Positive feedback loop between PI3K-Akt-mTORC1 signaling and the lipogenic pathway boosts Akt signaling: Induction of the lipogenic pathway by a melanoma antigen

Yoshio Yamauchi[1], Keiko Furukawa[2], Kazunori Hamamura[1], and Koichi Furukawa[1]

[1]Department of Biochemistry II, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai, Aichi, Japan

Running title: Positive feedback regulatory loop for Akt signaling

Key words: SREBP, Akt, mTOR, melanoma, ganglioside.

Corresponding author: Koichi Furukawa, Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan; tel: 81-52-744-2070; fax: 81-52-744-2069; e-mail: koichi@med.nagoya-u.ac.jp

1

Abstract

The lipogenic phenotype is a metabolic hallmark of cancer cells. Sterol regulatory element-binding proteins (SREBPs) are key transcriptional factors to regulate biosynthesis of cholesterol and fatty acids. It has been poorly understood how the lipogenic phenotype in cancer cells are regulated and how it augments their malignant properties. Here we describe roles of the melanoma antigen ganglioside GD3 and phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin 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 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 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.

2

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 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 (SREBPs) -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 biosyntheses (9). Sterols are well-known regulators of SREBPs, whereas recent studies have shown that SREBP is 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 SREBP, however, are largely unknown.

3

Changes in carbohydrate moieties of certain proteins and glycosphingolipids (GSLs) 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 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 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, Year 1988). SK-MEL-28-N1 cells (referred as N1, a gift from Drs. K. O. Lyoyd and J. Nakano, Year 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)

4

were previously established (19). Melanoma cells were maintained in DMEM containing

7.5% fetal bovine serum (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 (Fig S1).

Immunoblotting and antibodies. Whole cell lysate (WCL) was prepared by

lysing cells with urea buffer (8M urea, 10 mM Tris-HCl pH 8.0, 50 mM sodium phosphate,

150 mM 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 h and treated as described in Figure Legends. Post-nuclear

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 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) containing protease inhibitor

cocktail (Sigma) for 30 min at 4ºC, 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 % Opti-Prep (1.25 ml) and TNE buffer (0.75 ml) were

sequentially layered. The tubes were spun at 200,000 g for 3 h at 4 ºC using a Beckman

MLS50 rotor. Eight 0.5 ml-fractions were collected from the top. Lipids were extracted from

5

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 Legend. Cells were fixed with 2% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. After specimens were blocked with 4% FBS for 1 h, they were incubated with anti-p-Akt (Ser473) 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 non-targeting sequences were from Dharmacon. Transfection of siRNAs was performed 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 subcutaneously into the right flank of 6-week-old female BALB/c nu/nu mice (Japan SLC) with the indicated compound. 25HC, compactin, or vehicle (suspended in

6

100 µl of PBS) was injected into the tumor every 2 days. The tumor volume was calculated as length $\times$ width^2 $\times$ 0.5.

Statistical Analyses. Data are presented as means ± S.D.. Statistical analyses were performed using the two-tailed Student's *t* test. The difference between two 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 melanoma cells tested (Fig. 1). HMGR and FAS, two 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 to parental SK-MEL-28 cells and other melanoma cell lines expressing GD3 (Fig. S1). We thus asked whether GD3 expression induces lipogenic pathway. We employed N1 cell clones with or without GD3 expression (Fig. S1). GD3-positive G5 and G11 cells showed increases in both SREBP-1 and SREBP-2 mature forms compared to 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.

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

7

oncogenic BRAF induces the lipogenic pathway.

SREBP pathway is regulated by a PI3K-Akt-mTORC1 signaling. We previously showed that GD3 enhances cellular signalings including Akt (19, 20). We thus asked whether GD3-induced signalings are 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 are 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 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 two independent mechanisms (7); the two-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

8

were treated with or without LY294002 and/or MG132, a proteasomal inhibitor. LY294002 reduced mature forms of SREBP-1 and SREBP-2 within 2 h, 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 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 h (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 h. 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 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 syntheses in the presence

9

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 (Fig. S2), which impairs the balance of cholesterol distribution between lipid rafts and non-lipid 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. 25-hydroxycholesterol (25HC) inhibits SREBP processing by sequestering the SREBP/Scap complex in the ER. It reduced both SREBP mature forms in SK-MEL-28 cells (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 (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 cytosolic p-Akt. To further examine subcellular localization of p-Akt, we prepared lipid rafts. 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

10

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 a 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 (a HMGR inhibitor) all inhibited melanoma cell proliferation in the presence of FBS (Fig. 6A). In addition, all these three 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 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 demonstrate a crucial role of SREBP pathway in tumorigenesis *in vivo*.

Discussion

11

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 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, demonstrated 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% 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

12

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 indicated 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 elucidated 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

suggested 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 hepatocytes (43). Düvel *et al*. (44) also demonstrated an involvement of mTORC1 in

SREBP regulation by genetic and bioinformatic approaches. The current results strengthened

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

13

a short-lived protein with a half-life of about 20 min (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 others (44), however suggest a common mechanism

that 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 since 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 half-life of about 60 min (47),

whereas half-life of FAS is about 12 h (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 h) by Akt inhibition

(49) is much slower than that in HMGR expression (3 h). Therefore cholesterol biosynthesis

can be regulated more timely by Akt signaling. This tight regulation of cholesterol

14

biosynthesis could promptly control lipid rafts on physiological 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 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 physiological 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.

15

16

References

1.   Hsu P, Sabatini D. Cancer cell metabolism: Warburg and beyond. Cell. 2008;134:703-7.

2.   Vander Heiden M, Cantley L, Thompson C. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009;324:1029-33.

3.   Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell. 2008;13:472-82.

4.   Menendez J, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer. 2007;7:763-77.

5.   Katz M. Therapy insight: Potential of statins for cancer chemoprevention and therapy. Nat Clin Pract Oncol. 2005;2:82-9.

6.   Demierre M, Higgins P, Gruber S, Hawk E, Lippman S. Statins and cancer prevention. Nat Rev Cancer. 2005;5:930-42.

7.   Horton J, Goldstein J, Brown M. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest. 2002;109:1125-31.

8.   Goldstein J, DeBose-Boyd R, Brown M. Protein sensors for membrane sterols. Cell. 2006;124:35-46.

9.   Horton J, Shah N, Warrington J, Anderson N, Park S, Brown M, et al. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc Natl Acad Sci U S A. 2003;100:12027-32.

10.   Krycer J, Sharpe L, Luu W, Brown A. The Akt-SREBP nexus: cell signaling meets lipid metabolism. Trends Endocrinol Metab. 2010;21:268-76.

11.   Guo D, Prins R, Dang J, Kuga D, Iwanami A, Soto H, et al. EGFR signaling through an Akt-SREBP-1-dependent, rapamycin-resistant pathway sensitizes glioblastomas to antilipogenic therapy. Sci Signal. 2009;2:ra82.

12.   Ettinger S, Sobel R, Whitmore T, Akbari M, Bradley D, Gleave M, et al. Dysregulation of sterol response element-binding proteins and downstream effectors in prostate cancer during progression to androgen independence. Cancer Res. 2004;64:2212-21.

13.   Regina Todeschini A, Hakomori S. Functional role of glycosphingolipids and gangliosides in control of cell adhesion, motility, and growth, through glycosynaptic microdomains. Biochim Biophys Acta. 2008;1780:421-33.

14.   Thampoe I, Furukawa K, Vellvé E, Lloyd K. Sialyltransferase levels and ganglioside expression in melanoma and other cultured human cancer cells. Cancer Res. 1989;49:6258-64.

17

15.   Pukel C, Lloyd K, Travassos L, Dippold W, Oettgen H, Old L. GD3, a prominent ganglioside of human melanoma. Detection and characterisation by mouse monoclonal antibody. J Exp Med. 1982;155:1133-47.

16.   Merritt W, Casper J, Lauer S, Reaman G. Expression of GD3 ganglioside in childhood T-cell lymphoblastic malignancies. Cancer Res. 1987;47:1724-30.

17.   Siddiqui B, Buehler J, DeGregorio M, Macher B. Differential expression of ganglioside GD3 by human leukocytes and leukemia cells. Cancer Res. 1984;44:5262-5.

18.   Okada M, Furukawa K, Yamashiro S, Yamada Y, Haraguchi M, Horibe K, et al. High expression of ganglioside alpha-2,8-sialyltransferase (GD3 synthase) gene in adult T-cell leukemia cells unrelated to the gene expression of human T-lymphotropic virus type I. Cancer Res. 1996;56:2844-8.

19.   Hamamura K, Furukawa K, Hayashi T, Hattori T, Nakano J, Nakashima H, et al. Ganglioside GD3 promotes cell growth and invasion through p130Cas and paxillin in malignant melanoma cells. Proc Natl Acad Sci U S A. 2005;102:11041-6.

20.   Ohkawa Y, Miyazaki S, Hamamura K, Kambe M, Miyata M, Tajima O, et al. Ganglioside GD3 Enhances Adhesion Signals and Augments Malignant Properties of Melanoma Cells by Recruiting Integrins to Glycolipid-enriched Microdomains. J Biol Chem. 2010;285:27213-23.

21.   Fukumoto S, Mutoh T, Hasegawa T, Miyazaki H, Okada M, Goto G, et al. GD3 synthase gene expression in PC12 cells results in the continuous activation of TrkA and ERK1/2 and enhanced proliferation. J Biol Chem. 2000;275:5832-8.

22.   Zeng G, Gao L, Yu R. Reduced cell migration, tumor growth and experimental metastasis of rat F-11 cells whose expression of GD3-synthase is suppressed. Int J Cancer. 2000;88:53-7.

23.   Nakashima H, Hamamura K, Houjou T, Taguchi R, Yamamoto N, Mitsudo K, et al. Overexpression of caveolin-1 in a human melanoma cell line results in dispersion of ganglioside GD3 from lipid rafts and alteration of leading edges, leading to attenuation of malignant properties. Cancer Sci. 2007;98:512-20.

24.   Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997;387:569-72.

25.   Nakano J, Raj B, Asagami C, Lloyd K. Human melanoma cell lines deficient in GD3 ganglioside expression exhibit altered growth and tumorigenic characteristics. J Invest Dermatol. 1996;107:543-8.

26.   Yamauchi Y, Reid P, Sperry J, Furukawa K, Takeya M, Chang C, et al. Plasma membrane rafts complete cholesterol synthesis by participating in retrograde movement of precursor sterols. J Biol Chem. 2007;282:34994-5004.

18

27.   Yamauchi Y, Hayashi M, Abe-Dohmae S, Yokoyama S. Apolipoprotein A-I activates protein kinase C alpha signaling to phosphorylate and stabilize ATP binding cassette transporter A1 for the high density lipoprotein assembly. J Biol Chem. 2003;278:47890-7.

28.   Davies H, Bignell G, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;417:949-54.

29.   Tsao H, Goel V, Wu H, Yang G, Haluska F. Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. J Invest Dermatol. 2004;122:337-41.

30.   Efeyan A, Sabatini DM. mTOR and cancer: many loops in one pathway. Curr Opin Cell Biol. 2010;22:169-76.

31.   Baserga R, Peruzzi F, Reiss K. The IGF-1 receptor in cancer biology. Int J Cancer. 2003;107:873-7.

32.   Gao X, Zhang J. Spatiotemporal analysis of differential Akt regulation in plasma membrane microdomains. Mol Biol Cell. 2008;19:4366-73.

33.   Lasserre R, Guo X, Conchonaud F, Hamon Y, Hawchar O, Bernard A, et al. Raft nanodomains contribute to Akt/PKB plasma membrane recruitment and activation. Nat Chem Biol. 2008;4:538-47.

34.   Fedida-Metula S, Elhyany S, Tsory S, Segal S, Hershfinkel M, Sekler I, et al. Targeting lipid rafts inhibits protein kinase B by disrupting calcium homeostasis and attenuates malignant properties of melanoma cells. Carcinogenesis. 2008;29:1546-54.

35.   Adam R, Mukhopadhyay N, Kim J, Di Vizio D, Cinar B, Boucher K, et al. Cholesterol sensitivity of endogenous and myristoylated Akt. Cancer Res. 2007;67:6238-46.

36.   Zhuang L, Kim J, Adam R, Solomon K, Freeman M. Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. J Clin Invest. 2005;115:959-68.

37.   Pitroda S, Khodarev N, Beckett M, Kufe D, Weichselbaum R. MUC1-induced alterations in a lipid metabolic gene network predict response of human breast cancers to tamoxifen treatment. Proc Natl Acad Sci U S A. 2009;106:5837-41.

38.   Chang T, Chang C, Ohgami N, Yamauchi Y. Cholesterol sensing, trafficking, and esterification. Annu Rev Cell Dev Biol. 2006;22:129-57.

39.   Radhakrishnan A, Goldstein J, McDonald J, Brown M. Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance. Cell Metab. 2008;8:512-21.

40.   Porstmann T, Griffiths B, Chung Y, Delpuech O, Griffiths J, Downward J, et al. PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis

19

via activation of SREBP. Oncogene. 2005;24:6465-81.

41. Du X, Kristiana I, Wong J, Brown A. Involvement of Akt in ER-to-Golgi transport of SCAP/SREBP: a link between a key cell proliferative pathway and membrane synthesis. Mol Biol Cell. 2006;17:2735-45.

42. Porstmann T, Santos C, Griffiths B, Cully M, Wu M, Leevers S, et al. SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. Cell Metab. 2008;8:224-36.

43. Li S, Brown M, Goldstein J. Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. Proc Natl Acad Sci U S A. 2010;107:3441-6.

44. Düvel K, Yecies J, Menon S, Raman P, Lipovsky A, Souza A, et al. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell. 2010;39:171-83.

45. Gong Y, Lee J, Lee P, Goldstein J, Brown M, Ye J. Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake. Cell Metab. 2006;3:15-24.

46. Yellaturu C, Deng X, Cagen L, Wilcox H, Mansbach Cn, Siddiqi S, et al. Insulin enhances post-translational processing of nascent SREBP-1c by promoting its phosphorylation and association with COPII vesicles. J Biol Chem. 2009;284:7518-32.

47. Gil G, Faust J, Chin D, Goldstein J, Brown M. Membrane-bound domain of HMG CoA reductase is required for sterol-enhanced degradation of the enzyme. Cell. 1985;41:249-58.

48. Graner E, Tang D, Rossi S, Baron A, Migita T, Weinstein L, et al. The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. Cancer Cell. 2004;5:253-61.

49. Wang H, Altomare D, Skele K, Poulikakos P, Kuhajda F, Di Cristofano A, et al. Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells. Oncogene. 2005;24:3574-82.

50. Karbowniczek M, Spittle C, Morrison T, Wu H, Henske E. mTOR is activated in the majority of malignant melanomas. J Invest Dermatol. 2008;128:980-7.

Figure legends

Figure 1. Expression of SREBPs 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 h. 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.

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 in Fig. 1. On day 3, WCL was prepared for immunoblotting with the indicated antibodies. B and C, Biosynthesis of cholesterol (B) and phosphatidylcholine (PC) (C) was examined. Cells were set up as above. On day 3, cells were incubated in the presence of [^{3}H]acetate (20 μCi/ml) for 2 h in medium F. Cellular lipids were analyzed by TLC. * $P < 0.002$ (vs. N1 and V9).

Figure 3. PI3K, mTORC1 and mTORC2 are required for SREBP processing. A, SK-MEL-28 cells were set up as in Fig. 1. After incubation for 12 h in medium F, cells were treated with LY294002 or rapamycin at the indicated concentrations for 8 h. 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 nM siRNAs targeting Raptor (siRap), Rictor (siRic) or both (siRap/siRic), or control siRNAs (siCTR)

21

were transfected. On day 4, cells were switched to medium F and incubated for 21 h. Cells

were then treated with MG132 (20 µM) in medium F for 3 h 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; C, SK-MEL-28 cells were set up as panel A. The cells were

treated with or without LY294002 (50 µM) and/or MG132 (20 µM) for up to 6 h in medium F.

SREBP processing was examined as above.

Figure 4. PI3K-Akt-mTORC1 pathway regulates IGF-1-induced SREBP processing. A,

SK-MEL-28 cells were set up as in Fig. 1. Cells were treated with IGF-1 (100 ng/ml) for up

to 4 h. 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 h, cells were

pretreated with 50 µM LY294002 (LY), 100 nM rapamycin (Rap), or vehicle (0.1% DMSO)

for 30 min. Cells were then stimulated with IGF-1 (100 ng/ml) for 3 h 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 h, cells were treated with or without 50 µM

LY294002 for 4 h in the presence or absence of IGF-1. Cells were then incubated with

[^{3}H]acetate (20 µCi/ml) in the same condition for 2 h. 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, cells were switched to

medium F containing 0.1% DMSO as a control (CTR), 25 µM LY294002 (LY), or 50 nM

rapamaycin (Rap). After incubarion for 18 h, lipid rafts were prepared. Free cholesterol (FC)

22

and phospholipid (PL) contents in each fraction were determined. Ratios of lipid rafts (LR, fraction 2) to non-lipid rafts (NLR, fractions 4-8) for FC and PL are shown. * $P < 0.05$.

Figure 5. SREBP inhibition suppresses Akt activation in lipid rafts. A, On day 0, SK-MEL-28 cells were set up as in Fig. 4D. On day 2, cells were switched to medium F containing 0.1% EtOH (CTR) or 5 μM 25HC (HC). After 18 h of incubation, cells were stimulated or not with IGF-1 (100 ng/ml) for 15 min, and post-nuclear supernatant (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 μM 25HC. After incubation for 18 h, cells were incubated in the presence or absence of 50 μM LY294002 (LY) for 1 h and then stimulated with or without IGF-1 for 15 min. Cells were stained for p-Akt and GD3. Bar, 10 μm.

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 performed 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 μM), rapamycin (20 nM), 25HC (2 μM), NB598 (1 μM), compactin (5 μM), or the indicated combinations. MTT assay

23

was performed 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.

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 two

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.

24

Figure 1. Yamauchi et al.
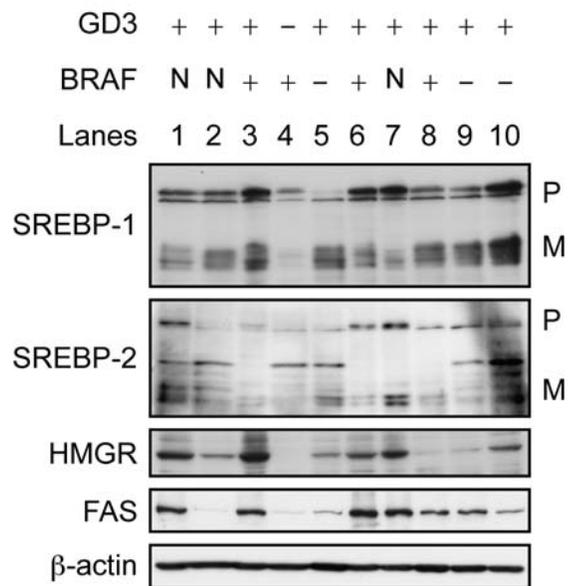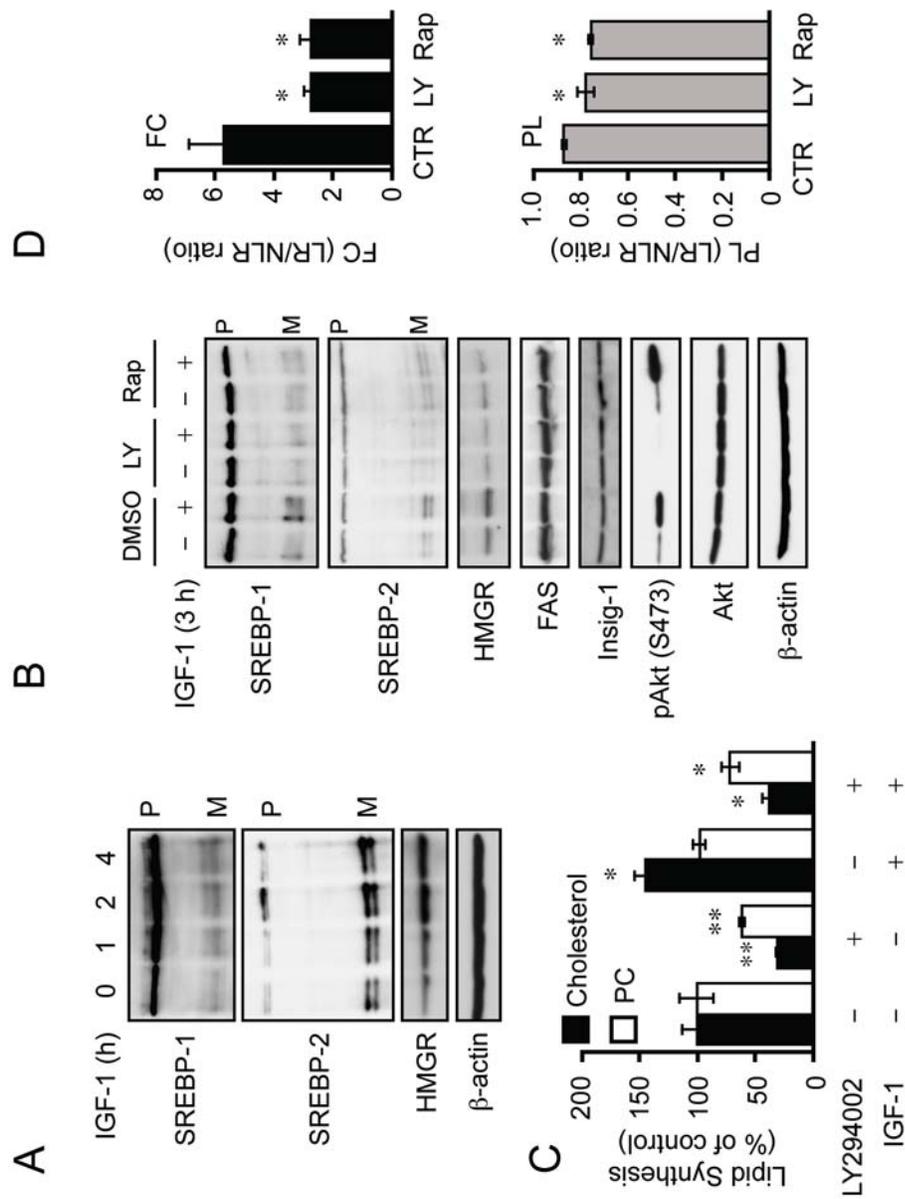
Figure 2. Yamauchi et al.
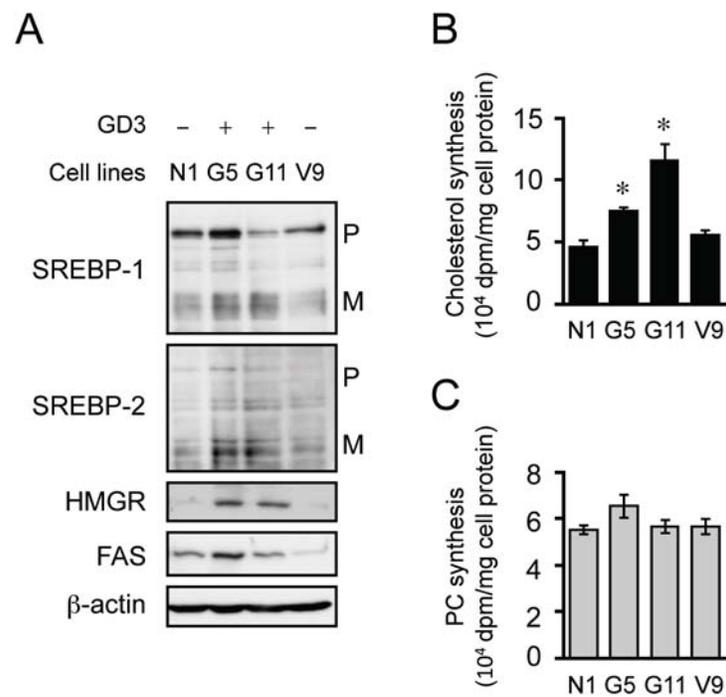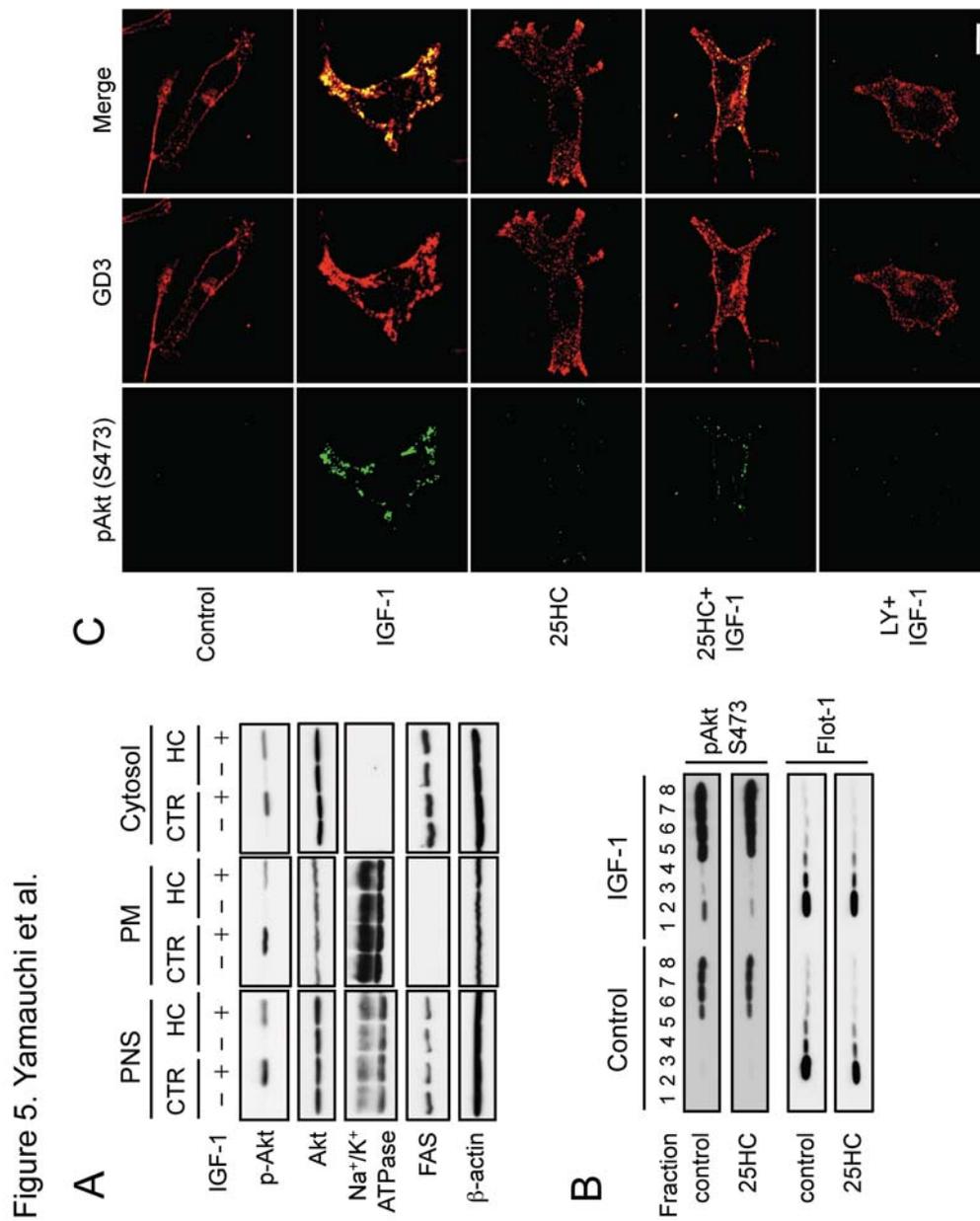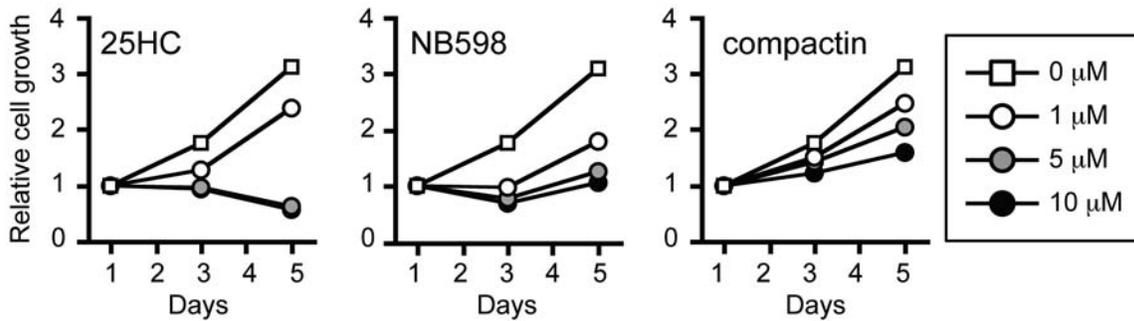
Figure 3. Yamauchi et al.
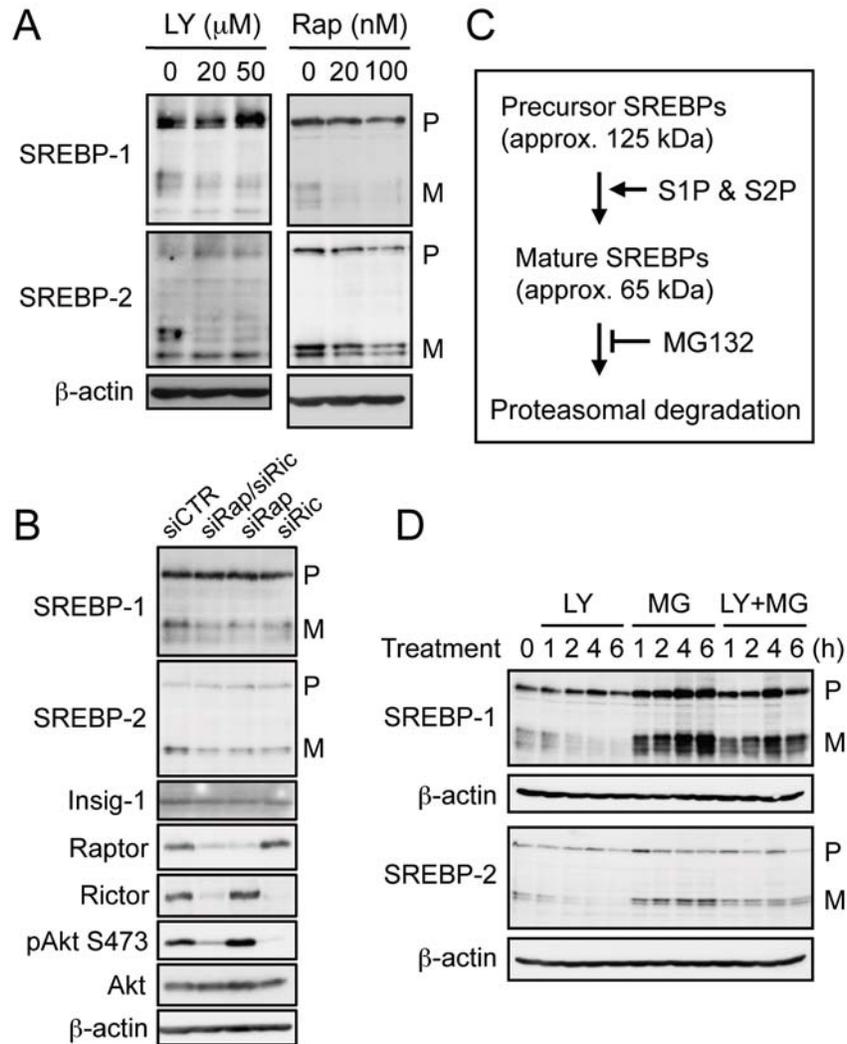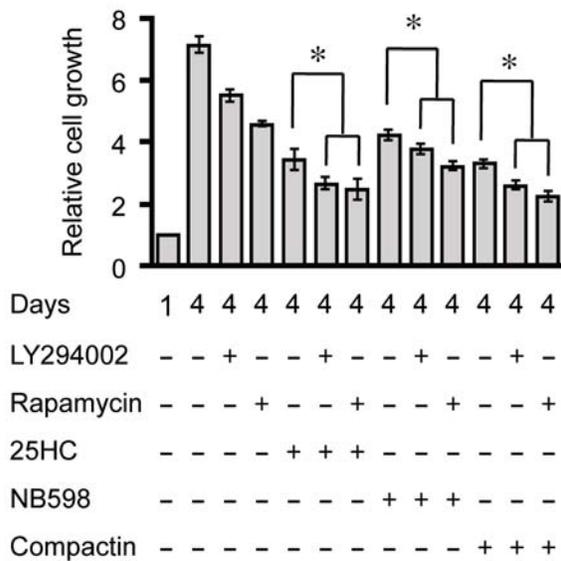
Figure 4. Yamauchi et al.

Figure 5. Yamauchi et al.

Figure 6. Yamauchi et al.
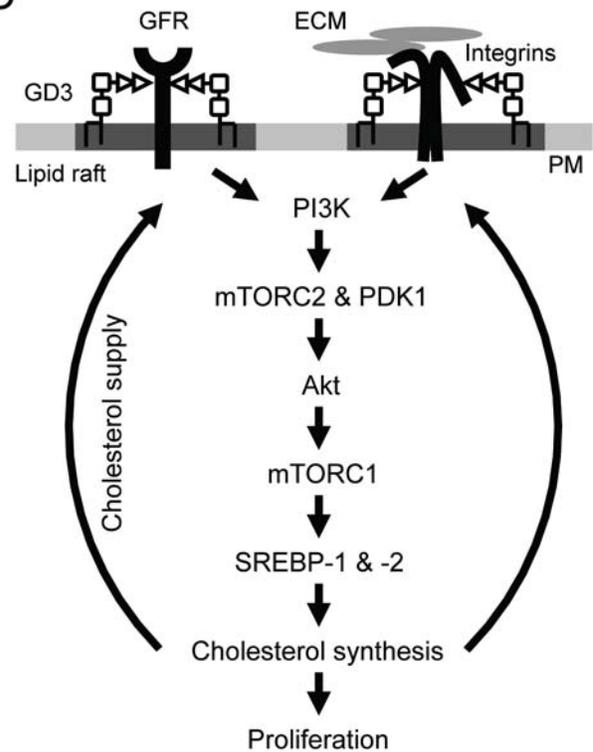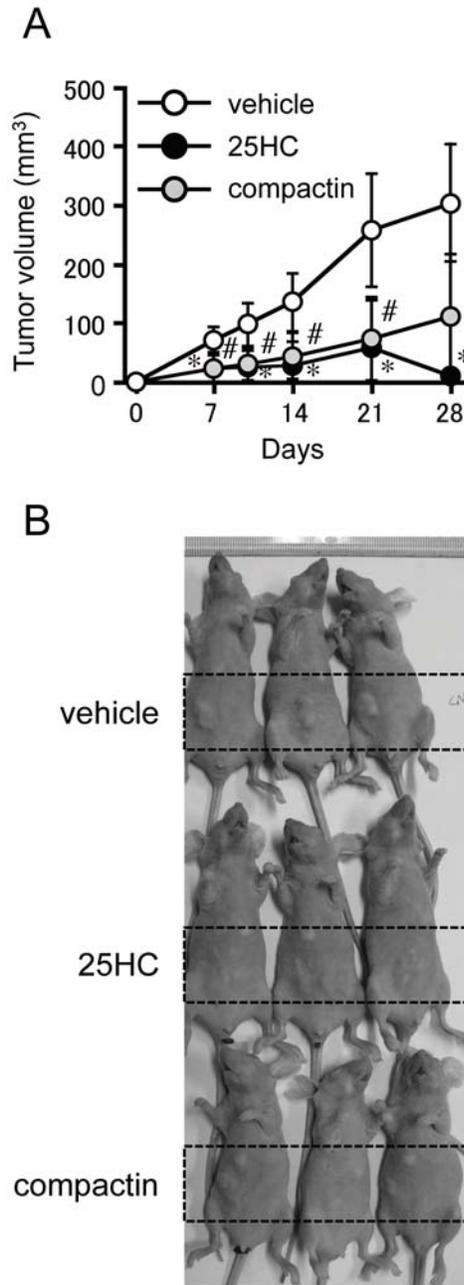
Figure 7. Yamauchi et al.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for 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

Yoshio Yamauchi, Keiko Furukawa, Kazunori Hamamura, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-10-4108
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2011/06/01/0008-5472.CAN-10-4108.DC1
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/early/2011/05/31/0008-5472.CAN-10-4108. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.