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Tumor and Stem Cell Biology

Cancer Research

Positive Feedback Loop Between PI3K-Akt-mTORC1 Signaling and the Lipogenic Pathway Boosts Akt Signaling: Induction of the Lipogenic Pathway by a Melanoma Antigen

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Abstract

The lipogenic phenotype is a metabolic hallmark of cancer cells. Sterol regulatory element–binding proteins (SREBP) are key transcriptional factors to regulate biosynthesis of cholesterol and fatty acids. It has been poorly understood how the lipogenic phenotype in cancer cells is regulated and how it augments their malignant properties. Here we describe roles of the melanoma antigen ganglioside GD3 and phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR complex 1 (mTORC1) signaling in the regulation of SREBP activity, cholesterol biosynthesis, and the integrity of lipid rafts in human melanoma cells. GD3 expression induced the activation of both SREBP-1 and SREBP-2. Consequently, HMG-CoA reductase expression and cholesterol biosynthesis increased. The activation of the SREBP pathway was independent of the oncogenic BRAF mutation. On the other hand, it was regulated by PI3K-Akt-mTORC1 signaling in human melanoma cells. Disruption of the signaling pathway resulted in the reduction of cholesterol in lipid rafts. Inhibition of the SREBP pathway attenuated Akt activation in lipid rafts and suppressed the growth of human melanoma cells *in vitro* and *in vivo*. These results suggest that PI3K-Akt-mTORC1 signaling is important for the integrity of lipid rafts by regulating SREBP activation and subsequent cholesterogenesis. We thus propose a positive feedback circuit in which PI3K-Akt-mTORC1-SREBP signaling boosts Akt signaling in human melanoma cells expressing GD3. *Cancer Res; 71(14); 4989–97. ©2011 AACR.*

Introduction

Altered metabolism is a hallmark of cancer cells (1–3). Cancer cells show increases in glucose uptake and glycolysis. The metabolic changes play an important role in their malignant properties. Cancer cells also display the lipogenic phenotype (4). The expression of key lipogenic enzymes such as fatty acid synthase (FAS) is elevated in various tumor tissues and cancer cell lines, including melanomas (4). Inhibition of FAS suppresses malignant properties of cancer cells. Epidemiologic studies have shown that statins, HMG-CoA reductase (HMGR) inhibitors, reduce the risk of certain cancers, including melanomas, indicating that the cholesterol biosynthetic pathway also plays an important role in malignancies (5, 6).

Lipid synthesis is tightly controlled by transcriptional regulation of lipogenic enzymes. Sterol regulatory element–binding proteins (SREBP) -1 and -2 are master transcriptional factors that regulate cholesterol and fatty acid biosynthesis (7). SREBPs are localized in endoplasmic reticulum (ER) membrane as a precursor and form a complex with SREBP-cleavage activating protein (Scap) and Insig (8). Upon a decrease in cellular sterol level, Insig dissociates from the complex and SREBP/Scap is transported to the Golgi apparatus where the precursors are cleaved to release the mature forms. The mature forms then enter into the nucleus and transactivate target genes. SREBP-1 and -2 regulate both cholesterol and fatty acid biosynthesis (9). Sterols are well-known regulators of SREBPs, whereas recent studies have shown that SREBPs are also regulated by phosphatidylinositol 3-kinase (PI3K)-Akt signaling (10), which is often hyperactivated in carcinomas. SREBP mature forms are increased in human cancers (11, 12). Roles of cancer-associated molecules in the regulation of SREBPs, however, are largely unknown.

Changes in carbohydrate moieties of certain proteins and glycosphingolipids (GSL) have frequently been observed in carcinomas. GSLs modulate cell signaling through growth factor receptors and/or integrins (13). In melanomas, one of the most aggressive cancers, disialylganglioside GD3 is

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widely expressed as a melanoma antigen (14, 15). GD3 expression has also been reported in other cancers (16–18). We and others showed that GD3 enhances proliferation and invasive activity of melanoma and other cells (19–22) although the molecular mechanism remains poorly understood. GD3 mediates such functions in membrane microdomains known as lipid rafts where cholesterol and sphingolipids are enriched (23). Lipid rafts are essential for various cellular functions (24). How the microdomains are properly maintained on cellular demands is not well understood.

In this study, we examined a role of the melanoma antigen GD3 in the lipogenic phenotype in human melanoma cells. Signaling pathway regulating the lipogenic pathway and a role of the lipogenic phenotype in their malignant properties were also investigated. Based on the current results, we propose a positive feedback regulatory loop in which the activation of the SREBP pathway by PI3K-Akt-mammalian target of rapamycin complex 1 (mTORC1) signaling is crucial for the reinforcement of Akt signaling.

Materials and Methods

Cell culture

Human melanoma cell lines (SK-MEL-25, -26, -28, -31, -37, -130, -131, and -173, and MeWo) were obtained from Dr. L. J. Old (Memorial Sloan-Kettering Cancer Center, 1988). SK-MEL-28-N1 cells (referred to as N1, a gift from Drs. K. O. Lyoyd and J. Nakano, 2002) were isolated from SK-MEL-28 as a GD3-deficient clone (25). N1 cell clones expressing GD3 synthase (G5 and G11) and a mock transfectant (V9) were previously established (19). Melanoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% FBS (medium A). Where indicated, cells were incubated in serum-free medium (medium F). Cells were frozen down at an early passage, and fresh vials were thawed every 2 months and/or 20 passages. Cells were routinely verified by morphology and growth rate. Melanoma cells were also authenticated by the expression of GD3 by FACS analysis (Supplementary Fig. S1).

Immunoblotting and antibodies

Whole cell lysate (WCL) was prepared by lysing cells with urea buffer (8 mol/L urea, 10 mmol/L Tris-HCl pH 8.0, 50 mmol/L sodium phosphate, 150 mmol/L NaCl, and protease inhibitor cocktail). Protein concentration was determined by BCA Protein Assay (Pierce). Aliquots of WCL were subjected to SDS-PAGE and immunoblot analysis. SREBP processing was examined by using anti-SREBP-1 (2A4; gift from Dr. T.-Y. Chang) or anti-SREBP-2 (1D2; MBL) antibodies. Other antibodies used are described in Supplemental Materials and Methods.

Cell fractionation and lipid raft preparation

Cells grown in 100-mm dishes were incubated in medium F for 18 hours and treated as described in figure legends. Postnuclear supernatant (PNS) and plasma membrane (PM)-rich and cytosolic fractions were prepared as described (26). To prepare lipid rafts, cells were lysed with 1% Lubrol WX (Serva) in TNE buffer (25 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA) containing protease inhibitor cocktail (Sigma) for 30 minutes at 4°C, and homogenized with a stainless homogenizer for 10 strokes. PNS was adjusted to 37.5% Opti-Prep (2 mL; Axis-Shield), and placed in the bottom of a centrifuge tube (Beckman). Thirty percent of Opti-Prep (1.25 mL) and TNE buffer (0.75 mL) was sequentially layered. The tubes were spun at 200,000 *g* for 3 hours at 4°C using a Beckman MLS50 rotor. Eight 0.5-mL fractions were collected from the top. Lipids were extracted from equal amounts of each fraction by chloroform/methanol (2:1, v/v).

Lipid analyses

Lipid synthesis was measured by incorporation of [^{3}H]acetate (150 mCi/mmol, American Radiolabeled Chemicals) into cholesterol and phosphatidylcholine (PC) as described (26). Amounts of cholesterol and choline-phospholipids were determined by colorimetric enzymatic assay systems (Kyowa Medex) as described (27).

Immunofluorescence staining

Cells grown on poly-L-lysine–coated glass coverslips were treated as described in the figure legends. Cells were fixed with 2% paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes. After specimens were blocked with 4% FBS for 1 hour, they were incubated with anti-phospho-Akt [p-Akt (Ser173)] and anti-GD3 antibodies (15). Specimens were then stained with Alexa 488-conjugated anti-rabbit IgG and with Alexa 555-conjugated anti-mouse IgG antibodies (Invitorgen), respectively. They were mounted with ProLong Gold Antifade Reagent (Invitrogen). Cell images were acquired by using a confocal microscopy FV500 (Olympus).

siRNA and transfection

siGenome SMART Pools targeting human Raptor, human Rictor, or nontargeting sequences were from Dharmacon. Transfection of siRNAs was conducted by using Lipofectamine 2000 (Invitrogen).

Cell proliferation

Cell proliferation was determined by MTT assay (23). On day 0, cells were seeded in 96-well plates (3,000 cells/well) in medium A. On day 1, medium was switched to DMEM containing 1% FBS and the indicated compounds. Medium was changed every 2 days.

Xenograft experiments

Human melanoma cells suspended in medium F (150 μL) were injected s.c. into the right flank of 6-week-old female BALB/c nu/nu mice (Japan SLC) with the indicated compound. 25-Hydroxycholesterol (25HC), compactin, or vehicle (suspended in 100 μL of PBS) was injected into the tumor every 2 days. The tumor volume was calculated as length × width^2 × 0.5.

Statistical analyses

Data are presented as means ± SD. Statistical analyses were conducted using the 2-tailed Student *t* test. The difference

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between 2 sets of values was considered significant when the P value was < 0.05.

Results

Effects of GD3 expression and BRAF mutation on SREBP pathway in human melanoma cells

Expression of SREBPs and key lipogenic enzymes in various human melanoma cells was examined. Mature forms of SREBP-1 and SREBP-2 were detected in most of the melanoma cells tested (Fig. 1). HMGR and FAS, 2 major SREBP-regulated gene products, were also expressed. In contrast, expression of SREBP-1 and SREBP-2 mature forms, HMGR and FAS in GD3-deficient N1 cells were much lower compared with parental SK-MEL-28 cells and other melanoma cell lines expressing GD3 (Supplementary Fig. S1). We thus asked whether GD3 expression induces the lipogenic pathway. We used N1 cell clones with or without GD3 expression (Supplementary Fig. S1). GD3-positive G5 and G11 cells showed increases in both SREBP-1 and SREBP-2 mature forms compared with parental N1 and control V9 cells (Fig. 2A). Both G5 and G11 cells also expressed higher levels of HMGR. Accordingly, cholesterol biosynthesis was substantially increased in G5 and G11 cells (Fig. 2B). However, FAS expression (Fig. 2A) and PC biosynthesis (Fig. 2C) were not affected by GD3 expression.

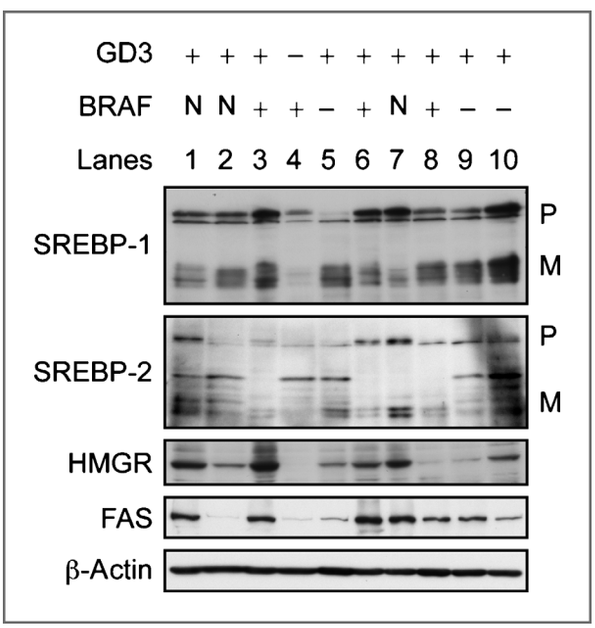

Figure 1. Expression of SREBP and lipogenic enzymes in human melanoma cells. On day 0, human melanoma cells were seeded in 6-well plates and grown in medium A. On day 2, cells were switched to medium F and incubated for 18 hours. Equal amounts of WCL proteins were subjected to immunoblotting with the indicated antibodies. β-Actin was used as a loading control. GD3 expression and BRAF mutation were indicated by + or −. Lanes 1: SK-MEL-25, 2: SK-MEL-26, 3: SK-MEL-28, 4: SK-MEL-28-N1, 5: SK-MEL-31, 6: SK-MEL-37, 7: SK-MEL-130, 8: SK-MEL-131, 9: SK-MEL-173, 10: MeWo. P, precursor form; M, mature form; N, not determined.

Human melanoma cells often express oncogenic BRAF V600E (28), which constitutively activates ERK signaling. Levels of SREBP mature forms (Fig. 1) were not correlated to BRAF genotypes previously identified (28, 29). It thus seems unlikely that the oncogenic BRAF induces the lipogenic pathway.

SREBP pathway is regulated by a PI3K-Akt-mTORC1 signaling

We previously showed that GD3 enhances cellular signaling including Akt (19, 20). We thus asked whether GD3-induced signaling is involved in SREBP regulation in human melanoma cells. As reported (10), the specific PI3K inhibitor LY294002 reduced mature forms of SREBP-1 and SREBP-2 in SK-MEL-28 (Fig. 3A) and G11 cells (data not shown). Wortmannin, another PI3K inhibitor, also produced similar results (data not shown). The mTORC1 kinase is regulated downstream of Akt. Whether mTORC1 is involved in the regulation of SREBP activity has been open to debate (10). Furthermore, its involvement in SREBP-2 processing has remained unknown. The mTORC1-specific inhibitor rapamycin reduced mature forms of both SREBPs with higher sensitivity toward SREBP-1 (Fig. 3A). These results suggest that mTORC1 is involved in the regulation of SREBPs in melanoma cells. However, prolonged treatment with or treatment at high concentration of rapamycin is known to inhibit mTORC2, which phosphorylates Akt at serine 473 (30). To validate the involvement of mTOR complexes in SREBP processing, the mTORC1 component Raptor, the mTORC2 component Rictor, or both of them were silenced by specific siRNAs, and SREBP cleavage was

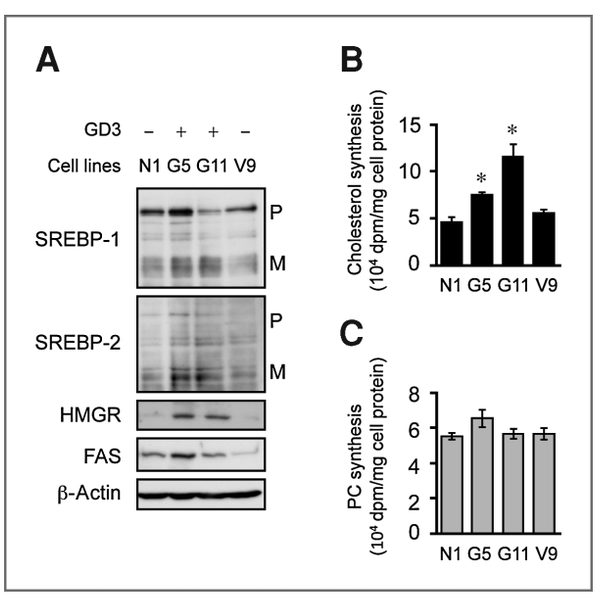

Figure 2. GD3 expression induces SREBP pathway. A, on day 0, GD3-deficient N1 cells, and N1 cells expressing GD3 synthase (G5 and G11) or vector (V9) were set up as shown in Fig. 1. On day 3, WCL was prepared for immunoblotting with the indicated antibodies. B and C, biosynthesis of cholesterol (B) and PC (C) was examined. Cells were set up as above. On day 3, the cells were incubated in the presence of [^{3}H]acetate (20 μCi/mL) for 2 hours in medium F. Cellular lipids were analyzed by TLC. *, $P < 0.002$ (vs. N1 and V9).

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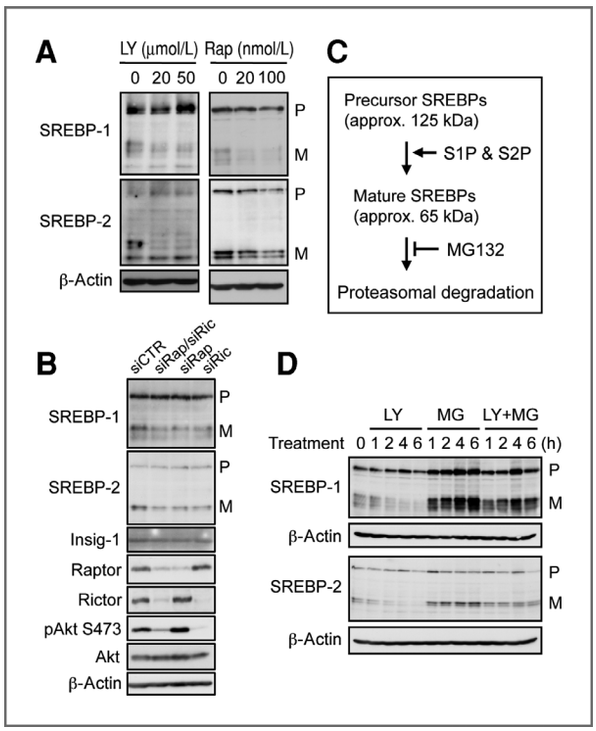

Figure 3. PI3K, mTORC1, and mTORC2 are required for SREBP processing. A, SK-MEL-28 cells were set up as shown in Fig. 1. After incubation for 12 hours in medium F, cells were treated with LY294002 or rapamycin at the indicated concentrations for 8 hours. Equal amounts of proteins were subjected to immunoblotting with the indicated antibodies. B, on day 0, SK-MEL-28 cells (1.2×10^5 cells/well) were seeded into 6-well plates. On days 1 and 3, 75 nmol/L siRNAs targeting Raptor (siRap), Rictor (siRic) or both (siRap/siRic), or control siRNAs (siCTR) were transfected. On day 4, cells were switched to medium F and incubated for 21 hours. The cells were then treated with MG132 (20 μmol/L) in medium F for 3 hours before harvesting. Cell lysates were subjected to immunoblotting with the indicated antibodies. C, schematic diagram of SREBP processing. MG132 protects mature forms from proteasomal degradation. S1P, site 1 protease; S2P, site 2 protease; D, SK-MEL-28 cells were set up as A. The cells were treated with or without LY294002 (50 μmol/L) and/or MG132 (20 μmol/L) for up to 6 hours in medium F. SREBP processing was examined as above.

examined. Silencing Raptor and/or Rictor markedly reduced both SREBP-1 and SREBP-2 mature forms (Fig. 3B), indicating that both mTORC1 and mTORC2 play a role in the SREBP processing. Insig-1 expression was affected neither by Raptor nor Rictor knockdown.

Amounts of SREBP mature forms are regulated by 2 independent mechanisms (7); the 2-step proteolytic cleavage and proteasomal degradation (Fig. 3C). To determine which process is regulated by Akt signaling in human melanoma cells, SK-MEL-28 cells were treated with or without LY294002 and/or MG132, a proteasomal inhibitor. LY294002 reduced mature forms of SREBP-1 and SREBP-2 within 2 hours, whereas MG132 rapidly increased the mature forms (Fig. 3D). When the cells were treated with both compounds, increases in mature forms of both SREBPs were suppressed, suggesting that PI3K-Akt signaling is involved in the processing step in melanoma cells.

To further explore the signaling cascade that regulates SREBP processing, SK-MEL-28 cells were stimulated with insulin-like growth factor-1 (IGF-1), which activates Akt signaling and is an important growth factor for malignant properties of melanoma cells (31). IGF-1 increased mature forms of SREBPs and HMGR expression within 2 hours (Fig. 4A). To determine whether inhibition of PI3K-Akt-mTORC1 signaling also prevents the IGF-1–induced SREBP processing, SK-MEL-28 cells were stimulated with IGF-1 in the presence or absence of LY294002 or rapamycin only for 3 hours. The increase in mature forms of SREBPs by IGF-1 was suppressed by LY294002 and rapamycin (Fig. 4B). Accordingly, HMGR expression did not increase by IGF-1 when PI3K or mTORC1 was inhibited. FAS expression was not induced by IGF-1. Moreover, Insig-1 expression was not influenced by IGF-1 and by these inhibitors, suggesting that the regulation of SREBP processing by the PI3K-Akt-mTORC1 pathway is independent of Insig-1. The increase in p-Akt by IGF-1 in rapamycin-treated cells could be because rapamycin inhibits the negative feedback regulation of PI3K-Akt signaling by S6 kinase (30).

To address whether the above changes influence lipid biosynthesis, SK-MEL-28 cells were incubated with IGF-1 and/or LY294002 in the presence of [^{3}H]acetate. IGF-1 increased cholesterol biosynthesis (Fig. 4C). On the other hand, PC biosynthesis was unchanged by IGF-1. LY294002 reduced both cholesterol and PC synthesis in the presence and absence of IGF-1, but cholesterol biosynthesis was more sensitive to the compound.

Effect of SREBP inactivation on lipid raft–associated signaling

Cholesterol is essential for the formation of lipid rafts. We explored whether PI3K-Akt-mTORC1–SREBP axis regulates the integrity of lipid rafts. The results showed that disruption of PI3K-Akt-mTORC1 signaling significantly reduces cholesterol contents in lipid rafts (Supplementary Fig. S2), which impairs the balance of cholesterol distribution between lipid rafts and nonlipid rafts (Fig. 4D). Changes in phospholipid distribution by these inhibitors were only modest.

Lipid rafts play an important role in Akt signaling (32–36). We next examined a role of SREBPs in Akt activation. 25HC inhibits SREBP processing by sequestering the SREBP/Scap complex in the ER. It reduced both SREBP mature forms in SK-MEL-28 cells (Supplementary Fig. S3). SK-MEL-28 cells pretreated with or without 25HC were stimulated with IGF-1, and Akt phosphorylation was assessed. In the cells treated with 25HC, phosphorylation of Akt by IGF-1 was suppressed (Fig. 5A). Inhibition of cholesterol biosynthesis also resulted in decreases in Akt phophorylation (Supplementary Fig. S4). SK-MEL-28 cells with or without the 25HC pretreatment were subjected to subcellular fractionation, and distribution of p-Akt was examined. The PM protein Na^+/K^+ ATPase was enriched in the PM-rich fraction, but undetectable in the cytosol fraction. In contrast, the cytosolic protein FAS was detected only in the cytosol fraction (Fig. 5A). Akt phosphorylation by IGF-1 was strongly suppressed in the PM-rich fraction prepared from the 25HC-treated cells. The 25HC treatment also slightly reduced

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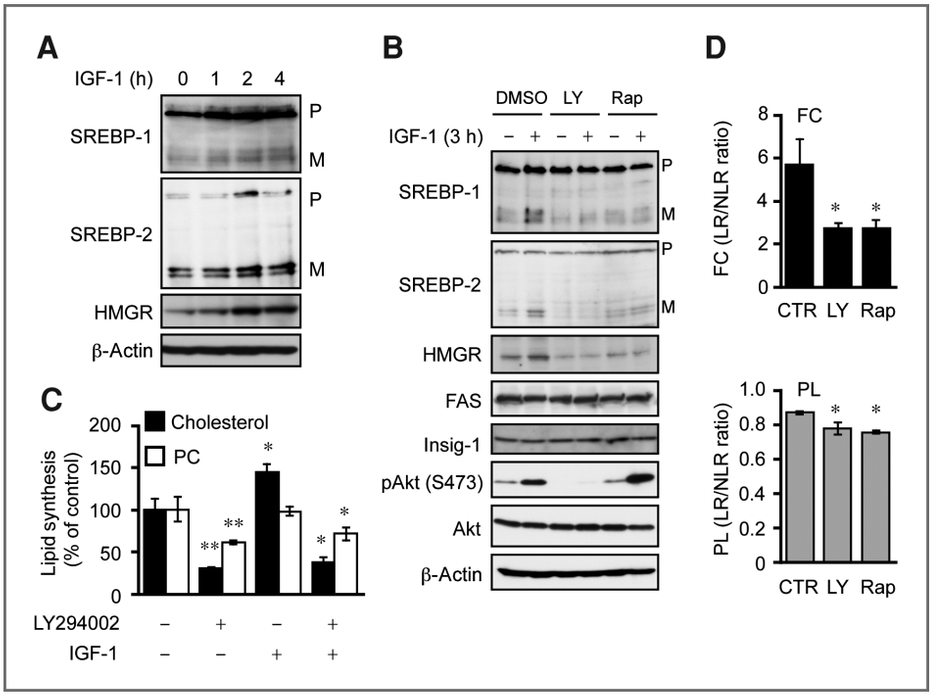

Figure 4. PI3K-Akt-mTORC1 pathway regulates IGF-1–induced SREBP processing. A, SK-MEL-28 cells were set up as shown in Fig. 1. Cells were treated with IGF-1 (100 ng/mL) for up to 4 hours. Aliquots of WCL were subjected to immunoblotting with the indicated antibodies. B, SK-MEL-28 cells were set up as above. After incubation in medium F for 18 hours, cells were pretreated with 50 μmol/L LY294002 (LY), 100 nmol/L rapamycin (Rap), or vehicle (0.1% DMSO) for 30 minutes. The cells were then stimulated with IGF-1 (100 ng/mL) for 3 hours in the presence of the inhibitors. WCL was subjected to immunoblot analysis. C, SK-MEL-28 cells were set up as above. After incubation in medium F for 12 hours, cells were treated with or without 50 μmol/L LY294002 for 4 hours in the presence or absence of IGF-1. The cells were then incubated with [^{3}H]acetate (20 μCi/mL) in the same condition for 2 hours. Radioactivities in cellular cholesterol and PC were determined. *, $P < 0.05$; **, $P < 0.01$. D, effects of inhibiting PI3K-Akt-mTORC1 signaling on the integrity of lipid rafts. On day 0, SK-MEL-28 cells were seeded into 100-mm dishes and grown in medium A. On day 2, the cells were switched to medium F containing 0.1% DMSO as a control (CTR), 25 μmol/L LY294002 (LY), or 50 nmol/L rapamycin (Rap). After incubation for 18 hours, lipid rafts were prepared. Free cholesterol (FC) and phospholipid (PL) contents in each fraction were determined. Ratios of lipid rafts (LR, fraction 2) to nonlipid rafts (NLR, fractions 4–8) for FC and PL are shown. * $P < 0.05$.

cytosolic p-Akt. To further examine subcellular localization of p-Akt, we prepared lipid rafts. The majority of the lipid raft marker flotillin-1 was recovered in fraction 2 (Fig. 5B). When the cells were stimulated with IGF-1, significant amounts of p-Akt were found in lipid raft fractions. In contrast, the level of p-Akt in lipid rafts was much lower in the IGF-1–stimulated cells pretreated with 25HC. To confirm these results, SK-MEL-28 cells pretreated with 25HC or vehicle were stimulated by IGF-1, and cellular localization of p-Akt and GD3 was examined under confocal microscopy. IGF-1 treatment increased p-Akt signals at cell peripheries, which was suppressed by pretreatment of the cells with LY294002 (Fig. 5C). p-Akt well colocalized with GD3, indicating that Akt is activated at or near GD3-enriched membrane microdomains. In contrast, p-Akt signals were not significantly increased upon IGF-1 stimulation in the cells pretreated with 25HC. 25HC seemed not to affect GD3 expression and distribution.

Roles of SREBP pathway in melanoma cell proliferation and tumorigenesis

The effect of SREBP or cholesterol biosynthesis inhibitors on melanoma cell proliferation was assessed. 25HC, NB598 (a squalene monooxygenase inhibitor), and compactin (an HMGR inhibitor) all inhibited melanoma cell proliferation in the presence of FBS (Fig. 6A). In addition, all these 3 compounds potentiated the inhibitory effect of LY294002 and rapamycin on their proliferation (Fig. 6B). These results indicate that melanoma cells require high levels of SREBP-dependent cholesterol biosynthesis to maintain their rapid proliferation (Fig. 6C).

Finally, we examined whether the SREBP pathway plays a role in tumor growth *in vivo*. We injected SK-MEL-28 cells into nude mice and treated the tumors with compactin or 25HC. The treatment with compactin or 25HC significantly suppressed the growth of melanoma cells in a xenograft model (Fig. 7A and B). These results clearly show a crucial role of SREBP pathway in tumorigenesis *in vivo*.

Discussion

Almost all human melanomas express GD3 (14, 15). We reported that GD3 expression in the GD3-negative mutants reinforces malignant properties of human melanoma cells (19, 20). In this work, we investigated whether GD3 is involved in the lipogenic phenotype, a metabolic change often observed in cancer cells, and how the lipogenic pathway is regulated in

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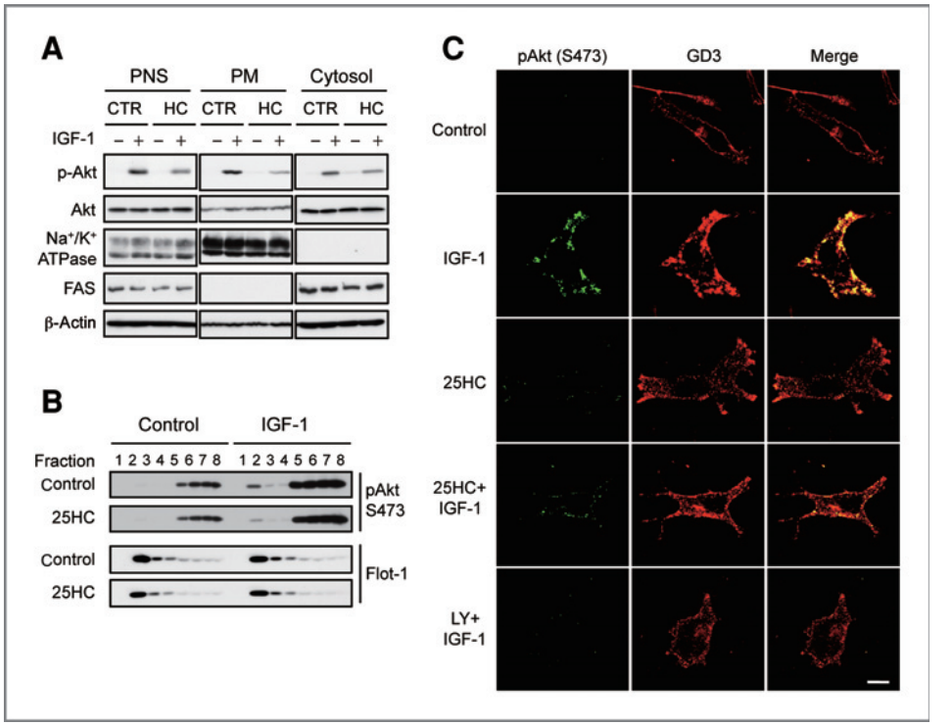

Figure 5. SREBP inhibition suppresses Akt activation in lipid rafts. A, on day 0, SK-MEL-28 cells were set up as shown in Fig. 4D. On day 2, cells were switched to medium F containing 0.1% EtOH (CTR) or 5 μmol/L 25HC (H). After 18 hours of incubation, cells were stimulated or not with IGF-1 (100 ng/mL) for 15 minutes, and PNS, PM-rich fraction (PM) and cytosol were prepared. Equal amounts of proteins were subjected to immunoblot analysis with the indicated antibodies. B, SK-MEL-28 cells were set up and treated as above. After lipid raft preparation, equal amounts of each fraction were subjected to immunoblot analysis with the indicated antibodies. C, on day 0, SK-MEL-28 cells were seeded on glass coverslips and grown in medium A. On day 2, cells were switched to medium F with or without 5 μmol/L 25HC. After incubation for 18 hours, cells were incubated in the presence or absence of 50 μmol/L LY294002 (LY) for 1 hour and then stimulated with or without IGF-1 for 15 minutes. Cells were stained for p-Akt and GD3. Bar, 10 μm.

human melanoma cells. The current study was also designed to address how the lipogenic phenotype augments malignant properties of melanomas.

We found that the SREBP pathway was activated in most of human melanoma cells. The activation did not correlate with BRAF status, suggesting that ERK signaling is not involved in the induction of the lipogenic pathway. On the other hand, the forced expression of GD3 induced the processing of SREBPs, HMGR expression, and cholesterol biosynthesis. We could not determine why FAS expression and PC synthesis were not altered by GD3 expression despite SREBP activation. The results, however, show that the melanoma antigen GD3 promotes SREBP processing and cholesterol biosynthesis. A recent study also showed a role of a tumor antigen in lipogenesis. The oncoprotein mucin 1 (MUC1), a glycoprotein overexpressed in 90% or more of breast cancers, induces expression of lipogenic enzymes regulated by SREBPs (37). Therefore, certain tumor antigens including GD3 and MUC1 mediate the induction of the lipogenic phenotype.

SREBP processing is tightly regulated by sterols. Cellular sterol levels are sensed and regulated by various mechanisms (38). A small change in the ER membrane cholesterol levels regulates the ER-to-Golgi transport of SREBP-2 (39). On the other hand, an involvement of PI3K-Akt signaling in the SREBP pathway has recently been highlighted (10). The constitutively active, myristoylated Akt (myr-Akt) induced the expression of various SREBP-target genes including HMGR and FAS (40). In gliobastomas, expression of a constitutive active form of epidermal growth factor receptor (EGFR), EGFRviii, causes hyperactivation of Akt and leads to SREBP-1 activation in an Akt-dependent manner (11). In human melanoma, GD3 enhances Akt activation (19, 20). We showed that GD3 expression promotes SREBP-1 and SREBP-2 processing through PI3K-Akt signaling. An important role of Akt was further confirmed by the finding that mTORC2 inhibition by silencing Rictor reduces SREBP processing. Our results also indicate that PI3K-Akt signaling regulates a step involved in the generation of the mature forms, which is consistent with a previous report (41). Our current results together with previous studies thus elucidate that PI3K-mTORC2-Akt signaling regulates the processing of both SREBPs.

Whether mTORC1 is involved in the regulation of SREBP processing is an important issue to be clarified. In patients with gliobastoma, levels of SREBP-1 mature form in the tumor tissues were not affected by rapamycin treatment (11). Rapamycin also failed to prevent SREBP-1 processing in glioblastoma cells expressing EGFRviii (11). These results suggest that mTORC1 is not involved in SREBP-1 processing in glioblastomas. In contrast, it was shown that mTORC1 plays a role in SREBP-1 processing in cells expressing myr-Akt (42) and hepa-

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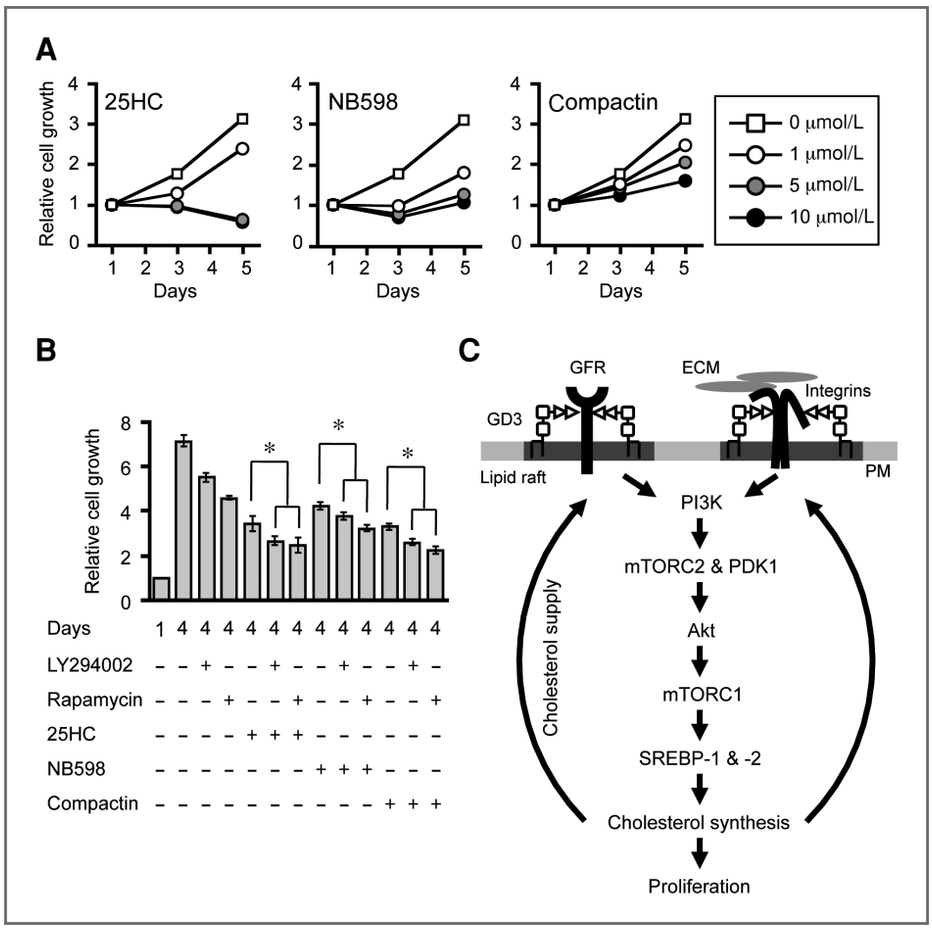

Figure 6. Melanoma cell proliferation is inhibited by 25HC and cholesterol synthesis inhibitors. A, on day 0, SK-MEL-28 cells were seeded in 96-well plates. On day 1, cells were switched to medium containing 25HC, NB598, or compactin at the indicated concentration. MTT assay was conducted on days 1, 3, and 5. The results for vehicle-treated control were obtained from the same set of experiments. B, SK-MEL-28 cells were set up as above. On day 1, the cells were switched to medium containing LY294002 (5 μmol/L), rapamycin (20 nmol/L), 25HC (2 μmol/L), NB598 (1 μmol/L), compactin (5 μmol/L), or the indicated combinations. MTT assay was conducted on days 1 and 4. *, $P < 0.001$. C, proposed model of a positive feedback loop between PI3K-Akt-mTORC1 signaling and SREBP pathway. See text for detail. ECM, extracellular matrix; GFR, growth factor receptor; PM, plasma membrane.

tocytes (43). Düvel and colleagues (44) also showed an involvement of mTORC1 in SREBP regulation by genetic and bioinformatic approaches. The current results strengthen an involvement of mTORC1 in the regulation of SREBP-1 processing. Furthermore, we showed that mTORC1 also plays a role in SREBP-2 processing.

How the PI3K-Akt-mTORC1 signaling regulates SREBP processing remains unknown. Involvement of the mTORC1 substrate ribosomal protein S6 kinase 1 in the SREBP processing is controversial at present (43, 44). Insig-1 is an important regulator for SREBP processing by acting as an ER retention factor of SREBP/Scap complex (8). Insig-1 is a short-lived protein with a half-life of about 20 minutes (45). Sterol-dependent stabilization of Insig-1 creates a convergent control of SREBP-2 processing for cholesterol homeostasis (45). However, we found that Insig-1 expression is not affected by PI3K-mTORC2-Akt-mTORC1 signaling. These results suggest that Insig-1 degradation is not involved in the regulation of SREBP processing by this signaling. PI3K-Akt signaling regulates the ER-to-Golgi transport of SREBP. A recent study showed that Akt phosphorylates SREBP-1 upon insulin stimulation, which in turn promotes its association to coatomer protein (COP) II vesicles and its transport to the Golgi (46). The association of SREBP-2 with COPII vesicles is not affected by insulin (46). Results from our current work and work by others (44), however, suggest a common mechanism through which PI3K-Akt-mTORC1 signaling regulates both SREBPs. Whether PI3K-Akt-mTORC1 signaling regulates ER cholesterol level is currently unknown.

In this study, we also investigated a role of PI3K-Akt-mTORC1-SREBP signaling in the integrity of lipid rafts because cholesterol biosynthesis was more promptly regulated by this signaling. IGF-1 rapidly promoted SREBP-1 and SREBP-2 processing, HMGR expression and cholesterol biosynthesis through PI3K-Akt-mTORC1 signaling. On the other hand, FAS expression and PC synthesis did not change by IGF-1. The different responses between the expression of HMGR and FAS or between cholesterol and PC synthesis could be explained as follows. HMGR is a short-lived protein with a half-life of about 60 minutes (47), whereas the half-life of FAS is about 12 hours (48). A positive feedback regulation between Akt signaling and FAS expression in ovarian cancer cells was reported (49). However, consistent with its longer half-life, a decrease in FAS expression (longer than 24 hours) by Akt inhibition (49) is much slower than that in HMGR expression (3 hours). Therefore cholesterol biosynthesis can be regulated in a more timely manner by Akt signaling. This tight regulation of cholesterol biosynthesis could promptly control lipid rafts on physiologic demands. In agreement with this hypothesis, we showed that the inhibition of PI3K-Akt-mTORC1 signaling disrupts lipid rafts. Activation of Akt requires proper lipid

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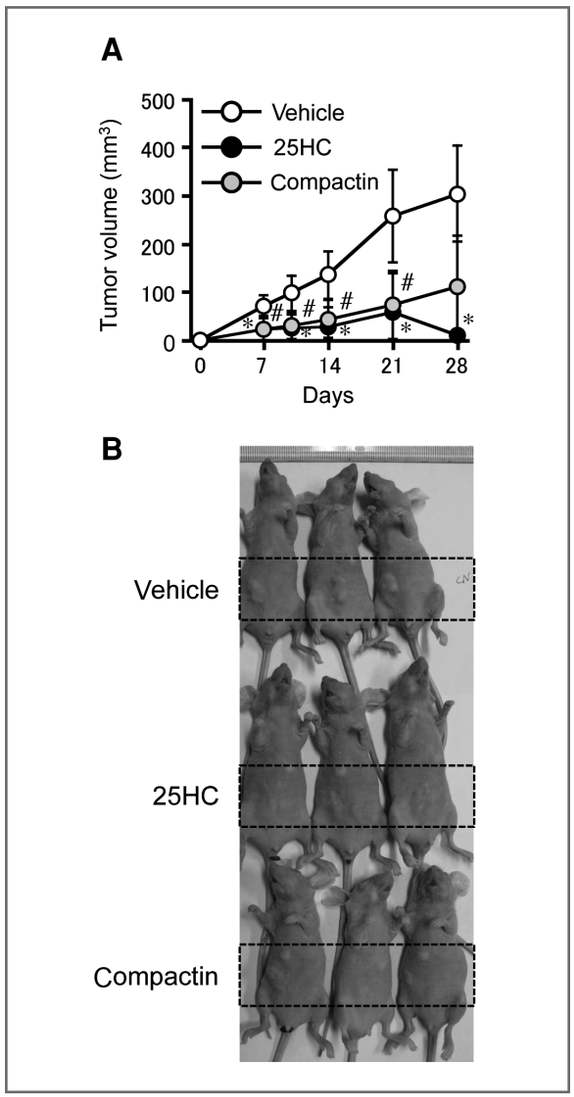

Figure 7. Tumor growth is SREBP pathway-dependent in a xenograft model. A, SK-MEL-28 cells (5×10^6 cells/animal) were injected into nude mice ($n = 4$–6). Mice were treated with compactin (20 μg/tumor), 25HC (20 μg/tumor), or vehicle (2% DMSO) every 2 days. Tumor volume was monitored periodically up to 4 weeks. The results were derived from 2 separate experiments except the compactin treatment. *, $P < 0.01$ vehicle vs. 25HC; #, $P < 0.05$ vehicle vs. compactin. B, typical images of tumors after the 4-week treatments.

raft domains (32–36). We also found that the inhibition of SREBP processing or cholesterol biosynthesis suppresses Akt activation in lipid rafts. We thus conclude that the PI3K-Akt-mTORC1-SREBP signaling plays an important role in the maintenance of integrity of lipid rafts (Fig. 6D). Although the change in lipid raft cholesterol we observed was not as large as that caused by cyclodextrin-mediated cholesterol depletion, a widely used method to disrupt the microdomains, this small change could affect their physiologic functions. In addition to cholesterol, other SREBP-regulated products such as isoprenoids and certain fatty acids could play a role in the structure and functions of lipid rafts.

Metabolic reprogramming occurs in many carcinomas. The mTORC1 signaling regulates not only lipogenesis but also glucose metabolism (44). mTORC1 is activated in malignant human melanomas (50). Given that GD3 enhances Akt signaling (19, 20) and promotes SREBP pathway, GD3 could be an important factor to induce metabolic reprogramming through PI3K-Akt-mTORC1 signaling independently of oncogenic BRAF signaling in human melanomas.

In conclusion, the current work shows that a positive feedback regulatory loop exists between PI3K-Akt-mTORC1 signaling and SREBP pathway, which boosts Akt signaling in human melanomas expressing GD3.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Positive Feedback Loop Between PI3K-Akt-mTORC1 Signaling and the Lipogenic Pathway Boosts Akt Signaling: Induction of the Lipogenic Pathway by a Melanoma Antigen

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