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Remodeling of Actin Cytoskeleton in Lupeol-Induced B16 2F2 Cell Differentiation

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Lupeol induces the formation of dendrites in B16 2F2 melanoma cells. The remodeling of cytoskeletal components contributes to the dendricity of melanoma cells. We studied the effects of lupeol on the remodeling of cytoplasmic filaments in B16 2F2 cells. Western blotting revealed no change in the levels of actin and tubulin. Lupeol attenuated stress fiber assembly, but did not promote the remodeling of microtubular networks. We examined the activation of cofilin, an actin-depolymerizing factor, in lupeol-treated B16 2F2 cells by western blotting. The level of phospho-cofilin was found to decrease in a time-dependent manner. Inhibition of p38 MAPK by SB203580 blocked tyrosinase induction by lupeol, but did not influence the disruption of stress fiber assembly or the dephosphorylation of cofilin. Furthermore, we studied the effects of lupeol on cell migration. At 10 μM, lupeol markedly inhibited the haptotaxis of B16 2F2 cells to fibronectin. Additionally, lupeol strongly inhibited the migration of human melanoma and neuroblastoma cells, and weakly suppressed the migration of lung adenocarcinoma cells. However, lupeol did not affect the motility of other cancer cells. The results suggest that lupeol suppresses the migration of malignant melanoma cells by disassembling the actin cytoskeleton.

Key words: B16 2F2 cell differentiation, cofilin, dendritic outgrowth, lupeol, stress fiber.

Abbreviations: MAPK, mitogen-activated protein kinase; α-MSH, α-melanocyte stimulating hormone; PKA, protein kinase A.

Melanocytes are derived from the neural crest, and contain melanosomes, lysosome-related organelles containing all the components required to synthesize melanin pigments such as tyrosinase, a key enzyme in melanin biosynthesis. In response to hormones and UV irradiation, melanin pigments are synthesized in melanosomes, and the melanosomes are transferred from the tips of melanocyte dendrites to surrounding keratinocytes to protect against UV damage or carcinogenic effects (*1–3*). Studies have demonstrated that the dendritic outgrowth of melanocyte/melanoma cells is promoted by UV irradiation, cAMP-elevating agents and growth factors, as well as by the induction of melanogenesis. Therefore, dendrites are recognized as a morphological indicator of melanocyte/melanoma cell differentiation (*4–6*). Recently, it was suggested that dendritic outgrowth accompanies the remodeling of cytoplasmic filaments, and the signaling mechanisms involved in the formation of dendrites in melanocyte/melanoma cells have been studied. In B16 mouse melanoma cells, stimulation by cAMP-elevating agents attenuates the assembly of actin stress fibers through the inactivation of Rho, a small GTP-binding protein. This stress fiber disassembly leads to morphological changes, including in dendrites (*4*, *7*, *8*).

Lupeol, a lupane triterpene, is widely distributed in the plant kingdom, and its biological activities have attracted a great deal of attention. Many studies on its anti-inflammatory properties have been reported (*9–11*). More recently, it was shown that lupeol inhibits phosphatidyl inositol 3-kinase and nuclear factor kappa B signaling induced by 12-*O*-tetradecanoyl-phorbol-13-acetate, and these inhibitory effects blocked the skin tumor promotion in CD-1 mice (*12*). Previously, we reported that lupeol induces the differentiation of B16 2F2 mouse melanoma cells *in vitro*, and up-regulates melanogenesis. Furthermore, we demonstrated that the activation of the p38 MAPK pathway is involved in lupeol-induced B16 2F2 cell differentiation as a downstream target of protein kinase A (PKA) (*13*, *14*). In the present study, we investigated the effects of lupeol on the formation of dendrites in B16 2F2 cells. It was found that the disassembly of the actin cytoskeleton is induced, and that the disruption leads to the formation of dendrites in B16 2F2 cells. Studies have demonstrated that the assembly and disassembly of the actin cytoskeleton are involved in cell migration, and that modulators of the actin cytoskeleton inhibite the movement of cancer cells (*15*, *16*). We evaluated the effects of lupeol on the migration of different cancer cells.

MATERIALS AND METHODS

Materials—Horseradish peroxidase (HRP)–conjugated anti-mouse IgG, mouse anti-actin monoclonal antibody (A4700), mouse anti–α-tubulin monoclonal antibody (T9026), α-MSH, SB203580 and tetramethyl-rhodamine isothyanate (TRITC)–conjugated anti-mouse IgG were

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purchased from Sigma Chemical Co. Ltd. Alexa Fluor 488–conjugated phalloidin was from Molecular Probes Inc. Rabbit anti-cofilin polyclonal antibody and rabbit anti–phospho-cofilin antibody were from CHEMICON International. Goat anti-tyrosinase antibody (M-19) was from Santa Cruz Biotechnology Inc. HRP-conjugated anti-rabbit IgG (H+L chain specific) was from Southern Biotech., and HRP-conjugated anti-goat IgG was from PROMEGA.

Cell Culture—ACHN, B16 2F2, HeLa, HT1080, MIA Paca2, NB-1 and T24 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. A549, G361 Saos2 and SH-10-TC cells were grown in RPMI-1640 medium supplemented with 10% FBS and antibiotics.

Dendritic Formation—Aliquots (1 ml) of B16 2F2 cells (2×10^4 cells/ml) were seeded in 24-well microplates, and cultured for 24 h. Then, various concentrations of lupeol were added, and the cell mixtures were incubated for 12 h. B16 2F2 cells were defined as having dendrites if the length of a dendrite was greater than the diameter of the cell bearing it.

Western Blotting—Aliquots (10 ml) of B16 2F2 cells (1×10^5 cells/ml) were incubated with or without a stimulant for an appropriate period. The cells were collected by pipetting, washed twice with PBS, and then lysed with 5 mM Tris-HCl buffer, pH 6.8, containing 1% SDS, 1 mM EDTA and 10% sucrose (100 μl). The proteins (10 μg) were separated by SDS–polyacrylamide gel electrophoresis using a 10–20% gel (PAGEL 1020N; ATTO), and then transferred to a nitrocellulose membrane. The membrane was immersed in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20 (TBST) containing 5% skim milk, and then incubated with mouse anti-actin antibodies (diluted 1:5,000 with TBST), mouse anti–α-tubulin antibodies (diluted 1:5,000 with TBST), goat anti tyrosinase (diluted 1:200 with TBST), rabbit anti-cofilin antibodies (diluted 1:1,000 with TBST) or rabbit anti–phospho-cofilin (diluted 1:1,000 with TBST) for 2 h at room temperature, washed three times with TBST, and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The membrane was washed three times with TBST, and the signals were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech.).

Fluorescence Microscopy—Cells grown on glass coverslips (ASAHI TECHNO GLASS Co.) pre-coated with fibronectin were cultured with or without lupeol for 12 h. The cells were rinsed twice with PBS, fixed in 4 % formaldehyde, and permealized with 0.1% Triton X-100 for 10 min. For the detection of stress fibers, the cells were stained with Alexa-488–conjugated phalloidin. For observation of the microtubular network, the microtubules in B16 2F2 cells were visualized using anti–α-tubulin antibody (diluted 1:500 with TBST) as primary antibody and TRIC-conjugated anti-mouse IgG as a secondary antibody.

Migration Assay—Cancer cell migration assays were performed using Transwell cell culture chambers (Corning Coaster Co.) according to Murata's method (*17*). The Transwell cell culture chambers contained polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8.0 μm (Whatman) were pre-coated with 2 μg of fibronectin (ASAHI TECHNO GLASS Co.) on the lower surface. Cancer cells (5×10^4 cells) in enriched RDF medium (KYOKUTO Pharmaceutical Industrial Co., Ltd.) supplemented with 0.1% BSA were added to the upper compartment, the chambers were and incubated at 37°C for 6 h. The cells on the lower surface of the filter were fixed with methanol and stained with hematoxylin. The cells on the upper surface were removed by wiping with cotton swabs. The average number of migrated cells in 4 areas was counted manually under a microscope, and each assay was repeated four times.

RESULTS

Briefly, we investigated the formation of dendrites in B16 2F2 cells incubated with 10 μM lupeol for 12 h. Untreated B16 2F2 cells were mainly round without dendrites (Fig. 1A-a). The morphology of B16 2F2 cells incubated with 10 μM lupeol for 12 h was markedly changed, and the dendricity was promoted (Fig. 1A-b). We manually counted the percentage of dendritic cells after treatment with various concentrations of lupeol for 12 h (Fig. 1B). The percentage of dendritic cells was increased by the addition of lupeol at between 5 and 20 μM, and dendrites were observed in 70.2% of B16 2F2 cells incubated with 10 μM lupeol.

We studied whether the remodeling of cytoskeletal components such as microfilaments and microtubules,

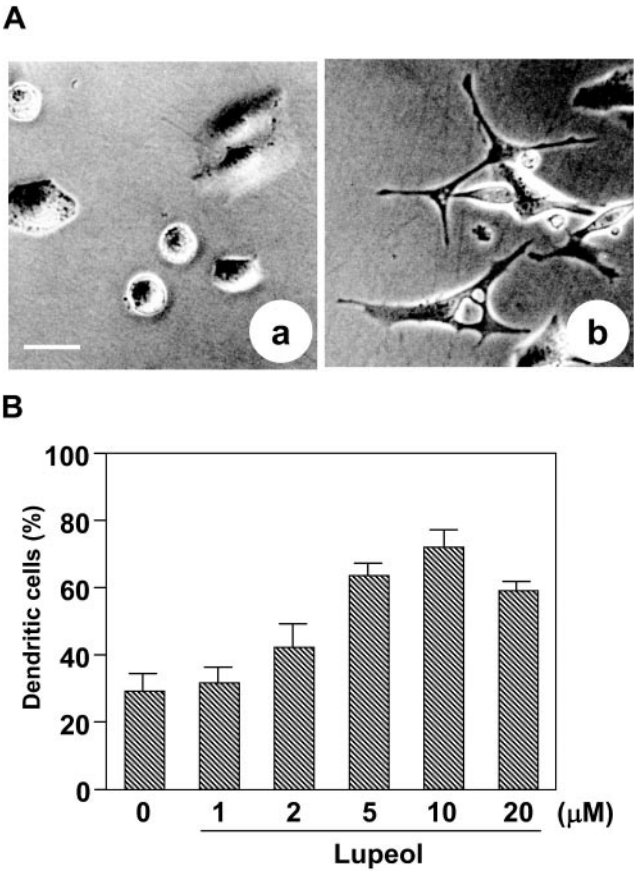

Fig. 1. **Morphological changes in B16 2F2 cells induced by lupeol**. A: B16 2F2 cells were incubated without lupeol (a) or with 10 μM lupeol (b) for 12 h, and the morphological changes were confirmed under a phase-contrast microscope (bar, 50 μm). B: Following the treatment of B16 2F2 cells with various concentrations of lupeol for 12 h, the percentage of dendritic cells was calculated manually for a minimum of 200 cells ($n = 4$).

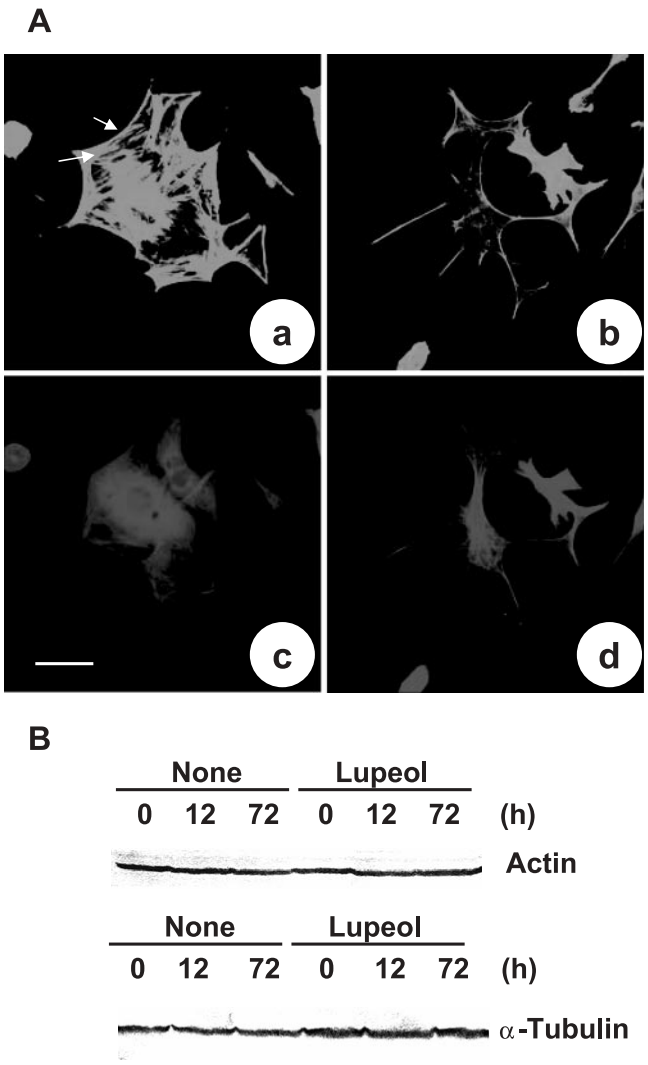

Fig. 2. **Effect of lupeol on the remodeling of microfilaments and microtubules in B16 2F2 cells.** A: B16 2F2 cells were cultured without lupeol (a and c) or with 10 μM lupeol (b and d) for 12 h, stress fibers were stained with Alexa Fluor 488 phalloidin (a and b), and the microtubular network was detected with anti–α-tubulin antibody using TRIC-conjugated secondary antibody (c and d), respectively (bar, 50 μm). The white arrows represent stress fibers. B: B16 2F2 cells were incubated with 10 μM lupeol for the periods indicated, and the proteins in the cell lysates were separated by SDS-PAGE, and subjected to western blotting with anti-actin antibody or anti–α-tubulin antibody.

was involved in the formation of dendrites in B16 2F2 cells. In untreated B16 2F2 cells, actin appeared organized in stress fibers crossing the cytoplasm (Fig. 2A-a). By treatment with 10 μM lupeol for 12 h, stress fiber assembly in the cytoplasm was disrupted, leaving phalloidin-labeled F-actin only in the dendrites (Fig. 2A-b). However, lupeol did not influence the remodeling of the microtubular network (Fig. 2A, c and d). Furthermore, we examined the effects of lupeol on the levels of actin and α-tubulin in B16 2F2 cells. Western blotting showed that the expression levels of actin and α-tubulin in B16 2F2 cells remained constant with 10 μM lupeol for 72 h (Fig. 2B). Recently, it was reported that the inactivation of cofilin, an actin depolymerization factor, was involved in the assembly of

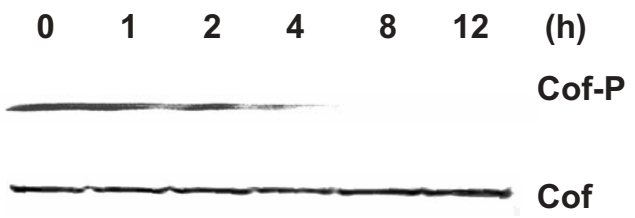

Fig. 3. **Effect of lupeol on the activation of cofilin in B16 2F2 cells.** B16 2F2 cells were incubated with 10 μM lupeol for 0–12 h, and then cell lysates were analyzed by western blotting using anti–phospho cofilin (COF-P) antibody (upper panel) or anti-cofilin (COF) antibody (lower panel).

actin stress fibers as a downstream target of Rho signaling (*18*, *19*). Using phosphospecific antibodies, we examined the levels of phospho-cofilin in B16 2F2 cells stimulated with lupeol (Fig. 3). We found that the levels of cofilin in B16 2F2 cells remained constant upon treatment with 10 μM lupeol for 12 h. The levels of phospho-cofilin in B16 2F2 cells decreased by the treatment with 10 μM lupeol in a time-dependent manner. Following incubation with lupeol for 12 h, the morphology of B16 2F2 cells was markedly changed (Fig. 1), and the phospho-cofilin in the cells had completely disappeared. Previously, we demonstrated that the activation of the p38 MAPK pathway by lupeol results in the induction of melanogenesis in B16 2F2 cells. Therefore, we studied whether the activation of p38 MAPK is involved in the remodeling of the actin cytoskeleton. Western blotting revealed that lupeol induced the expression of tyrosinase in B16 2F2 cells as compared with untreated cells (Fig. 4A). SB203580, a selective inhibitor of p38 MAPK, did not influence the expression of tyrosinase in B16 2F2 cells, and blocked the induction by lupeol (Fig. 4A). Stress fiber assembly in B16 2F2 cells was disrupted by lupeol (Fig. 4B, a and b). The actin cytoskeleton in B16 2F2 cells treated with SB203580 was maintained, and SB203580 did not block the remodeling of the actin cytoskeleton by lupeol (Fig. 4B, c and d). Furthermore, we examined the levels of phosho-cofilin in B16 2F2 cells treated with 10 μM lupeol. Treatment with 10 μM lupeol for 12 h resulted in a decrease in the levels of phospho-cofilin in B16 2F2 cells as compared with untreated cells (Fig. 4C). Moreover, SB203580 did not influence the levels of phospho-cofilin, and did not abolish the dephosphorylation of cofilin by lupeol (Fig. 4C).

It has been reported that disruptions in actin fiber assembly are involved in cancer cell motility and invasion (*15*). Therefore, we investigated the effect of lupeol on B16 2F2 cell motility in a cell migration assay. Lupeol suppressed the haptotaxis of B16 2F2 cells to fibronectin at concentrations over 5 μM, and at 10 μM, inhibited the migration of cells by 37.1%. We investigated the effects of lupeol on several types of human cancer cells (Fig. 5). The results are summarized in Table 1. At 10 μM, lupeol did not influence the growth of nine types of cancer cells, and weakly inhibited HeLa cell proliferation. Lupeol at 10 μM markedly suppressed G361 melanoma and NB-1 neuroblastoma cell migration, and weakly suppressed A549 lung adenocarcinoma cell migration. However, 10 μM lupeol did not inhibit the migration of other types of human cancer cells. Furthermore, we studied the actions of lupeol on the remodeling of the actin cytoskeleton in G361 melanoma and

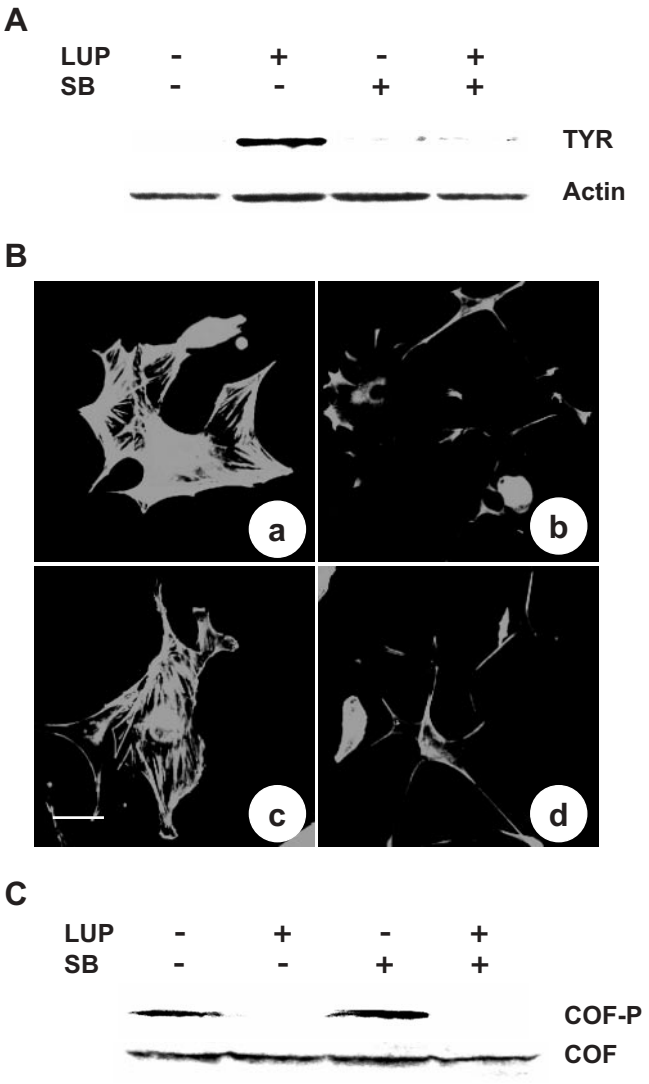

Fig. 4. **Effects of a p38 MAPK inhibitor on markers of B16 2F2 cell differentiation.** A: B16 2F2 cells were cultured alone or with 10 μM lupeol (LUP), 5 μM SB203580 (SB) or lupeol + SB203580 for 48 h, and the cell lysates were analyzed by western blotting using anti-tyrosinase (TYR) antibody (upper panel) or anti-actin antibody (lower panel). B: B16 2F2 cells were incubated alone (a) or with 10 μM lupeol (b), 5 μM SB203580 (C) or lupeol + SB203580 (d) for 12 h. The actin cytoskeleton was stained with fluorescent-labeled phalloidin (bar, 50 μm). C: B16 2F2 cells were incubated alone, or with 10 μM lupeol (LUP), 5 μM SB203580 (SB) or lupeol + SB203580 for 12 h, and the cell lysates were analyzed by western blotting using anti-phospho cofilin (COF-P) antibody (upper panel) or anti-cofilin (COF) antibody (lower panel).

Saos2 osteogenic sarcoma cells, which are sensitive and resistant to the inhibition of cell migration by lupeol, respectively. Lupeol at 10 μM caused a disruption of stress fiber assembly in G361 cells (Fig. 6, a and b), but did not influence the actin cytoskeleton of Saos2 cells (Fig. 6, c and d).

DISCUSSION

Lupeol has been found to be a differentiation-inducing compound in B16 2F2 cells, and to up-regulate the melanogenesis of these cells (*13*). In the present study, we

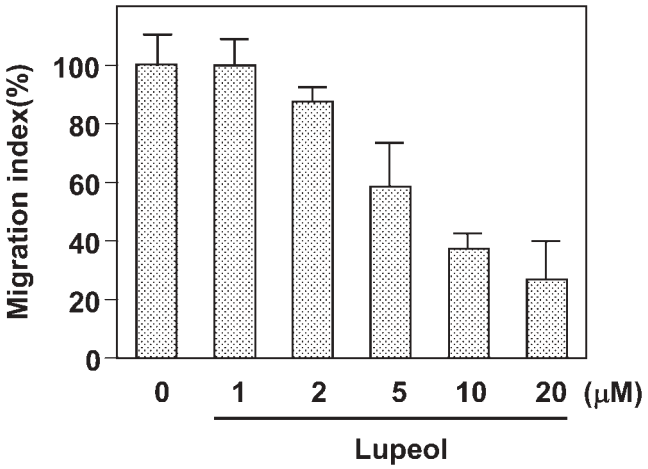

Fig. 5. **Inhibitory activity of lupeol on the migration of B16 2F2 cells.** B16 2F2 cells were seeded onto the filters of a Transwell chamber in the presence of various concentrations of lupeol. After 6 h, the cells that had migrated to the lower surfaces of the filters were manually counted ($n = 4$). The migration index represents a percentage of the control value.

Table 1. **Effects of lupeol on growth and migration of cancer cells.**

Cell line (origin)	Cell growth (%)	Migration index (%)
G361 (melanoma)	97.5 ± 3.8	40.5 ± 3.1
NB-1 (neuroblastoma)	96.0 ± 2.5	39.7 ± 6.1
A549 (lung adenocarcinoma)	100.1 ± 7.4	87.3 ± 5.0
ACHN (renal adenocarcinoma)	106.3 ± 6.9	103.4 ± 4.8
HeLa (cervical carcinoma)	72.4 ± 2.3	101.4 ± 4.1
HT1080 (fibrosarcoma)	91.6 ± 6.5	100.6 ± 10.8
MIA Paca2 (pancreatic cancer)	99.1 ± 4.9	93.1 ± 4.7
Saos2 (osteogenic sarcoma)	100.0 ± 9.8	101.3 ± 5.0
SH-10-TC (stomach cancer)	99.6 ± 5.2	94.6 ± 4.1
T24 (urinary bladder carcinoma)	90.7 ± 5.5	101.5 ± 2.3

The effects of 10 μM lupeol on cell growth for 72 h and migration for 6 h were examined. The values represent percentages, relative to the control ($n = 4$).

demonstrated that lupeol promotes the formation of dendrites in B16 2F2 cells. To characterize the morphological changes in the cells, the behavior of the actin cytoskeleton and microtubular networks in lupeol-treated B16 2F2 cells were examined under fluorescence microscopy. Our experiments reveal that lupeol induces the disassembly of actin stress fibers in the cytoplasm of B16 2F2 cells, but does not influence actin fibers in the dendrites or the expression levels of actin. Additionally, lupeol does not change the behavior of microtubular networks, or tubulin expression levels in B16 2F2 cells. These results show that rearrangement of the actin cytoskeleton by lupeol results in the formation of dendrites in B16 2F2 cells, and that lupeol induces the functional (melanogenesis) and morphological (dendritic formation) differentiation of B16 2F2 cells. Recently, the signaling mechanisms involved in the disassembly of actin stress fibers have been studied. Rho up-regulates the formation of stress fibers through the activation of downstream effectors such as Rock and Rho-kinase. Rock and Rho-kinase activate LIM-kinases,

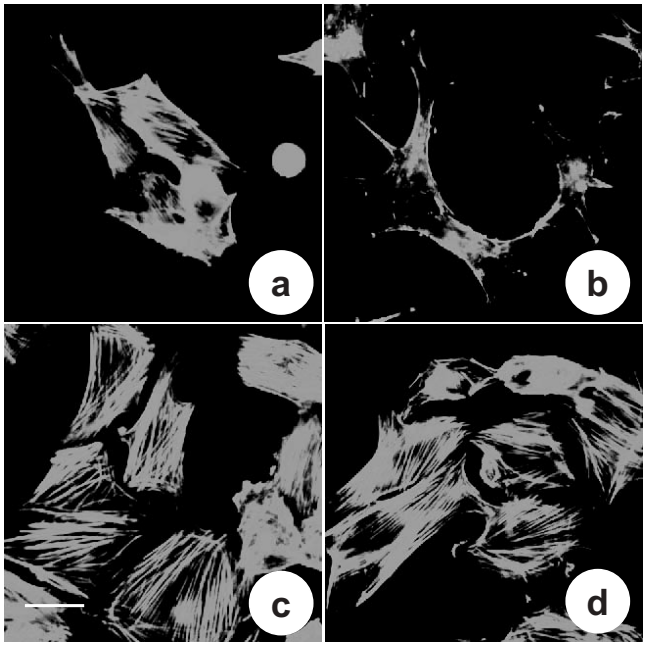

Fig. 6. **Actin cytoskeleton in human cancer cells treated with lupeol.** G361 melanoma (a and b) and Saos2 osteogenic sarcoma (c and d) cells were incubated without lupeol (a and c), or with 10 μM lupeol (b and d) for 12 h. The actin cytoskeleton was stained with fluorescent-labeled phalloidin (bar, 50 μm).

which are responsible for the phosphorylation and inactivation of actin depolymerization factors such as cofilin (*18–20*). In contrast to the mechanisms of inactivation of cofilin by LIM-kinases, Slingshot was found to dephosphorylate phospho-cofilin and reactivate cofilin to depolymerize F-actin (*21*). In the present study, it was revealed that the levels of phospo-cofilin in B16 2F2 cells decrease upon stimulation with lupeol, suggesting that lupeol might attenuate stress fiber assembly in B16 2F2 cells following the inhibition of Rho signaling and/or the activation of phosphatase such as Slingshot.

Previously, we demonstrated that p38 MAPK signaling is involved in the melanogenesis of B16 2F2 cells induced by lupeol (*14*). In lupeol-treated B16 2F2 cells, the induction of tyrosinase, a hallmark of melanoma cell differentiation, is significant, and the inhibition of p38 MAPK signaling by SB203580 abolishes the induction of tyrosinase. However, SB203580 does not affect the disruption of stress fibers and dephosphorylation of phospho-cofilin by lupeol. These results suggest that both melanogenesis and dendritic formation are hallmarks of melanoma cell differentiation, but that they were regulated separately by the activation of p38 MAPK signaling and cofilin, respectively.

Moreover, we examined the effect of lupeol on haptotaxis to fibronectin. It has been reported that other melanogens, such as forskolin and α-MSH, block the migration of B16 melanoma cells (*22*). Our results suggest that lupeol inhibits B16 2F2 cell migration in a dose-dependent manner. Our previous study revealed the IC_{50} of lupeol for B16 2F2 cell growth (38.0 μM), and that 10 μM lupeol does not influence melanoma cell growth (*23*). These results indicate that the remodeling of the actin cytoskeleton in B16 2F2 cells by lupeol, not cell growth inhibition, produce the inhibitory effects on melanoma cell migration. In the examination of the inhibitory effects of lupeol on different types of human cancer cells, lupeol selectively suppressed the migration of melanoma and neuroblastoma cells, which are derived from the neural crest (*24*, *25*), but had no influence on the migration or morphological changes of other cancer cells. These results suggest that lupeol might selectively disrupt the assembly of actin stress fibers in neural crest–derived cells, and this action results in its inhibitory effects on cell migration. Recently, it was shown that lupeol exhibits an anti-angiogenic effect through the inhibition of HUBEC tube formation (*26*). In this paper, we demonstrate the anti-migration activity of lupeol against melanoma and neuroblastoma cells. These results suggest that lupeol could be an effective compound to prevent tumor metastasis.

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