Lovastatin suppresses invasiveness of anaplastic thyroid cancer cells by inhibiting Rho geranylgeranylation and RhoA/ROCK signaling

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Abstract

Lovastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, inhibits the conversion of mevalonate from HMG-CoA. Previously, we have reported that lovastatin treatment induced the occurrence of apoptosis and differentiation in ARO anaplastic thyroid cancer cells. Here, we demonstrated that lovastatin inhibited the ARO cell invasiveness and delineated the underlying molecular mechanism. Lovastatin significantly suppressed the EGF-induced cell adhesion, actin filament reorganization and transmigration. Lovastatin also reduced EGF-induced increases in the levels of phosphorylated p125^{FAK} and paxillin. These inhibitory effects mediated by lovastatin can be prevented by pretreatment of the cells with mevalonate or geranylgeraniol (GGOH), but not farnesol (FOH). Accordingly, the consuming and depletion of geranylgeranyl pyrophosphate and consequent suppression of the protein geranylgeranylation, which is essential for activation of Rho GTPases, might account for the lovastatin-induced inhibition of cell motility and invasion. Western blot analysis showed that lovastatin inhibited membrane translocation of Rho (e.g. RhoA and Rac1) through decreasing post-translational geranylgeranyl modification of Rho. In addition, treatment of the cells with specific inhibitors against Rho (*Clostridium botulinum* C3 transferase) or ROCK (Y-27632) abolished the GGOH-mediated prevention of, and restored the lovastatin-induced decrease of cell invasion. Taken together, our results suggested that lovastatin suppressed EGF-induced ARO cell invasiveness through the reduction of Rho geranylgeranylation, which in turn suppressed the membrane translocation, and subsequent suppression of Rho/ROCK and FAK/paxillin signaling.

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Introduction

Anaplastic thyroid cancer is one of the most aggressive malignant tumors. Patients with anaplastic thyroid cancer have a poor prognosis with a mean survival time of 2–6 months (Tan *et al*. 1995). It is generally believed that the invasion outward into surrounding tissues and the distant metastasis cause its poor prognosis. Surgery, radiotherapy and chemotherapy are not very helpful in improving the survival time and the life quality of such patients (Venkatesh *et al*. 1990).

Failure of chemotherapy is due to the development of resistance to chemotherapeutic agents, such as doxorubicin (Tennvall *et al*. 1994). Recently, antimetastatic or antiinvasive therapy has been suggested as an approach to decrease the mortality of anaplastic thyroid cancer patients.

Cancer cell migration is a complicated process including cytoplasmic membrane protrusion in leading lamellipodia, new adhesions at this leading edge, construction through the cell body and detachment from the substratum at the tailing edge (Stupack &

Cheresh 2002). In general, these actions are controlled by a cell signaling cascade initiated by adhesion molecules (e.g. integrins) or growth factor receptors, such as epidermal growth factor (EGF). Overexpression of EGF or EGF receptor has been found in most thyroid cancers, including anaplastic thyroid cancer cells (Bergstrom *et al.* 2000). EGF can trigger the cytoskeletal reorganization of actin microfilaments through regulating a member of the Rho family of GTPase, such as Rho, Rac and Cdc42 (Chan *et al.* 2000). At the leading edge of the migrating cell, Rac and Cdc42 regulate lamellipodia and filopodia formation respectively. Rho is required for the formation and maintenance of focal adhesions (Nobes & Hall 1999). Moreover, it has been shown that RhoA, a member of the Rho subfamily, plays a central role in cellular functions related to survival, motility, apoptosis and invasion (Gomez *et al.* 1998). Previously, it has been demonstrated that RhoA/RhoA-associated kinase (ROCK, a downstream effector of RhoA)-coupled signaling is essential for controlling cell apoptosis, migration and angiogenesis (Riento & Ridley 2003). ROCK activates the myosin light chain (MLC) phosphorylation through the activation of MLC kinase or inactivation of MLC phosphatase. The activation of MLC results in an increase in actomyosin-based contractility, which is important for cell migration (Totsukawa *et al.* 2000).

Focal adhesions, the other important element for promoting cell motility, promote cell adherence to extracellular matrix (ECM) via specific integrin molecules (members of the ECM receptor family) at the attaching surface of culture cells. Alteration of such adherence interactions between cells and ECM affects the attachment and migration of the cells through surrounding tissues (Hood & Cheresh 2002). Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase associated with integrin within the focal adhesion complex, plays an important role in integrin-mediated cell adhesion and cell spreading for the cancer invasion processes (Kornberg *et al.* 1992). FAK-associated proteins, such as p130[Cas], Crk and paxillin, have been shown to function as positive regulators of integrin-mediated cell migration (Panetti 2002).

HMG-CoA reductase inhibitors have been widely used to reduce cardiovascular morbidity and mortality (Plosker & Wagstaff 1996, Sacks *et al.* 1996). In addition to a marked beneficial action on the blood lipid profile, HMG-CoA reductase inhibitors also exert some effects unrelated to lipid metabolism (Massy *et al.* 1996, Vaughan *et al.* 1996). The inhibitory effects of HMG-CoA reductase inhibitors on the growth of cultured malignant cells are related to their ability to block the isoprenylation of small GTP-binding proteins, including Ras and Rho (Goldstein & Brown 1990). Recently, we have demonstrated that lovastatin, an HMG-CoA reductase inhibitor, can inhibit cellular proliferation and induce the occurrence of apoptosis in cultured anaplastic thyroid cancer cells (Zhong *et al.* 2003). We also showed that lovastatin induced differentiation in human anaplastic thyroid carcinoma cells (Wang *et al.* 2003). Although HMG-CoA reductase inhibitors have been demonstrated to decrease the migration of vascular smooth muscle cells (Yasunari *et al.* 2001) and the metastatic invasion in pancreatic cancer cells *in vitro* and *in vivo* (Kusama *et al.* 2001), the molecular mechanisms underlying lovastatin-mediated inhibition on cancer cell invasion remain unclear.

Here, we showed that lovastatin effectively suppressed the ECM-associated adhesion and EGF-induced invasion processes, including cell spreading, tyrosine phosphorylations of FAK and paxillin, and reorganization of actin stress fibers. These lovastatin-mediated inhibitory effects observed in anaplastic thyroid cancer cells resulted from exhausting of the intracellular GGPP pools, which in turn decreased the geranylgeranyl modification of Rho GTPases (RhoA and Rac1) and their membrane targeting.

Materials and methods

Reagents

Lovastatin was a gift from Standard Chemical and Pharmacy Co. (Taiwan). Fetal bovine serum (FBS), culture medium RPMI 1640, penicillin and streptomycin were obtained from Life Technologies (Grand Island, NY, USA). GGTI-298, FTI-277 and *Clostridium botulinum* C3 exoenzyme were obtained from Calbiochem (La Jolla, CA, USA). Y-27632 was purchased from Tocris Cookson Ltd (Avonmouth, UK). Recombinant human EGF was purchased from R&D Systems (Minneapolis, MN, USA). Matrigel and fibronectin were obtained from Becton Dickinson/Biocoat (Bedford, MA, USA). FITC-phalloidin was obtained from Molecular Probes (Eugene, OR, USA). Transwell chambers were obtained from Costar (Cambridge, MA, USA). Bovine serum albumin (BSA), mevalonate, GGOH, FOH and gelatin were purchased from Sigma. Antibodies against pan-Ras and phospho-FAK (Tyr397) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-RhoA, Rac1, FAK, paxillin and phospho-paxillin

(Tyr181) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The human anaplastic thyroid cancer ARO cells were cultured in RPMI 1640 medium supplemented with 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37° C with 5% CO_2. The cells were subcultured twice a week and only those in exponential growing phase were used in these experiments.

Matrigel invasion assay

Cell invasion through reconstituted basement membrane Matrigel was assayed by a method described previously (Hirohashi 1998). Briefly, polycarbonate membranes (8.0 μm pore size) of the upper compartment of Transwell culture chambers were coated with 10% Matrigel (50 μl/insert). Subconfluent cells were starved in serum-free medium with 0.1% BSA (fraction V) for 24 h, supplemented with lovastatin during the last 18-h incubation. These conditioned cells were harvested and resuspended (1×10^6 cells/ml) in serum-free medium (0.1% BSA) with or without lovastatin. The cell suspension (100 μl) was placed onto the upper compartment, and the lower compartment was filled with 600 μl serum-free medium containing 0.1% BSA and EGF at various concentrations as indicated. In some studies, mevalonate, GGOH or FOH was added to the upper compartment. After 24-h incubation, the membranes (cells) were fixed with 4% paraformaldehyde for 20 min and then stained with 1% methylene blue/20% methanol solution. The cells that had migrated through the membrane to the lower surface were counted in six different fields under a light microscope at ×400 magnification.

Separation of particulate and cytosolic fractions

Separation of cytosolic and membrane fraction was performed as described previously (Zhong *et al.* 2003). Briefly, the cells were washed with cold PBS and lysed by 5-cycle freeze-thawing in lysis buffer containing 50 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 10 mM NaF, 1 mM DTT, 10 mg/ml leupeptin, 10 mg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 100 000 *g* for 30 min at 4° C. The supernatant was collected as the cytosolic fraction. Pellets were washed with above-mentioned lysis buffer, and then homogenized in the lysis buffer containing 2% Triton X-100 plus 1% SDS, and finally centrifuged at 10 000 *g* for 30 min at 4° C. The supernatant was collected as the particulate (membrane) fraction.

Western blot analysis

Proteins of cytosolic and particulate fractions were electrophoresed on 12% polyacrylamide gels and electrophoretically transferred (350 mA, 2 h) to Hybond-P polyvinylidine difluoride (PVDF) membranes (Amersham). Western blot analysis was carried out with specific primary antibodies in 1% BSA/TBST, washed with TBST (3 × 10 min), and then probed with the peroxidase-conjugated secondary antibody. Target proteins were observed by the enhanced chemiluminescence (ECL) detection system (Amersham). The ECL blots were exposed on Fuji super RX film (Fuji, Tokyo, Japan) for 1–2 min.

F-actin staining and flow cytometry analysis

The cells were fixed for 15 min with 4% paraformaldehyde in PBS (pH 7.5), washed twice with PBS and then quenched in 1 mM glycine in PBS for 15 min. After an additional wash, the cells were permeabilized with 0.2% Triton X-100 (w/v) in PBS containing 1% BSA for 10 min, and nonspecific binding sites were blocked with 1% BSA. The cells were then treated for 30 min with 500 nM FITC-conjugated phalloidin at room temperature. After washing, the cells were detected with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed with CELLQuest software (Becton Dickinson). The F-actin staining intensity represents the degree of actin reorganization and polymerization.

Cell adhesion assay

Cell adhesion assays were performed in 24-well plates (Nunc, Napierville, IL, USA) pre-coated without or with ECM proteins (gelatin, fibronectin or recombinant Matrigel). Cell suspension (1×10^5/ml) was seeded onto the precoated wells and incubated for 1 h at 37° C. Nonadherent cells were removed by washing three times with PBS, and the adherent cells were fixed and then stained with 0.5% crystal violet/20% methanol for 15 min. For quantitative estimation of cell adhesiveness, crystal violet was eluted with 33% acetic acid, and absorbance at 640 nm was measured with ELISA reader (MRX II, Dynex Technologies, Chantilly, VA, USA).
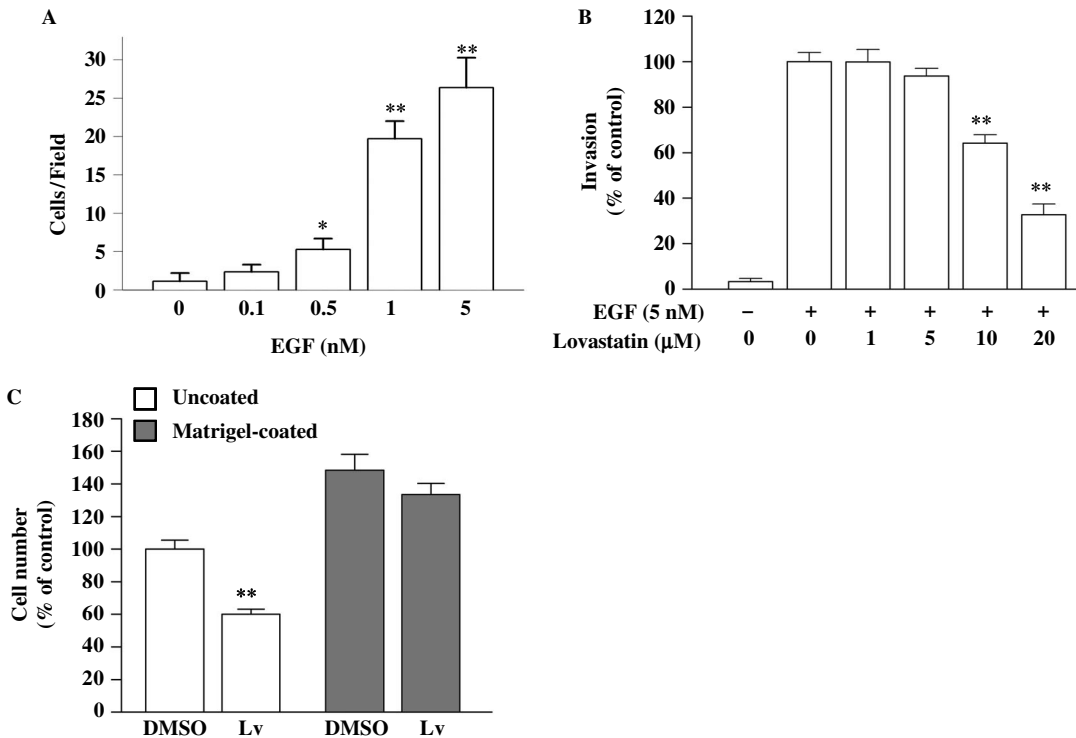
Figure 1 Inhibitory effects of lovastatin on EGF-induced invasion through Matrigel in ARO cells. (A) EGF dose-dependently increased ARO cell invasion through Matrigel. The invasion assay was conducted as described in the Materials and methods section. (B) Lovastatin dose-dependently inhibited EGF-induced ARO cell invasion through Matrigel. The ARO cells (1×10^5) were pretreated with various concentrations of lovastatin for 18 h before invasion assay was conducted. EGF (5 nM) was added to the lower chamber as a chemoattractant for cell invasion. After 24-h incubation, transmigrated cells were stained and counted. (C) The cell number was significantly inhibited by lovastatin when ARO cells were grown on the uncoated wells, but not on the Matrigel-coated wells used for invasion study. Lovastatin-treated cells were cultured on a dish coated with or without Matrigel for 24 h. Values represent the means $\pm$ s.e.m. ($n = 3-4$). *$P < 0.05$ different from control (without EGF stimulation); **$P < 0.01$ different from control. Lv, lovastatin.

Statistical analysis

All data were expressed as the mean value $\pm$ s.e.m. Comparisons were subjected to Student's two-tailed t-test. Significance was accepted at $P < 0.05$.

Results

Lovastatin inhibits the EGF-induced invasion in ARO cells

Initially, we tested the chemotactic efficacy of EGF. As illustrated in Fig. 1A, EGF at a range of concentrations (0.1–5.0 nM) dose-dependently increased the penetration of ARO cells across the Matrigel-coated membrane to the lower face of the membrane, suggesting that EGF can enhance the invasiveness of ARO cells. Accordingly, we used 5 nM EGF as a chemoattractant for subsequent studies of invasive

activity. To examine whether the lovastatin could suppress the EGF-induced increase in cell invasion, the ARO cells were treated with lovastatin at various concentrations (1.0–20 μM). As shown in Fig. 1B, lovastatin significantly decreased the EGF (5 nM)-induced increase in invasiveness of ARO cells in a dose-dependent manner. Importantly, lovastatin at the highest concentration (20 μM) used in this study did not cause detectable ARO cell death (data not shown). To rule out the possibility that lovastatin-mediated invasion inhibition was due to growth inhibition, ARO cells were pretreated with or without lovastatin for 18 h, and then cultured in Matrigel-coated wells for 24 h. As shown in Fig. 1C, the cell number was significantly decreased by lovastatin when ARO cells were grown on the uncoated wells, but not on the Matrigel-coated wells used for invasion studies.
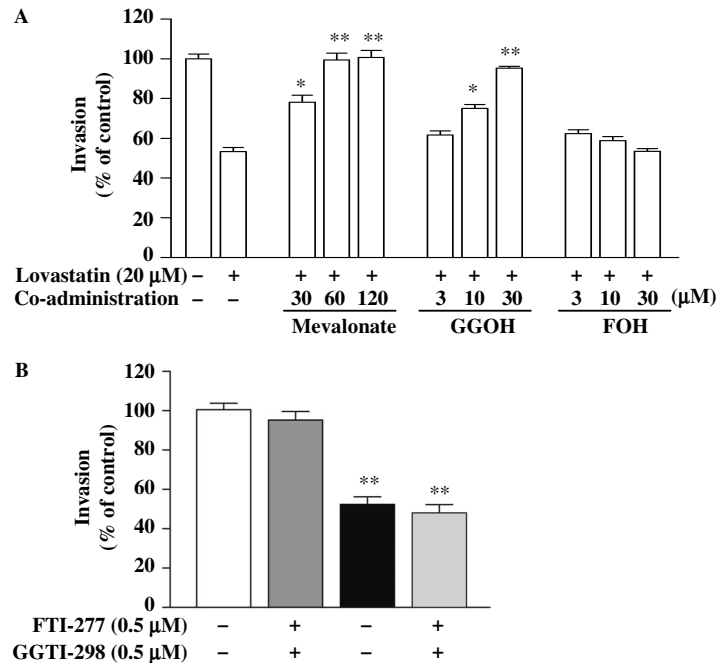
Figure 2 Involvement of protein geranylgeranylation in EGF-induced ARO cell invasion. (A) Mevalonate and GGOH, but not FOH, prevented the lovastatin-mediated inhibition of cell invasion. The ARO cells were pretreated with lovastatin together with mevalonate, GGOH, or FOH for 18 h before invasion assay was performed. Values represent the means ± S.E.M. ($n = 3$). *$P < 0.05$ different from treatment with lovastatin alone; **$P < 0.01$ different from treatment with lovastatin alone. (B) Rho is involved in the EGF-induced ARO cell invasion. Treatment of ARO cells with GTI-298 (GGTase inhibitor), but not FTI-277 (FTase inhibitor), inhibited EGF-induced ARO cell invasion. ARO cells were pretreated with FTI-277, GGTI-298, or FTI-277 plus GGTI-298 for 2 h before invasion assay. Values represent the means ± S.E.M. ($n = 3$). **$P < 0.01$ different from control. GGOH, geranylgeraniol; FOH, farnesol.

Geranylgeraniol and mevalonate impair the inhibitory effect of lovastatin on the EGF-induced invasiveness of ARO cells

To examine whether the farnesylated and geranylgeranylated proteins are involved in the lovastatin-induced inhibition in cell invasion, the ARO cells were treated with lovastatin (20 μM) in the presence of mevalonate or the mevalonate-derived isoprenoids, including FOH and GGOH. As shown in Fig. 2A, the lovastatin-induced inhibition of ARO cell invasion was completely reversed by cotreatment of the cell with mevalonate at a concentration of 60 μM, suggesting that inhibition of mevalonate production is responsible for the suppression effect of lovastatin on the ARO cell invasion. Moreover, cotreatment of the cells with lovastatin and GGOH (30 μM), which is metabolized to GGPP in the cells, also significantly reversed the lovastatin-induced suppression of ARO cell invasion up to 80%. In contrast, coadministration of lovastatin with 30 μM FOH did not affect the inhibitory effect of lovastatin on ARO cell invasion. FOH at a concentration higher than 30 μM, which induced the occurrence of synergistic cytotoxicity (Zhong *et al.* 2003), was not used in the study. Importantly, GGOH or FOH alone did not increase ARO cell invasion in the media with or without EGF stimulation (data not shown).

Protein geranylgeranylation is essential for EGF-induced ARO cell invasion

Since farnesylation and geranylgeranylation are important for subcellular distribution and functions of Ras and Rho, we further examined the involvement of geranylgeranylation of the Rho proteins in regulating the ARO cell invasion. As illustrated in Fig. 2B, treatment of the cells with GGTI-298 (an inhibitor of GGTase) significantly decreased the migration of ARO cells through Matrigel-coated polycarbonate membrane. In contrast, administration of FTI-277 (an inhibitor of FTase) did not affect the EGF-induced increase and GGTI-298-induced inhibition in ARO cell migration. These results suggested that protein geranylgeranylation is important for ARO cell transmigration to the EGF.
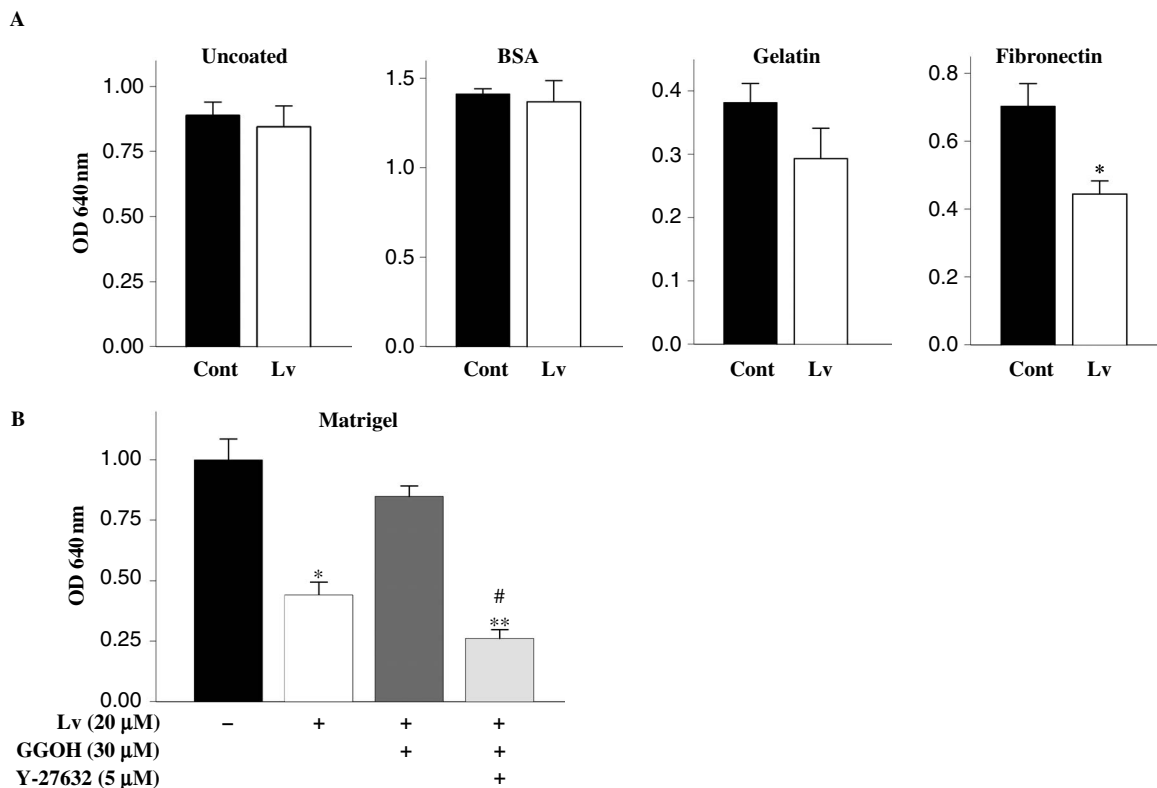
A

B

Figure 3 Lovastatin suppresses ARO cell adhesion through inhibiting the Rho/ROCK signaling pathway. (A) Effect of ECM components on lovastatin-induced cell adhesion. The ARO cells were cultured in serum-free medium in the absence (Cont) or presence of Lv for 24 h. Cell suspension (1×10^5) was seeded onto the wells coated without or coated with 1% BSA, gelatin, fibronectin, or Matrigel. After 1-h incubation, nonadherent cells were removed and washed with PBS. The adherent cells were then stained, eluted and measured by OD 640 nm. (B) Involvement of Rho/ROCK in the lovastatin-induced ARO cell adhesion. Cotreatment of the cells with GGOH reversed the Lv-induced decrease in ARO cell invasion. In contrast, treatment of the cells with Lv in combination with GGOH and Y-27632 (ROCK inhibitor) together abolished the GGOH-mediated prevention of Lv-induced inhibition of ARO cell invasion. Values represent the means ± S.E.M. ($n = 4$). *$P < 0.01$ different from control; $P < 0.05$ different from lovastatin treatment alone; **$P < 0.01$ different from cotreatment with lovastatin and GGOH. Lv, lovastatin; GGOH, geranylgeraniol.

Lovastatin suppresses ARO cellular adherence by inhibiting Rho/ROCK signaling

It has been reported that the degree of adhesion of cancer cells is correlated with the malignancy or metastatic activity (Hirohashi 1998). To evaluate the effect of lovastatin on cell adhesion to ECM proteins, the cells were grown in serum-free medium and the adhesion substratum coated with various ECM proteins, including gelatin, fibronectin and Matrigel. As shown in Fig. 3A, the amounts of adherent cells were not significantly different between control and the lovastatin-treated ARO cells in cell culture plate without coating or coated with BSA or gelatin. On the other hand, lovastatin reduced the amount of adherent cells in the fibronectin- and Matrigel-coated substratum to 60% and 50% respectively.

To further investigate the inhibitory effect of lovastatin on cell adhesion, the ARO cells were pretreated with GGOH and FOH followed by lovastatin, and then adhesion assay was performed on Matrigel-coated substratum for 1 h. Cotreatment of the cells with GGOH (30 μM) reversed the lovastatin-mediated suppression of adhesion up to 60% (Fig. 3B), while cotreatment with FOH had no effect (data not shown). Administration of Y-27632 (a selective inhibitor of ROCK) in serum free adhesion system, however, abolished the GGOH-mediated prevention of lovastatin-induced inhibition of ARO cell adherence (Fig. 3B). These results suggested that lovastatin attenuated the cellular adherence of the ARO cells to ECM proteins, such as fibronectin and recombinant Matrigel, through inhibiting the Rho/ROCK signaling pathway.
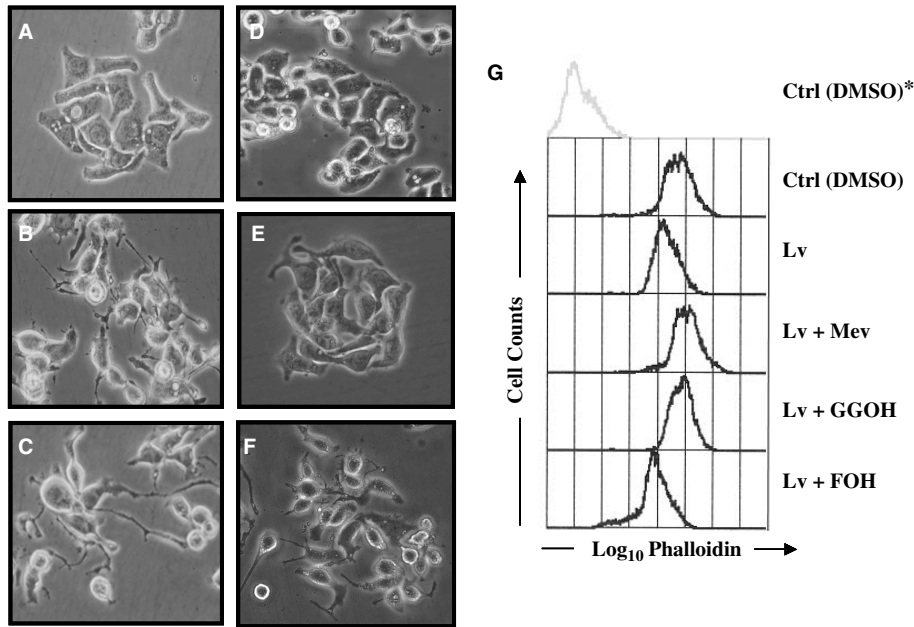
Figure 4 Effects of lovastatin on cell morphology and reorganization of actin stress fiber induced by EGF in ARO cells. The ARO cells were cultured in serum-free medium in the absence (A) or presence of 20 μM lovastatin for 24 (B) or 72 h (C). 100 μM Mev (D), 30 μM GGOH (E) or 30 μM FOH (F) were added to medium in the presence of lovastatin for 24 h. Cell morphologic alterations, including cell retraction from adhesion substratum, shrinkage of cytoplasm and rounding-up were visualized under light microscope. (G) Cells treated without or with lovastatin (Lv) in combination with Mev, GGOH, or FOH as indicated were fixed and stained with FITC-phalloidin after 15-min stimulation with EGF, and then analyzed by FACS technique. *Histogram represents the autofluorescence of the fixed control cells without FITC-phalloidin staining. Mev, mevalonate; GGOH, geranylgeraniol; FOH, farnesol.

Effects of lovastatin on cell morphology and cytoskeletal organization in ARO cells

Membrane protrusion and extension at the leading edge of cells are the initial processes for cell migration. The membrane protrusion is characterized by the polymerization and stabilization of actin filaments networks and the generation of adhesion complexes (Stupack & Cheresh 2002). In the absence of lovastatin, the ARO cells attached well to the culture dish (Fig. 4A). Treatment of the ARO cells with lovastatin (20 μM) for 24 h resulted in cell retraction from adhesion substratum and shrinkage of cytoplasm (Fig. 4B). When the incubation time was extended to 72 h, most of the cells rounded-up and sent out processes (Fig. 4C). These morphologic alterations can be reversed after removal of lovastatin for 48 h (data not shown). Lovastatin-induced morphologic changes could also be prevented by coadministration of the cells with mevalonate or GGOH for 24 h (Fig. 4D and E). The morphology of the cells treated with lovastatin and FOH together (Fig. 4F), however, is similar to those treated with lovastatin

alone (Fig. 4B). To further investigate the effects of lovastatin on the formation of actin stress fiber, which results from polymerization of actin to filamentous bundles, the ARO cells were challenged with 5 nM EGF for 15 min. As shown in Fig. 4G, lovastatin treatment caused a significant decrease in filamentous actin fibers. However, administration of mevalonate or GGOH reversed the lovastatin-induced reduction of actin filament assembly, while FOH treatment further decreased the intracellular F-actin. These results suggest that the inhibitory effect of lovastatin on the formation of stress fibers is through inhibiting the synthesis of the active geranyl-geranylated Rho.

Involvement of Rho signaling in lovastatin-induced inhibition of the focal adhesion complex formation

Since the focal adhesion complex is one of the important components promoting cell motility, the adhesive interactions between cells and ECM can influence the attachment and transmigration across the
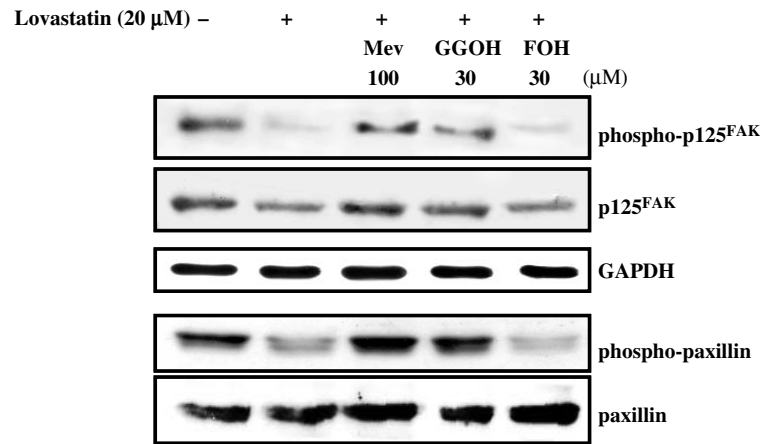
Lovastatin (20 µM) – + + + +
                         Mev GGOH FOH
                         100   30   30 (µM)

phospho-p125^{FAK}

p125^{FAK}

GAPDH

phospho-paxillin

paxillin

Figure 5 Involvement of protein geranylgeranylation in lovastatin-induced inhibitions of focal adhesion complex formation. Lovastatin at a concentration of 20 µM reduced p125^{FAK} protein level and phosphorylations of p125^{FAK} and paxillin. Pretreatment of ARO cells with Mev or GGOH, but not FOH, prevented these reductions. The ARO cells were cultured in serum-free medium and treated with DMSO (control), lovastatin or lovastatin plus Mev, GGOH or FOH. After 24-h incubation, the cells were stimulated with EGF (10 nM) for an additional 30 min followed by total protein extraction and Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also stained to serve as an internal loading control. Mev, mevalonate; GGOH, geranylgeraniol; FOH, farnesol.

surrounding cells (Hood & Cheresh 2002). To evaluate the effects of lovastatin on the EGF-stimulated formation of focal adhesion complexes, tyrosine phosphorylations of focal adhesion kinase (FAK) and paxillin, two elementary proteins for forming stable adhesion complexes and transducing the survival/motility signals, were examined in the lovastatin-treated ARO cells. As illustrated in Fig. 5, lovastatin downregulated the tyrosine phosphorylations of FAK and paxillin, and decreased the protein level of FAK, but not paxillin. These inhibitory effects induced by lovastatin were reversed by mevalonate and GGOH, but not FOH. These results suggest that the inhibitory effect of lovastatin on the phosphorylation of FAK and paxillin was through a reduction of the Rho geranylgeranylation, which is required for activating and transducing the Rho-mediated signals. Since translocation of Rho GTPases from the cytosol to the cytoplasmic membrane is required for their activations and functions, we further examined lovastatin treatment on membrane translocation of Rho GTPases. As illustrated in Fig. 6A, lovastatin dose-dependently decreased the protein levels of both RhoA and Rac1 (two members of Rho family GTPases) in the membrane (particulate) fraction of ARO cells. In contrast, lovastatin treatment did not cause any significant change in the subcellular distribution of Ras. These results suggested that lovastatin could deplete the intracellular GGPP reservoir, and subsequently block the geranylgeranyl modification of

Rho family members, such as RhoA and Rac1. To further investigate whether the preventive effects of mevalonate and GGOH on lovastatin-inhibited invasion are dependent on membrane translocation of Rho family, we analyzed subcellular localizations of Ras and Rho protein. As shown in Fig. 6B, lovastatin-mediated decrease of Rho proteins (RhoA and Rac1) in the particulate fraction was prevented by mevalonate and GGOH, but not FOH, whereas the protein levels of Ras in the particulate fraction were not significantly different among treatments. Since the post-translational process (such as isoprenylation) is essential for membrane translocation and sustained anchorage of G proteins, the processing status of Ras and Rho was examined with higher-percentage polyacrylamide gels. As shown in Fig. 6C, lovastatin blocked the processing modification of RhoA. However, mevalonate and GGOH, but not FOH, prevented the lovastatin-induced inhibition of RhoA protein processing. In contrast, protein modification of Ras was not influenced substantially even in the presence of FTI-277. To confirm that Ras protein is not involved in the lovastatin-mediated effects in ARO cells, we conducted an additional experiment in rat aortic smooth muscle cells (RASMC), showing that treatment of RASMC with lovastatin resulted in an inhibition of the processing modification of both Ras and RhoA proteins, which was prevented by mevalonate/FOH and mevalonate/GGOH respectively (Fig. 6D). These results indicated that administration
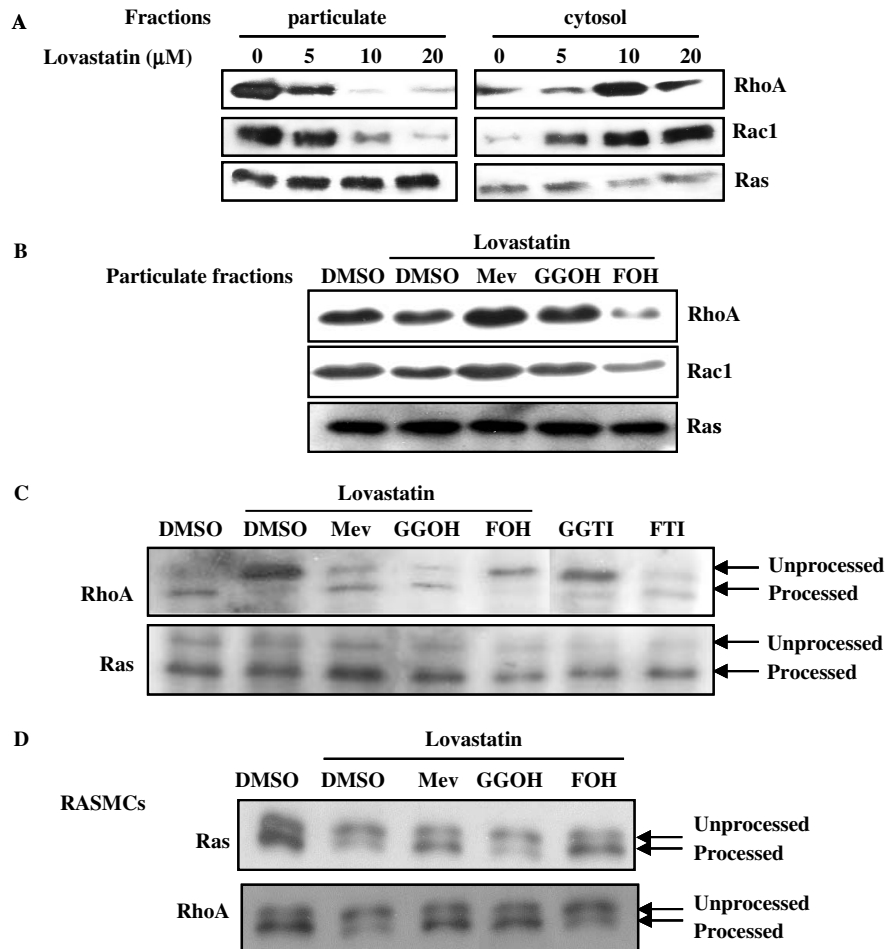
Figure 6 Lovastatin inhibits geranylgeranylation and membrane translocation of Rho GTPases induced by EGF in ARO cells. ARO cells were incubated without or with various concentrations of lovastatin (A) or coincubated for 24 h with 100 μM Mev, 30 μM GGOH or 30 μM FOH (B), and then stimulated with EGF (5 nM) for 15 min. Proteins in cytosolic and particulate fractions were detected for RhoA, Rac1 and Ras by Western blot analysis. (C) RhoA geranylgeranyl modification was inhibited by lovastatin. The cells were also treated with GGTI (0.5 μM) and FTI (0.5 μM) as post-translational isoprenyl processing controls for Rho and Ras respectively. In this experiment, 16% SDS-acrylamide gels were used. (D) Rat aortic smooth muscle cells were treated at a condition described in (B), and the isoprenyl processing status of Ras and RhoA was detected with 16% SDS–PAGE. Representative data from three independent experiments are shown. RASMCs, rat aortic smooth muscle cells; Mev, mevalonate; GGOH, geranylgeraniol; FOH, farnesol.

of GGOH/FOH to the lovastatin-treated cells exerts the same effect as GGPP/FPP in restoring the Ras/Rho isoprenylation.

Rho/ROCK signaling is required for EGF-induced invasion of the ARO cells

Since treatment of the ARO cells with lovastatin led to loss of Rho function, we hypothesize that activation of geranylgeranylated Rho and downstream signal molecules is essential for EGF-induced cell migration. To test this hypothesis, the ARO cells were treated with *Clostridium botulinum* C3 transferase, which inactivates Rho GTPase activity by catalyzing the ADP-ribosylation of RhoA, RhoB and RhoC. As shown in Fig. 7A, treatment of ARO cells with C3 transferase significantly reduced the EGF-induced transmigration up to 65%. Moreover, treatment with Y-27632, a ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor, dose-dependently inhibited the invasiveness of ARO cells. Nevertheless, pretreatment with C3 transferase or Y-27632 abolished the GGOH-induced prevention effect on lovastatin-mediated inhibition of ARO cell invasion (Fig. 7B).
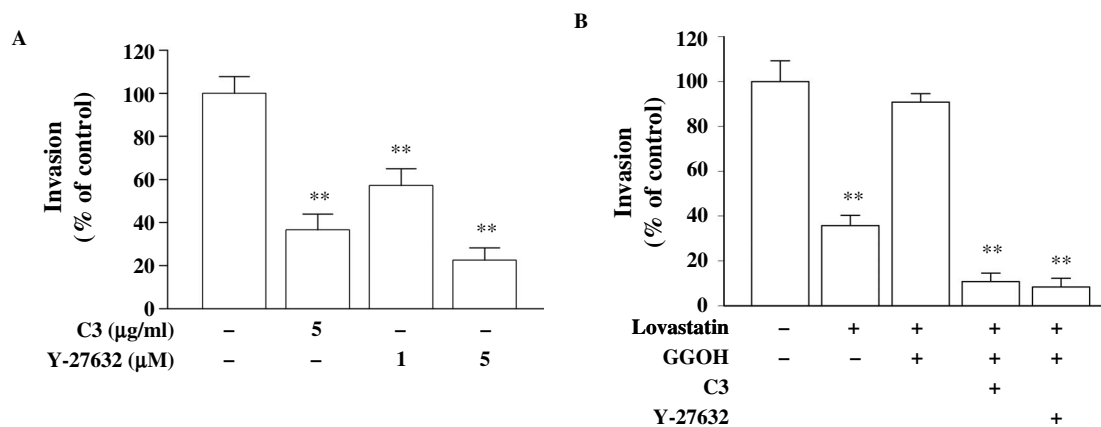
Figure 7 Involvement of Rho/ROCK signaling pathway in the lovastatin-mediated inhibition on EGF-induced ARO cell invasion. (A) Inhibition of RhoA/ROCK signaling decreased EGF-induced ARO cell invasion. Pretreatment of ARO cells with either C3 transferase (5 μg/ml) for 18 h or Y-27632 (1 and 5 μM) for 2 h reduced the EGF (5 nM)-induced ARO cell invasion. Values represent the means ± s.e.m. (n = 3). **P < 0.01 different from control. (B) Pretreatment of ARO cells with C3 transferase (5 μg/ml) or Y-27632 (5 μM) abolished the GGOH (30 μM)-induced prevention effect on lovastatin (20 μM)-mediated inhibition of ARO cell invasion. GGOH, geranylgeraniol.

Discussion

It has been reported that HMG-CoA reductase inhibitors can reduce cellular motility and migration in vascular smooth muscle cells (Yasunari et al. 2001), and suppress cell metastasis and invasion in various cancer cells, including mammary carcinoma (Farina et al. 2002) and pancreatic cancer (Kusama et al. 2001), in vitro and in vivo. However, the mechanisms underlying the inhibitory effects of HMG-CoA reductase inhibitors on malignant cell migration (or invasion) are still unclear. In the present study, we showed that lovastatin significantly inhibited the invasion of the ARO cells, and this inhibitory effect can be completely reversed by coadministration with mevalonate and GGOH, but not FOH (Fig. 2). Moreover, a profound decrease in EGF-induced invasion in the ARO cells was induced by GGTI-298, but not FTI-277, through blockade of geranylgeranylation (Fig. 6). Taken together, these results suggest that protein geranylgeranylation might play a crucial role in cell invasion through an ECM-based membrane barrier, and suppression of cell invasion induced by lovastatin may result from inhibition of protein geranylgeranylation.

Ras superfamily GTPases contain a CAAX box at the carboxyl-terminal (C, cysteine; A, aliphatic amino acid–leucine, isoleucine or valine; X, methionine, serine, leucine or glutamine). In the process of post-translational modification, prenyl transferase (FTase and GGTase) catalyzes and transfers an isoprenyl group (from FPP and GGPP) to cysteine (of the CAAX box) followed by cleavage of the three downstream amino acids (AAX), and finally causes palmitoylation of cysteine residuals in the region nearby (Clarke 1992). It has been well documented that translocation of GTPases from the cytosolic fraction to plasma membrane, where GTPase is stimulated by growth factors or integrins, is very important for their activation. Lovastatin treatment dose-dependently decreased the membrane translocation of Rho (RhoA and Rac1), but not Ras (Fig. 6A) in ARO cells. This inhibitory effect can be prevented by co-treatment with mevalonate or GGOH, but not FOH (Fig. 6B). In contrast, the membrane association of Ras is neither inhibited by lovastatin treatment alone, nor by coadministration with mevalonate-derived isoprenoids. Mevalonate and its derivatives appear to be exhausted after treatment with HMG-CoA reductase inhibitor. Depletion of the GGPP pool impairs the geranylgeranylation of Rho. Depletion of the FPP pool, on the other hand, impairs not only the farnesylation of Ras but also the geranylgeranylation of Rho. However, failure of lovastatin to inhibit Ras farnesylation and membrane translocation in an FPP-depleted condition is still an unsolved issue in our model with ARO thyroid cancer cells. One possible explanation is that the cysteine(s) (palmitoylation residues) or polylysine (higher positive charge) motif in the c-terminal region near the CAAX box of Ras is (are) mutated, and this leads to the anchoring of Ras protein to the plasma membrane without farnesylation. It has been shown that genetic variants of Ras proteins, which lack a CAAX motif and instead contain cysteine residues (for palmitation) with

multiple basic residues at their carbonyl termini, are able to target the cytoplasmic membrane without farnesylation (Booden *et al*. 1999). Moreover, a previous report showed that yeast Ras2 mutant proteins were sufficient to maintain Ras-dependent growth by combined effects of carbonyl-terminal polylysine sequences and palmitoylation of cysteine residues without prenylation (Mitchell *et al*. 1994). The structure of polylysine tail at carbonyl termini has been suggested to be a cationic platform through which nonfarnesylated Ras was able to contact plasma membrane and undergo palmitoylation at cysteines, which in turn kept nonprenylated Ras protein anchor to the plasma membrane. In addition, Chp, which is a Cdc42 homologous protein and lacks a CAAX motif, shares functional identity (such as promotion of filopodia formation) with Cdc42 and is located to the plasma membrane that is dependent on pamitoylation, but not prenylation, at its carbonyl terminus (Chenette *et al*. 2005). Whether a mutation of the CAAX domain together with cysteine residues at the carbonyl terminus occurs in the ARO cells and contributes to the membrane translocation of Ras through palmitation without farnesylation needs further studies.

Rho functions as a molecular switcher, cycling between GDP-binding inactive state and GTP-binding active states. GTPase hydrolyzes Rho-GTP to form Rho-GDP, which in turn activates the downstream effectors, including ROCK localized in membrane (Riento & Ridley 2003). ROCK has been reported to be involved in various processes related to cell motility, including formation of stress fibers, focal adhesions, and smooth muscle contraction (Riento & Ridley 2003). In the present study, we demonstrated that blockade of ROCK functions by treating the ARO cells with Y-27632 resulted in inhibiting the cellular attachment to ECM, such as fibronectin and recombinant Matrigel (Fig. 3), and the transmigration of ARO cancer cells through Matrigel-coated membrane (Fig. 7). Similar to lovastatin, Y-27632 induced changes in cell morphology, including limited extension in cell boundary, shrinkage of cell body, and decrease of intercellular adhesions and cell-substratum adhesion (data not shown). Moreover, Y-27632 also reversed the GGOH-induced inhibition in lovastatin-mediated suppression of the cell-substratum adhesive activity (Fig. 3B). Taken together, our data suggest that RhoA/ROCK signaling is involved in various cell functions, including maintenance of cell morphology, adhesion and induction of migration in the ARO cells.

Regulation of the actin filament assembly by RhoA has been implicated in promoting a variety of cellular processes, including alterations in morphology, motility and adhesion, which contribute to invasion and metastatic activities of cancer (Nobes & Hall 1999, Jaffe & Hall 2002). Expression of the constitutively active form of RhoA causes a great promotion of invasive ability *in vitro* and *in vivo* (Yoshioka *et al*. 1998). In contrast, expression of each dominant negative form of Rho (Cdc42, Rac or RhoA) inhibited PDGF-stimulated fibroblast invasion (Banyard *et al*. 2000). In the present study, we demonstrated that lovastatin induced changes in ARO cell morphology, including retraction from adhesion substratum, limited extension of cell boundary and rounding-up. These morphologic alterations can be retrieved by removal of lovastatin (data not shown) or by administration of mevalonate or GGOH. Lovastatin also inhibited the EGF-induced actin filaments polymerization in the ARO cells. However, EGF-induced stress fiber assembly is preserved when the active Rho GTPases are restored by coincubation of GGOH or mevalonate with lovastatin.

Although various Rho GTPase family members (such as RhoA, RhoB and RhoC) are highly homologous and have been demonstrated to be affected by statins, our present data suggest that inhibition of RhoA/ROCK signaling is critical for suppressing metastatic activity in the lovastatin-treated ARO cells. Surprisingly, we found that lovastatin treatment induced an increase rather than a decrease of RhoB and RhoC translocation from cytosol fraction to particulate fraction, which was reversed by mevalonate and GGOH, but not FOH (unpublished data, Zhong *et al*.). Unlike RhoA protein, which is located in plasma membrane for regulating actin stress fiber formation and integrin signaling, RhoB is located in the endosome and nuclear membranes with a unique function in intracellular trafficking of growth factor receptors such as the EGF receptor (Wherlock *et al*. 2004). RhoB plays an inhibitory role during cell cycle regulation and is upregulated in response to stress stimuli (Fritz & Kaina 1997). Moreover, RhoB is the only protein known to be post-translationally modified as both farnesylated (f-RhoB) and geranylgeranylated (gg-RhoB) forms within the cell (Adamson *et al*. 1992); different RhoB isoforms have distinct cellular functions in modulating cell growth (Lebowitz *et al*. 1997). A loss of f-RhoB and a consequent increase of the gg-RhoB pool have been observed in the cells treated with FTI anticancer drugs, which have been designed mainly to block the farnesylation of oncogenic Ras protein. It has been suggested that f-RhoB has a progrowth activity, whereas gg-RhoB has a proapoptotic function triggered by FTI treatment (Du *et al*.
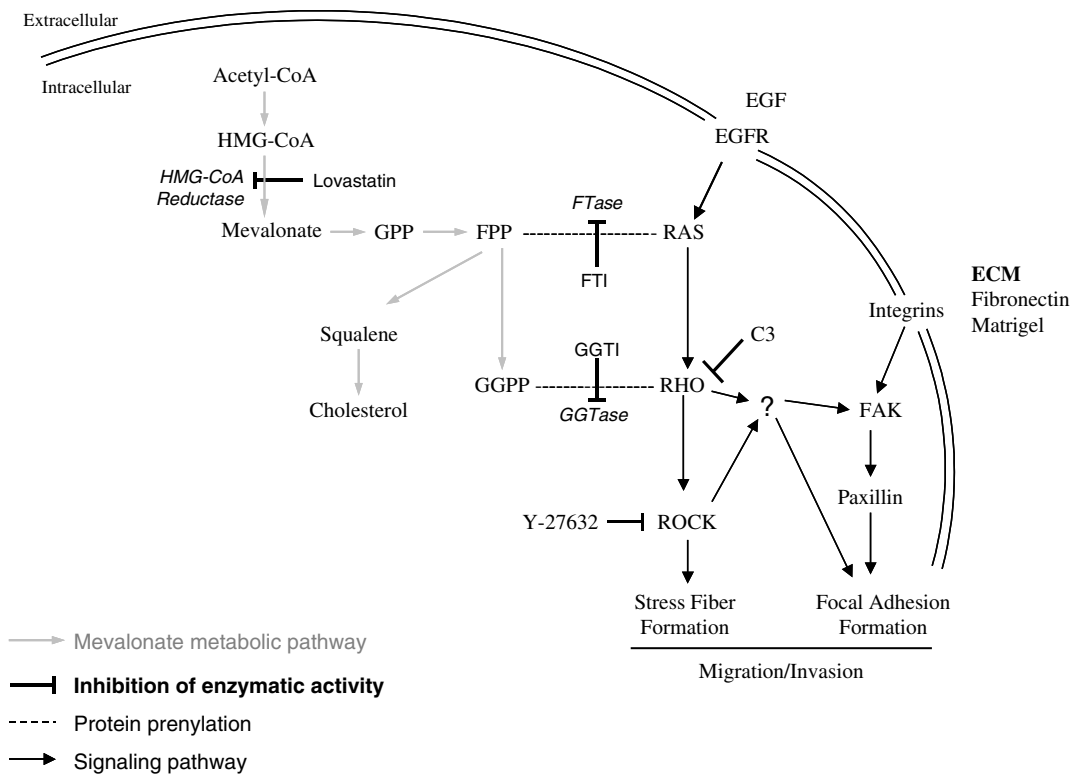
Figure 8 Model for lovastatin-induced suppression of invasion in anaplastic thyroid cancer. In response to lovastatin treatment, invasiveness of the thyroid cancer cell was suppressed via inhibition of cellular adhesion and migration. EGF-induced migration and focal adhesion were both inhibited by lovastatin administration via inhibitions of Rho/ROCK/stress fiber polymerization and phosphorylation of FAK/paxillin signaling molecules. ECM-mediated cellular adhesion was also decreased by lovastatin treatment through downregulation of the FAK protein level and the FAK/paxillin signaling. Rho inactivation caused by inhibiting the conversion of mevalonate and GGPP from HMG-CoA is the major factor causing the lovastatin-induced inhibition on thyroid cancer invasion.

1999, Du & Prendergast 1999, Liu *et al.* 2000). Although it has been indicated that RhoA and RhoC have overlapping functions, including promotion of cell motility, cytoskeletal alterations and metastasis, treatment of the ARO cells with lovastatin induced an increase instead of a decrease of RhoC translocation from cytosol fraction to particulate fraction (unpublished data, Zhong *et al.*), suggesting that RhoC might not be involved in the lovastatin-induced inhibition of the ARO cell invasiveness. RhoC has been implicated in the metastasis of various cancer cells, including bladder cancer (Kamai *et al.* 2003), breast cancer (Kleer *et al.* 2002), hepatocellular carcinoma (Wang *et al.* 2004), lung cancer (Ikoma *et al.* 2004) and melanoma (Eric *et al.* 2003). The discrepancy of a major role of RhoC in metastasis of cancer cells might be due to the cell phenotype. Moreover, whether the anti-invasiveness effect caused by lovastatin is specific to the ARO cells or generally occurs to all anaplastic thyroid cancers needs further investigation.

FAK was previously thought to facilitate focal adhesion complex formation on the basis of localization of the activated FAK in focal contacts. However, recent studies have suggested that FAK may regulate the turnover of focal adhesion complex proteins and cell motility. It has been demonstrated that the activity of FAK correlates with endothelial cell motility, and inhibition of FAK results in a decrease in endothelial cell motility (Gilmore & Romer 1996). Moreover, loss of FAK is associated with a decrease in cell migration and an increase in focal adhesion size (Ilic *et al.* 1995), whereas overexpression of FAK increases cell migration (Owen *et al.* 1999). These findings suggest that FAK might play an important role in the remodeling of focal adhesions and control of cytoskeletal reorganization. Interestingly, we found that lovastatin not only reduced the expression level of FAK but also decreased the EGF-induced phosphorylations of FAK and paxillin in the ARO cells. In the absence of lovastatin, however, the expression level of FAK was

not significantly altered in the EGF-treated ARO cells. Therefore, lovastatin treatment may elevate the turnover rate of FAK protein, which in turn contributed to downregulation of the FAK functions. These findings led us to hypothesize that post-translational regulation of protein degradation may be involved in the lovastatin-induced decrease in FAK protein in the ARO cells. Recently, it has been shown that calpain-mediated cleavage of FAK in adherent smooth muscle cells is correlated with disassembly of focal adhesion and cell rounding in response to matrix degradation (Carragher *et al.* 1999). The lovastatin-induced decrease in EGF-induced phosphorylation of FAK and its downstream effector, paxillin, can be reversed by GGOH and mevalonate, but not FOH (Fig. 5), suggesting that activations of FAK and paxillin are regulated directly or indirectly by the activated Rho GTPases. Similar to our observation, previous reports in the fibroblasts showed that lysophosphatidic acid (LPA) directly activates Rho GTPases, which in turn activate FAK phosphorylation localized to focal adhesions (Flinn & Ridley 1996).

Although our present study has demonstrated that lovastatin suppresses invasiveness of ARO cells *in vitro*, the effective anti-invasiveness-inducing concentrations of lovastatin relative to tissue concentrations achieved when this drug is used in humans needs further investigation. The concentrations of statins used in cell culture models are relatively high and may not be reached *in vivo*. The dose of lovastatin used for lowering serum cholesterol in human clinical treatment is about 0.25–1.0 mg/kg body weight (Agarwal *et al.* 1999). In a phase I study, however, the plasma concentrations of lovastatin measured in cancer patients were also relatively low (0.1–3.9 µM) (Thibault *et al.* 1996). It has been indicated that treatment of the patients with statins at the doses used for coronary artery disease prevention could cause a significant reduction of colon cancer incidence (Sacks *et al.* 1996). Moreover, lovastatin treatment induced cellular differentiation in ARO cells (at a dose of 10 µM or less for cell culture experiment) and in anaplastic thyroid cancer cells (at a dose of 80 mg/day for patients in clinical trial) (unpublished data, Chang *et al.*). The myopathy and hepatotoxicity caused by the statins treatment is seldom observed in clinical long-term therapy, suggesting that the use of lovastatin (even at a dose as high as 80 mg/day) for clinical therapeutic purpose in hypercholesterolemia is quite safe (Plosker *et al.* 1996, Davigono *et al.* 1998). All in all, it is most likely that statins at lower concentrations than those used in the present study may be possibly effective. We believe that the effective doses of statins could be reduced by combined treatment of cancer cells with statins and the well-established chemotherapeutic drugs. Moreover, in addition to the apoptosis-inducing activity, lovastatin could induce the differentiation in the thyroid cancer cells, and thereafter, made them more sensitive to radioactive iodine therapy, suggesting the potential application of lovastatin in the treatment of thyroid cancer. Based on the results of the present study, we propose a model of the molecular mechanisms through which lovastatin supresses the invasiveness of anaplastic thyroid cancer cells. A depiction of this model is shown in Fig. 8.

Acknowledgements

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