MYC Phosphorylation, Activation, and Tumorigenic Potential in Hepatocellular Carcinoma Are Regulated by HMG-CoA Reductase

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Abstract

MYC is a potential target for many cancers but is not amenable to existing pharmacologic approaches. Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) by statins has shown potential efficacy against a number of cancers. Here, we show that inhibition of HMG-CoA reductase by atorvastatin (AT) blocks both MYC phosphorylation and activation, suppressing tumor initiation and growth in vivo in a transgenic model of MYC-induced hepatocellular carcinoma (HCC) as well as in human HCC-derived cell lines. To confirm specificity, we show that the antitumor effects of AT are blocked by cotreatment with the HMG-CoA reductase product mevalonate. Moreover, by using a novel molecular imaging sensor, we confirm that inhibition of HMG-CoA reductase blocks MYC phosphorylation in vivo. Importantly, the introduction of phosphorylation mutants of MYC at Ser62 or Thr58 into tumors blocks their sensitivity to inhibition of HMG-CoA reductase. Finally, we show that inhibition of HMG-CoA reductase suppresses MYC phosphorylation and tumorigenic properties. The inhibition of HMG-CoA reductase may be a useful target for the treatment of MYC-associated HCC as well as other tumors. Cancer Res; 71(6): 2286–97. ©2011 AACR.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and generally incurable malignancies with an estimated 9% 5-year survival rate (1). Hepatocellular carcinogenesis is strongly associated with hepatitis B and C virus (HBV and HCV) infection and other pathologic conditions resulting in liver regeneration (2), which, in turn, facilitates the activation of specific oncogenes, most notably MYC (c-MYC; 3). Thus, targeted inactivation of MYC may be an effective therapy for HCC (4–6). Indeed, we have reported recently that the conditional inactivation of MYC can be sufficient to induce sustained regression of HCC (7). However, there is no existing therapy that targets MYC for the treatment of any cancer.

The MYC protooncogene family is composed of c-MYC, N-MYC, and L-MYC and has been shown to impact almost every aspect of tumorigenesis, including promoting unrestricted cell proliferation, inhibiting cell differentiation, reducing cell adhesion, and enhancing metastasis, genomic instability, and angiogenesis (8, 9). MYC functions as an oncogene upon overexpression, either due to increased expression of the myc gene or due to increased stability of the MYC protein. MYC protein stability is regulated as follows (10): through the ubiquitin/26S proteasome pathway and the sequential phosphorylation of MYC at serine 62 (S62) and threonine 58 (T58). The phosphorylation of S62 is mediated by the MAPK/ERK (mitogen activated protein kinase/extracellular signal regulated kinase) pathway and contributes to the stabilization of MYC. Subsequent phosphorylation of T58, mediated by GSK3β, promotes ubiquitin-dependent MYC degradation once S62 is dephosphorylated (11–13). Mutations in these phosphorylation sites that stabilize MYC protein have been identified in human cancers, thereby highlighting the importance of S62 and T58 phosphorylation as regulators of MYC in tumorigenesis (14, 15). Hence, targeting MYC phosphorylation could be useful as an anticancer therapy.

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) controls the rate-limiting step in the mevalonate (MV) pathway that is essential for cholesterol biosynthesis (16). Statins were initially utilized to inhibit HMG-CoA reductase as a means to reduce the serum cholesterol level (16). However, many studies have shown that inhibition of
HMG-CoA reductase also has antitumor efficacy both in vitro and in vivo in multiple tumor types (17–20), including colorectal cancer (21, 22), breast cancer (23), melanoma (24), and lymphoma (25). Statins have been suggested to block tumor cell growth through the inhibition of proliferation and angiogenesis, induction of apoptosis, and repression of tumor metastasis (26).

Statins may mediate their anticancer properties through inhibition of the synthesis of lipid isoprenoid intermediates, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are produced downstream of the MV pathway (19). Generally, FPP activates the Ras GTPase family whereas GGPP activates the Rho/Rac family by prenylating and anchoring them on the cell membrane (27). Both Ras (12, 27) and Rho/Rac family members (28) are required to phosphorylate MYC. Hence, we speculated that by inhibiting these pathways, statins might therefore block MYC activation. Here we show that the inhibition of HMG-CoA reductase by atorvastatin (AT) inhibits MYC phosphorylation and activation and thereby blocks MYC-induced HCC onset and tumor maintenance. Moreover, by using a novel molecular imaging sensor that noninvasively detects MYC phosphorylation, we found, both in vitro in human HCC cells and in vivo in mice, that AT inhibits MYC phosphorylation. Furthermore, the introduction of mutant MYC alleles that cannot be phosphorylated on S62 or T58 prevented AT from inhibiting the in vivo tumor growth of HCC. Finally, we provide evidence suggesting that AT may mediate these effects on MYC phosphorylation and activation by inhibiting Rac GTPase. Therefore, HMG-CoA reductase seems to be a critical regulator of MYC activation and may have potent activity against MYC-induced cancers.

Materials and Methods

**Antibodies**

The antibody to Ki67 was obtained from BD Biosciences; c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Santa Cruz Biotechnology; phospho-c-MYC was obtained from Cell Signaling Technology; antibodies to Tubulin and GAPDH were obtained from Cell Signaling Technology; antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Santa Cruz Biotechnology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology; antibodies to Tubulin and GAPDH were obtained from Cell Signaling Technology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology. Antibodies to c-MYC, Cdk4, E2F1, and Rac1 antibodies were obtained from Sigma-Aldrich. Horseradish peroxidase conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG were obtained from Cell Signaling Technology.

**Cell lines**

The Huh7 and HepG2 cell lines were obtained from the American Type Culture Collection originally characterized by DNA profile and cytogenetic analysis and were passaged for less than 6 months in vitro.

**Transgenic mice**

The Tet System was used previously to generate transgenic mice that conditionally express human c-MYC cDNA in hepatocytes, as described (7). MYC expression was induced by removing doxycycline (Dox, 100 μg/mL) from the drinking water of mice. All animals were maintained and treated in accordance with the policies of Stanford University.

**AT treatment**

AT (prescription formulation; Pfizer Inc.) was suspended in PBS. It was administered orally in 100 mg/kg doses with or without 20 mg/kg MV 3 times a week, using 20-mm feeding needles (Popper and Sons). PBS was administered as a negative control. Purified AT (Sequoia Research Products) was suspended in 100% DMSO and used for in vitro studies.

**Histology and immunohistochemistry**

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections of 5 μm were stained with hematoxylin-eosin (H&E) or analyzed by immunohistochemistry using the antibody to Ki67. DAB (3,3’-diaminobenzidine; Vector Laboratories) was used to achieve color development.

**Proliferation assay**

Cells were seeded in 24-well plates (5,000 cells/well) and incubated overnight. Next, cells were treated with PBS, AT (0.5, 1.0, 2.5, 5.0, 10, or 25 μmol/L), 10 or 25 μmol/L AT, AT and 100 μmol/L MV, 0.5, 10, or 25 μmol/L AT and 0.5 μmol/L MV, 10 or 25 μmol/L AT and 10 μmol/L FU, or 0.5 or 10 μmol/L AT and 10 μmol/L GGPP for 96 hours. Cell proliferation was evaluated using the MTT assay. Data were from 6 replicated experiments.

**Quantitative real-time PCR**

HCC cells were treated with PBS, 20 ng/mL Dox, 10 μmol/L AT, or 10 μmol/L AT and 100 μmol/L MV for 24 hours. Total mRNA from HCC cells was extracted and purified using the RNeasy Mini Kit from Qiagen and quantified by spectrophotometer (Beckman Coulter). cDNA was reverse-transcribed from 2 μg of total mRNA using oligo-d(T) primers. Real-time PCR analysis was done in an ABI PRIZM analyzer (Applied Biosystems).

**Cell membrane fractionation and protein isolation**

Cells (2 × 10³) were washed 3 times with PBS and extracted in lysis buffer (50 mmol/L Tris, 50 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L MgCl₂, 10 mmol/L NaF, 1 mmol/L DTT, pH 7.4). Lysates were centrifuged at 36,000 rpm for 40 minutes, using Beckman L8-70M ultracentrifuge. The membrane pellet was solubilized in immunoprecipitation buffer (0.15 mol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10 mmol/L Tris-HCl, pH 7.4) and incubated at 4°C for 30 minutes. The solution was centrifuged at 15,000 rpm for 10 minutes, and the supernatant was collected as the membrane protein fraction.

**Viral infection**

Ad-c-MYCWT, Ad-c-MYC S62A, and Ad-c-MYC T58A viruses were kindly provided as a gift from Dr. Rosalie C. Sears (Oregon Health & Science University, Portland, OR). Briefly, Ad-c-MYCWT, Ad-c-MYC S62A, and Ad-c-MYC T58A adenovirus were cloned by inserting c-MYCWT (Ad-c-MYCWT), c-MYC S62A (Ad-c-MYC S62A), and c-MYC T58A (Ad-c-MYC T58A) cDNA into the pAdEasy-1 backbone (Stratagene). Transplanted tumor cells were infected as previously described (29). Briefly, SCID mice were injected with hepatocellular...
carcinoma cells isolated from LAP-tTA/TRE-MYC transgenic mice. Tumor masses were injected at 3 sites with Ad-c-MYCWT or Ad-c-MYC<sup>TSSA</sup> once every week. Successful infection was confirmed by green fluorescent protein (GFP) coexpression in tumors (Supplementary Information, Fig. S9). Mice were treated with 100 μg/mL Dox to inactivate transgenic MYC expression. Tumor growth was measured using calipers 3 times a week for 3 weeks after viral injection.

**Immunoprecipitation and immunoblotting**

Cells were lysed in immunoprecipitation buffer (0.15 mol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10 mmol/L Tris-HCl, pH 7.4) and cleared lysates were immunoprecipitated with 2 μg HA antibody. The precipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with the antibodies indicated in the figures. Total MYC and phospho-MYC level were detected by immunoblotting, and their optical density (OD) was measured and normalized to actin band OD. The MYC phosphorylation level was determined by the ratio between phospho-MYC and total MYC.

**Molecular imaging of MYC phosphorylation**

A bioluminescent sensor system that can noninvasively detect c-MYC phosphorylation was utilized to detect the AT inhibitory effect in intact cells and living mice. The sensor system utilizes the fact that S62 phosphorylation of MYC is required for its interaction with GSK3β and detects the protein interaction between GSK3β and MYC to indirectly report the MYC phosphorylation, using a split Firefly luciferase (FL) complementation system (30). Specific GSK3β and MYC fragments are fused with the inactive C-terminal and N-terminal fragment of the split FL, respectively (GSK35–433-CFL/NFL-c-Myc). Phosphorylation-induced interaction between GSK3β and MYC brings the 2 split fragments into close proximity and recovery of the luciferase activity.

The sensor system has been validated both in intact cells and in mouse xenograft model, which is described in an independent article (31). For <i>in vitro</i> imaging, the sensor plasmids were transiently transfected into HuH7 and HepG2 cells, using Superfect (Qiagen) and Lipofectamine 2000 (Invitrogen) reagent, respectively. Renilla luciferase (RL) gene was cotransfected for the control of the transfection efficiency. Twenty-four hours after transfection, cells were treated with AT with different concentration as indicated for 18 hours. Bioluminescent imaging (BLI) was conducted in IVIS 50 (Caliper Life Science) after adding 45 μg/mL <i>v</i>-Luciferin (Promega) to the cells. Cells were lysed for RL assay (Promega) and Western blotting analysis after imaging. For <i>in vivo</i> liver tumor imaging, we used a hydrodynamic injection method as previously described (32). Briefly, 2 mL of saline solution containing 25 μg of the MYC sensor plasmid with CMV promoter was injected into the tail vein within 8 seconds. Mice were imaged in IVIS 200, 22 hours after injection, based on time course of the sensor expression as determined in control experiments (Supplementary Information, Figs. S6 and S7).

**Results**

**Inhibition of HMG-CoA reductase by AT suppresses MYC-induced HCC**

The effects of inhibition of HMG-CoA reductase were examined on HCC growth <i>in vitro</i> and <i>in vivo</i> by administering AT to multiple HCC tumor cell lines derived from the LAP-tTA/TRE-MYC transgenic mice, a previously described conditional transgenic model of MYC-induced HCC, using the Tet-system (7), in addition to human HCC cell lines. The murine cell lines were all generated from HCC isolated from dual transgenic animals. These cell lines are dependent upon high levels of human MYC expression, which can be inactivated upon treatment with Dox. Hence, in these transgenic tumor-derived cell lines, as a positive control for MYC inactivation, we used Dox to induce the suppression of transgenic MYC. Doses of AT used for our experiments were comparable with previously published studies (33, 34). Moreover, as control for nonspecific effects of statins, we confirmed that effects were reversed with cotreatment with the enzyme product of HMG-CoA reductase MV. Note that because of marked differences in the pharmacokinetics between mouse and human, AT doses in mice are approximately 50-fold higher than the pharmacologically equivalent dose in humans (35, 36).

First, AT inhibited the <i>in vitro</i> proliferation of the MYC-induced murine cell line HCC 3–4 as measured by the MTT assay (Fig. 1A and Supplementary Information, Fig. S2A; 75% decrease at 10 μmol/L and 92% decrease at 25 μmol/L AT at 96 hours of AT treatment, <i>P</i> < 0.0001 each). Also, AT decreased the number of cells in S and G<sub>2</sub>/M phases from 38% to 26%, as assessed by propidium iodide (PI) staining (Fig. 1B, <i>P</i> = 0.01), and reduced Ki67 immunofluorescence from 81.4% to 31.2% following 48 hours of 10 μmol/L AT treatment (Fig. 1C, <i>P</i> < 0.0001). Importantly, the effects of AT on proliferation and cell-cycle arrest were rescued by cotreatment with MV, the immediate downstream target of HMG-CoA reductase (Fig. 1A–C), confirming these effects are specific to inhibition of the cholesterol biosynthesis pathway. Moreover, AT was found to similarly inhibit proliferation and induce cell-cycle arrest and apoptosis in 2 independently derived murine HCC cell lines, EC4 and HCC 4–4, in a dose-dependent manner (Supplementary Information, Figs. S1–S3). Thus, AT inhibits proliferation and induces apoptosis of murine HCC tumor cells <i>in vitro</i>.

Second, AT inhibited the growth of the murine HCC 3–4 cell line transplanted into syngeneic mice by up to 80% compared with treatment by PBS or AT and MV (Fig. 1D and E, <i>P</i> = 0.0003). Note, that FVB/N mice treated with 100 mg/kg AT did not exhibit any general toxicity and, in particular, had normal liver histology and serum bilirubin levels, showing that the clinical effects are not secondary nonspecific hepatotoxicity (Supplementary Information, Fig. S4). Therefore, inhibition of HMG-CoA reductase by AT has potent <i>in vivo</i> antitumor activity against murine MYC-induced HCC.

Third, we interrogated the ability of AT to suppress growth in HuH7 cells, a human HCC cell line. AT blocked the <i>in vitro</i> growth of HuH7 cells over a 96-hour time course (Fig. 2A and Supplementary Information, Fig. S2B; 69% decrease at 10
Figure 1. Inhibition of HMG-CoA reductase by AT suppresses growth of MYC-induced HCC in vitro and in vivo. A, AT inhibits the proliferation of a MYC-induced tumor-derived cell line, HCC 3-4. MTT assay was conducted every 24 hours for 4 days on HCC cells treated with 10 μmol/L AT, AT plus 100 μmol/L MV, or DMSO as a vehicle control. Cells were also treated with Dox to inactivate transgenic MYC as a positive control. All experiments were repeated 3 times (P < 0.0001). Error bars, SD. B, AT induces cell-cycle arrest in murine HCC as assessed by fluorescence-activated cell-sorting analysis of PI-stained cells (P = 0.01). Cells were treated with 10 μmol/L AT, AT plus MV, DMSO, or Dox for 48 hours. C, immunofluorescence for Ki67 on HCC cells treated with 10 μmol/L AT for 48 hours shows that statin treatment inhibits HCC proliferation (P < 0.0001). D, AT inhibits growth of MYC-induced HCC cells in vivo. Murine HCC cells were subcutaneously transplanted into FVB/N mice treated with PBS (n = 5) or AT (n = 5). P = 0.0003. Error bars, SD. E, representative images of mice treated with PBS (left), AT (middle), or AT plus MV (right) show that AT suppresses growth of MYC-induced HCC in vivo.

Finally, we evaluated the ability of HMG-CoA reductase inhibition to block the initiation of MYC-induced HCC growth in vivo in the LAP-tTA/TRE-MYC transgenic mice treated with PBS, AT, or AT with MV (Fig. 3A, left). Treatment with 100 mg/kg AT versus PBS significantly delayed tumor onset and increased survival (Fig. 3A; median survival increased from 80 to 147 days, P < 0.0005). Importantly, MV treatment prevented AT from inhibiting tumorigenesis (Fig. 3A). Gross pathology revealed that AT markedly reduced the size and frequency of tumor nodules (Fig. 3B, left). H&E staining

μmol/L and 86% decrease at 25 μmol/L AT, P < 0.0001 each) while inhibiting cell-cycle progression (Fig. 2B; 75% reduction in S phase, P = 0.003) and reducing Ki67 positivity (Fig. 2C; 58% reduction, P < 0.0001) following 48 hours of 10 μmol/L AT treatment. Moreover, AT suppressed the in vivo growth of HuH7 cells (Fig. 2D; PBS vs. AT, P = 0.03; AT vs. AT + MV, P = 0.04; PBS vs. AT + MV, P = 0.8). Cotreatment with MV blocked the effects of statin treatment, confirming that the inhibition of human HCC by AT is specific to the suppression of HMG-CoA reductase (Fig. 2A–D).
revealed histologically normal liver tissue in AT-treated mice, suggesting a robust inhibition of disease onset (Fig. 3B, middle). AT-treated animals also exhibited evidence of inhibited cell proliferation, as indicated by reduced Ki67 staining compared with PBS-treated mice (Fig. 3B, right; 8 ± 3 positive cells vs. 422 ± 23 positive cells per field, \( P < 0.02 \)). Hence, inhibition of HMG-CoA reductase by AT is potent at inhibiting MYC-induced liver tumorigenesis.

**Inhibition of HMG-CoA reductase suppresses MYC phosphorylation, stability, and transactivation**

MYC activation has been shown to be regulated by phosphorylation (12). Thus, we considered that AT might exert its antineoplastic effects by inhibiting MYC phosphorylation. Indeed, AT, but not AT with MV, was found to induce a dose-dependent downregulation of MYC phosphorylation in vitro upon 24 hours treatment (Fig. 4A; 29% reduction at 0.5 \( \mu \text{mol/L} \), 34% at 1.0 \( \mu \text{mol/L} \), 79% at 2.5 \( \mu \text{mol/L} \), 83% at 5.0 \( \mu \text{mol/L} \), 94% at 10 \( \mu \text{mol/L} \), and 97% at 25 \( \mu \text{mol/L} \); AT; \( P = 0.004 \)) as well as in vivo (Fig. 4B; 93% reduction). Moreover, AT blocked MYC phosphorylation in MYC-induced murine lymphoma, osteosarcoma, and lung cancer, as well as in human breast cancer cell lines (data not shown). In turn, the dephosphorylation was associated with a reduction in MYC protein levels in vitro (Fig. 4A; 17% reduction at 0.5 \( \mu \text{mol/L} \), 40% at 1.0 \( \mu \text{mol/L} \), 9% at 2.5 \( \mu \text{mol/L} \), 41% at 5.0 \( \mu \text{mol/L} \), 69% at 10 \( \mu \text{mol/L} \), and 91% at 25 \( \mu \text{mol/L} \); AT, \( P = 0.002 \)) and in vivo (Fig. 4B; 57% reduction, \( P = 0.04 \)). Importantly, the inhibition of MYC phosphorylation by AT not only reduced MYC levels but also seemed to dramatically inhibit MYC transcriptional activation, as illustrated by the reduced mRNA expression of canonical target genes \( \text{ODC} \) and \( \text{nucleolin} \), both in murine (Fig. 4C; 72% reduction for \( \text{ODC} \), \( P = 0.003 \); 76% reduction for \( \text{nucleolin} \), \( P = 0.03 \)) and in human HCC upon 24 hours of 10 \( \mu \text{mol/L} \) AT treatment (Fig. 4D; 64% reduction for \( \text{ODC} \), \( P = 0.016 \); 59% reduction for \( \text{nucleolin} \), \( P = 0.008 \); Supplementary Information, Fig. S8B). Thus, the inhibition of HMG-CoA reductase blocks MYC phosphorylation, reduces MYC protein levels, and inhibits MYC transactivation.

**Noninvasive molecular imaging to measure in vivo MYC phosphorylation**

To evaluate the effects of inhibition of HMG-CoA reductase on MYC phosphorylation in situ in a living host, we utilized a novel molecular imaging sensor system (31). The sensor system consists of 2 parts: (i) a peptide corresponding to the phosphorylated domain of MYC fused to the N-terminal domain of FL and (ii) the C-terminal domain of FL fused to a peptide fragment of GSK3\( \beta \) that recognizes phospho-MYC (Fig. 5A). When coexpressed in an intact cell, MYC phosphorylation can be detected via interaction between the MYC and GSK3\( \beta \) peptides, thereby localizing the N- and C-termini of FL in close proximity, conferring luciferase activity. RL is cotransected as a control for transfection efficiency. The full-length FL was also transfected independently into these cells as a control for the direct effect of AT on luciferase activity. We confirmed that this imaging method could detect the dose-dependent...
reduction of MYC phosphorylation in human Huh7 and HepG2 cells upon 18 hours of AT treatment (Fig. 6B and C and Supplementary Information, Fig. S5, $P < 0.0001$).

Next, this imaging sensor was used to monitor MYC phosphorylation in vivo. The sensor system was introduced in liver cells of LAP-tTA/TRE-MYC mice by hydrodynamic injection. Two groups of transgenic mice ($n = 3$ each) had MYC activated at the same time and were treated with either AT or PBS. The MYC sensor was imaged at days 0 and 15 posttreatment. The PBS-treated group showed no significant change of the sensor signal, whereas the AT-treated group showed 72% reduction of the sensor signal at day 15 of treatment (Fig. 5D and E; AT-treated mice day 0 vs. day 15, $P = 0.038$; PBS-treated day 0 vs. day 15, $P = 0.638$). Notably,
AT-mediated inhibition of MYC phosphorylation in vivo was associated with a 44% and 56% downregulation in the expression of downstream target genes, E2F1 and Cdk4 (Supplementary Information, Fig. S8A), further showing the inhibition of MYC activity. Hence, a novel imaging sensor was used to show that the inhibition of HMG-CoA reductase by AT inhibits MYC phosphorylation in vivo.

MYC phosphomutants confer resistance to the inhibition of HMG-CoA reductase

To examine whether inhibition of MYC phosphorylation mediates the anticancer effect of AT, we introduced phosphomutants of MYC into HCC tumor cell lines. First, recombinant adenovirus was used to express MYC that is mutated with either an alanine substitution at either S62 or T58 (Ad-MYCS62A and Ad-MYCT58A) and hence cannot be regulated by phosphorylation at these sites (37). AT treatment at 10 μmol/L for 24 hours dramatically suppressed WT MYC but failed to significantly inhibit protein levels of either S62A MYC or T58A MYC (Fig. 6A, 62% reduction for MYCWT, P = 0.01; 68% reduction for MYCT58A, P = 0.008). These data show that the reduction of phospho-MYC by AT seems to occur by preventing S62 phosphorylation. However, the reduction of total MYC protein cannot occur without T58 phosphorylation. In addition, HCC cells expressing the S62A MYC mutant were less sensitive whereas cells expressing T58A MYC were completely insensitive to the inhibition of proliferation upon AT treatment (Fig. 6B, 26% reduction for WT, P < 0.001; 17% reduction for S62, P < 0.0001; no reduction for T58, P = 0.8). Thus, inhibition of HMG-CoA reductase suppresses MYC activation in a phosphorylation-dependent manner.

Next, we investigated whether the MYC phosphomutants could suppress the ability of the inhibition of HMG-CoA reductase to block HCC tumor growth in vivo. Syngeneic hosts were transplanted with MYC-induced HCC cells that were then injected with Ad-MYCWT, Ad-MYCS62A, or Ad-MYCT58A. Tumor
growth was monitored in response to AT, PBS, or AT with MV treatment (Fig. 6C). The adenoviral delivery of the MYC phosphomutants was confirmed by coexpression of GFP (Supplementary Information, Fig. S9). To suppress the conditional transgenic MYC expression, mice were treated with Dox, thereby resulting in the effective knock-in of the Ad-MYCWT, Ad-MYCS62A, or Ad-MYCT58A constructs into the HCC cells. HCC growth upon injection of Ad-MYCWT showed 66% inhibition by 100 mg/kg AT but not by PBS or AT and MV treatment (Fig. 6D, left, PBS vs. AT, $P = 0.01$; AT vs. AT + MV, $P = 0.007$). However, HCC tumors that were injected with Ad-MYCS62A exhibited only 44% inhibition of tumor growth upon treatment with AT (Fig. 6D, middle, PBS vs. AT, $P = 0.02$; AT vs. AT + MV, $P = 0.03$). Tumors that were injected with Ad-MYCT58A showed complete rescue from sensitivity to AT treatment (Fig. 6D, right, PBS vs. AT, $P = 0.56$; AT vs. AT + MV, $P = 0.03$). Therefore, MYC phosphorylation is necessary for AT to inhibit MYC-induced HCC tumor growth.

**Inhibition of HMG-CoA reductase may block MYC activity through Rac GTPase**

We examined whether the inhibition of HMG-CoA reductase blocks MYC activation through Rac GTPases. Statins block production of the isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate (23, 38, 39). FPP prenylates the Ras, Rheb, and PTP4A3 family whereas GGPP prenylates the Rac, Rho, and Cdc42 family of small GTPases (16). Previous studies suggest that Ras and Rac/Rho families of GTPases may contribute to the regulation of MYC phosphorylation (25). Thus, the inhibition of HMG-CoA reductase is likely to prevent MYC phosphorylation through these GTPases.

To explore the role of GTPases in mediating inhibition of MYC phosphorylation, we conducted several experiments. First, to control GTPase activity, we supplemented growth media with either FPP or GGPP before 96 hours AT treatment of MYC-induced murine HCC cells in vitro. Both MV and GGPP restored HCC cell proliferation to levels similar to those of DMSO controls (Fig. 7A; DMSO vs. AT + MV, $P = 0.07$; DMSO vs. GGPP, $P = 0.053$), whereas FPP showed significantly less reduction in AT-mediated growth inhibition (DMSO vs. FPP, $P < 0.002$). Similarly, in Huh7 cells, GGPP was more efficient in rescuing the transcription of multiple MYC.
target genes, including \( \text{CdK4} \) and \( \text{E2F1} \), than FPP following 24 hours of \( 10 \mu \text{mol/L} \) AT treatment (Supplementary Information, Fig. S10, 40%–45% reduction for FPP, \( P < 0.001 \); 25%–60% increase for GGPP, \( P < 0.03 \)). MYC phosphorylation was restored to levels similar to PBS-treated controls when media containing \( 10 \mu \text{mol/L} \) AT was supplemented with GGPP but not with FPP (Fig. 7C; 76% reduction for FPP, \( P = 0.001 \); 21% reduction for GGPP, \( P = 0.02 \)). Therefore, inhibition of HMG-CoA reductase seems to inactivate MYC through the inhibition of the Rho/Rac pathway.

Finally, we examined the potential role of the Rac/Rho/Cdc42 pathway as a mechanism by which AT suppresses MYC activation. First, we investigated whether AT was influencing the membrane localization of Rac1. Indeed, 24-hour treatment with \( 10 \mu \text{mol/L} \) AT resulted in the delocalization of Rac1 from the plasma membrane (Fig. 7D; 83% reduction for AT, \( P < 0.001 \)). As a control, we showed that treatment with MV blocked these effects, as did treatment with GGPP (Fig. 7D; no change for PBS vs. AT + MV, \( P = 0.08 \); 37% increase for PBS vs. GGPP, \( P < 0.03 \)). Notably, AT had little effect on Ras localization (data not shown). Second, using a Rac pull-down assay, 24 hours of \( 10 \mu \text{mol/L} \) AT treatment was shown to reduce Rac1 activity by 77% (Fig. 7E). Thus, the inhibition of HMG-CoA reductase by AT seems to suppress activation of the Rac pathway, suggesting that AT in blocks MYC phosphorylation and activation via inhibition of Rac.

Discussion

Here, we show that MYC phosphorylation, activation, and thereby tumorigenic potential are regulated by HMG-CoA reductase. In particular, the inhibition of HMG-CoA reductase by statins suppresses MYC phosphorylation and activation. The consequences of these effects on MYC include preventing HCC initiation as well as inhibiting the in vivo growth of established murine and human HCC tumors. Moreover, statins, by blocking HMG-CoA reductase, inhibit GTPase activity, thereby resulting in MYC dephosphorylation and inactivation, which is essential for their anticancer therapeutic effect (Fig. 7F). Hence, the inhibition of HMG-CoA reductase by AT may be an effective strategy for the inhibition of MYC in the treatment and prevention of HCC.

Importantly, we confirmed the antitumor effects of statins that we observed both in vitro and in vivo were specific to HMG-CoA reductase because they could be readily reversed by cotreatment with MV. We note that the AT doses we used in

Figure 6. HCC transduced with S62A or T58A MYC phosphomutants show reduced sensitivity to HMG-CoA reductase inhibition. A, murine HCC cells were infected with Ad-MYC\(^{\text{WT}}\) (WT), Ad-MYC\(^{\text{S62A}}\) (S62A), or Ad-MYC\(^{\text{T58A}}\) (T58A) adenovirus, and HA-tagged MYC was immunoprecipitated using an antibody to the HA tag. Immunoblot analysis suggests that AT-dependent phosphoregulation of MYC is via S62. However, the inhibition of protein stability requires both S62 and T58 phosphorylation. Cells were treated with \( 10 \mu \text{mol/L} \) AT for 24 hours. B, S62A partially and T58A completely abrogated AT inhibition of cell proliferation (S62A: PBS vs. AT, \( P < 0.0001 \); T58A: PBS vs. AT, \( P = 0.8 \)). Error bars, SD. C, HCC cells isolated from transgenic animals were transplanted into SCID mice, injected with Ad-MYC\(^{\text{WT}}\), Ad-MYC\(^{\text{S62A}}\), or Ad-MYC\(^{\text{T58A}}\) once every week, and orally treated with PBS (\( n = 6 \)), AT (\( n = 5 \)), or AT with MV (\( n = 5 \)) together with Dox. Tumor growth was measured 3 times per week. D, in vivo growth kinetics of HCC infected with Ad-MYC\(^{\text{WT}}\) show that AT inhibits tumor growth in vivo (left, PBS vs. AT, \( P = 0.01 \); AT vs. AT + MV, \( P = 0.007 \)). Error bars, SD. Infection with Ad-MYC\(^{\text{S62A}}\) partially rescues growth inhibition due to AT (middle, PBS vs. AT, \( P = 0.02 \); AT vs. AT + MV, \( P = 0.03 \)). Error bars, SD. Ad-MYC\(^{\text{T58A}}\) completely rescues AT-mediated growth inhibition of HCC (right, PBS vs. AT, \( P = 0.56 \); AT vs. AT + MV, \( P = 0.03 \)). Error bars, SD.
mice seem to be higher than generally used in humans. However, it is well known that, because of differences in the pharmacokinetics, murine doses have to be 50-fold higher than in humans (35, 36). Moreover, we confirmed that the antitumor dose of AT did not exhibit toxicity (Supplementary Information, Fig. S4) and hence is likely to be achievable in humans.

The inhibition of HMG-CoA reductase was found to block MYC phosphorylation. First, AT treatment blocks MYC phosphorylation in murine and human HCC cells both in vitro and in vivo. Concomitant treatment with MV abrogates the ability of AT to block MYC phosphorylation and activation and mediates its suppressive effect on HCC growth, indicating that the antineoplastic effect of AT is via the inhibition of HMG-CoA reductase (Fig. 4). Second, a novel phosphorylation sensor was used to show in situ in human HCC cells and in vivo in living mice (Fig. 5). This phosphosensor may be a useful approach to develop therapies that target MYC phosphorylation. Third, mutations in 2 MYC phosphorylation sites, specifically S62 and T58, blocks the ability of AT to inhibit tumor growth in vivo. Therefore, HMG-CoA reductase activity is important to the regulation of MYC phosphorylation. We infer that MYC phosphorylation is an essential component of the mechanism by which statins mediate their antineoplastic properties. The introduction of mutant MYC alleles in HCC tumor cells reduced their sensitivity to statins in vitro and in vivo (Fig. 6).

Figure 7. HMG-CoA reductase influences MYC phosphorylation through a Rac GTPase–dependent mechanism. A, suppression of murine HCC growth in vitro upon 10 μmol/L AT treatment for 96 hours is rescued by GGPP treatment, as assessed by MTT (DMSO vs. AT + MV, P = 0.07; DMSO vs. GGPP, P = 0.053, DMSO vs. FPP, P = 0.002). Error bars, SD. B, GGPP treatment rescues the AT-dependent suppression in human HCC cell growth upon 10 μmol/L AT treatment (DMSO vs. AT + MV, P = 0.01; DMSO vs. GGPP, P = 0.01, DMSO vs. FPP, P < 0.001). Error bars, SD. C, GGPP treatment rescues AT-dependent inhibition of MYC phosphorylation. Representative immunoblots are shown. Error bars, SD. D, GGPP treatment prevents the decrease in the membrane accumulation of Rac induced by 24 hours of 10 μmol/L AT. E, AT treatment inhibits Rac activity, which was reduced by 77% as measured by pull-down assay. F, our results suggest a model in which inhibition of HMG-CoA reductase by AT blocks prenylation and activation of small GTPases, specifically including Rac. AT-mediated inhibition of Rac likely results in reduction of phospho-S62 MYC. Dephosphorylation at S62 in the context of phospho-T58 thereby results in the ubiquitin-mediated degradation of MYC. As such, AT treatment ultimately results in the inhibition of MYC oncogenic activity and suppressed hepatocellular carcinogenesis.
suggests that AT-mediated reduction in phospho-S62 would therefore result in MYC phosphorylated at only T58, which is rapidly degraded in a ubiquitin-dependent manner (12, 13). Our results are consistent with a role of S62 and T58 phosphorylation in MYC stability and transcriptional activity and, most important, their role in tumorigenesis (12). However, we note that S62/T58 phosphorylation has not always been found to regulate MYC stability (40). We conclude that the inhibition of MYC phosphorylation may be important to the mechanism by which the inhibition of HMG-CoA reductase by statins exerts its antineoplastic properties.

Many reports suggest that statins have antineoplastic properties (25, 41–44). Many mechanisms have been proposed including the inhibition of the ErbB2 pathway (45), the blocking of the interaction between the lymphocyte function–associated antigen and intercellular cell adhesion molecule–1 (46), the suppression of geranylgeranylation of the Rho family proteins (47), and the prevention of the prenylation of RhoA and downstream activation of focal adhesion kinase, AKT, and β-catenin (23). Although we cannot preclude any of these possibilities, our results are consistent with the notion that the inhibition of HMG-CoA reductase by AT in HCC cells blocks MYC phosphorylation likely through the inhibition of small GTPases in the Rac pathway (Fig. 7).

Our results are the first to suggest that HMG-CoA reductase regulates MYC activation via Rac. We are currently investigating the signaling intermediates that may function between Rac and MYC to mediate the antineoplastic effect of AT. Previously, it has been suggested that Rac regulates MYC (28). Ral1 can inhibit protein phosphatase PP2A (48), which has been shown to dephosphorylate MYC at S62 (13). One possible mechanism suggested by our work is that AT inhibition of Rac can result in activation of PP2A, which subsequently dephosphorylates MYC at S62 and induces the ubiquitin-dependent degradation of T8-phosphorylated MYC (Fig. 7F).

Our results illustrate that the inhibition of HMG-CoA reductase by statins may be useful in the treatment and prevention of human HCC. HCC is increasing in incidence, has a generally dismal prognosis, and there are few treatment options (49). Statins were developed to inhibit HMG-CoA reductase by statins may be useful in the treatment and prevention of human HCC. HCC is increasing in incidence, whereas other studies have not found clinical benefit (52). A possible explanation for this possible discrepancy in the benefit from statins is that clinical activity could depend upon the activation status of MYC. Also, AT may be a more effective statin for the treatment of HCC.

We conclude that MYC phosphorylation is a critical mechanism by which the inhibition of HMG-CoA reductase by statins mediates their antineoplastic effects. We have shown that a novel molecular imaging sensor may be useful for the identification through high-throughput methods of new therapeutic agents that inhibit MYC phosphorylation and activation. Importantly, statins may be effective agents to inhibit MYC function as a treatment for HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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20. Wong WW, Dimitroulakos J, Minden MD, Penn LZ. HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as potential therapeutic agents. Cancer Res 1997;57:1


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