenic signaling pathways and downregulation of cell signaling-related proteins including vascular endothelial growth factor receptor-2 (VEGFR-2), integrin β1, integrin-linked kinase and matrix metalloproteinases-2. Furthermore, marmesin suppressed the expression and secretion of VEGF in both NSCLC cells, leading to inhibition of capillary-like structure formation in human umbilical vein endothelial cells. Collectively, these findings demonstrate the pharmacological roles and molecular targets of marmesin in regulating NSCLC cell responses and tumor angiogenesis.

Introduction

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and the 5-year relative survival rate is less than 20% (1,2). Dysregulated activation of receptor tyrosine kinases (RTKs) and their downstream signaling molecules is closely associated with NSCLC growth and progression. Among RTKs, epidermal growth factor receptor (EGFR) is highly expressed or constitutively activated in NSCLC patients with poor prognosis (3), suggesting the rational strategy and pharmacological efficacy of EGFR-targeted therapeutics in the treatment of NSCLC. However, inhibition of RTKs including EGFR in the clinic often leads to recurrent and metastatic phenotypes of NSCLC. In addition, anti-angiogenic therapy such as inhibition of vascular endothelial growth factor-A (VEGF-A)/VEGF receptor-2 (VEGFR-2) signaling pathways shows a transient therapeutic effect and subsequent development of NSCLC resistance (4-6). Thus, a further understanding of the molecular mechanism of RTK-mediated signaling pathways in NSCLC growth and progression in the tumor microenvironment is a prerequisite for the identification of therapeutic targets and the development of highly effective anticancer drugs.

Broussonetia kazinoki (*B. kazinoki*) has been used as a traditional medicine for the treatment of blurred vision and inflammatory and infectious diseases as well as a raw material for paper production in Northeastern Asia including Korea. Previous investigations have demonstrated that *B. kazinoki* extract and its bioactive components such as flavan derivatives have anti-diabetic, anti-allergic, anti-inflammatory and anti-tumor properties (7-12). We recently reported that an ethanolic extract of *B. kazinoki* and marmesin regulate VEGF-A-induced endothelial cell fates *in vitro* and angiogenic sprouting *ex vivo* (13,14). Marmesin, a furanocoumarin component isolated from a variety of plants including *Peucedanum japonicum*, *Dystaenia takeshimana*, *Feronia limonia* and *Ferula lutea* as well as *B. kazinoki*, has been reported to exert a variety of pharmacological functions such as anti-inflammatory, anti-hepatotoxic, anti-angiogenic and antitumor activities (14-18). However, the effects and molecular mechanisms of marmesin on NSCLC cell responses have never been elucidated, to date. In the present study, we report for the first time the regulatory effects and molecular mechanisms of marmesin on NSCLC cell fates and tumor-derived angiogenic responses.

Materials and methods

Cell culture conditions. Human NSCLC cell lines (A549 and H1299) were obtained from the American Type Culture

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Key words: marmesin, non-small cell lung cancer, VEGFR-2, VEGF, integrin β1

Collection (ATCC; Manassas, VA, USA) and were grown in 10% fetal bovine serum-Dulbecco's modified Eagle's medium (FBS-DMEM) (HyClone Laboratories, Logan, UT, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA) and used between passages 4 and 6 for all experiments. Cells were cultured in EGM-2® BulletKit media, according to the manufacturer's instructions (Lonza).

Reagents. Marmesin was isolated in an ethyl acetate fraction partitioned from the ethanolic extract of *B. kazinoki*. The following pharmacological agents and antibodies were purchased from commercial sources: anti-phospho-Src (Y416), anti-Src, anti-phospho-MEK (S217/S221), anti-MEK, anti-phospho-ERK (T202/Y204), anti-phospho-Akt (S473), anti-phospho-$p70^{S6K}$ (T421/S424) and phospho-pRb (S780) (Cell Signaling Technology, Beverly, MA, USA); anti-ERK, anti-Akt, anti-$p70^{S6K}$, anti-VEGFR-2, anti-integrin α3, anti-integrin β1, anti-ILK, anti-Cdk4, anti-Cdk2, anti-actin antibodies, and mouse and rabbit IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell viability and proliferation assay. Subconfluent A549 and H1299 cells, plated on 6-well plates ($5x10^4$ cells/well; SPL Life Sciences, Gyeonggi-do, Korea), were serum-starved for 24 h in basal DMEM to synchronize cells in the G_1/G_0 phase of the cell cycle, and pretreated with marmesin (0.1-10 μM) for 30 min prior to 10% FBS stimulation for 24 h. In some experiments, quiescent cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for 12 h. After stimulation, cells were thoroughly rinsed with phosphate-buffered saline (PBS; pH 7.4) to remove any residual marmesin, and further incubated with 10% FBS for another 12 h until the end of the 24 h time point. Cell viability was determined by a Muse™ Cell Analyzer using cell count and viability assay kit (Merck Millipore, Billerica, MA, USA), and the cell proliferation was quantified as previously described (19). The results from triplicate determinations (mean ± standard deviation) are presented as the percentage of viable cells of total cell count or the fold-increase of the untreated controls.

Western blot analysis. Quiescent cells were pretreated with marmesin for 30 min, followed by 10% FBS stimulation for different time points, as indicated. 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 μg/ml AEBSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 80 mM β-glycerophosphate, 25 mM sodium fluoride and 1 mM sodium orthovanadate for 30 min at 4˚C. Cell lysates were clarified at 13,000 x g for 20 min at 4˚C, and the supernatants were subjected to western blotting as previously described (20). All western blot analyses are representative of at least three independent experiments. Bands of interest were integrated and quantified by the use of National Institutes of Health (NIH) ImageJ version 1.34s software.

Cell invasion assay. The upper side of the Transwell insert (6.5-mm diameter insert, 8-μm pore size) (Corning Costar, Inc., Corning, NY, USA) was coated with 50 μl of 1 mg/ml Matrigel® (BD Biosciences, Bedford, MA, USA) diluted in serum-free DMEM. Aliquots (100 μl) of cells ($5x10^5$ cells/ml) resuspended in serum-free DMEM were added to the upper compartment of the Matrigel-coated Transwell and 600 μl of serum-free DMEM was added to the lower compartment. After serum starvation for 2 h, the cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for 16 h. The inserts were fixed with methanol and using a cotton-tipped swab the non-invasive cells were removed from the top of the membrane. After staining with 0.04% Giemsa staining solution (Sigma-Aldrich Co., St. Louis, MO, USA), the numbers of invasive cells (mean ± standard deviation) were determined from six different fields using x200 objective magnification (21,22).

RNA purification and reverse transcriptase-polymerase chain reaction. Total RNA was purified with PureHelix™ RNA extraction solution (Nanohelix Co., Daejeon, Korea). Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as template for each reverse transcriptase (RT)-mediated polymerase chain reaction (PCR) using First Strand cDNA Synthesis kit (BioAssay Co., Ltd., Daejeon, Korea). Primers for PCR were synthesized by Bioneer Corporation (Daejeon, Korea). Primer sequences were as follows: MMP-2 forward, 5'-GCTCAGATCCGTGGTGAGAT-3' and reverse, 5'-GGTG CTGGCTGAGTAGATCC-3'; VEGFR-2 forward, 5'-TGCCT ACCTCACCTGTTTCCT-3' and reverse, 5'-TACACGGTGG TGTCTGTGTCA-3'; VEGF-A forward, 5'-TCGGGCCTCCG AAACCATGA-3' and reverse, 5'-CCTGGTGAGAGATCTG GTTC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3'. Bands of interest were integrated and quantified by the use of NIH ImageJ version 1.34s software.

VEGF enzyme-linked immunosorbent assay (ELISA). Quiescent cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for 12 h. Cells were washed with PBS to remove any residual marmesin, and then further stimulated with 10% FBS for another 12 h. ELISA assay was performed to measure the concentration of VEGF in the conditioned media using VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Secreted VEGF levels were analyzed at 450 nm using BioTek Synergy Mx microplate reader (BioTek Instruments, Winooski, VT, USA).

Tube formation assay. Tube formation assay was performed to examine the ability of conditioned media from marmesin-treated NSCLC cells to regulate angiogenic responses *in vitro*. Quiescent NSCLC cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for 12 h. Cells were washed with PBS to remove any residual marmesin, and then further stimulated with 10% FBS for another 12 h. After stimulation, conditioned media were collected. Quiescent HUVECs ($4x10^4$ cells/ml) were added to Matrigel®-coated plates and treated with conditioned media for 9 h. Tube formation was observed with an Olympus CKX41 inverted microscope (CAchN 10/0.25php objective)

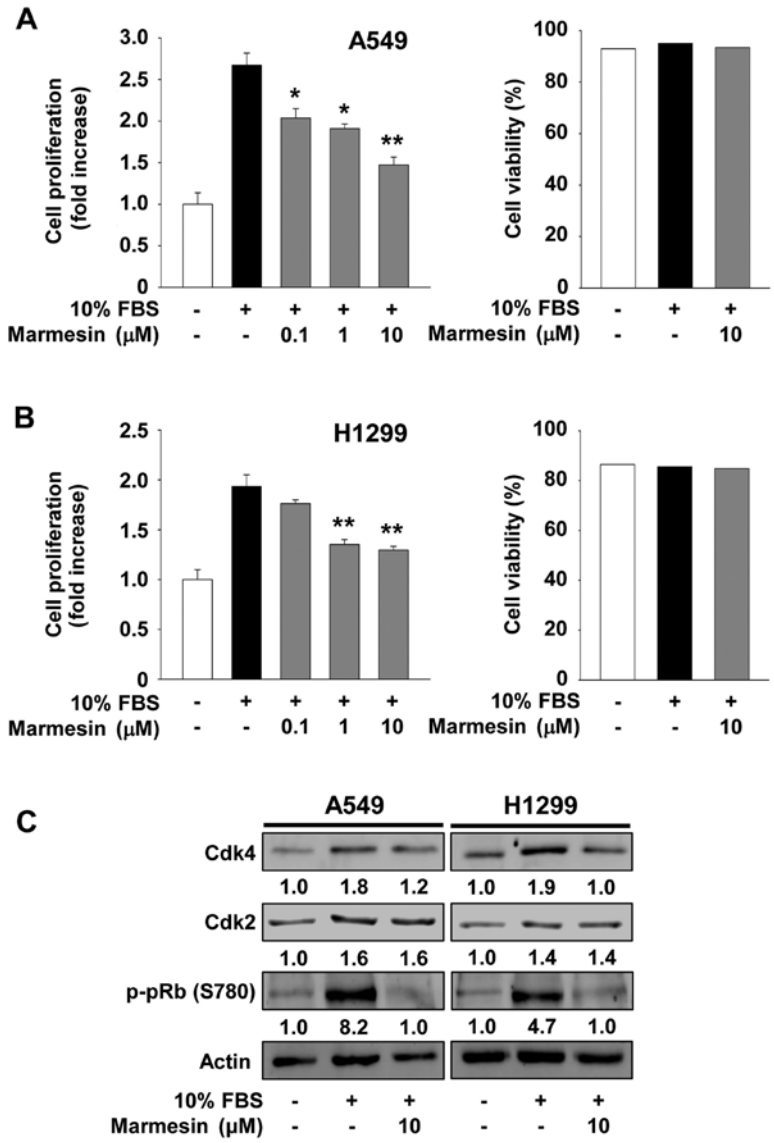

Figure 1. Effects of marmesin on cell proliferation and viability in NSCLC cells. Quiescent A549 and H1299 cells were pretreated with marmesin (0.1-10 μM) for 30 min, followed by 10% FBS stimulation for 24 h. (A and B) Cell proliferation (left panel), viability (right panel) and (C) western blot analyses were performed as described in Materials and methods. The results from at least three independent experiments (mean ± SD) are presented as the fold-increase of the untreated controls (for proliferation) or the percentage of the viable cells of the total cell counts (for viability). Statistical significance is indicated (*P<0.05, **P<0.01, compared with 10% FBS-treated cells). (C) Cell lysates were subjected to western blot analysis with anti-Cdk4, anti-Cdk2, anti-phospho-pRb (S780) or anti-actin antibodies. Integrated density values were normalized to the untreated controls.

and ToupTek Toupview software (version x36, 3.5.563; Hangzhou ToupTek Photonics Co., Zhejiang, China).

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 at P<0.05.

Results

Marmesin inhibits NSCLC cell proliferation. We first investigated the ability of marmesin to regulate cell proliferation in p53 wild-type A549 and p53-deficient H1299 NSCLC cells (Fig. 1A and B). Marmesin treatment inhibited mitogen-stimulated cell proliferation in a dose-dependent manner and did not alter cell viability and morphology at the highest concentration used in the present study, indicating that marmesin-mediated inhibition of cell proliferation was not mediated by induction of apoptosis or cytotoxicity. Based on these findings, we next analyzed the changes in the cell cycle regulatory proteins in the marmesin-treated NSCLC cells (Fig. 1C). Marmesin treatment markedly suppressed mitogen-induced expression of cyclin-dependent kinase 4 (Cdk4), but not Cdk2, to levels observed in the untreated controls, resulting in inhibition of pRb phosphorylation in both NSCLC cell lines. Marmesin has previously been reported to inhibit proliferation by downregulation of Cdk4, Cdk2 and cyclin D in VEGF-A-treated HUVECs (14). Although the molecular mechanism of marmesin in regulating cell cycle-related proteins appears slightly different in cell types, these findings demonstrate the antiproliferative activity of marmesin against various types of cells, independently of p53 expres-

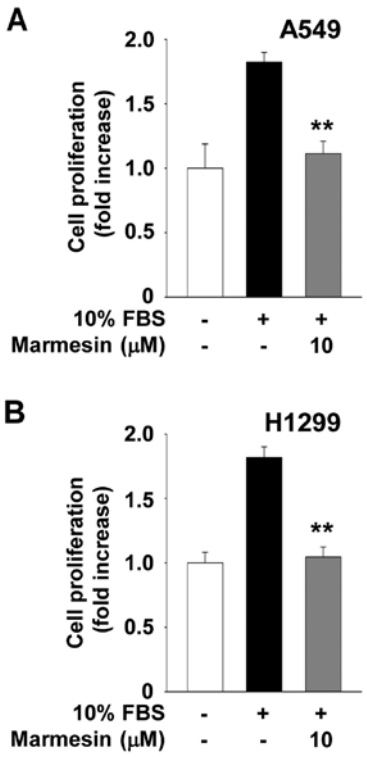

Figure 2. Irreversible effect of marmesin on cell proliferation in NSCLC cells. Quiescent (A) A549 and (B) H1299 cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for 12 h. Following stimulation, cells were washed thoroughly with PBS to remove any residual marmesin, and further incubated with 10% FBS for another 12 h until the end of the 24 h time point. The results from at least three independent experiments (mean ± SD) are presented as the fold-increase of the untreated controls. Statistical significance is indicated (**P<0.01, compared with the 10% FBS-treated cells).

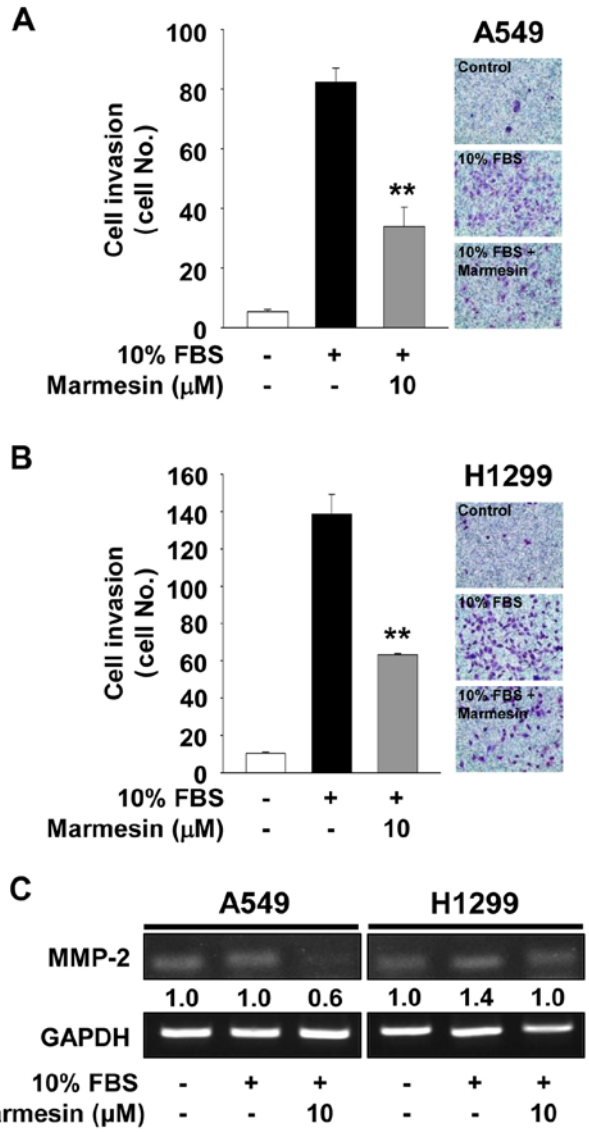

Figure 3. Effects of marmesin on cell invasion and MMP-2 expression in NSCLC cells. (A and B) *In vitro* Transwell invasion and (C) RT-PCR analyses were performed as described in Materials and methods. (A) A549 and (B) H1299 cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for (A and B) 16 or (C) 24 h. The number of invasive cells was determined by counting six different fields of cells (magnification, x200). Results from six independent experiments (mean ± SD) are presented as the number of invasive cells. Statistical significance is indicated (**P<0.01, compared with 10% FBS-treated cells). (C) Results shown are representative of at least three independent experiments. Integrated density values were normalized to untreated controls.

sion status. In addition, the inhibitory effect of marmesin on NSCLC cell proliferation was not changed after the withdrawal of marmesin at the 12 h time point, and was sustained up to 24 h, suggesting that this effect may be irreversible until the end of this experiment (Fig. 2).

Marmesin inhibits NSCLC cell invasion. We next examined the effect of marmesin on cell invasion in the A549 and H1299 cells. Marmesin treatment markedly blocked mitogen-stimulated cell invasion (Fig. 3A and B). The regulatory pattern of marmesin on NSCLC cell invasion was very similar to that on NSCLC cell migration (data not shown). Based on these findings, we analyzed the changes in expression of matrix metalloproteinases (MMPs) in the marmesin-treated NSCLC cells. Expression and activity of MMPs have been known to modulate cell migration, invasion and angiogenesis by degrading extracellular matrix components and cell surface molecules (23-26). As shown in Fig. 3C, marmesin treatment suppressed mitogen-induced expression of MMP-2 in both cell lines. In contrast, the levels of tissue inhibitor of metalloproteinases-2, an endogenous inhibitor of MMPs, were not altered in the mitogen- or marmesin-treated NSCLC cells (data not shown) (27-29). Collectively, these findings suggest that inhibition of cell invasion by marmesin is mediated, at least in part, through the suppression of MMP-2 expression (Fig. 3C).

Marmesin-mediated inhibition of NSCLC cell proliferation and invasion is mediated through inactivation of mitogen-stimulated signaling pathways and downregulation of cell surface signaling molecules. To elucidate the molecular mechanisms and therapeutic targets of marmesin in regulating NSCLC cell proliferation and invasion, we examined the changes in activation of mitogen-stimulated signaling pathways including Src kinase, mitogen-activated protein kinase (MEK), extracellular signal-regulated kinase (ERK), Akt and p70S6 kinase ($p70^{S6K}$) in marmesin-treated NSCLC cells (14,30). As expected, mitogenic stimulation significantly increased the phosphorylation of MEK, ERK, Akt and $p70^{S6K}$,

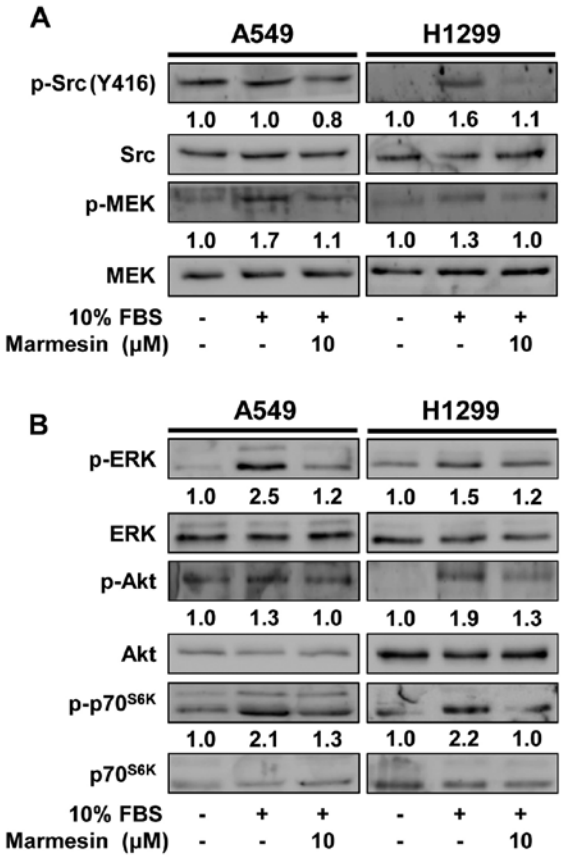

Figure 4. Effect of marmesin on mitogen-stimulated signaling pathways in NSCLC cells. Quiescent NSCLC cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for (A) 5 or (B) 15 min. Results shown are representative of at least three independent experiments. Integrated density values were normalized to untreated controls.

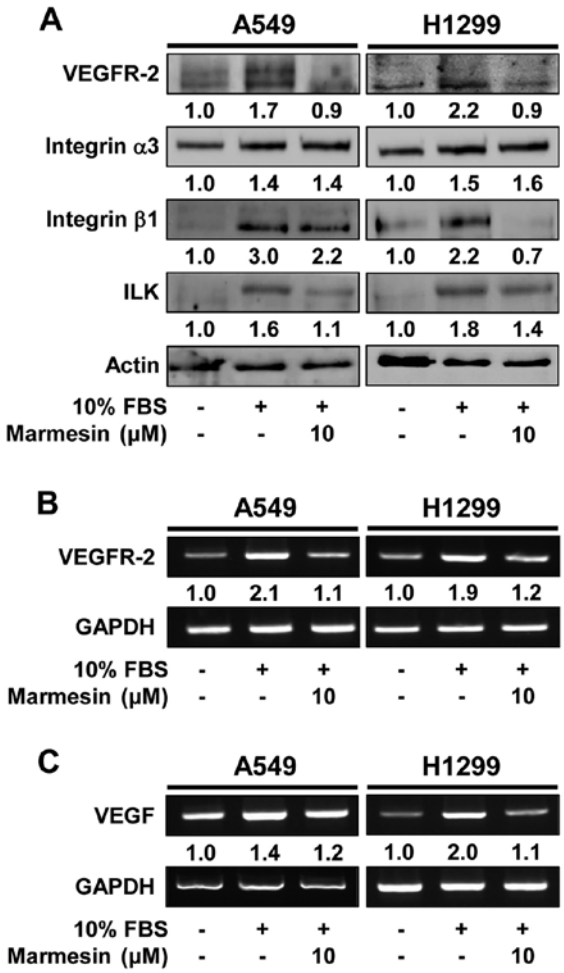

Figure 5. Effect of marmesin on the expression of mitogen-stimulated signaling molecules in NSCLC cells. (A) Western blot and (B and C) RT-PCR analyses were performed as described in Materials and methods. Quiescent NSCLC cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for 24 h. Results shown are representative of at least three independent experiments. Integrated density values were normalized to untreated controls.

as compared with unstimulated controls (Fig. 4). In contrast, marmesin treatment markedly inhibited mitogen-stimulated phosphorylation of Src, MEK, ERK, Akt and $p70^{S6K}$ in both NSCLC cells. Moreover, marmesin treatment markedly suppressed mitogen-induced expression of cell signaling molecules such as VEGFR-2, integrin β1 and integrin-linked kinase (ILK), and a key angiogenic factor VEGF, which play important roles in cancer growth and progression associated with angiogenesis (Fig. 5) (31-33). In addition to direct antitumor activity, these findings suggest the possibility that marmesin may regulate angiogenic responses through inhibition of VEGF expression and secretion in NSCLC cells.

Marmesin inhibits endothelial cell tube formation by downregulation of NSCLC-derived VEGF secretion. In the tumor microenvironment cancer cells secrete a variety of biological molecules including cytokines and growth factors, which play important roles in cellular responses such as proliferation, migration, invasion and angiogenesis (5,34). Based on inhibitory effect of marmesin on VEGF expression, we thus analyzed the secreted levels of VEGF in conditioned media collected from marmesin-treated NSCLC cells. As shown in Fig. 6A, marmesin treatment significantly inhibited the secretion of VEGF from both NSCLC cell lines in response to mitogenic stimulation. H1299 cells were found to be more responsive to marmesin-mediated inhibition of VEGF secretion, as compared with A549 cells. These findings are similar to the patterns of VEGF transcription levels in marmesin-treated NSCLC cells (Fig. 5C), demonstrating that marmesin inhibits VEGF secretion through downregulation of VEGF mRNA expression. To determine whether secreted biomolecules including VEGF from marmesin-treated NSCLC cells affect the cellular fate of adjacent and/or other cells, we next performed *in vitro* angiogenesis assay using conditioned media from NSCLC cells treated with or without marmesin (Fig. 6B). The conditioned media from marmesin-treated NSCLC cells significantly inhibited the formation of capillary-like structures by HUVECs. Although the types and levels of biomolecules secreted from marmesin-treated NSCLC cells remain to be further identified, these observations suggest that marmesin-mediated inhibition of VEGF expression and secretion in NSCLC cells may be one of the major factors in the modulation of tumor-derived angiogenesis (Fig. 6B).

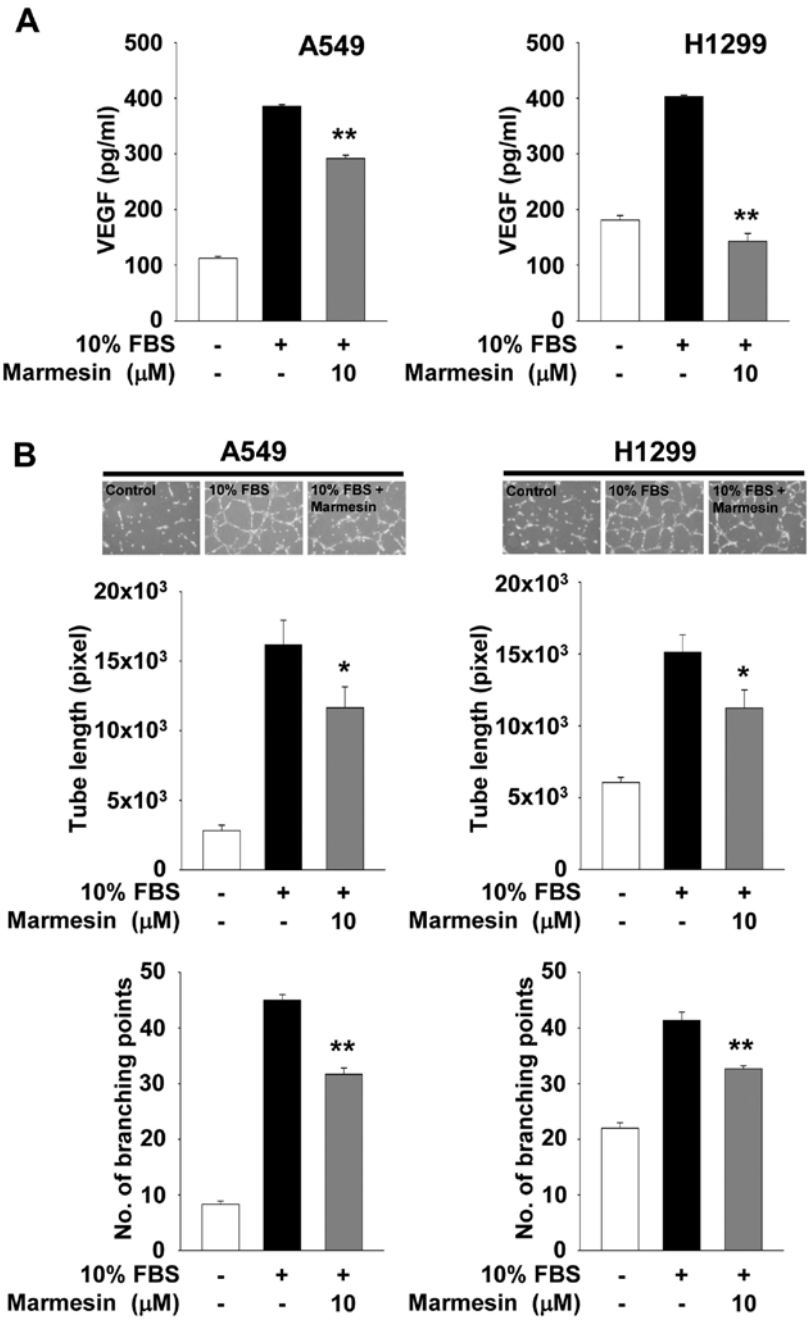

Figure 6. Effects of marmesin on VEGF secretion in NSCLC cells and angiogenic responses *in vitro*. Quiescent NSCLC cells were pretreated with marmesin (10 μM) for 30 min, followed by 10% FBS stimulation for 12 h. Cells were washed with PBS to remove any residual marmesin, and then further stimulated with 10% FBS for another 12 h. After stimulation, conditioned media were collected. (A) VEGF ELISA and (B) tube formation assays were performed using conditioned media as described in Materials and methods. Values represent the mean ± SD of at least three independent experiments. Statistical significance is indicated (*P<0.05, **P<0.01, compared with 10% FBS-treated cells).

Discussion

Dysregulated activation of RTKs and/or cross-talk between RTKs and integrins have been known to play important roles in cancer growth, progression and poor prognosis in human lung cancer (3,4,20,31,34). Therefore, highly activated RTKs or integrins are considered as key targets of antitumor agents in clinical trials and use. Numerous investigations indicate that drugs targeting RTKs are more effective than conventional therapeutics for the treatment of lung cancer. However, molecular-targeted therapy in the clinic eventually develops recurrent and metastatic lung cancers, suggesting that the identification of key molecular targets in RTK/integrin signaling pathways is absolutely required for the development of effective therapeutic strategies and agents to treat aggressive types of lung cancer.

Marmesin has been known to exert antitumor activity against several types of cancer cells including colon cancer (18). Recently, we reported that marmesin, a coumarin component isolated from *Broussonetia kazinoki*, inhibits VEGF-A-induced endothelial cell responses *in vitro* and angiogenic sprouting *ex vivo* (14). These findings led us to investigate the effects of

marmesin on lung cancer cell fate and lung cancer cell-derived angiogenesis. In the present study, we showed that marmesin inhibited proliferation and invasion of NSCLC cells, independently of p53 expression status. Antiproliferative activity of marmesin appeared to be irreversible, since withdrawal of marmesin did not reverse or affect cell proliferation in the NSCLC cells. In addition, marmesin suppressed VEGF expression and secretion in NSCLC cells, leading to inhibition of capillary-like structure formation of HUVECs. The mechanism of these effects involved inactivation of mitogenic signaling pathways such as Src, MEK, ERK, Akt and $p70^{S6K}$, and downregulation of VEGF, VEGFR-2, integrin β1, ILK and MMP-2. In conclusion, these findings provide important insights into the regulatory roles and therapeutic potential of marmesin in NSCLC, and warrant preclinical evaluation and development of marmesin as a potent antitumor agent for the treatment of NSCLC associated with pathological angiogenic responses.

Acknowledgements

The present study was supported by the R&D Program for Forestry Technology (S121313L070100) through the Korea Forest Service, and by the Basic Science Research Program (2014R1A1A2058015) through the National Research Foundation of Korea, Ministry of Education.

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