学位論文

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D-Allose Inhibits Cancer Cell Growth by Reducing GLUT1 Expression

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Glucose is a major energy source for mammalian cells and is transported into cells via cell-specific expression of various glucose transporters (GLUTs). Especially, cancer cells require massive amounts of glucose as an energy source for their dysregulated growth and thus over-express GLUTs. D-allose, a C-3 epimer of D-glucose, is one of rare sugars that exist in small quantities in nature. We have shown that D-allose induces the tumor suppressor gene coding for thioredoxin interacting protein (TXNIP) and inhibits cancer cell growth by G1 cell cycle arrest. It has also been reported that GLUTs including GLUT1 are over-expressed in many cancer cell lines, which may contribute to larger glucose utilization. Since D-allose suppresses the growth of cancer cells through the upregulation of TXNIP expression, our present study focused on whether D-allose down-regulates GLUT1 expression via TXNIP expression and thus suppresses cancer cell growth. Western blot and real-time PCR analyses revealed that D-allose significantly induced TXNIP expression and inhibited GLUT1 expression in a dose-dependent manner in three human cancer cell lines: hepatocellular carcinoma (HuH-7), Caucasian breast adenocarcinoma (MDA-MB-231), and neuroblastoma (SH-SY5Y). In these cell lines, D-allose treatment inhibited cell growth. Importantly, D-allose treatment decreased glucose uptake, as measured by the uptake of 2-deoxy D-glucose. Moreover, the reporter assays showed that D-allose decreased the expression of luciferase through the hypoxia response element present in the tested promoter region. These results suggest that D-allose may cause the inhibition of cancer growth by reducing both GLUT1 expression and glucose uptake.

Keywords: D-allose; glucose transporter 1; hypoxia response element; rare sugar; thioredoxin interacting protein
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Introduction

Glucose is a major energy source for mammalian cells and is transported into cells via cell-specific expression of various glucose transporters (GLUTs). So far, 14 GLUTs have been found in humans and include transporters for fructose, urate, and myoinositol (Chen et al. 2015). Of those, GLUT1 is involved in the facilitated diffusion of glucose into cells and maintains basal glucose transport in most types of cells. Over-expression of GLUT1 as well as GLUT2, 3, 4, and 5 has been reported in many cancer cell lines such as HeLa (cervical cancer cells), Caco-2 (colorectal cancer cells), HL-60 (leukemia), HepG2 (hepatocellular carcinoma cells), and HTB63 (skin cancer cells) (Medina and Owen 2002). Therefore, the detection of increased glucose (^{18}F-deoxy-glucose) uptake by positron emission tomography is used for diagnosing tumors and their metastases (Reske et al. 1997; Hiyoshi et al. 2009). It was reported that GLUT1 expression alters cell growth especially in tumor cells (Young et al. 2011). In many cancer cells, for example bladder, brain, breast, lung and more, the expression of GLUT1 seems to be predominant (Medina and Owen 2002). GLUT1 expression is regulated by a number of different mechanisms, for example, transcription factors such as Sp1, Sp3, and p53 (Behrooz and Ismail-Beigi 1997; Schwartzenberg-Bar-Yoseph et al. 2004; Hwang and Ismail-Beigi 2006). In addition, GLUT1 expression and glucose uptake are stimulated by hypoxia via induction of transcription factors, such as hypoxia induction factor 1-alpha (HIF-1-alpha), which increases GLUT1 promoter activity (Hayashi et al. 2004). The binding site of HIF-1-alpha, namely the hypoxia response element (HRE), is present in the human and rat GLUT1 promoter regions (Behrooz and Ismail-Beigi 1997; Zelzer et al. 1998). Recently, Wu et al. (2013) reported that knockdown of thioredoxin interacting protein (TXNIP) expression increased GLUT1 expression in HepG2 hepatocellular carcinoma cells, but the details of the regulatory mechanism

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are unknown. TXNIP was originally cloned as a 1α,25-dihydroxy vitamin D_3-inducible protein in HL-60 cells (Chen and DeLuca 1994). TXNIP interacts with thioredoxin via mixed disulfide formation and thus inhibits thioredoxin function (Nishiyama et al. 1999). TXNIP is also a tumor suppressor, and its expression is down-regulated in many cancer cells such as colorectal and gastric cancer cells (Ikarashi et al. 2002; Takahashi et al. 2007).

We have previously shown that the rare sugar D-allose strongly induces TXNIP and inhibits cancer cell growth (Sui et al. 2005a). Both D-allose and D-glucose are monosaccharides, and D-allose is a C-3 epimer of D-glucose with 80% of the sweetness of sucrose. D-allose is one of many rare sugars that exist in very small quantities in nature (Izumori 2002), and our group has reported its unique properties such as anti-inflammatory (Gao et al. 2013), anti-oxidative (Ishihara et al. 2011), and inhibitory effects on osteoclast differentiation (Yamada et al. 2012). D-allose inhibits the growth of many types of cancer cells, such as human hepatocellular carcinoma (HuH-7), HepG2, human gastric cancer (OVCAR3), and HeLa cells (Sui et al. 2005a, b) and reduces the tumor volume in *in vivo* experiments (Hoshikawa et al. 2010). This inhibitory effect has been partially explained by induction of TXNIP and subsequent stabilization of $p27^{kip1}$, a cell-cycle regulatory protein that causes cell cycle arrest at the G1/S transition phase (Yamaguchi et al. 2008). As D-allose significantly induces TXNIP, we hypothesized that it may also affect GLUT1 expression and glucose uptake into cancer cells, resulting in growth suppression.

In this study, we examined the anti-cancer effect of D-allose using human cancer cell lines: HuH-7, Caucasian breast adenocarcinoma (MDA-MB-231), and neuroblastoma (SH-SY5Y). These three cancer cell lines were used for *in vitro* cancer research including GLUT1 expression (Medina and Owen 2002; Gunton et al. 2003; Russo et al. 2004). We also used HuH-7 cells to study the regulatory mechanism of GLUT1 expression.

Materials and Methods

Chemicals

Sugars used in this study including D-allose, D-allulose, and D-glucose were supplied by the Rare Sugars Research Center, Kagawa University, Kagawa, Japan. Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan) and Sigma (St. Louis, MO, USA).

Cell culture

HuH-7 cells were purchased from Riken Cell Bank (Tsukuba, Japan). MDA-MB-231 and SH-SY5Y cells were obtained from ATCC (Manassas, VA, USA). These cells were maintained in DMEM (Sigma) supplemented with 10% (v/v) fetal bovine serum (Japan Bioserum, Hiroshima, Japan) and 1% penicillin-streptomycin (GE Healthcare, Chalfont St. Giles, England) at 37°C in a humidified atmosphere of 5% CO_2.

Construction of expression vectors and transfection

The entire coding region of human TXNIP cDNA was amplified from human fetal brain cDNA using primers that incorporate restriction enzyme sites (single underline: EcoRI, double underline: XbaI): sense primer, 5′-GTGAATTCATGGTGATGTTCAAGAAGATCAAG-3′; antisense primer, 5′-TCCTCTAGATCACTGCACATTGTTGTTGAG GAT-3′. The PCR fragment was digested and cloned into the pME-FLAG expression vector (pME-FLAG-TXNIP). For the gene reporter assay, we used the rat GLUT1 promotor region containing HRE consensus sequence that was well characterized (Behrooz and Ismail-Beigi 1997). The rat genomic DNA was isolated from normal rat liver using Quick-gDNA MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. A 200-bp fragment of the rat GLUT1 promoter region that is located ~3.5 kbp upstream of the GLUT1 major transcription start site containing the HRE consensus sequence (Fig. 7C) was amplified by PCR using specific primers that incorporate restriction enzyme sites (single underline: KpnI, double underline: HindIII): 5′-GCCCGGTACCCAACAGAGCCT TTTCCGGGGTGTC-3′; antisense: 5′-GCCAAGCTTGAGCTC CCAGGAACTTGGGGGGAG-3′. The PCR fragment was digested and cloned into the pGL4.16 plasmid (pGL4.16-GLUT1-WT) (Promega, Madison, WI, USA). The mutant HRE consensus site (pGL4.16-GLUT1-MUT) was generated by PCR using this plasmid as a template with the following primers: sense: 5′-CACAGGATA TCTGGCTGACACGCATCAG-3′; antisense: 5′-AGCCAGATATCC TGTGGACCCAAGGCCC-3′. For the HRE reporter assay, the HRE luciferase reporter vector (pGL4.42-HRE) and pGL4.74 vector were purchased from Promega.

Gene knock-down by small interfering RNA (siRNA)

HuH-7 cells (20,000 cells) were cultured in 35 mm dish. For the knock-down experiment, 5 nM TXNIP siRNA (5′-UGCUCGAAU UGACAGAAAATT-3′, Cosmo Bio Co. Ltd., Tokyo, Japan) or negative control siRNA (Scramble siRNA, Ambion, Applied Biosystems) was transfected with Hiperfect transfection reagent (Qiagen) according to the manufacture's protocol. After 18 hours, we added D-allose where the final concentration was 50 mM. 72 hours after adding D-allose, the plasmids were transfected again to increase the transfection efficiency and the cells were further incubated for another 4 days (altogether, D-allose treatment was for 7 days).

Western blotting analysis

Sugars were added, and cells were cultured for 7 days at 37°C. Then, cells were washed with PBS, scraped into lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton-X100, 0.5% NP-40) containing protease inhibitor cocktail (Sigma), and sonicated. Samples were centrifuged for 10 min at 15,000 rpm at 4°C, and the supernatants were collected. Proteins were separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose membranes, blocked with 5% (w/v) non-fat dried milk in Tris-Buffered Saline and Tween 20 (TTBS), and incubated with anti-GLUT1 (Millipore, Billerica, MA, USA) in 5% (w/v) non-fat dried milk in TTBS at a dilution of 1:2,000, anti-TXNIP (MBL, Nagoya, Japan) in Can Get Signal Immunoreaction Enhancer Solution (TOYOBO, Osaka, Japan) at a dilution of 1:1,000, or anti-β-actin antibody (Sigma) in 5% (w/v) non-fat dried milk in TTBS at a dilution of 1:5,000. Membranes were washed and probed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (GE Healthcare) at a dilution of 1:5,000 each, and signals were detected using Immobilon Western chemilumines-

cent horseradish peroxidase substrate (Millipore).

Real-time quantitative PCR

Total RNA was isolated using an RNeasy Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using an Omniscript RT Kit (Qiagen) with random hexamers. Real-time quantitative PCR was carried out using Taqman gene expression assay primers and the 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in duplicate. The β-actin gene was used to normalize across the assay and runs, and the threshold value (Ct) for each sample was used to determine the expression level of the gene.

Cell proliferation assay

The cells were grown in 96-well plates at 5,000 cells/well in 0.1 mL medium and cultured for 24 hours. D-allose (50 mM) was added to the medium, and the cells were cultured up to 7 days. Cell proliferation was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

Plasmid transfection and Luciferase reporter gene assay

For the TXNIP over-expression study, HuH-7 cells were transfected with empty vector or pME-FLAG-TXNIP plasmid using FuGENE 6 transfection reagent. After 72 hours, these plasmids were transfected again to increase the transfection efficiency (Ishikawa and Homcy 1992), and cells were further incubated for 4 days (7 days in total).

For the gene reporter assay, HuH-7 cells (20,000 cells) were seeded in 24-well plates in 500 μL medium and co-transfected with pGL4.16-GLUT1-WT, pGL4.16-GLUT1-MUT, or pGL4.42-HRE (200 ng/well) plus pGL4.74 (20 ng/well). After 24 hours, the medium was changed to with or without 50 mM D-allose. Cells were further incubated for 24 hours. Luciferase activity was measured using cell lysates and the Dual-Glo Luciferase Assay System (Promega). Values for luciferase activity were normalized using Renilla luciferase data. The luciferase activity was compared with the control group (without D-allose treatment) and was shown as % ratio to the control group (Farrell et al. 2010).

To study the effect of TXNIP over-expression on GLUT1 or HRE promoter activity, HuH-7 cells (20,000 cells/well) were seeded in 24-well plates and transfected with empty or pME-FLAG-TXNIP plasmid (1,000 ng/well). After 48 hours, the medium was changed, and the transfection was carried out using the same plasmids. Cells were further cultured for 24 hours, and the luciferase activity was measured.

Glucose uptake measurement

HuH-7 cells were incubated with or without 50 mM D-allose for 7 days in a 6-well plate. After 7 days, the medium was removed and cells were incubated with serum-free DMEM for 6 hours and then washed twice with Krebs-Ringer-phosphate-HEPES (KRPH) buffer (30 mM HEPES, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 118 mM NaCl, 5 mM KCl at pH 7.5, warmed to 37°C). The cells were further incubated with KRPH buffer containing 2% bovine serum albumin (Sigma) and 1 mM 2-deoxy glucose (2-DG; Sigma) for 20 min. Then, cells were washed three times with ice-cold PBS containing 200 μM phloretin and collected in 3 mL of 10 mM Tris-HCl buffer (pH 8.0). Collected cells were sonicated, heated at 80°C for 15 min, and centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was diluted five times with 10 mM Tris-HCl buffer, and the concentration of 2-DG was measured using the 2-DG Uptake Measurement Kit (Cosmo Bio) according to the manufacturer's protocol. To examine the effect of TXNIP over-expression, HuH-7 cells were transfected with empty vector or pME-FLAG-TXNIP plasmid. After transfection, cells were cultured for 7 days and used for the assay. The amount of 2-DG was measured by the same method as following D-allose treatment.

Statistical analyses

Data are presented as the means ± standard error (SE) of at least three independent experiments. Significant differences among the groups were determined by one-way analysis of variance, and differences between groups were analyzed by the Student's t-test. A P-value of < 0.05 was considered significant.

Results

D-allose up-regulates TXNIP and down-regulates GLUT1 expression in cancer cells

We first analyzed the expression of GLUT1 and TXNIP in HuH-7, MDA-MB-231, and SH-SY5Y cells after 7 days of sugar treatment. Western blot analysis showed that 50 mM D-allose significantly decreased the GLUT1 level (42.32 ± 11.41% in HuH-7, 62.58 ± 5.49% in MDA-MB-231, and 64.40 ± 9.43% in SH-SY5Y cells; n = 3 each). Individual treatment with 50 mM D-allulose or 50 mM D-glucose slightly modulated GLUT1 expression (113.24 ± 8.30% in HuH-7, 77.25 ± 12.47% in MDA-MB-231, and 92.68 ± 4.77% in SH-SY5Y cells; n = 3 each and 95.41 ± 3.13% in HuH-7, 62.76 ± 15.93% in MDA-MB-231, and 86.03 ± 20.36% in SH-SY5Y cells; n = 3 each, respectively). On the other hand, the TXNIP level was increased dramatically by 50 mM D-allose compared with that of control (no sugar) in all cell lines examined (1070.01 ± 297.10% in HuH-7, 537.26 ± 65.18% in MDA-MB-231, and 456.36 ± 69.72% in SH-SY5Y cells; n = 3 each) (Fig. 1). However, individual treatment with D-allulose or D-glucose (50 mM each) slightly increased TXNIP expression (147.39 ± 52.07% in HuH-7, 143.02 ± 25.80% in MDA-MB-231, and 181.06 ± 56.51% in SH-SY5Y cells; n = 3 each and 395.09 ± 182.42% in HuH-7, 242.04 ± 70.17% in MDA-MB-231, and 199.55 ± 66.37% in SH-SY5Y cells; n = 3 each, respectively).

Next, we examined the dose-dependent effect of D-allose and observed that both the decrease in GLUT1 and increase in TXNIP expression were largely dose dependent. The levels of GLUT1 expression following 12.5 and 25 mM D-allose treatment were 78.45 ± 21.14% and 59.67 ± 15.68% in HuH-7, 83.80 ± 27.32% and 90.22 ± 13.99% in MDA-MB-231, and 105.89 ± 25.26% and 86.33 ± 10.28% in SH-SY5Y cells, respectively (n = 3 each). The levels of TXNIP expression following 12.5 and 25 mM D-allose treatment were 578.47 ± 48.13% and 759.88 ± 97.33% in HuH-7, 345.22 ± 44.79% and 425.83 ± 35.98% in MDA-MB-231, and 394.72 ± 103.95% and 435.17 ± 93.18% in SH-SY5Y cells, respectively (n = 3 each).

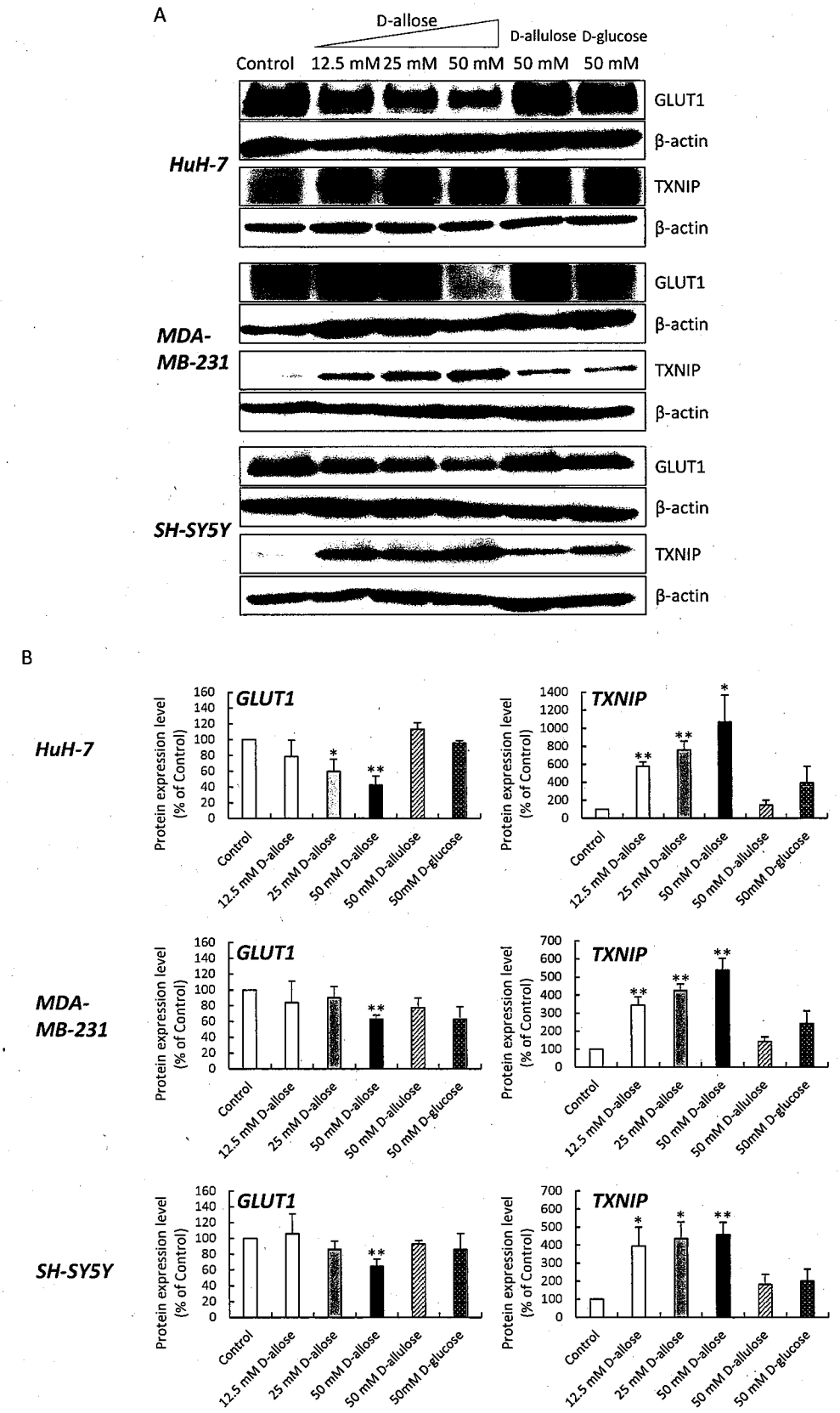

Fig. 1. The effect of sugars on the expression levels of GLUT1 and TXNIP proteins.
(A) HuH-7 cells, MDA-MB-231 cells, and SH-SY5Y cells were treated with various concentrations of D-allose (12.5, 25, and 50 mM), D-allulose (50 mM), or D-glucose (50 mM) and cultured for 7 days. Western blot analysis was performed using anti-GLUT1, anti-TXNIP, and anti-β-actin antibodies. (B) Densitometric analysis. The protein level was quantified using ImageJ software. Data are shown as the value relative to the control group, which was defined as 100%. Results are expressed as the means ± SE (n = 3 each). *P < 0.05, **P < 0.01.

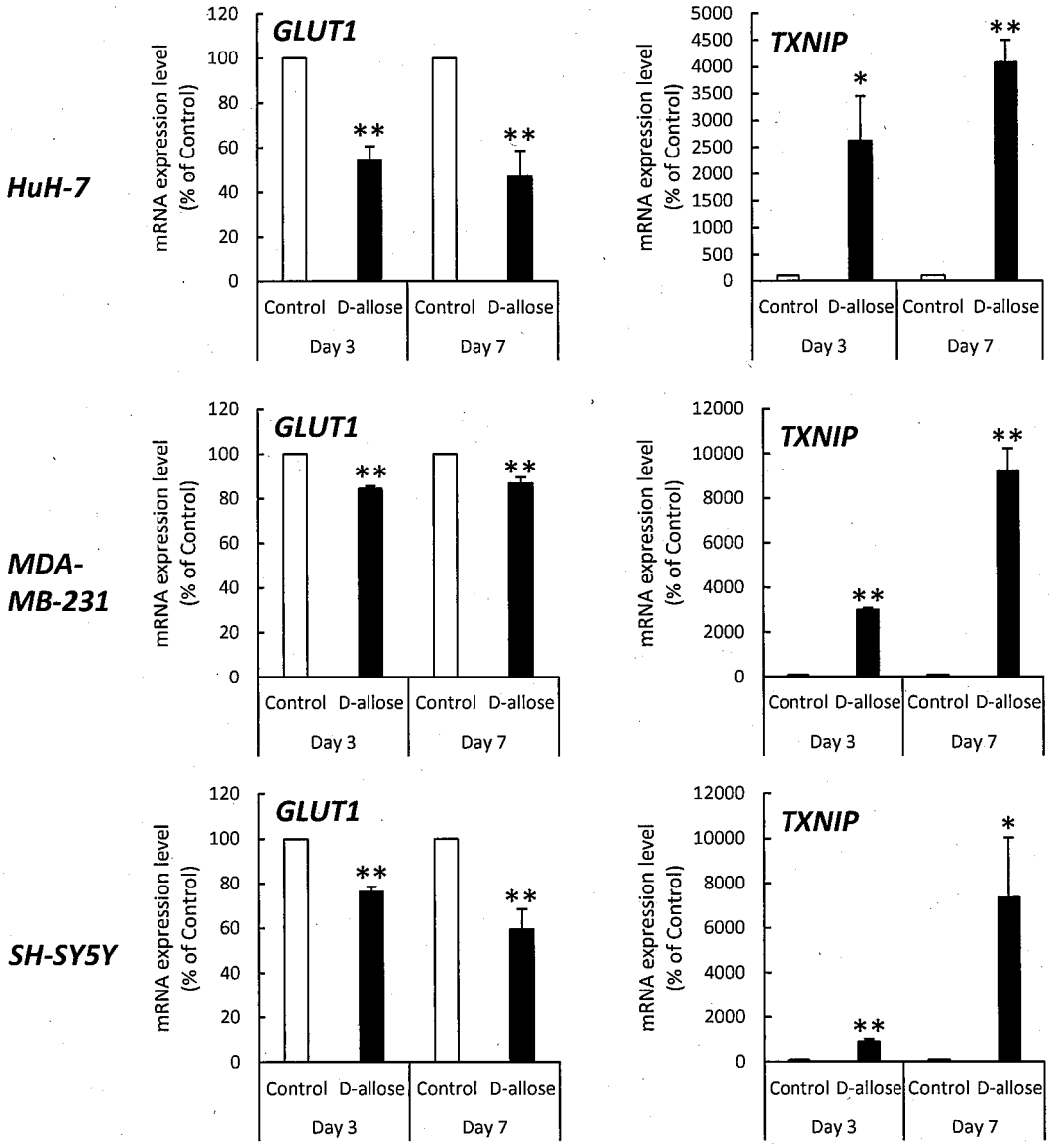

Fig. 2. The effect of D-allose on the expression levels of GLUT1 and TXNIP mRNAs.
Cells were treated with or without D-allose (50 mM) and cultured for 3 or 7 days (n = 3-4 each). Real-time PCR was carried out using Taqman gene expression assays. Data are the means ± SE (n = 3 each). *P < 0.05, **P < 0.01.

We also examined the mRNA expression of GLUT1 and TXNIP with real-time PCR (Fig. 2). HuH-7, MDA-MB-231, and SH-SY5Y cells were treated with 50 mM D-allose, and the cells were incubated for 3 and 7 days. The levels of GLUT1 were significantly decreased (54.20 ± 6.50% in HuH-7, 84.29 ± 1.20% in MDA-MB-231, 76.22 ± 2.29% in SH-SY5Y cells on Day 3, and 47.03 ± 11.54% in HuH-7, 86.79 ± 2.76% in MDA-MB-231, 59.30 ± 9.13% in SH-SY5Y cells on Day 7) compared with control, and TXNIP expression was significantly increased (2,619.39 ± 832.74% in HuH-7, 2,999.08 ± 67.01% in MDA-MB-231, 898.90 ± 112.73% in SH-SY5Y cells on Day 3, 4,082.39 ± 420.31% in HuH-7, 9,215.41 ± 1,008.17% in MDA-MB-231, 7,345.26 ± 2,673.77% in SH-SY5Y cells on Day 7; n = 3-4 each).

The knock-down of TXNIP by siRNA markedly reduced the TXNIP level (Fig. 3A). When the protein level of TXNIP of the control (without D-allose treatment) of the scramble siRNA-transfected sample (scramble group, n=3) was taken as 100%, D-allose markedly enhanced TXNIP expression to 35 fold (3,468.92 ± 876.13%, n=3) (Fig. 3B). TXNIP siRNA significantly reduced both basal and D-allose-induced TXNIP levels to 34.72 ± 12.87% and 261.34 ± 145.78%, respectively.

When the protein level of GLUT1 of the control (without D-allose treatment) of the scramble group was taken as 100%, D-allose significantly reduced GLUT1 to 76.31 ± 2.17%. TXNIP knock-down significantly increased GLUT1 level to 130.60 ± 4.43%, and D-allose treatment reduced GLUT1 level to 85.15 ± 0.71%. This GLUT1 level was

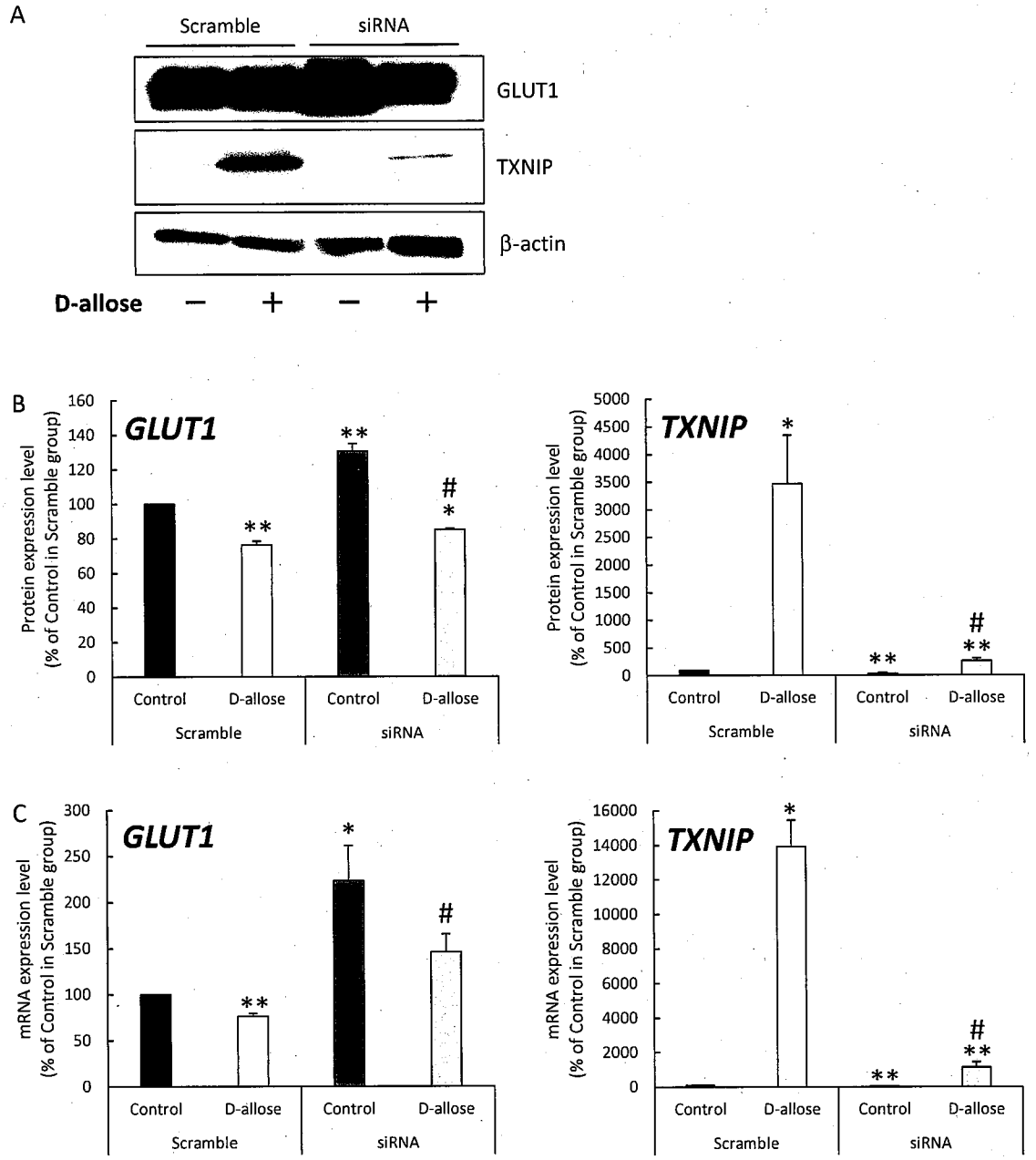

Fig. 3. The effect of D-allose on the expression of GLUT1 and TXNIP in TXNIP-knock-down HuH-7 cells.
HuH-7 cells were transfected with siRNA or negative control (scramble group) and were further cultured with or without D-allose (50 mM) and cultured for 7 days (n = 3 each). (A) Samples were used for western blot analysis with anti-GLUT1 or anti-TXNIP antibody. (B) The protein level was quantified using ImageJ software. Data are the percentage of control in the scramble group. Results are expressed as the means ± SE (n = 3 each). *P < 0.05, **P < 0.01 when compared with control in the scramble group. #P < 0.05 when compared with D-allose in the scramble group. (C) Real-time PCR analysis was carried out using Taqman gene expression assays. Data are shown as a percentage of control in the scramble group. Results are expressed as the means ± SE (n = 3 each). *P < 0.05, **P < 0.01 when compared with control in the scramble group. #P < 0.05 when compared with D-allose in the scramble group.

significantly higher than the one of D-allose in the scramble group. The analysis of mRNA levels of TXNIP and GLUT1 supported the changes of both proteins (Fig. 3C). D-allose significantly enhanced TXNIP mRNA level to 140 fold in the scramble group and to only 4.4 fold in the presence of TXNIP siRNA. GLUT1 mRNA level was significantly reduced by D-allose to 0.76 fold. In the presence of TXNIP siRNA, basal GLUT1 mRNA level was increased to 2.2 fold than that of the scramble group and D-allose treatment made this enhancement lower to 1.5 fold.

D-allose inhibits cancer cell proliferation

As D-allose down-regulated GLUT1 expression in the cancer cell lines examined, we next measured the effect of

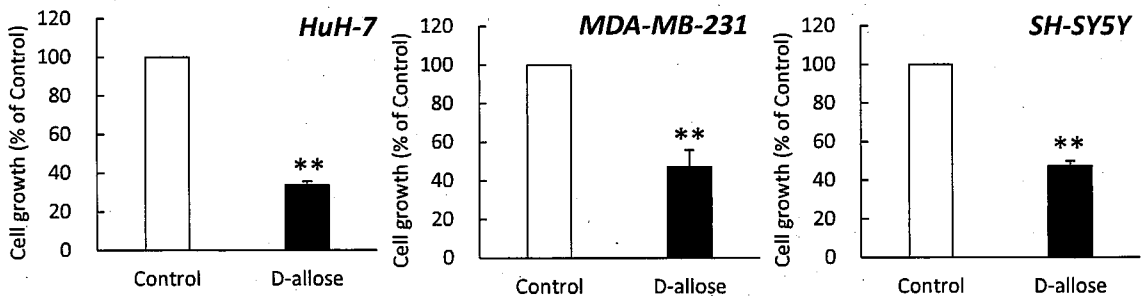

Fig. 4. Cell proliferation assay.
D-allose (50 mM) was added to the culture medium of HuH-7, MDA-MB-231, and SH-SY5Y cells. Cells were incubated for 7 days, and cell proliferation was measured using the Cell Counting Kit-8. Data are shown as the value relative to the control group. Results are expressed as the means ± SE (n = 3 each). **P < 0.01.

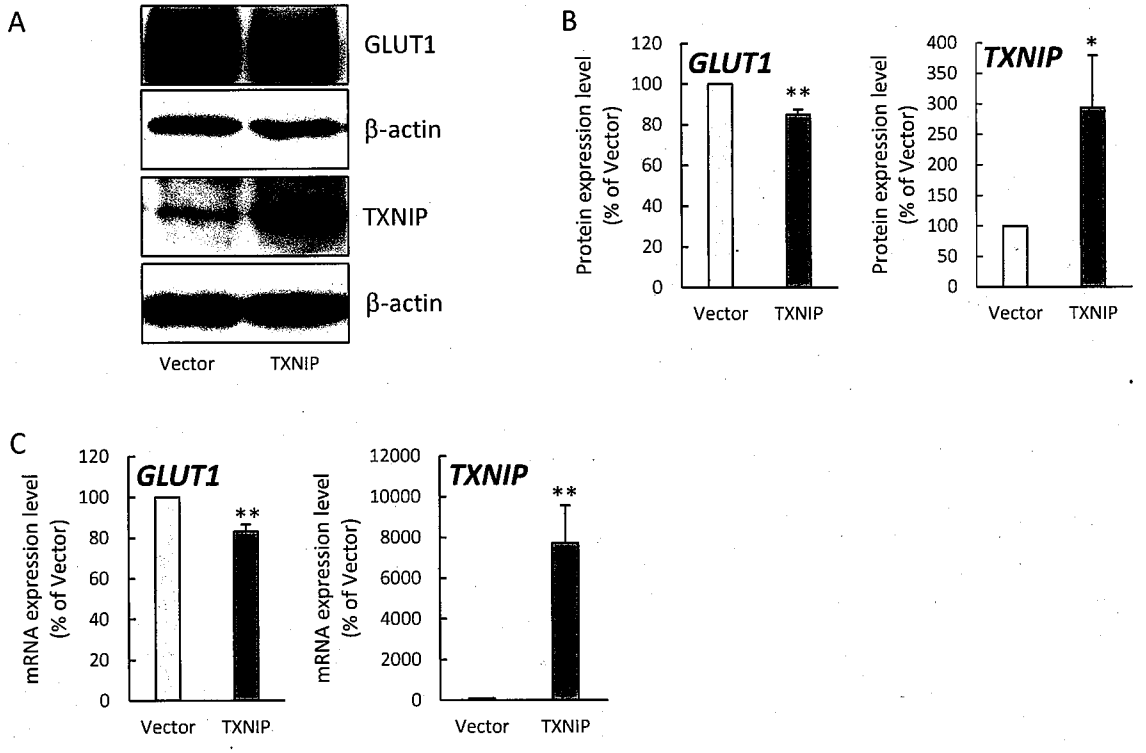

Fig. 5. The effect of TXNIP over-expression on GLUT1 expression.
HuH-7 cells were transfected with pME-FLAG-TXNIP or empty plasmid and were further cultured for 7 days. (A) Samples were used for western blot analysis with anti-GLUT1 or anti-TXNIP antibody. (B) The protein level was quantified using ImageJ software. Data are the percentage of the empty vector group. Results are expressed as the means ± SE (n = 3 each). *P < 0.05, **P < 0.01. (C) Real-time PCR analysis was carried out using Taqman gene expression assays. Data are shown as a percentage of the empty vector group. Results are expressed as the means ± SE (n = 3 each). *P < 0.05, **P < 0.01.

D-allose on cell proliferation after 7 days of D-allose treatment (Fig. 4). D-allose significantly suppressed the proliferation of HuH-7, MDA-MB-231, and SH-SY5Y cells compared with that of control (33.66 ± 2.07%, 47.07 ± 8.66%, and 47.18 ± 2.66% of control, respectively; n = 3 each).

Over-expression of TXNIP down-regulates GLUT1 expression

As D-allose strongly induced TXNIP expression, we further analyzed the effect of TXNIP over-expression on HuH-7 cells because the reduction in GLUT1 expression by D-allose was prominent in this cell line. After transfection with pME-FLAG-TXNIP or empty plasmid, cells were cultured for 7 days and examined. Western blot analysis showed that over-expression of TXNIP significantly decreased the level of GLUT1 protein to 84.84 ± 2.58% of control (n = 4) (Fig. 5A, B). The mRNA level of GLUT1 was decreased to 83.08 ± 3.40% of control (n = 4) (Fig. 5C). These data indicated that TXNIP is involved in the negative regulation of GLUT1 expression via its transcrip-

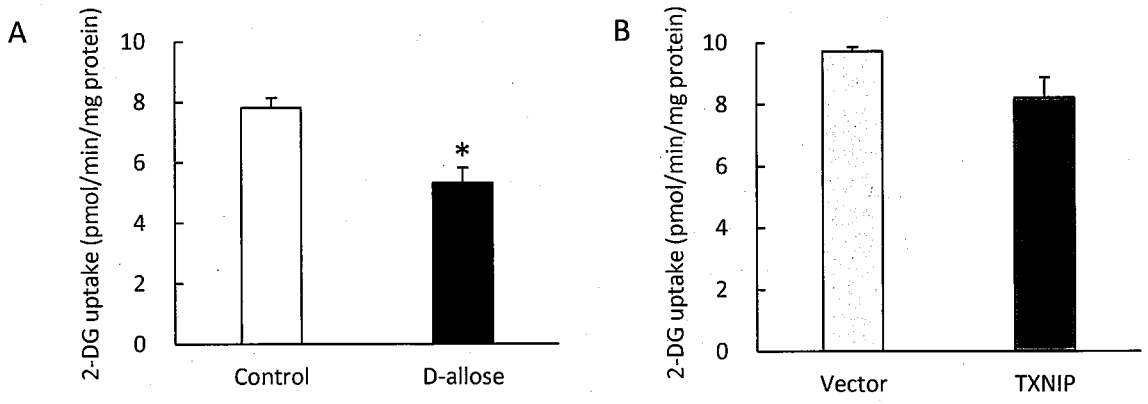

Fig. 6. The effect of D-allose and TXNIP over-expression on glucose uptake.
(A) HuH-7 cells were treated with or without D-allose (50 mM) and cultured for 7 days. (B) Cells were transfected with pME-FLAG-TXNIP or empty plasmid and cultured for 7 days. Glucose uptake was measured, and the data were normalized to the protein level. Data are presented as the means ± SE (n = 3 each). *P < 0.05.

tional activity.

D-allose treatment decreases glucose uptake

As D-allose or TXNIP over-expression decreased GLUT1 expression, we measured glucose uptake in HuH-7 cells. D-allose treatment significantly decreased glucose uptake from 7.81 ± 0.33 to 5.33 ± 0.50 pmol/min/mg protein ($P < 0.05$, n = 3 each) (Fig. 6A). Over-expression of TXNIP also decreased glucose uptake from 9.73 ± 0.13 to 8.23 ± 0.65 pmol/min/mg protein (n = 3 each) although the difference was not significant (Fig. 6B).

D-allose or TXNIP over-expression inhibits the GLUT1 promoter activity via HRE

Wu et al. (2013) reported that TXNIP down-regulates the HRE-driven promoter activity of vascular endothelial growth factor (VEGF). Malm et al. (2015) reported that D-allose decreased the HRE luciferase activity. In addition, the promoter region of GLUT1 contains an HRE consensus sequence (Behrooz and Ismail-Beigi 1997). From these observations, we have hypothesized that D-allose or TXNIP over-expression inhibits the HRE-driven promoter activity, which in turn causes the decrease in GLUT1 expression. Accordingly, we used two types of the model promoter containing the HRE: the HRE-luciferase reporter plasmid and the rat GLUT1 gene promoter. Each construct was introduced into HuH-7 cells, and the luciferase activity was measured. D-allose treatment decreased the HRE luciferase activity to 61.87 ± 11.76% of the control, indicating negative regulation by D-allose ($P < 0.01$, Fig. 7A). We also studied the effect of TXNIP over-expression on HRE-driven promoter activity. Over-expression of TXNIP significantly decreased the HRE luciferase activity to 60.97 ± 4.73% of control ($P < 0.01$, Fig. 7B). Next, we examined the effect of D-allose or TXNIP over-expression on GLUT1 promoter activity. D-allose treatment significantly decreased the GLUT1 promoter activity to 47.18 ± 6.56% of control ($P < 0.01$, n = 3 each), but not the GLUT1 promoter activity containing the mutated HRE (Fig. 7D). In contrast, TXNIP over-expression significantly decreased GLUT1 promoter activity to 60.35 ± 1.81% of control ($P < 0.01$) and the mutated HRE consensus sequence activity to 68.14 ± 4.17% of control (n = 3 each) (Fig. 7E). These results suggest that D-allose or TXNIP over-expression decreased the expression of luciferase under the control of the rat GLUT1 gene promoter probably through the different mechanism.

Discussion

As we hypothesized, D-allose down-regulated the GLUT1 protein level in all three cell lines examined (HuH-7, MDA-MB-231, and SH-SY5Y). In these cell lines, a massive induction of TXNIP by D-allose was observed (Fig. 1). The data showed a negative correlation between GLUT1 and TXNIP expression, and D-allose dose dependently increased these effects. On the contrary, the effects of neither D-allulose nor D-glucose were significant. D-glucose induces TXNIP expression (Minn et al. 2005), and similarly, D-glucose treatment in the present study induced a non-significant increase in the expression of TXNIP and a slight decrease in GLUT1 expression. However, strong expression of TXNIP was observed with D-allose treatment, although D-glucose treatment had a stronger effect in comparison to D-allulose treatment.

Beside protein levels, D-allose down-regulated the GLUT1 mRNA level in all three cell lines after 3 and 7 days. In contrast, the TXNIP mRNA level was dramatically increased by D-allose treatment in all three cell lines (Fig. 2). This result suggests that the TXNIP induction by D-allose may cause down-regulation of GLUT1 expression at the transcriptional level.

Next, we examined cell proliferation after D-allose treatment (Fig. 4) and observed significant inhibition of cell proliferation in all these cell lines with a prominent effect in HuH-7 cells. The reduction in GLUT1 expression in HuH-7 cells significantly decreased the 2-DG uptake (Fig. 6), indicating that D-allose likely affects cancer cell growth

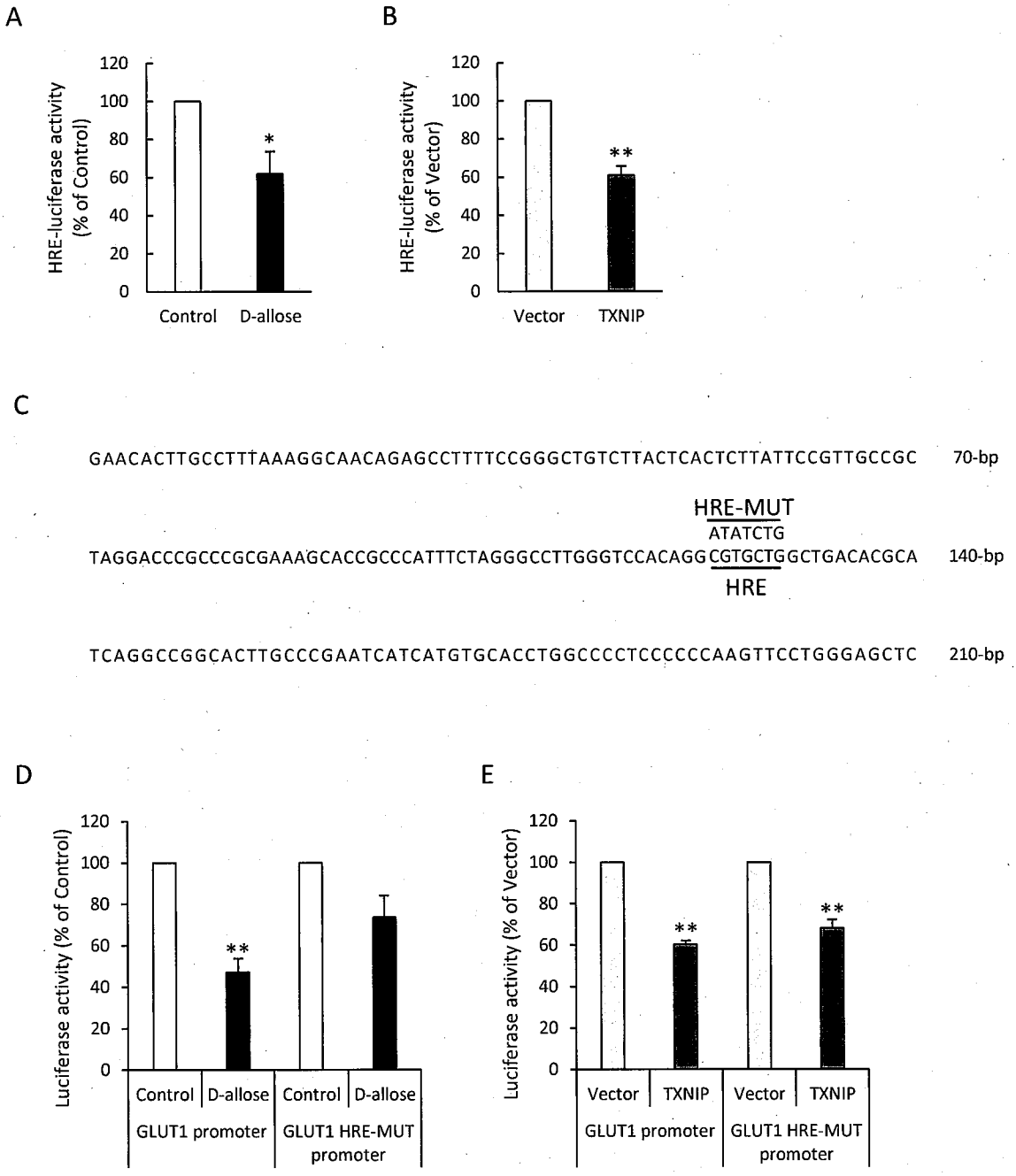

Fig. 7. The effect of D-allose or TXNIP over-expression on HRE or GLUT1 promoter activity.
(A) HuH-7 cells were seeded in 24-well plates and co-transfected with pGL4.42-HRE or empty plasmid plus the pGL4.74 vector for normalization. D-allose (50 mM) was added, and the luciferase activity was measured after 24 hours using a Dual-Glo Luciferase Assay System. Data are the means ± SE (n = 3-4). (B) HuH-7 cells were transfected with pME-FLAG-TXNIP or empty plasmid. After 48 hours, luciferase activity was measured. Data are the means ± SE (n = 3-4). (C) The promoter region of the rat GLUT1 gene containing the HRE consensus site is shown. HRE-MUT: The consensus sequence of the HRE site was destroyed by four nucleotide mutations. (D) The wild-type GLUT1 gene reporter plasmid (pGL4.16-GLUT1-WT) or HRE mutated GLUT1 gene reporter plasmid (pGL4.16-GLUT1-MUT) was transfected, D-allose was added to the medium, and luciferase activity was measured. (E) HuH-7 cells were transfected with the wild-type GLUT1 gene reporter plasmid (pGL4.16-GLUT1-WT) or HRE mutated GLUT1 gene reporter plasmid (pGL4.16-GLUT1-MUT), along with pME-FLAG-TXNIP, and luciferase activity was measured. *P < 0.05, **P < 0.01.

via a reduction in GLUT1 expression. Expression of other GLUTs such as GLUT2, 3, 4, and 5 may occur to compensate for the reduction in GLUT1. However, the result of the glucose uptake assay indicated that such compensation, if present, was not sufficient to overcome the attenuation of glucose uptake via a significant reduction in GLUT1

expression. Notably, we have previously shown the effect of D-allose using normal rat primary hepatocyte, where D-allose did not show any effect on the normal cells (Yamaguchi et al. 2008).

GLUT1 expression is regulated by different mechanisms, and the cis-element for transcription factors such as Sp1, Sp3, and p53 is present in the GLUT1 promoter region (Behrooz and Ismail-Beigi 1997). The Sp1 site is essential for the positive response to hyperosmolarity and is located in the proximal region of the GLUT1 promoter. Another report showed that the Sp1 site is necessary for basal expression of the GLUT1 gene (Hwang and Ismail-Beigi 2006). In contrast, the function of the Sp3 site is to repress GLUT1 promoter activity (Fandos et al. 1999). These effects are opposite, and an increase in the Sp3/Sp1 ratio reduces transcriptional activity of the GLUT1 gene. P53 is a well-known tumor suppressor protein that induces apoptosis (Oren 1999), and p53 expression may repress GLUT1 promoter activity (Schwartzenberg-Bar-Yoseph et al. 2004).

A previous study showed that GLUT1 promoter activity was modulated by HIF transcriptional activity (Chen et al. 2001). HRE-driven luciferase activity was enhanced by hypoxic conditions, and GLUT1 expression was stimulated by up-regulated HRE activity (Hayashi et al. 2004). Farrell et al. (2010) reported that TXNIP inhibited HRE-driven luciferase activity in a SV-40 transformed alveolar epithelial cell line (MLE-12) and the expression of the HIF target gene, VEGF. In addition to D-allose treatment, TXNIP over-expression also induced a significant decrease in GLUT1 expression (Fig. 5). Furthermore, the TXNIP knock-down using siRNA cancelled the D-allose-induced suppression of GLUT1 expression (Fig. 3). Thus, these data confirm that TXNIP plays an important role in the regulation of GLUT1 expression.

In the rat GLUT1 promoter region, a 490-bp segment located at approximately −3.5 kbp is highly homologous to the mouse GLUT1 enhancer and mediates the response to cobalt chloride (Behrooz and Ismail-Beigi 1997). This 490-bp segment also contains several regulatory elements including HRE, two phorbol ester response elements, two SP1s, a serum response element, and p53 binding sites. We thus used the rat GLUT1 promoter containing the HRE to see whether D-allose or TXNIP over-expression inhibits the promoter activity. D-allose treatment significantly decreased GLUT1 promoter activity via the HRE (Fig. 7D), whereas TXNIP significantly decreased GLUT1 promoter activity even through the mutated HRE (Fig. 7E). Thus, TXNIP may also reduce the expression of luciferase. Moreover, since TXNIP binds to thioredoxin, the TXNIP-mediated modulation of GLUT1 expression may be repressed by the increase of thioredoxin expression. However, the effect of thioredoxin and other associated factors that can bind to TXNIP will have to be studied.

We previously reported the growth inhibitory effect of D-allose via stabilization of the $p27^{kip1}$ protein induced by TXNIP and induction of G1 cell cycle arrest (Yamaguchi et al. 2008). In addition, the present study revealed the reduction in GLUT1 expression by D-allose treatment, suggesting that this effect may contribute to the growth inhibitory effect of D-allose. However, the treatment with D-allose did not cause apoptosis (Yamaguchi et al. 2008). By tunnel staining with TXNIP transfection, we also confirmed no apoptotic change (data not shown).

In conclusion, the down-regulation of GLUT1 expression by D-allose is mediated by the up-regulation of TXNIP and the suppression of HRE-driven promoter activity of GLUT1. As GLUT1 is over-expressed in various types of cancer cells and D-allose is a strong inducer of TXNIP, D-allose may inhibit GLUT1 expression in cancer cells and may thus be an effective treatment for cancer. Further research regarding the effect of D-allose on HIF target genes such as VEGF will be helpful for understanding the detailed mechanism of the effects of D-allose on cancer cell growth.

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Conflict of Interest

The authors declare no conflict of interest.

References

Behrooz, A. & Ismail-Beigi, F. (1997) Dual control of glut1 glucose transporter gene expression by hypoxia and by inhibition of oxidative phosphorylation. *J. Biol. Chem.*, **272**, 5555-5562.

Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F. & Maity, A. (2001) Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. *J. Biol. Chem.*, **276**, 9519-9525.

Chen, K.S. & DeLuca, H.F. (1994) Isolation and characterization of a novel cDNA from HL-60 cells treated with 1,25-dihydroxyvitamin D-3. *Biochim. Biophys. Acta*, **1219**, 26-32.

Chen, L.Q., Cheung, L.S., Feng, L., Tanner, W. & Frommer, W.B. (2015) Transport of sugars. *Annu. Rev. Biochem.*, **84**, 865-894.

Fandos, C., Sanchez-Feutrie, M., Santalucia, T., Vinals, F., Cadefau, J., Guma, A., Cusso, R., Kaliman, P., Canicio, J., Palacin, M. & Zorzano, A. (1999) GLUT1 glucose transporter gene transcription is repressed by Sp3. Evidence for a regulatory role of Sp3 during myogenesis. *J. Mol. Biol.*, **294**, 103-119.

Farrell, M.R., Rogers, L.K., Liu, Y., Welty, S.E. & Tipple, T.E. (2010) Thioredoxin-interacting protein inhibits hypoxia-inducible factor transcriptional activity. *Free Radic. Biol. Med.*, **49**, 1361-1367.

Gao, D., Kawai, N., Nakamura, T., Lu, F., Fei, Z. & Tamiya, T. (2013) Anti-inflammatory effect of D-allose in cerebral ischemia/reperfusion injury in rats. *Neurol. Med. Chir. (Tokyo)*, **53**, 365-374.

Gunton, J.E., Delhanty, P.J., Takahashi, S. & Baxter, R.C. (2003) Metformin rapidly increases insulin receptor activation in human liver and signals preferentially through insulin-receptor substrate-2. *J. Clin. Endocrinol. Metab.*, **88**, 1323-1332.

Hayashi, M., Sakata, M., Takeda, T., Yamamoto, T., Okamoto, Y., Sawada, K., Kimura, A., Minekawa, R., Tahara, M., Tasaka, K. & Murata, Y. (2004) Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under

hypoxic conditions in trophoblast-derived cells. *J. Endocrinol.*, **183**, 145-154.

Hiyoshi, Y., Watanabe, M., Imamura, Y., Nagai, Y., Baba, Y., Yoshida, N., Toyama, E., Hayashi, N. & Baba, H. (2009) The relationship between the glucose transporter type 1 expression and F-fluorodeoxyglucose uptake in esophageal squamous cell carcinoma. *Oncology*, **76**, 286-292.

Hoshikawa, H., Mori, T. & Mori, N. (2010) In vitro and in vivo effects of D-allose: up-regulation of thioredoxin-interacting protein in head and neck cancer cells. *Ann. Otol. Rhinol. Laryngol.*, **119**, 567-571.

Hwang, D.Y. & Ismail-Beigi, F. (2006) Control of Glut1 promoter activity under basal conditions and in response to hyperosmolarity: role of Sp1. *Am. J. Physiol. Cell Physiol.*, **290**, C337-C344.

Ikarashi, M., Takahashi, Y., Ishii, Y., Nagata, T., Asai, S. & Ishikawa, K. (2002) Vitamin D3 up-regulated protein 1 (VDUP1) expression in gastrointestinal cancer and its relation to stage of disease. *Anticancer Res.*, **22**, 4045-4048.

Ishihara, Y., Katayama, K., Sakabe, M., Kitamura, M., Aizawa, M., Takara, M. & Itoh, K. (2011) Antioxidant properties of rare sugar D-allose: Effects on mitochondrial reactive oxygen species production in Neuro2A cells. *J. Biosci. Bioeng.*, **112**, 638-642.

Ishikawa, Y. & Homcy, C.J. (1992) High efficiency gene transfer into mammalian cells by a double transfection protocol. *Nucleic Acids Res.*, **20**, 4367.

Izumori, K. (2002) Bioproduction strategies for rare hexose sugars. *Naturwissenschaften*, **89**, 120-124.

Malm, S.W., Hanke, N.T., Gill, A., Carbajal, L. & Baker, A.F. (2015) The anti-tumor efficacy of 2-deoxyglucose and D-allose are enhanced with p38 inhibition in pancreatic and ovarian cell lines. *J. Exp. Clin. Cancer Res.*, **34**, 31.

Medina, R.A. & Owen, G.I. (2002) Glucose transporters: expression, regulation and cancer. *Biol. Res.*, **35**, 9-26.

Minn, A.H., Hafele, C. & Shalev, A. (2005) Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces beta-cell apoptosis. *Endocrinology*, **146**, 2397-2405.

Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., Takagi, Y., Sono, H., Gon, Y. & Yodoi, J. (1999) Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J. Biol. Chem.*, **274**, 21645-21650.

Oren, M. (1999) Regulation of the p53 tumor suppressor protein. *J. Biol. Chem.*, **274**, 36031-36034.

Reske, S.N., Grillenberger, K.G., Glatting, G., Port, M., Hildebrandt, M., Gansauge, F. & Beger, H.G. (1997) Overexpression of glucose transporter 1 and increased FDG uptake in pancreatic carcinoma. *J. Nucl. Med.*, **38**, 1344-1348.

Russo, V.C., Kobayashi, K., Najdovska, S., Baker, N.L. & Werther, G.A. (2004) Neuronal protection from glucose deprivation via modulation of glucose transport and inhibition of apoptosis: a role for the insulin-like growth factor system. *Brain Res.*, **1009**, 40-53.

Schwartzenberg-Bar-Yoseph, F., Armoni, M. & Karnieli, E. (2004) The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. *Cancer Res.*, **64**, 2627-2633.

Sui, L., Dong, Y., Watanabe, Y., Yamaguchi, F., Hatano, N., Tsukamoto, I., Izumori, K. & Tokuda, M. (2005a) The inhibitory effect and possible mechanisms of D-allose on cancer cell proliferation. *Int. J. Oncol.*, **27**, 907-912.

Sui, L., Dong, Y., Watanabe, Y., Yamaguchi, F., Hatano, N., Izumori, K. & Tokuda, M. (2005b) Growth inhibitory effect of D-allose on human ovarian carcinoma cells in vitro. *Anticancer Res.*, **25**, 2639-2644.

Takahashi, Y., Masuda, H., Ishii, Y., Nishida, Y., Kobayashi, M. & Asai, S. (2007) Decreased expression of thioredoxin interacting protein mRNA in inflamed colonic mucosa in patients with ulcerative colitis. *Oncol. Rep.*, **18**, 531-535.

Wu, N., Zheng, B., Shaywitz, A., Dagon, Y., Tower, C., Bellinger, G., Shen, C.H., Wen, J., Asara, J., McGraw, T.E., Kahn, B.B. & Cantley, L.C. (2013) AMPK-dependent degradation of TXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. *Mol. Cell*, **49**, 1167-1175.

Yamada, K., Noguchi, C., Kamitori, K., Dong, Y., Hirata, Y., Hossain, M.A., Tsukamoto, I., Tokuda, M. & Yamaguchi, F. (2012) Rare sugar D-allose strongly induces thioredoxin-interacting protein and inhibits osteoclast differentiation in Raw264 cells. *Nutr. Res.*, **32**, 116-123.

Yamaguchi, F., Takata, M., Kamitori, K., Nonaka, M., Dong, Y., Sui, L. & Tokuda, M. (2008) Rare sugar D-allose induces specific up-regulation of TXNIP and subsequent G1 cell cycle arrest in hepatocellular carcinoma cells by stabilization of p27kip1. *Int. J. Oncol.*, **32**, 377-385.

Young, C.D., Lewis, A.S., Rudolph, M.C., Ruehle, M.D., Jackman, M.R., Yun, U.J., Ilkun, O., Pereira, R., Abel, E.D. & Anderson, S.M. (2011) Modulation of glucose transporter 1 (GLUT1) expression levels alters mouse mammary tumor cell growth in vitro and in vivo. *PLoS One*, **6**, e23205.

Zelzer, E., Levy, Y., Kahana, C., Shilo, B.Z., Rubinstein, M. & Cohen, B. (1998) Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. *EMBO J.*, **17**, 5085-5094.