

# Suppression in Mevalonate Synthesis Mediates Antitumor Effects of Combined Statin and $\gamma$ -Tocotrienol Treatment

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**Abstract** Statins directly inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) activity, while  $\gamma$ -tocotrienol, an isoform of vitamin E, enhances the degradation and reduces cellular levels of HMGR in various tumor cell lines. Since treatment with statins or  $\gamma$ -tocotrienol alone induced a dose-responsive inhibition, whereas combined treatment with subeffective doses of these agents resulted in a synergistic inhibition in +SA mammary tumor cell growth, studies were conducted to investigate the role of the HMGR pathway in mediating the antiproliferative effects of combined low dose statin and  $\gamma$ -tocotrienol. Treatment with 8  $\mu$ M simvastatin inhibited cell growth and isoprenylation of Rap1A and Rab6, and supplementation with 2  $\mu$ M mevalonate reversed these effects. However, the growth inhibitory effects of 4  $\mu$ M  $\gamma$ -tocotrienol were not dependent upon suppression in mevalonate synthesis. Treatment with subeffective doses of simvastatin (0.25  $\mu$ M), lovastatin (0.25  $\mu$ M), mevastatin (0.25  $\mu$ M), pravastatin (10  $\mu$ M), or  $\gamma$ -tocotrienol (2  $\mu$ M) alone had no effect on protein prenylation or mitogenic signaling, whereas combined treatment with these agents resulted in a significant inhibition in +SA cell growth, and a corresponding decrease in total HMGR, Rap1A and Rab6 prenylation, and MAPK signaling, and mevalonate supplementation reversed these effects. These findings demonstrate that the synergistic antiproliferative effects of combined low dose statin and  $\gamma$ -tocotrienol treatment are directly related to an inhibition in HMGR activity and subsequent suppression in mevalonate synthesis.

**Keywords** Statins ·  $\gamma$ -Tocotrienol · Breast cancer · Vitamin E · HMGR · Rab6 · Rap1A · Mevalonate · MAPK · p38

## Abbreviations

HMGR	3-Hydroxy-3-methylglutaryl-coenzyme A reductase
MAPK	Mitogen-activated protein kinase
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
ERK	Extracellular signal-regulated kinase
BSA	Bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
PBS	Phosphate buffered saline
MTT	3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide
SDS	Sodium dodecyl sulfate
PVDF	Polyvinylidene fluoride
TBST	10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4
TBS	0.05 M Tris-buffered saline (TBS) pH 7.6
PI3K	Phosphatidylinositol 3-kinase

## Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) is the rate limiting enzyme in the mevalonate biosynthetic pathway and is involved in the synthesis of sterol and non-sterol intermediates essential for cell survival and growth [1]. Specifically, HMGR catalyzes the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, which subsequently produces isoprenoid intermediates

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such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) that are required for posttranslational modification (isoprenylation) of various small GTP-binding proteins including Ras, Rap, Rab, Rho, nuclear lamins, and heterotrimeric G-protein  $\gamma$ -subunit [1–3]. Isoprenylation is needed for anchoring these signaling proteins to the cell membrane in close proximity to growth factor receptors so that these signaling proteins can be activated following receptor activation, and subsequently participate in turning on downstream mitogenic signaling pathways such as MAPK and Akt [2–4].

HMGR activity is characteristically elevated and/or unregulated in many forms of cancer and thereby acts to promote cancer cell survival and proliferation by providing an abundance of non-sterol intermediates [5]. As a result, a great deal of attention has been focused on the development of agents that target and inhibit HMGR activity for use in cancer chemotherapy. Statins represent a class of such agents that act as competitive inhibitors of HMGR and display potent anticancer activity in a variety of cell culture and animal tumor models [6, 7]. However, clinical use of statins in the treatment of cancer has been greatly limited by their high-dose toxicity that includes severe myotoxicity, liver toxicity, gastrointestinal dysfunction, and even death [8, 9].

$\gamma$ -Tocotrienol, a naturally occurring isoform within the vitamin E family of compounds, displays potent anticancer activity [10] and has been shown to reduce HMGR activity through the post-transcriptional down-regulation of this enzyme [11]. The vitamin E family of compounds is divided into two subgroups called tocopherols and tocotrienols. Tocopherols are commonly found in high concentrations in a wide variety of foods, whereas tocotrienols are relatively rare and found in appreciable levels only in a few specific vegetable fats, such as palm oil [10]. Although chemically very similar, tocopherols have a saturated, whereas tocotrienols have an unsaturated phytyl chain attached to a chroman ring structure. It is now clearly established that tocotrienols, but not tocopherols, display potent antiproliferative and apoptotic activity against mammary tumor cells at treatment doses that have little or no effect on normal cell growth and viability [10].

Previous studies demonstrated that combined treatment of subeffective doses of statins with subeffective doses of  $\gamma$ -tocotrienol, synergistically inhibited the growth of highly malignant +SA mammary epithelial cells in culture, and these effects were associated with a suppression in MAPK and Akt mitogenic signaling [12]. Other studies showed that similar combination therapy induced G1 cell cycle arrest in +SA mammary tumor cells and these effects were associated with an increase in p27 expression, decreased cyclin D1 expression, and hypophosphorylation of Rb [13]. However, the specific intracellular target responsible for

mediating the antiproliferative effects of combined statins and  $\gamma$ -tocotrienol treatment remains unclear. Since statins and  $\gamma$ -tocotrienol have both been shown to suppress HMGR activity, it was hypothesized that inhibition of mevalonate synthesis may be ultimately responsible for mediating the growth inhibiting effects of combined therapy with these agents. Therefore, studies were conducted to characterize the growth inhibitory effects of combined low dose statins and  $\gamma$ -tocotrienol treatment on mevalonate synthesis, isoprenylation of small G-proteins Rap1A and Rab6, and mitogenic signaling in +SA mammary tumor cells.

## Experimental Procedures

### Chemicals and Antibodies

All materials were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated. Isolated  $\gamma$ -tocotrienol (>98% purity) was generously provided as a gift by First Tech International Ltd. (Hong Kong). Antibodies for cyclin D1, phospho-p44/42 ERK, phospho-p38, total p44/42 ERK, and total p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for Ki-67 antigen, unprenylated Rap 1A and isoprenylated Rab 6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for HMGR and Ras were obtained from Upstate Biotechnology (Lake Placid, NY, USA) and BD Biosciences (San Jose, CA, USA), respectively. Anti- $\alpha$ -tubulin antibody was obtained from EMD Biosciences (La Jolla, CA, USA). Horseradish peroxidase-labeled goat anti-rabbit and anti-mouse secondary antibodies were purchased from PerkinElmer Biosciences (Boston, MA, USA) and donkey anti-goat secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 594 donkey anti-goat secondary antibody was obtained from Molecular Probes (Eugene, Oregon, USA).

### Cell Line and Culture Conditions

Experiments conducted in the present study represent a logical continuation of previous studies that have extensively characterized the antiproliferative and apoptotic effects of  $\gamma$ -tocotrienol in the highly malignant +SA mammary epithelial cell line [10]. The +SA mammary tumor cell line was derived from an adenocarcinoma that developed spontaneously in a BALB/c female mouse [14, 15]. +SA cells display anchorage-independent growth when cultured in soft agarose gels, and when injected back into the mammary gland fat pad of syngeneic female mice, +SA cells form anaplastic adenocarcinomas that

metastasize to the lung [15]. Cell culture conditions have been previously described in detail [12, 16, 17]. Briefly, +SA cells were maintained in serum-free defined medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 containing 5 mg/mL bovine serum albumin (BSA), 10  $\mu\text{g}/\text{mL}$  transferrin, 100 U/mL soybean trypsin inhibitor, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10  $\mu\text{g}/\text{mL}$  insulin, and 10 ng/mL epidermal growth factor. For subculturing, cells were rinsed twice with sterile  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free phosphate buffered saline (PBS) and incubated in 0.05% trypsin containing 0.025% EDTA in PBS for 5 min at 37°C. The released cells were centrifuged, resuspended in serum-free media and counted using hemocytometer.

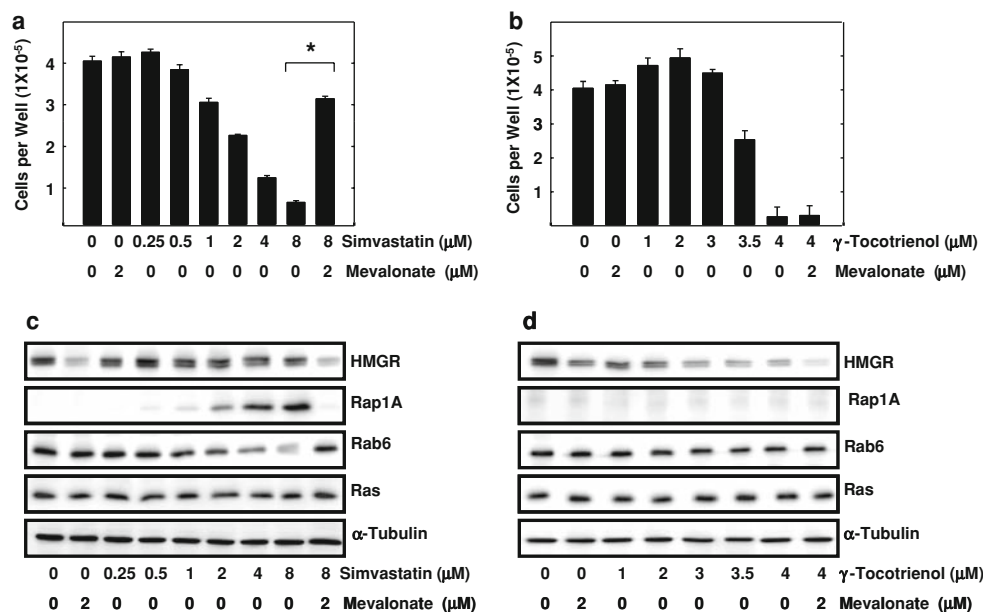
### Experimental Treatments

To dissolve highly lipophilic  $\gamma$ -tocotrienol in aqueous culture media, a stock solution of  $\gamma$ -tocotrienol was first prepared by binding it to bovine serum albumin (BSA) as described previously [12, 13, 16, 17]. Simvastatin, lovastatin or mevastatin exist in their inactive lactone forms that do not inhibit HMGR, but are active as their corresponding open-ring hydroxy-derivatives [18, 19]. Hence,

these statins were activated prior to addition to treatment media as described previously [12, 13]. Briefly, statins were dissolved in a 70% ethanol solution containing 0.1 N NaOH and then incubated at 50°C for 1 h. The alkaline statin solution was then neutralized by the addition of 70% ethanol solution containing 0.1 N HCl to form a stock solution that was then used to prepare treatment media. Pravastatin has an active open ring structure and was therefore, directly dissolved in 70% ethanol to form a stock solution. All media was adjusted so that the final ethanol concentration was same in all the groups within a given experiment and never exceeded 0.05%, and all cells were fed fresh control or treatment media every day.

### Growth Studies and Viable Cell Number

In growth studies (Figs. 1, 4), +SA cells were initially seeded at a density of  $5 \times 10^4$  cells/well (6 wells/group) in 24-well culture plates in serum-free defined control media and allowed to attach overnight. Cells were then, exposed to respective experimental treatments containing simvastatin, lovastatin, mevastatin, pravastatin,  $\gamma$ -tocotrienol, mevalonate alone or in various combinations for 4 days. During this period, cells were fed fresh treatment or control



**Fig. 1** Effects of simvastatin and  $\gamma$ -tocotrienol treatment on +SA cell growth, HMGR and Ras levels, and Rap1A and Rab6 isoprenylation. Cells were plated at a density of  $5 \times 10^4$  cells/well (6 wells/group) in 24-well culture plates and exposed to 0–8  $\mu\text{M}$  simvastatin treatment alone or the combined treatment of 8  $\mu\text{M}$  simvastatin with 2  $\mu\text{M}$  mevalonate (a), 0–4  $\mu\text{M}$   $\gamma$ -tocotrienol treatment alone or the combined treatment of 4  $\mu\text{M}$   $\gamma$ -tocotrienol with 2  $\mu\text{M}$  mevalonate (b) for 4 days in culture. Afterwards, viable cell count was determined using MTT assay. Vertical bars indicate the mean cell count  $\pm$  SEM in each treatment group. \* $P < 0.05$  as compared with

8  $\mu\text{M}$  simvastatin-treated group. For Western blot studies (c, d), cells were plated at a density of  $1 \times 10^6$  cells/100-mm culture dishes and treated with the above doses of simvastatin and mevalonate (c), or  $\gamma$ -tocotrienol and mevalonate (d) for a 4-day culture period. Afterwards, whole cell lysates were prepared for subsequent separation by polyacrylamide gel electrophoresis, followed by Western blot analysis to measure total HMGR, Rap1A (unprenylated), Rab6 (isoprenylated), total Ras and  $\alpha$ -tubulin levels.  $\alpha$ -Tubulin was visualized to ensure equal sample loading in each lane

media every day. At the end of the treatment exposure period, the viable cell number was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously [16, 17]. Briefly, cells in 24-well plates were incubated at 37°C with fresh defined control media containing 0.42 mg/mL MTT. After a 4-h incubation period, the media was removed and MTT crystals were dissolved in isopropanol (1 mL/well), and the optical density of each sample was measured at 570 nm on a microplate reader (SpectraCount, Packard BioScience Company). The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by hemocytometry, at the beginning of each experiment.

#### Western Blot Analysis

For Western blot analysis, +SA cells were plated at a density of  $1 \times 10^6$  cells/100-mm culture dish and grown in serum-free defined control or treatment media. After a 4-day treatment period, cells were isolated with trypsin, washed with PBS, and the whole cell lysates were then prepared as previously described [13, 16, 20]. Protein concentration in each sample was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts (25 µg/lane) of each sample were subjected to electrophoresis through 7.5 to 15% SDS–polyacrylamide minigels. Minigels within a given experiment were run simultaneously using a AccuPower model 500 power supply unit (VWR, Suwanee, GA). Proteins separated on minigels were transblotted at 30 V for 12–16 h at 4°C onto a polyvinylidene fluoride (PVDF) membrane (PerkinElmer Lifesciences, Wellesley, MA, USA) in a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the methods of Towbin and these PVDF membranes were used for subsequent Western blot analyses as previously described [13, 16, 20, 21]. Briefly, membranes were then blocked with 2% BSA in 10 mM Tris–HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) and then, incubated with specific primary antibodies against HMGR, Rap1A, Rab6, Ras, phospho-p44/42 ERK, phospho-p38, total p44/42 ERK, total p38, cyclin D1, and  $\alpha$ -tubulin, diluted 1:2,000–1:10,000 in TBST/2% BSA for 2 h. Membranes were washed five times with TBST and then incubated with respective horseradish peroxidase-conjugated secondary antibodies diluted 1:5,000 in TBST/2% BSA for 1 h followed by rinsing with TBST. Chemiluminescence (Pierce, Rockford, IL, USA) was used to visualize the antibody bound proteins. Images of protein bands from all treatment groups within an experiment were acquired using Kodak Gel Logic 1500 Imaging System (Carestream Health Inc, New Haven, CT, USA). The visualization of  $\alpha$ -tubulin was used to ensure equal sample

loading in each lane. All experiments were repeated at least three times and a representative Western Blot image from each experiment is shown in Figs. 1, 3 and 5.

#### Ki-67 Immunofluorescence

+SA cells were initially plated at a density of  $2 \times 10^5$  cells/chamber in single chamber slides (Nalge Nunc International, Rochester, NY, USA) and allowed to attach overnight. Afterwards, cells were divided into different groups that fed their respective treatment media. After 4 days of treatment exposure, cells were rinsed with 0.05 M Tris-buffered saline (TBS) pH 7.6, and fixed with methanol previously cooled to  $-20^\circ\text{C}$ . Fixed cells were blocked with 2% donkey serum in TBS for 1 h, followed by overnight incubation with Ki-67 primary antibody at 4°C. Cells were washed five times with TBS followed by 45 min incubation with fluorescent labeled anti-goat secondary antibody at room temperature in dark. After washing the cells five times with TBS, cells were embedded with Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA) containing DAPI as the counterstain, followed by imaging by confocal microscopy (LSM Pascal confocal microscope, Carl Zeiss Microimaging Inc., Thornwood, NY, USA). Quantification of Ki-67 labeling was achieved by counting the number of +SA cells displaying positive staining versus the total number of cells within five randomly selected 80,000- $\mu\text{m}^2$  areas of each slide in each treatment group using LSM software ZEN 2007 (Carl Zeiss Microimaging Inc., Thornwood, NY).

#### Statistical Analysis

Differences among the various treatment groups in +SA cell growth studies were determined by analysis of variance (ANOVA) followed by Dunnett's *t* test. A difference of  $P < 0.05$  was considered to be statistically significant as compared with vehicle-treated controls or as defined in the figure legends.

## Results

#### Effects of Simvastatin and $\gamma$ -Tocotrienol on +SA Cell Proliferation, HMGR Levels and Protein Prenylation

Treatment with 0–0.5  $\mu\text{M}$  simvastatin or 2  $\mu\text{M}$  mevalonate had no effect, whereas treatment with 1–8  $\mu\text{M}$  simvastatin significantly inhibited +SA mammary tumor cell growth in a dose-dependent manner as compared with vehicle-treated controls (Fig. 1a). Simvastatin-induced growth inhibition was reversed in cells given 2  $\mu\text{M}$  mevalonate

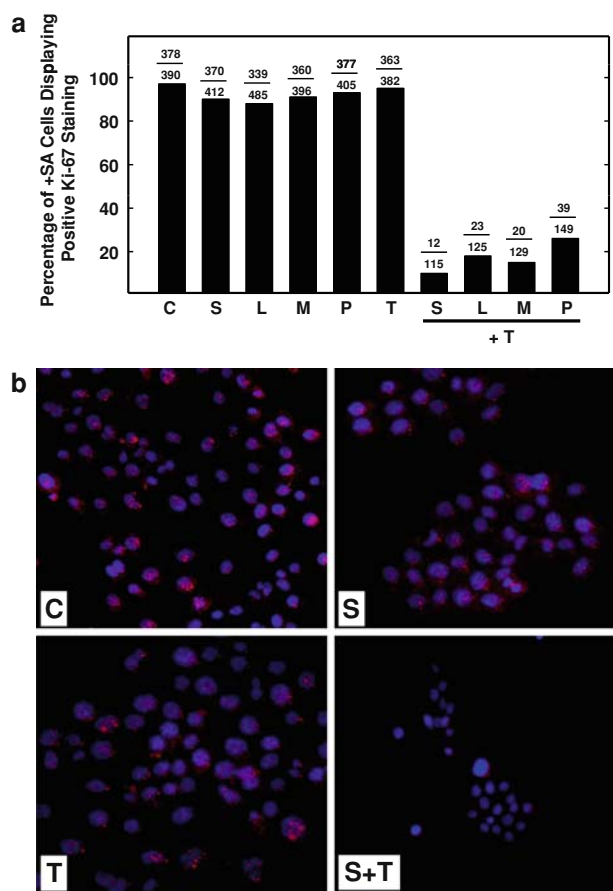


supplementation (Fig. 1a). Treatment with 1–8  $\mu\text{M}$  simvastatin had little or no effect on the relative levels of HMGR, while treatment with 2  $\mu\text{M}$  mevalonate decreased HMGR levels as compared with vehicle-treated controls (Fig. 1c). The anti-HMGR antibody used in these Western blots specifically detects two adjacent protein bands in +SA cell lysates and this doublet possibly indicates two isozymes of HMGR that have been reported previously [22]. Treatment with 1–8  $\mu\text{M}$  simvastatin was also found to reduce the level of protein prenylation as evidenced by a marked increase in unprenylated Rap1A and a marked decrease in isoprenylated Rab6 as compared with their respective vehicle-treated controls (Fig. 1b). Simvastatin-induced inhibition of Rap1A and Rab6 prenylation was blocked by 2  $\mu\text{M}$  mevalonate supplementation (Fig. 1c). Total Ras levels were similar in all treatments groups (Fig. 1c).

Treatment with 0–3  $\mu\text{M}$  of  $\gamma$ -tocotrienol had no effect, whereas treatment with 3.5–4  $\mu\text{M}$   $\gamma$ -tocotrienol treatment significantly inhibited +SA cell growth in a dose-responsive manner (Fig. 1b). However, simultaneous administration of 4  $\mu\text{M}$   $\gamma$ -tocotrienol and 2  $\mu\text{M}$  mevalonate did not reverse the growth inhibitory effects of  $\gamma$ -tocotrienol treatment (Fig. 1b). Treatment with 1–4  $\mu\text{M}$   $\gamma$ -tocotrienol or 2  $\mu\text{M}$  mevalonate alone decreased HMGR levels, and combined treatment of these agents further decreased HMGR levels as compared with vehicle-treated controls (Fig. 1d). Treatment with 4  $\mu\text{M}$   $\gamma$ -tocotrienol or 2  $\mu\text{M}$  mevalonate alone or in combination had no effect on Rap1A and Rab6 prenylation or total Ras levels as compared to vehicle-treated controls (Fig. 1d).

#### Combined Treatment Effects of Individual Statins and $\gamma$ -Tocotrienol on +SA Cell Proliferation

It has been established that combined treatment with low dose statins and  $\gamma$ -tocotrienol results in a synergistic inhibition in +SA mammary tumor cell growth [12, 13]. Figure 2a shows the effects of statins and  $\gamma$ -tocotrienol alone or in combination on the ratio of +SA cells demonstrating positive Ki-67 immunofluorescent staining versus the total number of cells within each treatment group. The Ki-67 protein is specifically expressed and localized in the nucleus of proliferating cells and can be used as a marker for cell proliferation. More than 88% of cells displayed positive Ki-67 staining (pink) in the vehicle-treated control group (C) and groups treated with subeffective doses of simvastatin (S; 0.25  $\mu\text{M}$ ), lovastatin (L; 0.25  $\mu\text{M}$ ), mevastatin (M; 0.25  $\mu\text{M}$ ), pravastatin (P; 10  $\mu\text{M}$ ) or  $\gamma$ -tocotrienol (T; 2  $\mu\text{M}$ ) alone (Fig. 2a). However, combined treatment with subeffective doses of individual statins with a subeffective dose of  $\gamma$ -tocotrienol displayed <26% positive Ki-67 staining (Fig. 2a). Fluorescent



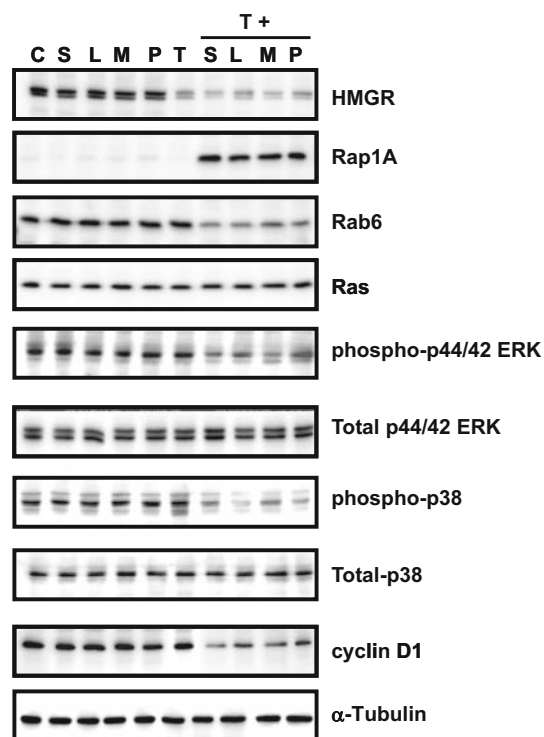
**Fig. 2** Effects of low dose statin and  $\gamma$ -tocotrienol treatment on Ki-67 expression in +SA mammary tumor cells. +SA cells were initially plated at a density of  $2 \times 10^5$  cells/chamber in single-well chamber slides and exposed to media containing vehicle (C), 0.25  $\mu\text{M}$  simvastatin (S), 0.25  $\mu\text{M}$  lovastatin (L), 0.25  $\mu\text{M}$  mevastatin (M), 10  $\mu\text{M}$  pravastatin (P), 2  $\mu\text{M}$   $\gamma$ -tocotrienol (T) or the combination of individual statins (S, L, M, or P) with 2  $\mu\text{M}$   $\gamma$ -tocotrienol (T) for a 4-day culture period. Afterwards, cells were fixed with methanol and stained with fluorescent Ki-67 antibody and counterstained with DAPI, and images were visualized by confocal microscopy. **a** Vertical bar graph shows percent of malignant +SA mammary epithelial cells displaying positive Ki-67 fluorescent staining in each treatment group. Numbers presented over each vertical bar represent the number of cells displaying positive Ki-67 staining over the total number of cells counted in each treatment group. Cells were counted in five squares of area equivalent to  $80,000 \mu\text{m}^2$ . **b** Confocal fluorescent images of positive Ki-67 staining in +SA mammary tumor cells. The pink color indicates positive Ki-67 staining in individual nuclei of +SA cells. Magnification of each image is  $200 \times$

confocal images of +SA cells in the vehicle-treated control (C), 0.25  $\mu\text{M}$  simvastatin (S), 2  $\mu\text{M}$   $\gamma$ -tocotrienol (T), and combined 0.25  $\mu\text{M}$  simvastatin and 2  $\mu\text{M}$   $\gamma$ -tocotrienol (S + T) treatment groups are shown in Fig. 2b. The majority of +SA cells in control (C), simvastatin alone (S) or  $\gamma$ -tocotrienol alone (T) treated groups showed abundant positive (pink) Ki-67 staining in their nuclei. In contrast, Ki-67 positive staining was greatly reduced in the +SA cell nuclei treated with the combination of simvastatin and

$\gamma$ -tocotrienol (S + T; Fig. 2b). Similar results were obtained when +SA cells were exposed to 0.25  $\mu$ M lovastatin, 0.25  $\mu$ M mevastatin, or 10  $\mu$ M pravastatin alone or in combination with 2  $\mu$ M  $\gamma$ -tocotrienol (images not shown).

#### Effects of Combined Statin and $\gamma$ -Tocotrienol Treatment on HMGR Levels, Protein Prenylation and Mitogenic Signaling

Figure 3 shows that treatment with 0.25  $\mu$ M simvastatin (S), 0.25  $\mu$ M lovastatin (L), 0.25  $\mu$ M mevastatin (M) or 10  $\mu$ M pravastatin (P) alone did not alter the relative levels of HMGR. However, treatment with 2  $\mu$ M tocotrienol (T) alone or in combination with the various statins caused a relatively large decrease in HMGR levels as compared to vehicle-treated controls (Fig. 3). Similar treatment with



**Fig. 3** Effects of statin and  $\gamma$ -tocotrienol treatment on the intracellular levels of HMGR, isoprenylated small G-proteins, MAPKs and cyclin D1 in +SA mammary tumor cells after a 4-day culture period. +SA cells were plated at a density of  $1 \times 10^6$  cells/100 mm culture dishes and exposed to control (C) simvastatin (S), 0.25  $\mu$ M lovastatin (L), 0.25  $\mu$ M mevastatin (M), 10  $\mu$ M pravastatin (P), or 2  $\mu$ M  $\gamma$ -tocotrienol (T) alone, or the combination of individual statins (S, L, M or P) with 2  $\mu$ M  $\gamma$ -tocotrienol (T). Afterwards, cells in all treatment groups were isolated and whole cell lysates were prepared and electrophoresed through polyacrylamide minigels, transblotted to PVDF membrane, followed by Western blot analysis to measure relative levels of total HMGR, Rap1A (unprenylated), Rab6 (isoprenylated), total Ras, phospho-p44/42 ERK, phospho-p38 levels, total p44/42 ERK, total p38, cyclin D1 and  $\alpha$ -tubulin levels.  $\alpha$ -Tubulin was visualized to ensure equal sample loading in each lane

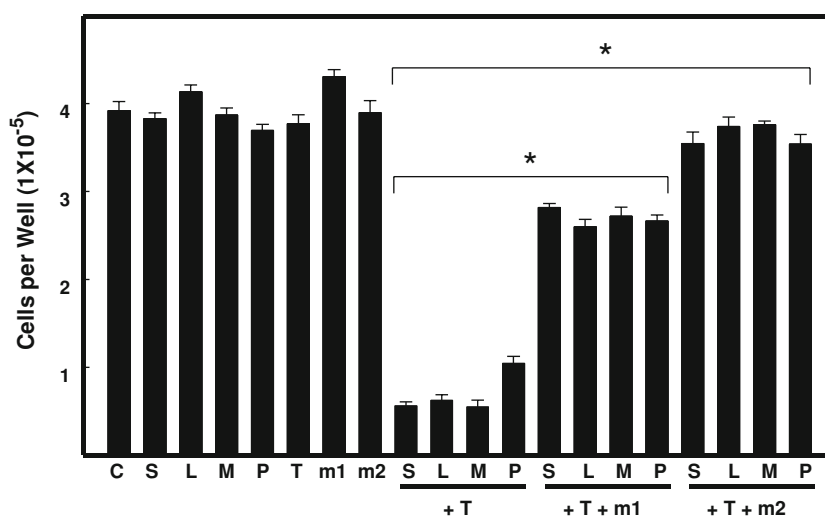
simvastatin, lovastatin, mevastatin, pravastatin, or  $\gamma$ -tocotrienol alone had no effect on the relative levels of unprenylated Rap1A, but combined treatment of  $\gamma$ -tocotrienol with individual statins dramatically increased unprenylated Rap1A levels as compared with vehicle-treated controls (Fig. 3). Likewise, the relative intracellular levels of isoprenylated Rab6, total Ras, phospho-p44/42 ERK, total ERK, phospho-p38, total p38 and cyclin D1 were unaffected by treatment with individual statins or  $\gamma$ -tocotrienol alone, whereas combined treatment of  $\gamma$ -tocotrienol with individual statins resulted in a reduction in the levels of isoprenylated Rab6, phospho-p44/42 ERK, phospho-p38 and cyclin D1, as compared with vehicle-treated controls (Fig. 3). These same combination treatments did not alter the relative levels of total Ras, total ERK, and total p38 (Fig. 3). The antibodies used to detect total and phosphorylated p44/42 ERK detect two bands at 44 and 42 kDa corresponding to the two protein kinases ERK1 and ERK2, respectively.

#### Effects of Mevalonate Supplementation on +SA Cell Growth

Treatment with 0.25  $\mu$ M simvastatin (S), 0.25  $\mu$ M lovastatin (L), 0.25  $\mu$ M mevastatin (M), 10  $\mu$ M pravastatin (P), 2  $\mu$ M  $\gamma$ -tocotrienol (T), 1  $\mu$ M (m1) mevalonate, or 2  $\mu$ M (m2) mevalonate alone had no effect on +SA mammary tumor cell growth as compared with vehicle-treated controls (C) following a 4-day culture period (Fig. 4). Combined treatment with similar doses of individual statins with  $\gamma$ -tocotrienol markedly suppressed growth of +SA cells. However, these growth inhibitory effects of combined statin and  $\gamma$ -tocotrienol treatment were partially reversed with 1  $\mu$ M mevalonate supplementation and completely reversed with 2  $\mu$ M mevalonate supplementation (Fig. 4). Interestingly, supplementation with 0–50  $\mu$ M FPP or GGPP was unable to reverse the inhibitory effect of combined statin and  $\gamma$ -tocotrienol treatment on the +SA mammary tumor cell growth (data not shown).

#### Effects of Statins, $\gamma$ -Tocotrienol, and Mevalonate Supplementation on HMGR, Protein Prenylation and Mitogenic Signaling

Figure 5 shows that combined treatment of 0.25  $\mu$ M simvastatin (S), 0.25  $\mu$ M lovastatin (L), 0.25  $\mu$ M mevastatin (M) or 10  $\mu$ M pravastatin (P) with 2  $\mu$ M  $\gamma$ -tocotrienol (T) resulted in a large increase in unprenylated Rap1A levels and a corresponding decrease in the levels of HMGR, isoprenylated Rab6, phospho-p44/42 ERK, phospho-p38 and cyclin D1, as compared with vehicle-treated controls (C). In contrast, +SA cells receiving 2  $\mu$ M mevalonate supplementation (m) alone or together with combined



**Fig. 4** Effects of mevalonate supplementation on the growth of +SA mammary tumor cells. Cells were plated at a density of  $5 \times 10^4$  cells/well (6 wells/group) in 24-well culture plates and exposed to control (C), 0.25  $\mu$ M simvastatin (S), 0.25  $\mu$ M lovastatin (L), 0.25  $\mu$ M mevastatin (M), 10  $\mu$ M pravastatin (P), 2  $\mu$ M  $\gamma$ -tocotrienol (T), 1  $\mu$ M mevalonate (m1) or 2  $\mu$ M mevalonate (m2) alone, or combination

with individual statins (S, L, M or P) and/or  $\gamma$ -tocotrienol (T) for a 4-day culture period. Afterwards, viable cell count was determined using the MTT assay. Vertical bars indicate the mean cell count  $\pm$  SEM in each treatment group. \* $P < 0.05$  as compared with respective combination statin and  $\gamma$ -tocotrienol treatment groups not exposed mevalonate supplementation

statins and  $\gamma$ -tocotrienol treatment showed no change in the relative levels of unprenylated Rap1A, isoprenylated Rab6, phospho-p44/42 ERK, phospho-p38 and cyclin D1 levels, as compared with vehicle-treated controls (Fig. 5). However, all groups receiving mevalonate supplementation displayed a decrease in HMGR levels as compared with vehicle-treated controls. The relative levels of total Ras, total ERK, and total p38 did not differ among any of the treatment groups (Fig. 5).

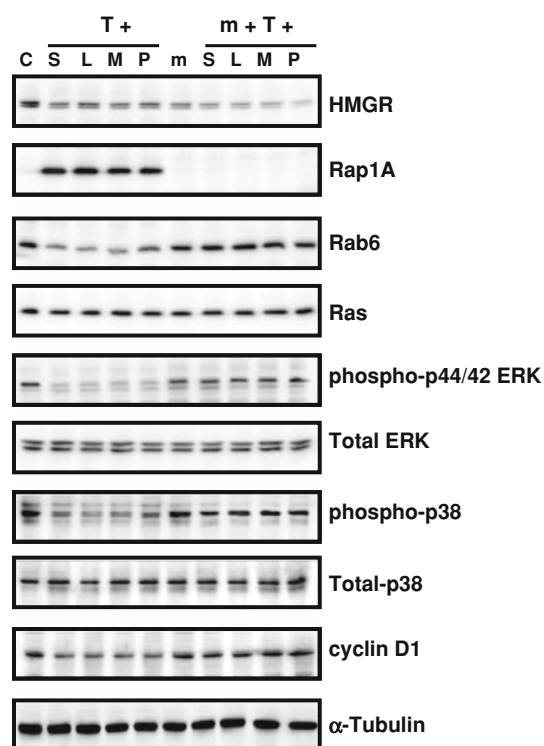
## Discussion

Results in this study demonstrate that the growth inhibitory effects of statins on +SA mammary tumor cells are directly related to suppression in HMGR activity, mevalonate synthesis, small G-protein prenylation and mitogenic signaling. In contrast, the antiproliferative effects of  $\gamma$ -tocotrienol are not dependent on a reduction in HMGR activity, as evidenced by the finding that treatment with growth inhibiting doses of  $\gamma$ -tocotrienol has little effect on protein prenylation, and mevalonate supplementation does not reverse the antiproliferative effects of this form of vitamin E. However, the synergistic suppression of +SA mammary tumor cell growth that occurs with combined treatment of subeffective doses of individual statins with subeffective doses of  $\gamma$ -tocotrienol appears to result from the action of  $\gamma$ -tocotrienol to potentiate statin-dependent inhibition of HMGR activity. This suggestion is supported by the finding that mevalonate supplementation was able to reverse

the inhibitory effects of combined low dose statin and  $\gamma$ -tocotrienol treatment on +SA cell growth, protein prenylation and mitogenic signaling. These findings demonstrate that the synergistic antiproliferative effects of combined low dose statin and  $\gamma$ -tocotrienol treatment result directly from an inhibition in HMGR activity and subsequent suppression in mevalonate synthesis.

Previous investigations have indicated that the anticancer effects of statins are mediated by both HMGR-dependent and -independent mechanisms [18, 23]. However, the growth inhibitory effects of statins were reversed by mevalonate supplementation and indicate that inhibition of HMGR activity is the primary mechanism mediating the antiproliferative effects of statins in +SA mammary tumor cells. It was also found that mevalonate supplementation alone and in combination with individual statins decreased HMGR levels in +SA cells. This observation agrees with earlier findings that showed mevalonate supplementation induces a down-regulation in HMGR through both transcriptional and post-transcriptional mechanisms [1, 24].

The HMGR pathway plays an essential role in the synthesis of isoprenoids which are required for the isoprenylation of key signaling proteins such as Rap1A and Rab6. Rap and Rab family members are small G proteins similar to Ras that are involved with activating signal transduction pathways important in regulating cellular proliferation, differentiation, adhesion and cancer progression [25–27]. Rap1 activates the MAPK pathways through the interaction with B-Raf [28]. Rab proteins are characteristically elevated in prostate, bladder, ovarian and



**Fig. 5** Effects of mevalonate supplementation on intracellular levels of HMGR, isoprenylation of small G-proteins, MAPKs and cyclin D1. +SA cells were plated at a density of  $1 \times 10^6$  cells/100 mm culture dishes and exposed to control media (C) or 2  $\mu$ M mevalonate (m) containing media, or treatment media containing the combination of 0.25  $\mu$ M simvastatin (S), 0.25  $\mu$ M lovastatin (L), 0.25  $\mu$ M mevastatin (M), or 10  $\mu$ M pravastatin (P), with 2  $\mu$ M  $\gamma$ -tocotrienol (T) alone or in combination with 2  $\mu$ M mevalonate (m) for a 4-day culture period. Afterwards, whole cell lysates were prepared and electrophoresed through polyacrylamide minigels, transblotted to PVDF membrane, followed by Western blot analysis to measure relative levels of total HMGR, unprenylated Rap1A, isoprenylated Rab6, total Ras, phospho-p44/42 ERK, phospho-p38, total p44/42 ERK, total p38, cyclin D1 and  $\alpha$ -tubulin.  $\alpha$ -Tubulin was visualized to ensure equal sample loading in each lane

breast cancers, and suppression of Rab6 prenylation can initiate apoptosis in many types of cancer cells [29, 30]. In contrast to statins, the growth inhibitory effects of  $\gamma$ -tocotrienol treatment alone occurs independently of HMGR activity and is not associated with a reduction in small G-protein prenylation. The reduction in HMGR levels observed following  $\gamma$ -tocotrienol treatment alone is apparently insufficient to decrease HMGR activity enough to block the prenylation of Rap1A and Rab6. However, combined low dose treatment of statins and  $\gamma$ -tocotrienol greatly reduced prenylation of Rap1A and Rab6, and this effect was reversed by mevalonate supplementation. Studies also found that unlike mevalonate, supplementation of FPP or GGPP did not reverse the inhibitory effects of combined low dose statin and  $\gamma$ -tocotrienol treatment (data not shown). It is possible that non-sterol products synthesized downstream of mevalonate, but upstream of FPP and

GGPP, such as isopentenyl pyrophosphate (IPP) and geranyl pyrophosphate (GPP), play an important role in the prenylation of Rap1A and Rab6 in +SA cells. Additional studies are required to determine the exact enzymatic step downstream of mevalonate that is targeted by combined statin and  $\gamma$ -tocotrienol treatment to prevent the prenylation of Rap1A and Rab6.

Previous studies have established that  $\gamma$ -tocotrienol decreases cellular levels of HMGR by accelerating the degradation of this enzyme [11]. However, the present findings clearly indicate that when given alone, the growth inhibitory effects of  $\gamma$ -tocotrienol are not dependent on its action to down regulate HMGR levels. The antiproliferative effects of  $\gamma$ -tocotrienol have been previously reported to be associated with a suppression in ErbB3/PI3K/Akt mitogenic signaling [31, 32]. ErbB/PI3K/Akt signaling pathway is important for cell survival and growth, and plays a pivotal role in tumorigenesis. Since Akt phosphorylates and stimulates the function of many intracellular proteins involved in proliferation and survival, enhanced Akt signaling has been shown to be associated with the development of breast cancer in humans [33]. The ability of subeffective doses of  $\gamma$ -tocotrienol to synergistically enhance the growth inhibitory effects of individual statins can be explained, at least in part, by the finding that statin inhibition of HMGR activity is associated in triggering a compensatory feedback mechanism that results in an up-regulation in HMGR expression [34, 35], and this self-limiting effect of statins is blocked when given in combination with  $\gamma$ -tocotrienol.

Mitogenic activation of small G-proteins like Ras, Rap and Rab stimulates MAPK pathways and promotes cell cycle progression [36–38]. MAPK signaling cascades such as Erk, p38, and JNK control the fundamental cellular processes of growth, proliferation, differentiation, migration and apoptosis, and elevated or unregulated MAPK signaling can play a critical role in the development and progression of cancer [39]. Over expression or enhanced Ras activation and MAPK signaling can result in enhanced expression of cyclin D1 and stimulate unregulated cell cycle progression in many types of human cancers [40]. Combined treatment of  $\gamma$ -tocotrienol with individual statins caused a reduction in Rap1A and Rab6 prenylation, decreased phosphorylation (activation) of p44/42-ERK and p38, and decreased expression of cyclin D1 which regulates cell cycle transition from G1 to S phase, and these effects were reversed by mevalonate supplementation. Although this same treatment was found to have no effect on total Ras levels, it is very likely that prenylated Ras levels were also reduced and contributed to the reduction in phosphorylated-MAPKs that was observed in these +SA mammary tumor cells. Further studies are necessary to determine the effects on combined low dose statin and  $\gamma$ -tocotrienol treatment on Ras prenylation.



HMGR activity and expression is characteristically elevated and often resistant to feedback regulation by sterols in many types of cancer cells [1, 41, 42]. Fortunately, HMGR activity has also been found to be highly sensitive to the feedback inhibition by non-sterol isoprenoids, and exogenous administration of plant derived isoprenoids such as  $\alpha$ -limonene, perillyl alcohol, and  $\beta$ -ionone has been shown to induce a down-regulation of HMGR in various types of cancer cells [5, 22, 43]. Chemically,  $\gamma$ -tocotrienol is also classified as an isoprenoid and this property might explain the mechanism involved in mediating  $\gamma$ -tocotrienol-induced down-regulation of HMGR levels in +SA mammary tumor cells [11, 22].

In conclusion, the present findings demonstrate that suppression in HMGR activity and mevalonate synthesis is primarily responsible for mediating the antiproliferative effect of combined low-dose statin and  $\gamma$ -tocotrienol treatment in +SA mammary tumor cells. The synergistic antiproliferative activity of these agents appears to be due to the cooperative action of  $\gamma$ -tocotrienol to cause a down-regulation in HMGR levels and statins to directly inhibit HMGR activity. These findings also suggest that combined low-dose statin and  $\gamma$ -tocotrienol treatment may greatly improve therapeutic responsiveness in the treatment of breast cancer, while at the same time greatly reduce or eliminate the severe myotoxicity and other adverse side effects associated with high-dose statin monotherapy.

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