

Table 3 Continued

		<i>P</i>	Risk ratio	95% CI
AFP (ng/mL)		0.9638		
	>1000	0.7967	1.069	0.645–1.772
	≤200	0.9361	1.021	0.621–1.676
	201–1000		1	
PIVKA-II (mAU/mL)		0.4997		
	>1000	0.7397	1.060	0.750–1.499
	≤100	0.3008	0.766	0.463–1.269
	101–1000		1	
AP-factor		0.0161		
	AP1	0.0431	1.731	1.017–2.947
	AP3	0.0553	1.474	0.991–2.191
	AP2		1	
Differentiation		0.0760		
	Well	0.2922	2.184	0.510–9.348
	Moderate	0.1192	3.050	0.750–12.404
	Poor	0.0768	3.577	0.872–14.675
	Unknown		1	
Microscopic portal vein invasion	Absent	0.0561	0.716	0.508–1.009
	Present		1	
Microscopic hepatic vein invasion	Absent	0.2297	0.749	0.468–1.200
	Present		1	
Microscopic intrahepatic metastasis	Absent	0.2509	0.832	0.608–1.139
	Present		1	
Non-cancerous liver	Cirrhosis	0.0120	1.356	1.069–1.720
	Non-cirrhosis		1	

AFP, α -fetoprotein; AP-factor, a product of the serum levels of AFP and PIVKA-II; CI, confidence interval; HBsAg, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICG-R15, indocyanine green retention rate at 15 min; PIVKA-II, protein induced by vitamin K absence or antagonism factor-II.

(AUC = 0.74607) is significantly higher than that of AFP (AUC = 0.69804, $P = 0.0271$) and PIVKA-II (AUC = 0.69130, $P = 0.0065$).

DISCUSSION

IN OUR PRESENT study, the AP-factor was found to be closely related to both tumor differentiation and vascular invasion and was also identified as an independent factor related to PS and RFS outcomes with a P -value lower or equal to that of microscopic portal invasion, although AFP and PIVKA-II were not found to be independent survival factors. ROC analysis to evaluate 2-year survival in our HCC patient subjects who had received a hepatectomy confirmed that the AP-factor is a significantly superior indicator compared with AFP and PIVKA-II. Hence, the AP-factor is suggested to be a more reliable marker than other well-known indicators including AFP, PIVKA-II and microscopic portal invasion for the accurate prediction of survival and recurrence in HCC patients after a hepatectomy.

Previous reports have shown that AFP is an independent predictor of prognosis,¹⁴ even in patients who have undergone a hepatectomy.¹⁵ However, in our present analyses when the AP-factor was simultaneously inputted, AFP was not found by multivariate analysis to be an independent factor related to survival and recurrence in HCC. Although high levels of AFP in fully developed HCC or in the serum of the host are associated with more aggressive behavior and increased anaplasia,¹⁶ it has been suggested that AFP regulates immune responses and induces either stimulatory or inhibitory growth activity.¹⁷ On the other hand, it is well established that the AFP levels may increase in some patients with acute and chronic hepatitis without HCC,¹⁸ and that the elevation of AFP levels correlates with the inflammation of background disease and hepatocyte regeneration.¹⁹ Hence, because AFP does not always directly reflect tumor malignancy, its levels did not influence survival and recurrence in HCC cases according to multivariate analysis in our current study.

Protein induced by vitamin K absence or antagonists-II is also known as DCP. The specificity of PIVKA-II is approximately 95%, which is higher than that of AFP.²⁰ Recently, a highly sensitive assay for PIVKA-II was developed.²¹ While sensitivity is still at approximately 50% for most small HCC,²² the frequency of HCC patients in our present study with a lower than 40-mAU/mL PIVKA-II level was 36.6%. It is reported that the elevation of PIVKA-II correlates with the presence of vascular invasion.^{9,23} DCP is reportedly an indicator of portal vein invasion of HCC,²⁴ as well as an independent prognostic indicator of recurrence and survival after hepatectomy.^{7,10} However, in our present study, when we simultaneously inputted the AP-factor into our multivariate analysis, the results suggested that PIVKA-II is not an independent factor related to survival and recurrence. In previous studies that have assessed the value of DCP in predicting recurrence and survival after hepatectomy, the assays used were not highly sensitive. Hence, most of the cases that tested positive in these earlier studies had widespread or advanced HCC, and the biological nature of PIVKA-II positivity might have been overstated. Moreover, PIVKA-II may not reflect all of the factors related to the malignancy of HCC as it mainly indicates vascular invasion and not differentiation. PIVKA-II was therefore not selected an independent factor for HCC patient outcomes after hepatectomy in our current study.

The AP-factor – a product of the serum levels of AFP and PIVKA-II – was found in our current analyses to be significantly associated with all of the pathological factors tested including differentiation, microvascular portal invasion, microvascular hepatic vein invasion and intrahepatic metastasis (all $P < 0.0001$). From these results, we revealed that the AP-factor may have a duality in its relationship with AFP and PIVKA-II. It was previously reported that AFP has prognostic limitations in the case of microvascular hepatic vein invasion, as does PIVKA-II in the case of differentiation, in HCC.^{8–10,23,25} The AP-factor overcomes these limitations because its P -value in relation to microvascular hepatic vein invasion was found to be very low ($P < 0.0001$). Because the AP-factor may represent the dual characteristics of both AFP and PIVKA-II, it may be a surrogate marker of both tumor differentiation and vascular invasion and more directly reflect tumor malignancy than either AFP or PIVKA-II individually. These findings may involve the fact that recurrence in AP1 patients tended to occur only in the liver, whereas in AP3 patients it tended to occur in extrahepatic sites, including or excluding the liver. Therefore, we identified the AP-factor as an inde-

pendent factor very closely related to survival following microscopic vascular invasion, and closely related to recurrence in cases of increased tumor number.

Shimada *et al.* have reported that the positivity of both DCP and AFP is an independent indicator of a poor prognosis in HCC in terms of disease-free survival and PS.⁷ For this reason, these authors suggested that both DCP and AFP produced by the HCC itself promote either tumor growth or tumor metastasis in an auto-crine and/or paracrine fashion. Kaibori *et al.* have also reported that a positive status for both AFP and DCP at recurrence is an important prognostic indicator for HCC recurrence after hepatic resection.²⁶ However, our current patients were classified mainly by their AP-factor (AFP × PIVKA-II) levels because we hypothesized that this factor may be a surrogate marker of both tumor differentiation and vascular invasion and will more directly reflect tumor malignancy than either AFP or PIVKA-II individually. Moreover, ROC analysis of 2-year survival outcomes in our patients showed a significant superiority of the AP-factor over AFP and PIVKA-II as a prognostic indicator. For these reasons, the AP-factor may be a more reliable prognostic marker of PS and RFS of patients with HCC. Moreover, the classification of AP1, AP2 and AP3 is meaningful because it was possible to determine that AP2 was also equal to AP1, which was hoped to have the best outcome, and AP3 had the worst outcome in these three groups. Kiriya *et al.* reported that triple positive tumor markers for HCC showed poor prognosis and invasive characteristics in pathological findings.²⁷ However, in this paper it was described that most of the patients in this study had less than the minimum detectable limit for *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3). Therefore, we evaluated the malignancy from AFP and PIVKA-II without AFP-L3.

Transplantation is considered to be the treatment of choice even for resectable small HCC in Child–Pugh class A patients.²⁸ Because the overall survival rates after hepatectomy for small HCC are shown to be equal to those after liver transplantation, hepatectomy before transplantation should be first performed for resectable HCC in patients with preserved liver function.²⁹ Moreover, because Poon *et al.* reported no differences in the cumulative survival curves of patients without microscopic venous invasion in resection and transplantation groups,³⁰ it is proposed that patients without microscopic portal invasion according to the Milan criteria should first be treated by hepatectomy. On the other hand, if HCC patients show microscopic portal invasion, the outcomes of liver transplantations are also

unfavorable³¹ and patients who may be rendered transplantable after hepatectomy may be selected. However, microscopic portal invasion cannot be diagnosed preoperatively and a pathological examination is required to evaluate this factor. From our current data, the AP-factor was shown to be very closely related to both tumor differentiation and vascular invasion and was selected as an independent factor related to survival with an equal *P*-value to microscopic portal invasion, and an independent factor related to recurrence with a lower *P*-value. Hence, the AP-factor is suggested to be a critical HCC marker with an accuracy that equals microscopic portal invasion at preoperatively predicting tumor malignancy. Hence, HCC patients in whom the same outcomes can be expected for hepatectomy as with transplantation or who may be rendered transplantable by hepatectomy could be selected by measuring their AP-factor.

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Suprabasin as a novel tumor endothelial cell marker

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Tumor angiogenesis is necessary for the progression of tumor growth and metastasis.^(1,2) Because tumor blood vessels supply tumor cells with nutrients and oxygen, anti-angiogenesis treatment is recognized as a new cancer therapy.⁽³⁾ Bevacizumab, anti-vascular endothelial growth factor (VEGF) antibody,⁽⁴⁾ and sorafenib or sunitinib, a VEGF receptor kinase inhibitor, have been used as anti-angiogenic drugs.⁽⁵⁾ However, there are negative reports regarding side effects and increases in metastasis have been observed. To overcome these problems, a new anti-angiogenic drug is required.⁽⁶⁾ The morphology of tumor blood vessels is different from that of normal blood vessels.^(7–9) Differences between tumor endothelial cells (TEC) and normal endothelial cells (NEC) in aspects, such as gene expression and biological behavior, have also been reported.^(7,8,10) Recently, we revealed that TEC were more resistant to anti-cancer drugs compared with NEC.⁽¹¹⁾ In addition, inhibition of cyclooxygenase-2 or lysyl oxidase in TEC suppressed tumor growth and lung metastasis *in vivo*.^(12,13) These findings indicate that TEC may be a good target for anti-cancer therapy. To identify specific TEC markers, we performed DNA microarray analysis and reported that some molecules were upregulated in TEC.^(14–16) Among these molecules, suprabasin (SBSN) showed very high expression levels in several TEC.

Recent studies have reported that stromal cells contribute to tumor progression. We previously demonstrated that tumor endothelial cells (TEC) characteristics were different from those of normal endothelial cells (NEC). Furthermore, we performed gene profile analysis in TEC and NEC, revealing that suprabasin (SBSN) was upregulated in TEC compared with NEC. However, its role in TEC is still unknown. Here we showed that SBSN expression was higher in isolated human and mouse TEC than in NEC. SBSN knockdown inhibited the migration and tube formation ability of TEC. We also showed that the AKT pathway was a downstream factor of SBSN. These findings suggest that SBSN is involved in the angiogenic potential of TEC and may be a novel TEC marker.

Suprabasin has been identified as an epidermal differentiation marker and has been detected in the suprabasal layers of the epithelia in the epidermis, stomach and tongue in mice.^(17,18) SBSN participates in the proliferation of normal small cell lung carcinoma cells.⁽¹⁹⁾ The SBSN expression is also correlated with the growth and invasiveness of salivary gland adenoid cystic carcinoma and glioblastoma.^(20,21) However, the details of SBSN's involvement in tumor malignancy and tumor angiogenesis are unknown.

In this study, we examined the SBSN expression and its function in TEC to determine whether SBSN is a potential TEC marker.

Materials and Methods

Cell lines and culture conditions. The human renal clear cell carcinoma cell OS-RC-2 was purchased from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. A375SM cells, a super-metastatic human melanoma cell line, were a gift from Dr Isaiah J Fidler (MD Anderson Cancer Center, Houston, TX, USA). The cells were cultured in minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL

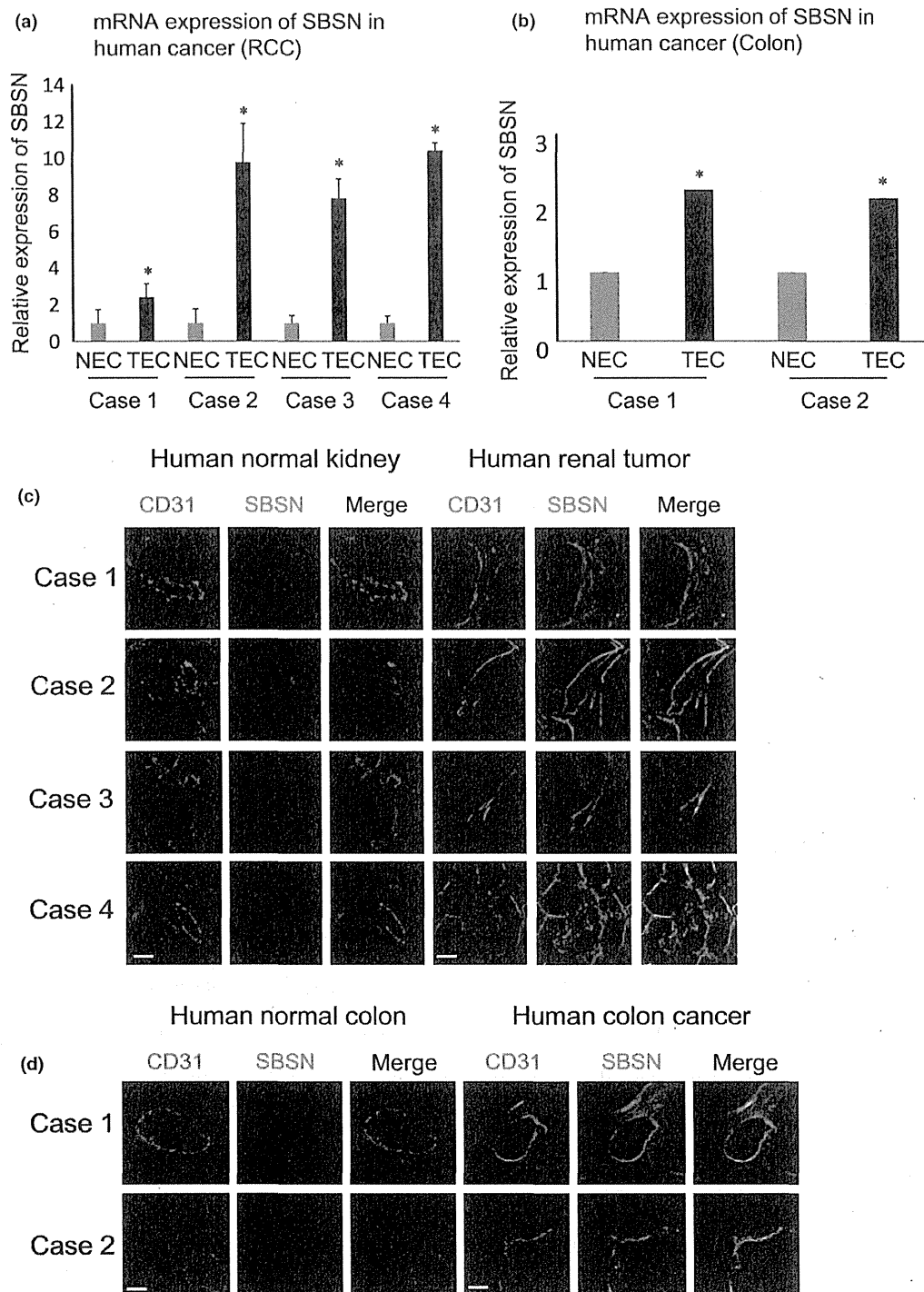


Fig. 1. Suprabasin (SBSN) expression in human tumor endothelial cells (hTEC). (a, b) Relative *SBSN* mRNA expression levels in hNEC and hTEC evaluated by quantitative PCR (a, RCC, $n = 4$; b, colon tumor, $n = 2$). * $P < 0.01$ versus control; two-sided Student's *t*-test. (c, d) Clinical samples of renal cell carcinoma (RCC) and colon cancer-derived tumor endothelial cells were double-stained with anti-CD31 and anti-SBSN antibodies. Scale bar: 50 μ m.

streptomycin, as described previously.⁽¹⁵⁾ In the growth factor experiments, NEC were treated with human EGF (AF-100-15; PeproTech, Rocky Hill, NJ, USA) at final concentrations of 5 and 15 ng/mL and human VEGF (100-20; PeproTech, Rocky Hill, NJ, USA) at final concentrations of 15 and 30 ng/mL for

12 h. These cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Isolation of tumor endothelial cells and normal endothelial cells. All procedures for animal experiments were approved by the local animal research authorities, and animal care

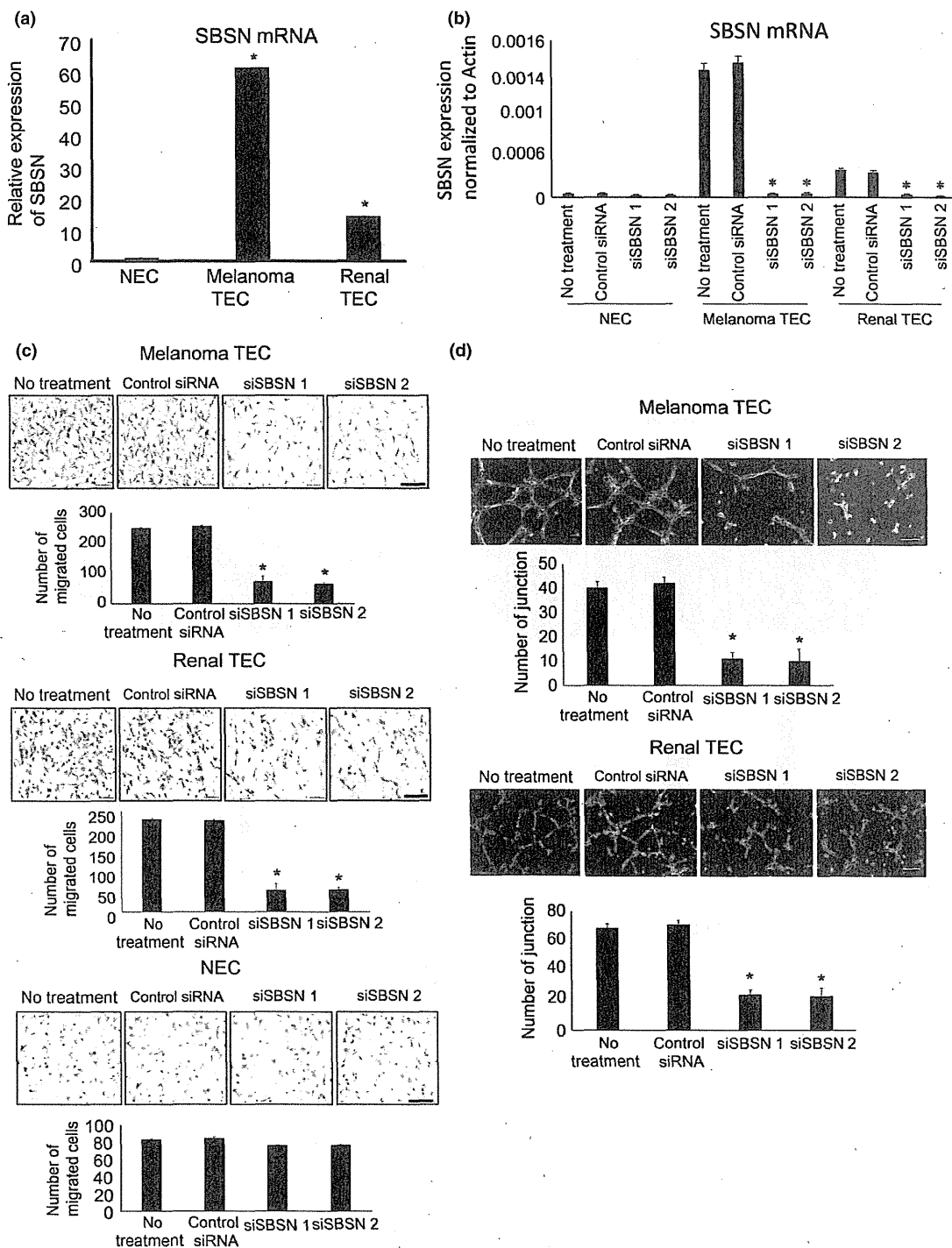


Fig. 2. Effect of *SBSN* knockdown on cell migration and tube formation in mouse tumor endothelial cells (mTEC). (a) Relative *SBSN* mRNA expression levels in mouse normal endothelial cells (mNEC) and mTEC (melanoma and renal) evaluated by quantitative PCR. (b) *SBSN* mRNA expression levels in mTEC and mNEC transfected with the control siRNA or *siSBSN*, determined by quantitative PCR. (c) Migration toward vascular endothelial growth factor (VEGF) of mTEC and mNEC transfected with control siRNA or *siSBSN* analyzed using a Boyden chamber. Scale bar: 100 μ m. (d) Tube number of mTEC transfected with control siRNA or *siSBSN*. Scale bar: 50 μ m. * $P < 0.01$ versus control; one-way ANOVA with the Tukey–Kramer multiple comparison test (mean \pm SD, $n = 3$).

was performed in accordance with institutional guidelines. Mouse TEC (mTEC) and NEC (mNEC) were isolated as previously described⁽¹²⁾ with some modifications. Diphtheria

toxin (500 ng/mL; Calbiochem, San Diego, CA, USA) was added to mTEC subcultures to kill any human tumor cells and to mNEC subcultures for technical consistency. Using

an anti-human CD31 antibody, human TEC (hTEC) and NEC (hNEC) were isolated from excised renal cell carcinoma (RCC) or colon cancer tissues from patients at Hokka-

ido University Hospital. Normal renal or colon tissues were obtained from areas that were adjacent to the tumor in the same patient. Clinical background information is described

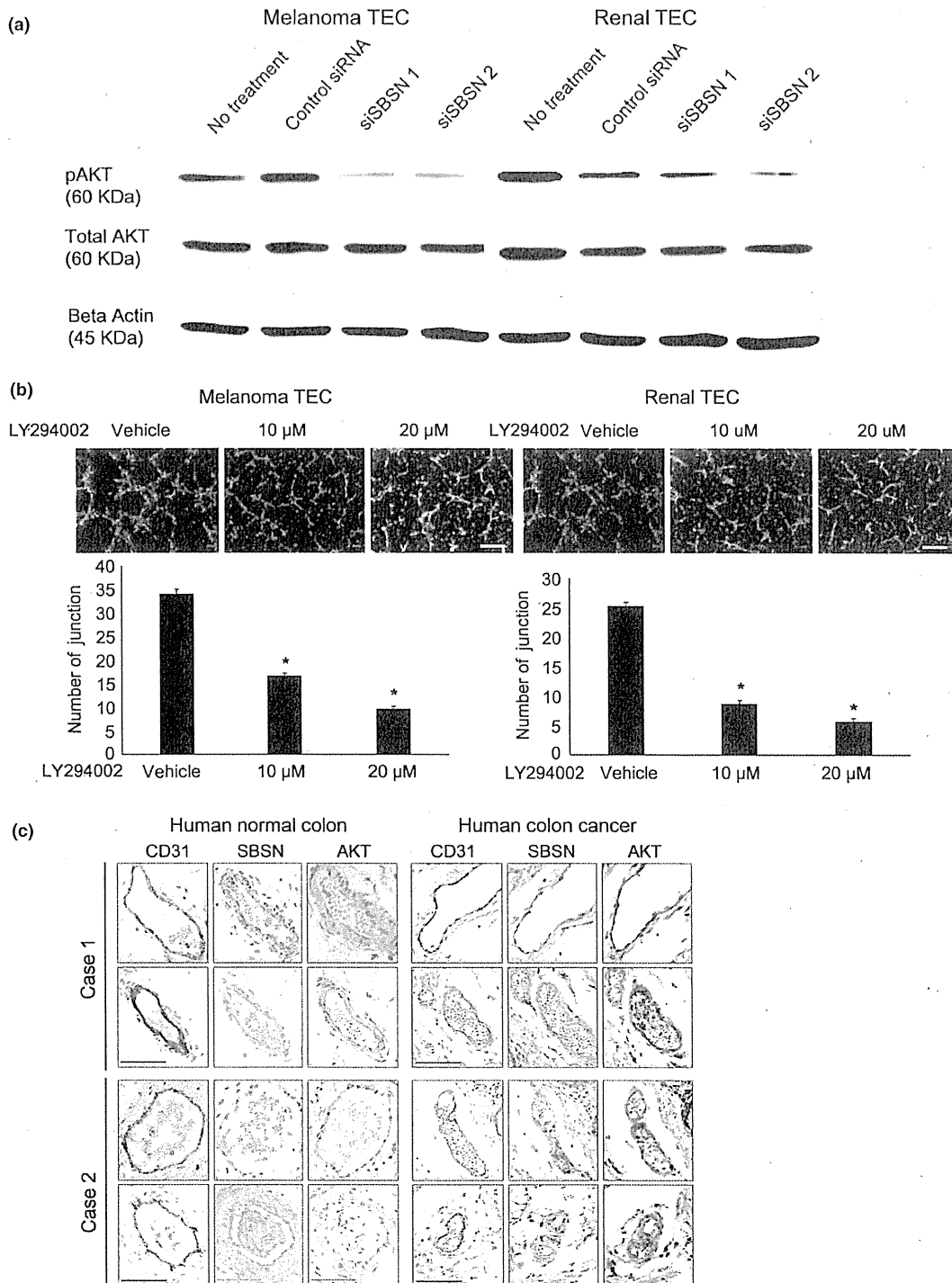


Fig. 3. Relationship between suprabasin (SBSN) knockdown and AKT activation in mouse tumor endothelial cells (mTEC) *in vitro* and *in vivo*. (a) Total AKT, phosphorylated AKT (p-AKT), and beta actin protein levels in mTEC treated with control siRNA or siSBSN, determined by western blotting. (b) Tube number of mTEC treated with or without LY294002 (10 or 20 μM) evaluated by the tube formation assay. Scale bar: 100 μm. **P* < 0.05 versus control; two-sided Student's *t*-test (mean ± SD, *n* = 3). (c) SBSN and AKT expression levels were determined by immunohistochemical analysis. CD31-positive blood vessels were stained with anti-SBSN and anti-AKT antibodies in two cases of human colon cancer (Cases 1 and 2), whereas those of normal tissues were weakly stained *in vivo*. Scale bar: 80 μm.

in Supplementary Table S1. These protocols were approved by the Ethics Committee of Hokkaido University, and written informed consent was obtained from each patient before surgery. Endothelial cells (EC) were cultured as previously described.^(22,23)

Reverse transcription and quantitative PCR. Total RNA was extracted from cells and human tumor and normal tissue samples using the ReliaPrep RNA Cell Miniprep System (Promega Corporation, Madison, WI, USA). Complementary DNA (cDNA) was synthesized using a ReverTra-Plus kit (Toyobo, Osaka, Japan). For relative quantification of target mRNA, we used SsoFast EvaGreen Supermix (CFX 96 Real-Time PCR Detection System; Bio-Rad, Hercules, CA, USA) for mouse EC and SYBR Green Real-time PCR Master Mix-Plus (Bio-Rad) for human EC (in triplicate) according to the manufacturer's instructions.⁽²⁴⁾ The quantitative PCR amplification program was performed at 95°C for 3 min and 45 cycles at 95°C for 10 s and 60°C for 30 s. Data were analyzed with CFX Manager software (Bio-Rad). The primers used are described in Supplementary Table S2. Each experiment included four PCR reactions, and each experiment was performed three times.

Western blotting. Western blotting analysis was performed as described previously.⁽²⁵⁾ This analysis used antibodies specific for total AKT, phosphorylated AKT (Cell Signaling Technology, Beverly, MA, USA), total Erk, phosphorylated Erk (Cell Signaling Technology), beta actin and an HRP-conjugated secondary antibody.

Immunostaining. Human tissue samples were obtained from excised RCC, normal renal tissue, colon cancer and normal colon tissues of patients at Hokkaido University Hospital. Frozen sections of excised tissues were prepared as previously described.^(14,26) Human sections were double-stained with anti-human CD31/Alexa Fluor 594 rat anti-mouse IgG and anti-SBSN/Alexa Fluor 488 goat anti-rabbit IgG. All samples were counterstained with DAPI (Roche Diagnostics, Mannheim, Germany) and examined using an Olympus FluoView FV10i confocal microscope (Olympus, Tokyo, Japan).

For AKT staining, formalin-fixed paraffin-embedded specimens from two cases of colon cancer were prepared. Immunohistochemical analysis was performed using serial sections that were stained with anti-SBSN (1:250 dilution), AKT (1:100 dilution) and CD31 (Leica Microsystems, UK; 1:500 dilution), followed by antibody detection using a peroxidase-conjugated streptavidin-diaminobenzidine (DAB) readout system (DAKO), and counterstaining with DAPI. Images were randomly captured using a nanozoomer slide scanner and NDPViewer (Hamamatsu, Japan).

Suprabasin knockdown. siSBSN was transfected into cells using Lipofectamine transfection reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. The sequence of siSBSN was 5'-UAUUGAUGCCUCAA GGGCCUUGCC-3' (siSBSN1) and 5'-UUCCCUUCCAGCU UGAGUGAUUCCG-3' (siSBSN2). A nontargeting control siRNA was used (Invitrogen).

Cell migration assay. Cell migration toward VEGF-A was analyzed using a Boyden chamber (Neuro Probe, Gaithersburg, MD, USA), as previously described.⁽²⁷⁾ VEGF-A (10 ng/mL) was added to the lower chamber as a chemoattractant. TEC were treated with the control siRNA (10 nM) or siSBSN (10 nM) in endothelial basal medium (EBM)-2 supplemented with 0.5% FBS for 24 h. In total, 1.5×10^4 cells were seeded in the upper chamber and

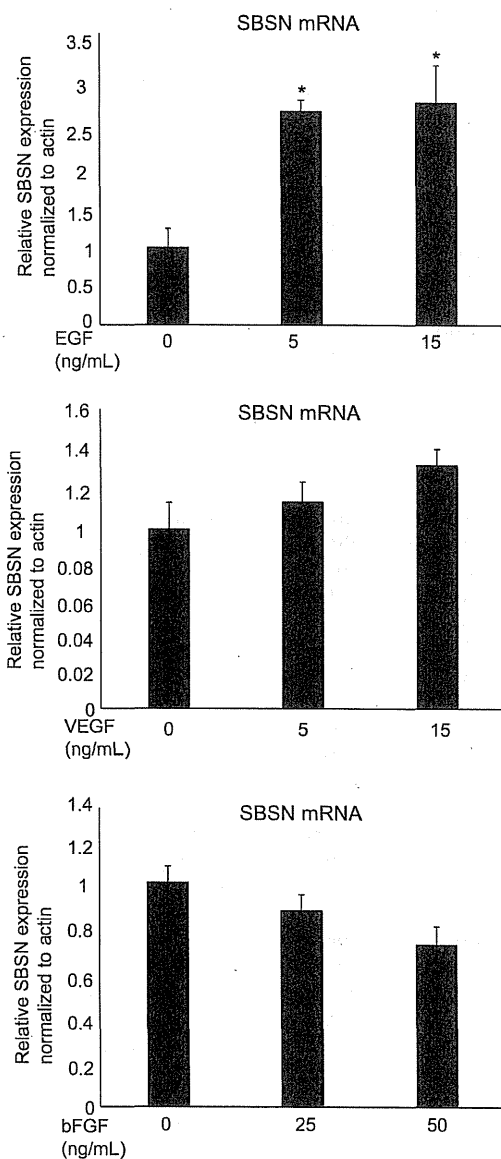


Fig. 4. Suprabasin (SBSN) expression after growth factor treatment. NEC were incubated in 0.5% EBM2 medium for 12 h, followed by treatment with endothelial growth factor (EGF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) for 12 h. The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. **P* < 0.05 versus control; two-sided student's *t*-test. After 12 h of incubation, mRNA was extracted from the cells and used in the RT-PCR analysis of SBSN expression.

incubated for 4 h at 37°C. The assays were independently performed three times.

Tube formation assay. A tube formation assay was performed as previously described.⁽²⁶⁾ EC were seeded at a density of 1.0×10^5 cells per well and incubated at 37°C on Matrigel (BD Biosciences, San Jose, CA, USA). Tube formation was observed using an inverted microscope by measuring the junction number of endothelial tubes. For inhibition experiments using the PI3 kinase inhibitor LY294002, TEC were preincubated for 2 h at 37°C in EBM2 supplemented with 0.5% FBS. To investigate the involvement of AKT in TEC tube formation, assays were performed with or without LY294002 (0, 10

or 20 μ M). The assays were independently performed three times.

Cell proliferation assay. Cell proliferation was assessed with an MTS assay as described previously.⁽¹¹⁾ TEC were treated with the control siRNA (10 nM) or si*SBSN* (10 nM) in EBM2 supplemented with 0.5% FBS for 24 h. After siRNA transfection, 1.0×10^3 cells per well were seeded into 96-well plates in EBM2 supplemented with 5% FBS. Cell proliferation was measured daily for 3 days by the MTS assay. The assays were independently performed three times.

Statistical analysis. Results are given as mean \pm SD. Group comparisons were made by one-way ANOVA with the Tukey–Kramer multiple comparison test. When only two groups were compared, a two-sided Student's *t*-test was used. $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant.

Results

Suprabasin was highly expressed in human tumor endothelial cells. To analyze the *SBSN* expression in hTEC and hNEC, we isolated hTEC from tissues of four cases of RCC and two cases of colon cancer. Furthermore, hNEC were isolated from the tissues of normal renal tissue and colon in the same patients.^(14,28) The *SBSN* mRNA expression levels in hTEC isolated from RCC and colon cancer tissues were higher than those of hNEC (Fig. 1a,b). Double-immunofluorescence staining with anti-*SBSN* and anti-CD31 antibodies revealed that *SBSN* was markedly expressed in tumor blood vessels both in RCC and colon cancer, whereas the *SBSN* expression was low in normal blood vessels (Fig. 1c,d). In addition, *SBSN* mRNA expression levels were higher in human renal tumor tissues than those in normal tissues (Suppl. Fig. S1). These findings showed that *SBSN* was upregulated in hTEC from several tumor types.

Suprabasin knockdown inhibited migration and tube formation of mouse tumor endothelial cells. To clarify the role of *SBSN* in TEC, we used mTEC isolated from human tumor xenografts (A375SM and OS-RC-2). mNEC were isolated from mouse dermis as a normal control. We verified that mNEC and mTEC had the characteristics of EC using an RT-PCR assay (Suppl. Fig. S2). The *SBSN* mRNA expression levels were upregulated in mTEC from melanoma and renal carcinoma compared with mNEC (Fig. 2a) and other mouse normal tissues (Suppl. Fig. S3). To evaluate the *SBSN* function in TEC, we examined the migration ability and tube formation of mTEC following the *SBSN* knockdown. The efficacy of RNA interference (RNAi) was confirmed using quantitative real-time PCR, which showed that si*SBSN*, unlike control siRNA, decreased the *SBSN* mRNA level in mTEC and mNEC (Fig. 2b). We next demonstrated that the *SBSN* knockdown significantly suppressed cell migration toward VEGF-A in mTEC but not in mNEC (Fig. 2c). However, si*SBSN* had no effect on cell proliferation in either mTEC or mNEC (Suppl. Fig. S4). In this study, we used two types of siRNA and obtained similar results. This suggests that the results are not off-target effects of the nucleic acids. In addition, the junction number of endothelial tubes in mTEC was reduced by si*SBSN* treatment (Fig. 2d). These findings revealed that *SBSN* contributed to the angiogenic phenotype, such as migration and tube formation in mTEC.

Suprabasin knockdown suppressed AKT pathway in mouse tumor endothelial cells. The PI3K/AKT pathway plays an essential role in the survival of TEC.⁽²⁹⁾ We previously reported that activation of AKT was involved in cell migration

of mTEC,^(13,23) and, therefore, we explored the interaction between the AKT pathway and *SBSN*. Phosphorylation of AKT in mTEC was suppressed by the PI3K inhibitor LY294002 treatment (Suppl. Fig. S5a). Moreover, we showed that the protein level of phosphorylated AKT was reduced by si*SBSN* treatment compared with control siRNA in both types of mTEC (melanoma and renal) (Fig. 3a), but not in NEC or in other cell types (Suppl. Fig. S5b). Moreover, we demonstrated that LY294002 inhibited tube formation in mTEC in a concentration-dependent manner (Fig. 3b). These findings indicate that *SBSN* regulated the migration and tube formation of mTEC via the AKT pathway. In addition, *SBSN*-positive blood vessels in human colon cancer tissues were positively stained by anti-AKT, but not those of normal colon tissues (Fig. 3c). This result suggests that *SBSN* may also be involved in AKT activation in human tumor blood vessels. To address how *SBSN* expression is regulated, endothelial cells were treated with growth factors such as endothelial growth factor (EGF), VEGF and fibroblast growth factor-2 (FGF-2). Among these growth factors, EGF significantly induced *SBSN* mRNA expression in NEC (Fig. 4).

Discussion

In this study, we demonstrated that the *SBSN* expression was markedly increased in human TEC (renal carcinoma and colon carcinomas) as well as mTEC (melanoma and renal carcinoma). These findings indicate that *SBSN* may be used as a common marker of TEC.

The *SBSN* mRNA expression levels tended to be higher in hTEC (isolated from renal carcinoma) with higher T classifications under the tumor-node-metastasis system (order: case 4 > 2 > 3 > 1) (Suppl. Table S1). In this study, because the number of clinical samples was small, further studies are required to explore the relationship between the *SBSN* expression and clinical background in larger numbers of patients.

Previously, we reported that mTEC demonstrate a pro-angiogenic phenotype compared with mNEC.^(11,30,31) *SBSN* plays a role in epidermal differentiation⁽¹⁸⁾ and the growth and invasiveness of tumors.^(19–21) For example, Shao *et al.*⁽²¹⁾ report that *SBSN* was upregulated because the *SBSN* gene promoter in adenocystic carcinoma was demethylated. However, our preliminary analysis of epigenetics showed that methylation levels in TEC did not differ from those in NEC, which suggests that there may be another mechanism that is responsible for the enhanced expression of the *SBSN* gene in TEC. We found that EGF upregulated the expression of *SBSN* in NEC. However, its mechanism of transcriptional regulation or its function in tumor angiogenesis is unknown. In this study, we demonstrated that the *SBSN* knockdown inhibited cell migration and tube formation in mTEC. These findings revealed the role of *SBSN* in tumor angiogenesis.

We previously reported that the VEGF receptor-2 (VEGFR-2) expression was high in TEC and that TEC were more sensitive to VEGF than NEC.⁽²⁷⁾ The *SBSN* knockdown had no significant effect on the *VEGFR* mRNA expression in mTEC, suggesting that involvement of *SBSN* in the angiogenic phenotype of mTEC is independent of VEGF/VEGFR-2 signaling.

There has been no report of *SBSN* signaling. We showed that the activation of AKT was suppressed by si*SBSN*. However, activation of the ERK pathway, which is related to angiogenesis, was not affected (Suppl. Fig. S6). Our finding revealed at least a part of downstream signaling of *SBSN* in TEC. Thus, these findings enhanced our understanding of TEC function.

Our data demonstrate that the number of tube junctions in TEC was decreased more by siSBSN than by a PI3K inhibitor.

These results suggest that other molecules besides AKT are involved in SBSN-related tube formation in mTEC. Additional studies are required to determine whether the AKT is directly involved in the downstream of SBSN.

In this study, to the best of our knowledge, we demonstrated for the first time that SBSN is upregulated in TEC and that SBSN plays significant roles in the pro-angiogenic phenotype in TEC, but not in NEC. In this study, we showed that SBSN could be a potential TEC marker. Thus, SBSN may be a novel target for anti-angiogenic therapy, which is specific for tumor blood vessels.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Suprabasin (SBSN) expression in human tumor tissues. Relative SBSN mRNA expression levels in both human colon normal and cancer tissues were analyzed by quantitative RT-PCR. * $P < 0.05$ versus control; two-sided Student's t -test. Clinical samples from three patients were collected.

Fig. S2. Characterization of isolated mouse tumor endothelial cells (mTEC) and mouse normal endothelial cells (mNEC). mRNA levels of CD31, CD105, VEGFR-1 (VR1), VEGFR-2 (VR2), CD11b, CD45, human HB-EGF (hHB-EGF) and GAPDH in mTEC and mNEC were evaluated by RT-PCR.

Fig. S3. SBSN expression in mouse tumor endothelial cells (mTEC) and other various tissue of mouse organs. Relative *SBSN* mRNA expression levels in various tissue of mouse organs besides ECs analyzed by quantitative PCR. * $P < 0.01$ versus control. (ND, not detected.)

Fig. S4. Effect of si*SBSN* on proliferation of mouse tumor endothelial cells (mTEC) transfected with control siRNA or si*SBSN* was analyzed using the MTS assay.

Fig. S5. (a) Effect of LY294002 treatment on mouse tumor endothelial cells (mTEC). (a) Total AKT, phosphorylated AKT (p-AKT), and beta actin protein levels in mTEC treated or not treated with LY294002 (10 or 20 μ M) were determined by western blotting. (b) Total AKT, phosphorylated AKT (p-AKT), and beta actin protein levels in mTEC were compared with those of the NEC, NIH3T3 and B16F10 cell lines transfected with control siRNA or si*SBSN*. Approximately 20 μ g of total protein was loaded into each lane for western blot analysis.

Fig. S6. Effect of ERK activation by suprabasin (SBSN) knockdown. ERK activation was determined by western blot analysis. Total ERK and phosphorylated ERK (p-ERK) protein expression levels were detected in mouse tumor endothelial cells (mTEC) transfected with control siRNA or si*SBSN* in melanoma tumor endothelial cells (TEC) and renal TEC. Approximately 20 μ g of total protein was loaded into each lane for western blot analysis. Beta actin antibody was used as internal control.

Table S1. Clinical background of renal cell carcinoma (RCC) and colon cancer specimens. M/F, male/female; †according to 1997 tumor-node-metastasis (TNM) staging guidelines; ††according to the Fuhrman system.

Table S2. List of primers. Primer sequences for RT-PCR and quantitative PCR.

Diacylglycerol Kinase δ Phosphorylates Phosphatidylcholine-specific Phospholipase C-dependent, Palmitic Acid-containing Diacylglycerol Species in Response to High Glucose Levels*

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Background: Diacylglycerol (DG) kinase (DGK) δ is activated by acute high glucose stimulation.

Results: DGK δ high glucose-dependently phosphorylates 30:0-, 32:0-, and 34:0-DG and interacts with phosphatidylcholine-specific phospholipase C (PC-PLC).

Conclusion: DGK δ utilizes palmitic acid-containing DG species and metabolically connects with PC-PLC.

Significance: The newly identified PC-PLC/DGK δ pathway could play an important role in insulin signaling and glucose uptake.

Decreased expression of diacylglycerol (DG) kinase (DGK) δ in skeletal muscles is closely related to the pathogenesis of type 2 diabetes. To identify DG species that are phosphorylated by DGK δ in response to high glucose stimulation, we investigated high glucose-dependent changes in phosphatidic acid (PA) molecular species in mouse C2C12 myoblasts using a newly established liquid chromatography/MS method. We found that the suppression of DGK δ 2 expression by DGK δ -specific siRNAs significantly inhibited glucose-dependent increases in 30:0-, 32:0-, and 34:0-PA and moderately attenuated 30:1-, 32:1-, and 34:1-PA. Moreover, overexpression of DGK δ 2 also enhanced the production of these PA species. MS/MS analysis revealed that these PA species commonly contain palmitic acid (16:0). D609, an inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC), significantly inhibited the glucose-stimulated production of the palmitic acid-containing PA species. Moreover, PC-PLC was co-immunoprecipitated with DGK δ 2. These results strongly suggest that DGK δ preferably metabolizes palmitic acid-containing DG species supplied from the PC-PLC pathway, but not arachidonic acid (20:4)-containing DG species derived from the phosphatidylinositol turnover, in response to high glucose levels.

Type 2 diabetes is expected to afflict over 300 million people worldwide by 2015 (1). The characteristic features of type 2 diabetes include insulin resistance, glucose intolerance, hyper-

glycemia, and often, hyperinsulinemia (2). Glucose-induced insulin resistance is associated with a temporal increase in the intracellular diacylglycerol (DG)² mass in skeletal muscle (3).

DG is metabolized, at least in part, by DG kinase (DGK), which phosphorylates DG to generate phosphatidic acid (PA) (4–8). To date, 10 mammalian DGK isozymes (α , β , γ , δ , η , κ , ϵ , ζ , ι , and θ) have been identified, and these isozymes are subdivided into five groups according to their structural features (6, 7). Type II DGKs consist of the δ , η , and κ isoforms (9, 10). Moreover, alternatively spliced forms of DGK δ (δ 1 and δ 2) (11) and η (η 1 and η 2) (12) have been found.

DGK δ is highly expressed in skeletal muscle (13), which is a major insulin-target organ for glucose disposal (14). Chibalin *et al.* (15) demonstrated that DGK δ regulates glucose uptake and that a decrease in DGK δ expression resulted in the aggravation of type 2 diabetes. Long term exposure (96 h) to high glucose medium decreased DGK δ protein levels in primary cultured skeletal muscle cells, and the transcription of DGK δ and the levels of DGK δ protein were also reduced in skeletal muscles from type 2 diabetes patients (15). Moreover, DGK δ haploinsufficient mice (DGK δ ^{+/-}) exhibited decreased total DGK activity, reduced DGK δ protein levels, and the accumulation of DG in skeletal muscle. The increase in the amount of DG caused the increase in the phosphorylation of protein kinase C (PKC) δ and a reduction in the expression of the insulin receptor and insulin receptor substrate-1 proteins involved in insulin signaling (15). Furthermore, Miele *et al.* (16) reported that acute high glucose exposure (within 5 min) increased DGK δ activity in skeletal muscle cells followed by a reduction of PKC α activity and transactivation of the insulin receptor signal.

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² The abbreviations used are: DG, diacylglycerol; DGK, DG kinase; D609, O-tricyclo[5.2.1.0^{2,6}]dec-9-yl dithiocarbonate; FIPI, 5-fluoro-2-indolyl deschlorhalopemide; ESI, electrospray ionization; PA, phosphatidic acid; PC, phosphatidylcholine; PC-PLC, PC-specific phospholipase C; PLD, phospholipase D; TOFA, 5-(tetradecyloxy)-2-furoic acid; PI, phosphatidylinositol; DMSO, dimethyl sulfoxide; AcGFP, GFP from *Aequorea coerulescens*.

Metabolic Linkage between PC-PLC and DGK δ

Hence, these studies indicate that DG consumed by DGK δ in response to high glucose exposure is a key regulator of glucose uptake in skeletal muscle cells. DGK δ 1 translocated from the cytoplasm to the plasma membrane in mouse myoblast C2C12 cells within 5 min of short term exposure to a high glucose concentration, whereas DGK δ 2 was located in punctate vesicles irrespective of the glucose concentration (17).

Mammalian cells contain at least 50 structurally distinct molecular DG species because DG contains a variety of fatty acyl moieties at positions 1 and 2 (18). In general, DGs containing arachidonic acid (20:4), especially 18:0/20:4-DG (38:4-DG), in the phosphatidylinositol (PI) turnover are important molecules that serve as second messengers for PKC activation (18). Moreover, previous studies have demonstrated that DGK ϵ preferably phosphorylates arachidonic acid-containing DGs derived from PI turnover (19, 20). Therefore, it is generally believed that all DGKs preferentially metabolize 38:4-DG for the regulation of signal transduction. However, the DG molecular species phosphorylated by DGK δ in response to glucose stimulation remain unknown.

In this study, we investigated the changes in the amounts of PA molecular species that are produced by DGK in glucose-stimulated C2C12 myoblasts using our previously developed liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) method (21) to identify the DG molecular species metabolized by DGK δ under short term high glucose conditions. Interestingly, the LC/ESI-MS analyses indicated that DGK δ preferably metabolizes limited DG molecular species, 30:0-, 30:1-, 32:0-, 32:1-, 34:0-, and 34:1-DG, commonly containing palmitic acid (16:0), but not DG species containing arachidonic acid in response to high glucose stimulation. Moreover, the 30:0-, 32:0-, and 34:0-DG species were suggested to be supplied by phospholipase C (PLC)-dependent phosphatidylcholine (PC) hydrolysis, indicating an unexpected linkage between PC-PLC and DGK δ .

EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 mouse myoblasts were maintained on 100-mm dishes in DMEM (Wako Pure Chemicals) containing 10% FBS (Biological Industries-Invitrogen) at 37 °C in an atmosphere containing 5% CO₂. For differentiation to myotubes, confluent C2C12 myoblasts were cultured in differentiation medium (DMEM containing 0.1% FBS and 5 μ g/ml insulin (Sigma-Aldrich)) for 4 days.

Establishment of a Stable Cell Line Overexpressing DGK δ —To establish C2C12 cells stably expressing human DGK δ 2, the cells were transfected with pAcGFP-DGK δ 2 (11, 17) using PolyPect (Qiagen) according to the instruction manual and were selected with 800 μ g/ml G418 for 2 weeks. Single colonies were isolated and then were then grown in DMEM containing 10% FBS.

RNA Interference—To silence the expression of mouse DGK δ , the following Stealth RNAi duplexes (Invitrogen) were used: DGK δ -siRNA-1, 5'-GAAUGUGAUGCUGGAUCUUAC-UAAA-3' and 5'-UUUAGUAAGAUCAGCAUCACAUUC-3'; DGK δ -siRNA-2, 5'-UGGCAUUGGCUUGGAUGCAAAGUA-3' and 5'-UAUCUUUGCAUCCAAGCCAAUGCCA-3'. The duplexes were transfected into C2C12 myoblasts by electropor-

ation (at 350 V and 300 microfarads) using the Gene Pulser Xcell™ electroporation system (Bio-Rad Laboratories). The transfected cells were then allowed to grow for 48 h in DMEM containing 10% FBS.

Glucose Stimulation and Treatment with Lipid Metabolism Enzyme Inhibitors—Glucose stimulation was performed as reported previously (16). Briefly, untransfected C2C12 myoblasts and C2C12 myoblasts transfected with Stealth RNAi duplexes were grown on poly-L-lysine (Sigma-Aldrich)-coated culture dishes. The cells were rinsed and incubated in glucose-free medium (16) in the absence or presence of PC-PLC inhibitor *O*-tricyclo[5.2.1.0^{2,6}]dec-9-yl dithiocarbonate (D609, 100 μ M, Calbiochem) (22), acetyl-CoA carboxylase inhibitor 5-(tetradecyloxy)-2-furoic acid (TOFA, 20 μ M, Calbiochem) (23, 24), or phospholipase D (PLD) inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI, 100 nM, Calbiochem) (25) for 3 h. The cells were incubated for 5 min in the same buffer supplemented with 25 mM glucose.

Lipid Extraction and Western Blot Analysis—The cells grown under each culture condition were harvested and lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, cOmplete™ EDTA-free protease inhibitor (Roche Diagnostics)) followed by centrifugation at 1,000 \times *g* for 5 min at 4 °C. Total lipids were extracted from the cell lysates (1.0 mg of protein), in which DGK δ expression was confirmed by Western blot analysis using an anti-DGK δ antibody (13), according to the method of Bligh and Dyer (26). The extracted lipids were used for subsequent MS analyses.

Analysis of PA Molecular Species—PAs in extracted cellular lipids (5 μ l) containing 40 pmol of the 14:0/14:0-PA internal standard (Sigma-Aldrich) were analyzed separately by LC/ESI-MS using an Accela LC system (Thermo Fisher Scientific) coupled online to an Exactive Orbitrap MS (Thermo Fisher Scientific) equipped with an ESI source as described previously (21). The MS peaks are presented in the form of X:Y, where X is the total number of carbon atoms and Y is the total number of double bonds in both acyl chains of the PA.

For the identification of fatty acid residues in PA molecular species by ESI-MS/MS, PA molecular species (28:0–40:0-PA) were fractionated using the above LC/ESI-MS system equipped with an FC 203B fraction collector (Gilson). The mixture of these isolated PA molecular species was infused into an Exactive Orbitrap MS (Thermo Fisher Scientific) equipped with a syringe pump (an infusion rate of 5 μ l/min) and an ESI source. A collision energy of 40 eV was used to obtain fragment ions.

Analysis of DG Molecular Species—The isolation of DG was performed according to previously reported procedures (27). The extracted cellular lipids (per 1 mg of protein) were developed on Silica Gel 60 high performance thin layer chromatography plates (Merck, 10 \times 20 cm) using hexane/diethyl ether/acetic acid (75:25:1, v/v). After development, DG was extracted from silica gel and redissolved in 200 μ l of methanol:chloroform (9:1, v/v) containing 1 μ g/ml 12:0/12:0-DG (Avanti Polar Lipids), and 10 μ l of 100 mM sodium acetate were added to each sample (28). MS analysis was performed on an Exactive Orbitrap MS (Thermo Fisher Scientific) equipped with a Fusion 100T syringe pump (an infusion rate of 5 μ l/min, Thermo Fisher Scientific) and an ESI source. The ion spray

voltage was set to 5 kV in the positive ion mode. The capillary temperature was set to 300 °C.

Measurement of DGK δ Activity—The octyl glucoside-mixed micellar assay of DGK activity was performed as described previously (12). COS-7 cells transfected with p3 \times FLAG-DGK δ 2 (29) were harvested and lysed in ice-cold lysis buffer followed by centrifugation at 1,000 \times *g* for 5 min at 4 °C. The cell lysates were added to octyl glucoside buffer containing 2 mM 16:0/16:0-, 16:0/18:1-, or 18:0/20:4-DG (Avanti Polar Lipids) and 10 mM phosphatidylserine (Avanti Polar Lipids).

Immunoprecipitation and Measurement of PC-PLC Activity—The glucose-stimulated cells stably expressing human DGK δ 2 were harvested and lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 1 mM dithiothreitol, cComplete™ EDTA-free protease inhibitor (Roche Diagnostics)) for immunoprecipitation. The mixtures were centrifuged at 12,000 \times *g* for 5 min at 4 °C to yield the cell lysates. 500 μ g of the cell lysates were incubated with normal rabbit IgG (2 μ g, Santa Cruz Biotechnology) or rabbit anti-DGK δ antibody (2 μ g) (13, 29) at 4 °C overnight and incubated with protein A/G PLUS-agarose (Santa Cruz Biotechnology) for an additional 1 h. The bead-bound proteins were washed with ice-cold wash buffer (50 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100, 10% glycerol, 20 mM NaF) four times and resolved in 70 μ l of 1 \times reaction buffer (50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 10 mM dimethylglutarate, 2 mM CaCl₂) in the Amplex Red® PC-PLC assay kit (Molecular Probes-Life Technologies). In this enzyme-coupled assay, PC-PLC activity is monitored indirectly using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red® reagent), a sensitive fluorogenic probe for H₂O₂. First, PC-PLC converts PC to form phosphocholine and DG. After the action of alkaline phosphatase, which hydrolyzes phosphocholine, choline is oxidized by choline oxidase to betaine and H₂O₂. Finally, H₂O₂, in the presence of horseradish peroxidase, reacts with Amplex Red® reagent in a 1:1 stoichiometry to generate the highly fluorescent product, resorufin. Resorufin has absorption and fluorescence emission maxima of ~571 nm and 585 nm, respectively. 50- μ l aliquots of the mixtures were used for the measurement of PC-PLC activities, and 10 μ l of the mixtures were used for Western blot analysis.

Statistics—All LC/ESI-MS data were normalized based on the protein content and the intensity of the internal standard. The data were represented as the mean \pm S.D. Statistical analysis was performed using the two-tailed *t* test or analysis of variance followed by Tukey's post hoc test.

RESULTS

Increase in the Amount of PA by Acute Stimulation with High Glucose—We first examined whether the amount of total PA was increased in C2C12 myoblasts stimulated with 25 mM glucose. As shown in Fig. 1A, LC/ESI-MS analysis indicated that exposure to high glucose levels (for 5 min) statistically increased the total PA amounts (1.23-fold, *p* < 0.005). In addition, the stimulation significantly increased the amounts of C30 to C36 PA molecular species, with the exception of 36:1-PA (Fig. 1B). However, the stimulation did not substantially affect

the production of C38 to C40 PA molecular species, including 38:4-PA, with the exception of 38:6-PA.

We investigated the high glucose-dependent increases of total PA amount and PA molecular species in C2C12 myoblasts at different time points. After 5 min of glucose stimulation, the levels of total PA and PA molecular species were significantly increased (Fig. 1, C and D). However, total PA and PA molecular species levels returned close to basal levels by prolonging the incubation with high glucose concentrations for up to 15 and 30 min. We confirmed that DGK activity *in vitro* was increased by glucose stimulation for 5 min (data not shown). These results strongly suggest that C2C12 myoblasts and L6 myotubes (16) have essentially the same lipid metabolism pathway to produce PA in response to acute glucose stimulation.

We confirmed the changes in the amounts of PA molecular species in C2C12 myotubes in response to acute high glucose stimulation (5 min). The glucose-stimulated C2C12 myotubes showed essentially the same results (Fig. 1, E and F) as those obtained with C2C12 myoblasts (Fig. 1, A and B). The results support that C2C12 myoblasts and myotubes possess essentially the same lipid metabolism pathway to produce PA in response to high glucose stimulation. Because C2C12 myoblasts were more efficiently transfected with siRNAs than C2C12 myotubes, C2C12 myoblasts were used for identification of PA molecular species produced by DGK δ in response to high glucose stimulation.

Effects of DGK δ -specific siRNAs on High Glucose-induced Increases in PA Molecular Species—To clarify whether the glucose-stimulated production of PA molecular species is catalyzed by DGK δ , we investigated the effects of a DGK δ -specific siRNA, DGK δ -siRNA-1. Of the two alternatively spliced DGK δ products, DGK δ 1 and DGK δ 2 (11), C2C12 myoblasts predominantly expressed DGK δ 2 (Fig. 2A). DGK δ -siRNA-1 efficiently suppressed DGK δ 2 expression in C2C12 myoblasts (Fig. 2A). To facilitate comparison, averages of the relative values (+glucose versus -glucose) from four independent experiments are displayed (Fig. 2B). Interestingly, the suppression of DGK δ expression by DGK δ -siRNA-1 significantly inhibited the glucose stimulation-dependent production of saturated fatty acid-containing C30-C34 PA species, 30:0-, 32:0-, and 34:0-PA, to their basal levels. In addition, one saturated and one monounsaturated fatty acid-containing PA, 34:1-PA, decreased as well. However, the amount of arachidonic acid (20:4)-containing PA, 38:4-PA, was not markedly changed (Fig. 2B). To rule out off-target effects of DGK δ -siRNA-1, we employed an independent siRNA targeted to a different region of DGK δ mRNA (DGK δ -siRNA-2). DGK δ -siRNA-2, which suppressed DGK δ 2 expression slightly less strongly than DGK δ -siRNA-1 (Fig. 2C), also statistically inhibited the production of 30:0-, 34:1-, and 34:0-PA and moderately attenuated 32:0-PA generation (Fig. 2D). These results suggest that DGK δ selectively phosphorylated 30:0-, 32:0-, 34:1-, and 34:0-DG, which contain either two saturated fatty acids or one saturated and one monounsaturated fatty acids, but not 38:4-PA.

We investigated whether the decreases in the amounts of 30:0-, 32:0-, 34:1-, and 34:0-PA by DGK δ -siRNAs were due to decreases in the substrates, the corresponding DG species, in high glucose-stimulated and -unstimulated C2C12 myoblasts.

Metabolic Linkage between PC-PLC and DGK δ

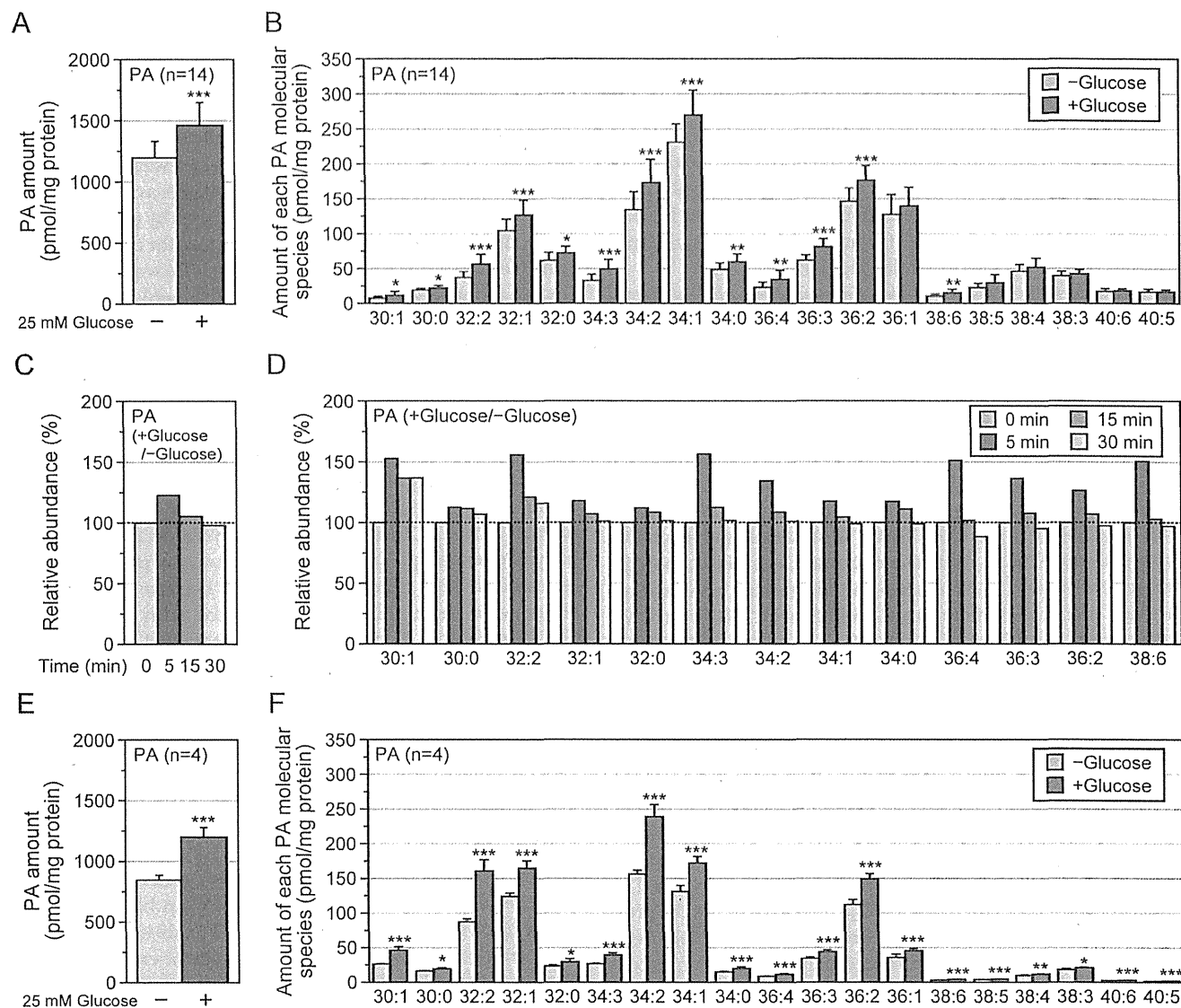


FIGURE 1. Changes in the total PA and PA molecular species by high glucose stimulation in C2C12 myoblasts and myotubes. A and B, the amounts of the total PAs (A) and major PA molecular species (B) in the glucose-unstimulated or glucose-stimulated C2C12 myoblasts were quantified using the LC/ESI-MS method. The values are presented as the mean \pm S.D. ($n = 14$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (no stimulation versus glucose stimulation). C and D, the amounts of the total PAs (C) and major PA molecular species (D) that statistically increased in A in the cells stimulated by glucose for 5, 15, or 30 min were detected using the LC/ESI-MS method. The results are presented as the percentage of the value of PA molecular species in glucose-unstimulated cells. The values are presented as the mean ($n = 2$). Essentially the same results were obtained in two independent experiments. E and F, the amounts of the total PAs (E) and major PA molecular species (F) in the glucose-unstimulated or glucose-stimulated C2C12 myotubes were quantified using the LC/ESI-MS method. The values are presented as the mean \pm S.D. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (no stimulation versus glucose stimulation).

Glucose stimulation substantially increased the amounts of various DG species (Fig. 3). However, DGK δ -siRNA-1 failed to significantly affect the amounts of 30:0-, 32:0-, 34:1-, and 34:0-DG molecular species both in the absence and in the presence of high glucose levels. Therefore, it is likely that the decreases in the amounts of 30:0-, 32:0-, 34:1-, and 34:0-PA were not caused by decreased amounts of the corresponding DG species.

Effect of Overexpression of DGK δ on the Production of PA Molecular Species—To confirm the results of the siRNA experiments, we evaluated the result of DGK δ 2 overexpression on high glucose-dependent production of PA species in C2C12 cells. In response to high glucose, the levels of 30:0-, 32:0-, and

34:0-PA statistically increased in C2C12 cells stably expressing DGK δ 2 when compared with control cells (Fig. 4B). Moreover, 30:1- and 32:1-PA were also augmented. In contrast, 38:4-PA did not increase. Taken together with the siRNA results (Figs. 2 and 4), these results support the hypothesis that DGK δ phosphorylated DG species with an apparent preference for 30:0-, 32:0-, and 34:0-DG, but not arachidonic acid-containing DG, 38:4-DG. Moreover, it is possible that this enzyme also generates 30:1-, 32:1-, and 34:1-PA.

Fatty Acid Composition of 30:0-, 32:0-, and 34:0-PA—We next determined the molecular identities of the two fatty acids included in 30:0-, 32:0-, and 34:0-PA, which were indicated to be selectively generated by DGK δ 2 in C2C12 cells. ESI-MS/MS

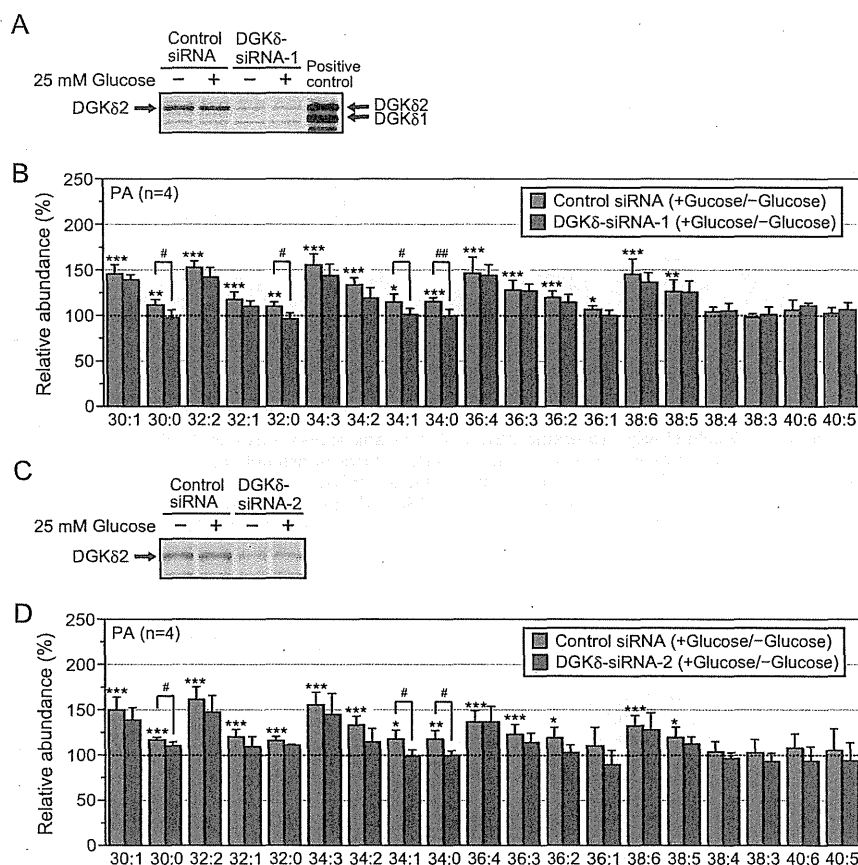


FIGURE 2. Effects of DGK δ -siRNA-1 and -2 on high glucose-induced increases of PA molecular species in C2C12 myoblasts. A and C, the suppression of DGK δ 2 expression by DGK δ -siRNA-1 (A) or DGK δ -siRNA-2 (C) was confirmed by Western blot analysis using the anti-DGK δ antibody. Human DGK δ 1 and DGK δ 2 (11) expressed in COS-7 cells were electrophoresed as a control (A). B and D, the major PA molecular species in the glucose-unstimulated or glucose-stimulated cells transfected with control siRNA or DGK δ -siRNA-1/2 were detected using the LC/ESI-MS method. The results are presented as the percentage of the value of PA molecular species in glucose-unstimulated cells transfected with control siRNA or DGK δ -siRNA-1/2. DGK δ -siRNA-1/2 did not significantly affect the value of PA molecular species in glucose-unstimulated cells. The values are presented as the mean \pm S.D. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (no stimulation versus glucose stimulation). #, $p < 0.05$; ##, $p < 0.01$ (control siRNA versus DGK δ -siRNA-1 or DGK δ -siRNA-2).

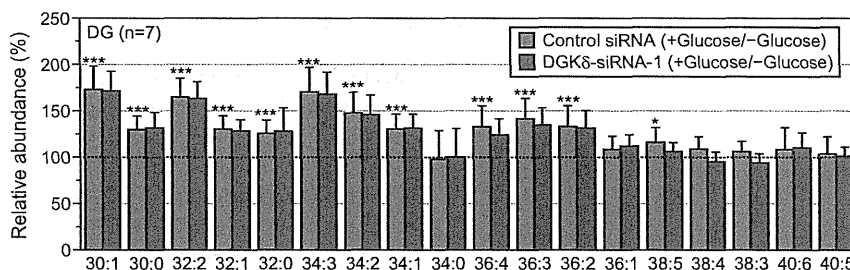


FIGURE 3. Effect of DGK δ -siRNA-1 on high glucose-induced increases of DG molecular species in C2C12 myoblasts. The major DG molecular species in the glucose-unstimulated or glucose-stimulated cells transfected with control siRNA or DGK δ -siRNA-1 were detected using the ESI-MS method. The results are presented as the percentage of the value of DG molecular species in glucose-unstimulated cells transfected with control siRNA or DGK δ -siRNA-1. DGK δ -siRNA-1 did not significantly affect the value of DG molecular species in glucose-unstimulated cells. The values are presented as the mean \pm S.D. ($n = 7$). *, $p < 0.05$; ***, $p < 0.005$ (no stimulation versus glucose stimulation).

analysis revealed that the main fatty acid residues were as follows: 30:0 consisted of 14:0 and 16:0 (100%), 32:0 included 16:0 and 16:0 (96.6%), and 34:0 contained 16:0 and 18:0 (99.7%) (Table 1). These results indicate that 30:0-, 32:0-, and 34:0-PA consist of relatively short saturated fatty acids and commonly contain palmitic acid (16:0). It is possible that DGK δ 2 also produces 30:1-, 32:1-, and 34:1-PA species (Figs. 2 and 4). These PA

species contain saturated fatty acids, 16:0 and 14:0, and mono-unsaturated fatty acids, 16:1 and 18:1 (Table 1).

In Vitro DGK δ Activity—We examined whether the preference of DGK δ 2 for palmitic acid (16:0)-containing DG species, 30:0-, 32:0-, and 34:0-DG, is an intrinsic catalytic feature of DGK δ . To this end, we measured DGK δ 2 activity *in vitro* using 32:0 (16:0/16:0)-, 34:1 (16:0/18:1)-, or 38:4 (18:0/20:4)-DG as

Metabolic Linkage between PC-PLC and DGK δ

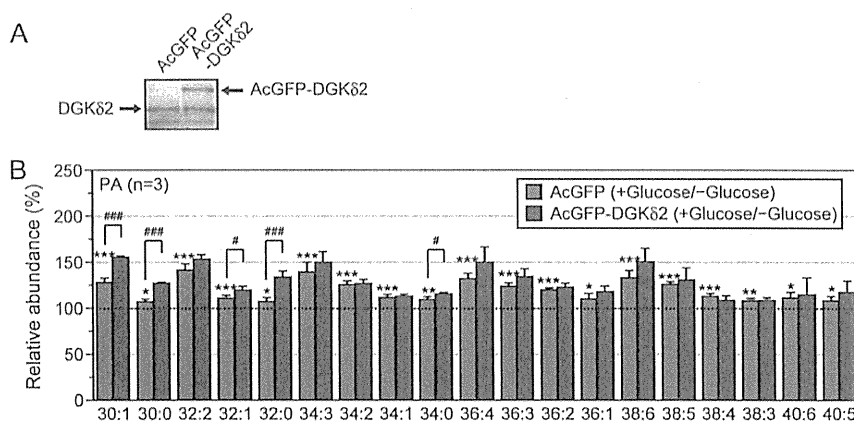


FIGURE 4. PA molecular species in C2C12 cells stably expressing DGK δ 2. *A*, the stable expression of AcGFP-DGK δ 2 in C2C12 cells was confirmed by Western blot analysis using the anti-DGK δ antibody. *B*, the major PA molecular species in the glucose-unstimulated or glucose-stimulated cells stably expressing human DGK δ 2 were identified and quantified using LC/ESI-MS. The results are presented as the percentage of the value of PA molecular species in glucose-unstimulated cells transfected with AcGFP alone or AcGFP-DGK δ 2. Overexpression of DGK δ 2 did not significantly affect the value of PA molecular species in glucose-unstimulated cells. The values are presented as the mean \pm S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (no stimulation versus glucose stimulation). #, $p < 0.05$; ##, $p < 0.005$ (no overexpression versus DGK δ overexpression).

TABLE 1

Identification of the acyl species in each PA molecular species

PA molecular species	Identified acyl chains ^a		
		%	
30:1	14:0/16:1 (86.0%)	16:0/14:1 (14.0%)	
30:0	14:0/16:0 (100%)		
32:2	16:1/16:1 (98.5%)	14:0/18:2 (1.5%)	
32:1	16:0/16:1 (88.7%)	14:0/18:1 (11.3%)	
32:0	16:0/16:0 (96.6%)	14:0/18:0 (3.4%)	
34:3	16:1/18:2 (67.2%)	16:2/18:1 (27.7%)	16:0/18:3 (5.1%)
34:2	16:1/18:1 (86.3%)	16:0/18:2 (13.6%)	16:2/18:0 (0.1%)
34:1	16:0/18:1 (93.2%)	18:0/16:1 (6.8%)	
34:0	16:0/18:0 (99.7%)	14:0/20:0 (0.3%)	
36:4	16:0/20:4 (83.0%)	16:1/20:3 (8.7%)	18:1/18:3 (7.2%)
36:3	18:1/18:2 (74.9%)	16:0/20:3 (23.6%)	16:1/20:2 (0.8%)
36:2	18:1/18:1 (91.6%)	18:0/18:2 (5.0%)	18:0/18:3 (0.7%)
36:1	18:0/18:1 (87.8%)	16:0/20:2 (2.5%)	16:1/20:1 (0.9%)
38:6	16:0/22:6 (68.0%)	16:0/20:1 (6.5%)	16:1/20:0 (5.6%)
38:5	16:0/22:5 (45.0%)	16:1/22:5 (30.9%)	18:1/20:5 (1.1%)
38:4	18:0/20:4 (80.1%)	18:1/20:4 (45.0%)	18:0/20:5 (8.5%)
38:3	18:0/20:3 (88.6%)	18:1/20:3 (19.5%)	16:0/22:4 (0.4%)
40:6	18:1/22:5 (51.5%)	18:1/20:2 (10.5%)	16:0/22:3 (0.9%)
40:5	18:0/22:5 (97.4%)	18:0/22:6 (47.6%)	16:0/24:6 (0.9%)
		18:1/22:4 (2.6%)	16:1/22:4 (1.6%)

^a The relative abundance (%) was based on the peak areas of the fragment ions (ESI-MS/MS) for each molecular ion.

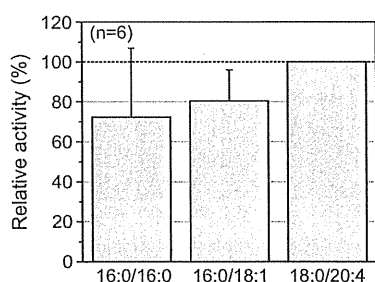


FIGURE 5. *In vitro* DGK δ activity. For measurement of *in vitro* DGK δ activity, 2 mM (5.4 mol%) 16:0/16:0-, 16:0/18:1-, and 18:0/20:4-DG were used as substrates. The activity of 3 \times FLAG-tagged DGK δ 2 in COS-7 cells was compared with the control. The results are presented as the percentage of the value of activity against 18:0/20:4-DG. The values are presented as the mean \pm S.D. ($n = 6$).

substrates. As shown in Fig. 5, the levels of 32:0- and 34:1-PA generated by DGK δ 2 were similar to or slightly lower than that of 38:4-PA. These results indicate that DGK δ 2 does not exhibit intrinsic substrate selectivity for particular DG molecular species, 32:0-DG, *in vitro*. Therefore, we hypothesized that DGK δ

accomplishes apparent substrate selectivity in C2C12 cells by accessing a DG pool containing only 30:0-, 32:0-, and 34:0-DG, and not based on the intrinsic properties of the enzyme.

Effects of Inhibitors of Lipid Metabolism Enzymes on High Glucose Level-induced PA Production—To test this hypothesis, we next searched for the lipid metabolic pathway that supplies 30:0-, 32:0-, and 34:0-DG species as a substrate for DGK δ 2. There are three pathways that produce DG, 1) the *de novo* pathway (30, 31), 2) the PLD/PA phosphatase pathway (32), and 3) the PC-specific PLC pathway (33). The treatment with 20 μ M TOFA, which inhibits acetyl-CoA carboxylase involved in the *de novo* synthesis of DG (23, 24), did not decrease the glucose-stimulated production of PA molecular species (Fig. 6A). Moreover, 100 nM FIPI, which inhibits PLD involved in DG generation from PC through the action of PA phosphatase (25), reduced the amounts of most of the PAs in the absence of high glucose stimulation (data not shown). However, this compound failed to attenuate the glucose-stimulated production of PA molecular species (Fig. 6B). These results strongly suggest that these pathways are not involved in the DG supply to DGK δ 2.

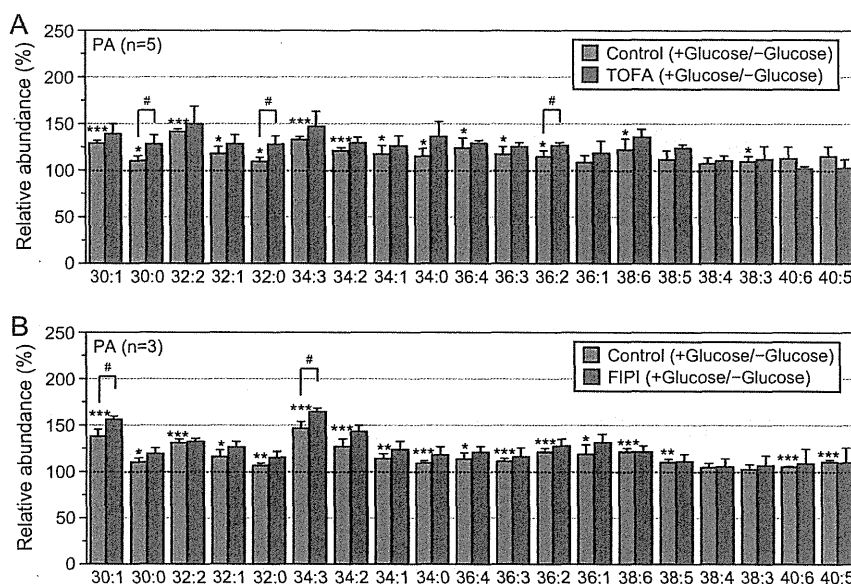


FIGURE 6. Effects of TOFA and FIPI on the production of PA molecular species in glucose-stimulated C2C12 cells. A, the major PA molecular species in glucose-unstimulated or glucose-stimulated cells treated with DMSO (control) or TOFA were detected using the LC/ESI-MS method. The results are presented as the percentage of the value of PA species in glucose-unstimulated cells treated with DMSO (control) or TOFA. The values are presented as the mean \pm S.D. ($n = 5$). *, $p < 0.05$; ***, $p < 0.005$ (no stimulation versus glucose stimulation). #, $p < 0.05$ (without TOFA versus with TOFA). B, the major PA molecular species in the glucose-unstimulated or glucose-stimulated cells treated with DMSO (control) or FIPI were detected using the LC/ESI-MS method. The results are presented as the percentage of the value of PA species in glucose-unstimulated cells treated with DMSO (control) or FIPI. The values are presented as the mean \pm S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (no stimulation versus glucose stimulation). #, $p < 0.05$ (without FIPI versus with FIPI).

D609 is an inhibitor of PC-PLC (22), which generates DG via PC hydrolysis (34). Treatment with 100 μ M D609 strongly inhibited the high glucose stimulation-responsive production of 30:0-, 32:0-, and 34:0-PA to their basal levels (Fig. 7A), suggesting that DGK δ utilizes DG species supplied from the PC-PLC pathway.

We next confirmed that D609 inhibited the production of DG molecular species, including 30:0-, 32:0-, and 34:0-DG. This inhibitor statistically attenuated the amounts of 30:0-, 32:0-, and 34:0-DG in the absence of high glucose stimulation (Fig. 7B). However, D609 inhibited high glucose-dependent increases for all of the C30-C34 DG species (Fig. 7C). These results suggest that, in response to acute high glucose stimulation (5 min), DGK δ can utilize DG species that are supplied from the PC-PLC pathway, in both high glucose-independent and high glucose-dependent manners.

Linkage between PC-PLC and DGK δ —To further examine the linkage between the PC-PLC pathway and DGK δ , we determined whether D609 and DGK δ -siRNA-1 additively affected the high glucose-dependent increases of 30:0-, 32:0-, and 34:0-PA. If DGK δ utilizes DG species supplied from the PC-PLC pathway, it would be expected that reduced expression of DGK δ via DGK δ -siRNA-1 would not enhance the effect of the PC-PLC inhibitor. It was confirmed that the expression of DGK δ was substantially reduced by DGK δ -siRNA-1, even in the presence of D609 (Fig. 8A). As shown in Fig. 8B, DGK δ -siRNA-1 failed to further inhibit the glucose-dependent increases of 30:0-, 32:0-, and 34:0-PA in the presence of D609. These results strongly suggest that 30:0-, 32:0-, and 34:0-DG phosphorylated by DGK δ in response to acute high glucose exposure are generated, at least in part, by PC hydrolysis catalyzed by PC-PLC.

We next examined whether DGK δ directly or indirectly interacted with PC-PLC. To this end, we used C2C12 cells stably overexpressing DGK δ 2 (Fig. 4) and stimulated the cells with high glucose. We confirmed that DGK δ 2 was immunoprecipitated with the anti-DGK δ antibody (Fig. 8C). Because the molecular identity of mammalian PC-PLC remains unclear (35), its antibody is unavailable. Therefore, we determined PC-PLC activity in the immunoprecipitates using the Amplex Red[®] PC-PLC assay kit, which detects phosphocholine generated by PC-PLC. As demonstrated in Fig. 8D, PC-PLC activity was clearly co-immunoprecipitated with DGK δ 2. The assay does not detect the activity of sphingomyelin synthase, which produces DG and sphingomyelin, but not phosphocholine. The contribution of PLD, which hydrolyzes PC to PA and choline, can be accounted for by elimination of alkaline phosphatase from the assay (see "Experimental Procedures"). However, when the assay was performed in the absence of alkaline phosphatase, the activity was not detectable. Taken together, these results strongly suggest that DGK δ 2 utilizes DG species supplied from PC-PLC-dependent PC hydrolysis in response to high glucose (Fig. 9).

DISCUSSION

The increase in PA molecular species by stimulation with high glucose levels has not been identified until now. Moreover, it has not been reported that high glucose induces total PA production. The main reasons for this are that PA species are minor components and it is difficult to quantify the amounts of PA molecular species using conventional LC/ESI-MS methods. To overcome this difficulty, we recently established an LC/ESI-MS method specialized for PA species (21). In this study, we revealed for the first time that acute high glucose

Metabolic Linkage between PC-PLC and DGK δ

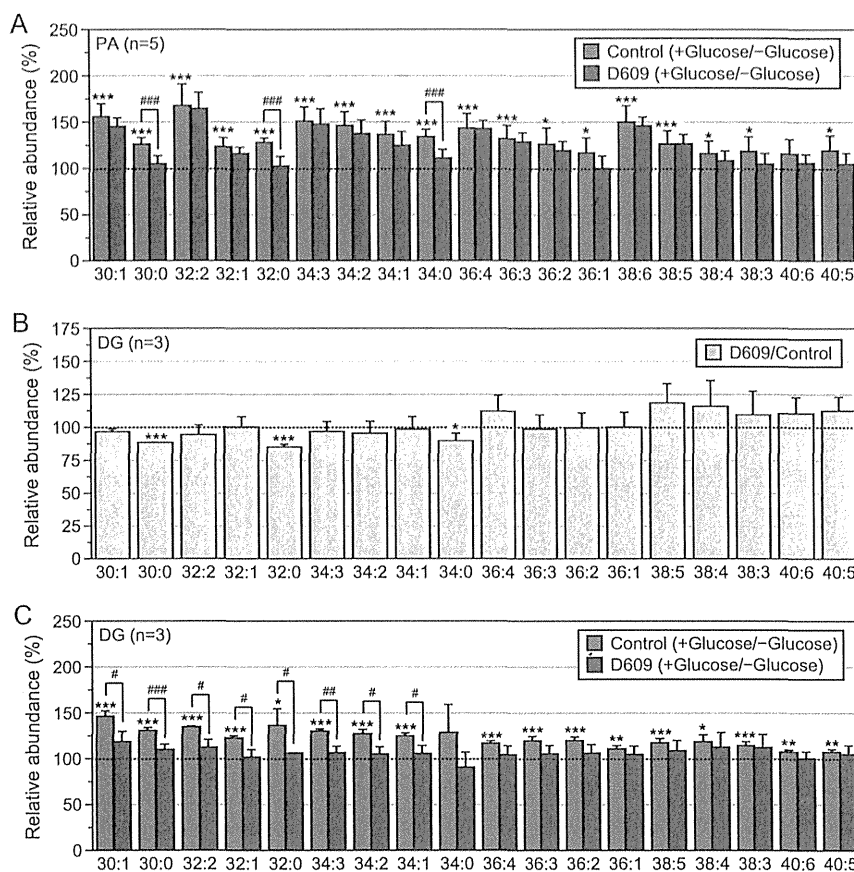


FIGURE 7. Effect of D609 on high glucose-induced increases in PA and DG molecular species in C2C12 myoblasts. *A*, the major PA molecular species in the glucose-unstimulated or glucose-stimulated cells treated with DMSO (control) or D609 were detected using the LC/ESI-MS method. The results are presented as the percentage of the value of PA molecular species in glucose-unstimulated cells treated with DMSO (control) or D609. D609 did not significantly affect the value of PA molecular species in glucose-unstimulated cells. The values are presented as the mean \pm S.D. ($n = 5$). *, $p < 0.05$; ***, $p < 0.005$ (no stimulation versus glucose stimulation). ###, $p < 0.005$ (without D609 versus with D609). *B* and *C*, the major DG molecular species in the glucose-unstimulated or glucose-stimulated cells treated with DMSO (control) or D609 were detected using the ESI-MS method. *B*, comparison of +D609 versus -D609 in the absence of glucose. The results are presented as the percentage of the value of DG species in glucose-unstimulated cells treated with DMSO (control). The values are presented as the mean \pm S.D. ($n = 3$). *, $p < 0.05$; ***, $p < 0.005$. *C*, comparison of +glucose versus -glucose in the absence or presence of D609. The results are presented as the percentage of the value of DG species in glucose-unstimulated cells treated with DMSO (control) or D609. D609 did not significantly affect the value of DG molecular species in glucose-unstimulated cells. The values are presented as the mean \pm S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (no stimulation versus glucose stimulation). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.005$ (without D609 versus with D609).

stimulation statistically increased the PA mass and number of molecular species using the newly developed method (Fig. 1). The results indicate that our LC/ESI-MS method is a powerful tool for detecting even small changes in PA molecular species.

The suppression of DGK δ expression by RNA silencing decreased the high glucose-induced production of 30:0-, 32:0-, 34:1-, and 34:0-PA in C2C12 myoblasts (Fig. 2). Moreover, the levels of 30:1-, 30:0-, 32:1-, 32:0-, and 34:0-PA were substantially increased in a high glucose-dependent manner in C2C12 cells stably expressing DGK δ 2 when compared with control cells (Fig. 4). Taken together, these results strongly suggest that DGK δ preferentially generates 30:0-, 32:0-, and 34:0-PA, which contain two saturated fatty acids, in the cells. The main fatty acid residues of these PA species were 14:0 and 16:0, 16:0 and 16:0, and 16:0 and 18:0, respectively (Table 1). These results suggest that DGK δ produces PA with an apparent preference for palmitic acid (16:0)-containing PA. Moreover, the suppression of DGK δ expression by siRNA-1 and -2 also decreased the high glucose-induced production of 34:1-PA (Fig. 2). The over-

expression of DGK δ 2 statistically increased the levels of 30:1- and 32:1-PA (Fig. 4). The DGK δ suppression also modestly attenuated 30:1- and 32:1-PA levels (Fig. 2), and the DGK δ 2 overexpression slightly augmented 34:1-PA production (Fig. 4). Therefore, it is possible that this enzyme also generates 30:1-, 32:1-, and 34:1-PA, which contain one saturated and one monounsaturated fatty acid, in addition to 30:0-, 32:0-, and 34:0-PA. 30:1-, 32:1-, and 34:1-PA contain saturated fatty acids, 16:0 and 14:0, and monounsaturated fatty acids, 16:1 and 18:1 (Table 1). The DGK δ -siRNAs and DGK δ overexpression failed to statistically affect the amounts of high glucose-induced increases of 32:2-, 34:3-, 34:2-, 36:4-, 36:3-, 36:2-, and 38:6-PA (Figs. 2 and 4), implying that these PA species were generated by other DGK isozyme(s).

DGK is a member of the PI turnover pathway and initiates resynthesis of PI (18). This fact led us to believe that DGK isozymes, including DGK δ , also exhibit selectivity against 38:4 (18:0/20:4)-DG derived from PI turnover. Indeed, it was reported that DGK ϵ preferentially phosphorylated DGs con-