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Impact of anti-diabetic sodium-glucose cotransporter 2 inhibitors on tumor growth of intractable hematological malignancy in humans

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ABSTRACT

Under the dysfunction of mitochondria, cancer cells preferentially utilize both glycolytic and pentose phosphate pathways rather than electron transport chains to desperately generate adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), classically recognized as the Warburg effect. Based on this background, the present study tested the hypothesis that anti-diabetic sodium-glucose cotransporter 2 (SGLT2) inhibitors would exert a tumor-suppressive impact on intractable human hematological malignancies *via* the modulation of glucose metabolism within cells and cell cycles. The level of mRNA for SGLT2 was remarkably elevated in leukemic cells from patients with adult T-cell leukemia (ATL), one of the most intractable blood cancers in humans, and as well as in two kinds of ATL cell lines (MT-1 and MT-2). Two kinds of SGLT2 inhibitors, Luseogliflozin and Tofogliflozin substantially suppressed the proliferation of MT-1 and MT-2 cells in both adherent and anchorage-independent culture conditions. Such a suppressive effect on tumor cell growth was reproduced by Luseogliflozin in leukemic cells in peripheral blood from patients with ATL. In MT-2 cells, both of SGLT2 inhibitors considerably attenuated glucose uptake, intracellular ATP levels, and NADPH production, resultantly enhancing cell cycle arrest at the G0/G1 phase. From the standpoint of metabolic oncology, the present study suggests that SGLT2 inhibitors would be a promising adjunctive option for the treatment of the most intractable human hematological malignancies like ATL.

1. Introduction

Cancer cells robustly modify their circumstance advantageous to promote growth and proliferation, thereby surviving severe environments including hypoxia and malnutrition [1]. Under the severe dysfunction of mitochondria, cancer cells preferentially utilize glycolytic and pentose phosphate pathways (PPPs) rather than electron transport chains to desperately generate adenosine triphosphate (ATP) [2] and nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) [3,4], recognized as the Warburg effect [5]. Due to the

Abbreviations: ATL, adult T-cell leukemia; ATP, adenosine triphosphate; CADM1, cell adhesion molecule 1; FDG-PET/CT, fluorodeoxyglucose-positron emission tomography/computed tomography; Gem, gemcitabine; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3; HK1, hexokinase 1; HK2, hexokinase 2; HTLV-1, human T cell lymphotropic virus type 1; Luse, Luseogliflozin; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); PBMC, peripheral blood mononuclear cells; PFK, phosphofructokinase; PK, pyruvate kinase; PPPs, pentose phosphate pathways; SGLT1, sodium-glucose cotransporter 1; SGLT2, sodium-glucose cotransporter 2; Tofo, Tofogliflozin.

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inconvenient substrate shift to glycolysis with poor efficiency for generating ATP, glucose uptake reciprocally increases in cancer cells [2]. Based on this notion, fluorodeoxyglucose-positron emission tomography/computed tomography (FDG-PET/CT) is widely employed for detecting malignancies in clinics [6–9].

We recently reported that FDG-PET/CT is advantageous for clinically grading human adult T-cell leukemia (ATL) [10], an extremely intractable hematologic malignancy caused by human T cell lymphotropic virus type 1 (HTLV-1) [11]. Our studies support the notion that disease activity of ATL is highly dependent on its potency of glucose uptake, exemplifying the Warburg effect in its glucose metabolism. Globally, HTLV-1 is endemic predominantly in southwestern part of Japan, sub-Saharan Africa, the Caribbean Basin and South America [12,13]. Cumulative incidence of clinically overt ATL is approximately 5% among healthy carriers with HTLV-1 [12]. In most cases, clinical manifestation of ATL develops after 40～ 60 years of latent period of time [12,13]. Considering the notion that T cells represent high demand for glucose by nature [14,15], we employed ATL cell lines as an experimental model to explore the impact of the Warburg effect in human hematological malignancies [16].

So far, a line of anti-cancer agents targeting exaggerated glucose demand in malignancies have been developed including 2-deoxyglucose (2-DG), lonidamine, 3-bromopyruvate (3-BrPA) and oxythiamine [17]. However, these agents are not yet successfully applied to clinical practice. On the other hand, it is well characterized that sodium-glucose cotransporter 2 (SGLT2) inhibitors potently prevent glucose reabsorption in renal tubule, increase glucose excretion to urine, thereby effectively lowering blood glucose level in patients with diabetes mellitus [18]. Recently, it has been demonstrated that SGLT2 inhibitors considerably improve cardiovascular and renal outcomes in patients with type 2 diabetes [19]. Furthermore, A couple of recent studies in rodents reported that pharmacological inhibition of SGLT2 was shown to exert a beneficial potential for solid malignancies in pancreas, colon, liver and lung [20–23].

Of note, Scafoglio et al. demonstrated that SGLT2 inhibitors substantially reduced the growth of human-derived pancreatic cancer in a xenograft murine model [21], and also provided convincing evidence that SGLT2 may play a pivotal role in growth of early staged lung adenocarcinoma in humans [23]. In this context, we aimed to examine the expression of SGLT2 in cancer cells from peripheral blood of patients with ATL as well as its cell lines, and explored whether pharmacological inhibition of SGLT2 would exert anti-cancer impact on ATL.

2. Materials and methods

2.1. Reagents

Clinically-applied SGLT2 inhibitors in Japan, Luseogliflozin (Taisho Pharmaceutical Co., Ltd.) [24] and Tofogliflozin (Kowa Pharmaceutical Co., Ltd.) [25] were dissolved in dimethyl sulfoxide (DMSO). Gemcitabine, an inhibitor against nucleic acid synthesis (Wako Pure Chemical Industries, Ltd.) were used as inducers for apoptosis.

2.2. Patients studied

Using Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden), mononuclear cells in peripheral blood (PBMCs) were isolated from 9 healthy volunteers, 5 clinically occult HTLV-1 carriers and 12 patients with ATL in university of the Ryukyus in Okinawa, Japan. Clinical subtype of ATL in each patient was determined according to the Shimoyama Criteria [26]. PBMCs from 12 patients with ATL (5 of chronic type and 7 of acute type) were subjected to *in vitro* experiments prior to a series of chemotherapies. The present study was approved by the institutional review board at University of the Ryukyus hospital (approval number 1030) and all participants gave written informed consent.

2.3. Cell culture

HTLV-1-infected cell lines, MT-1and MT-2 [27] were cultured in a RPMI 1640 medium (Sigma-Aldrich, St. Louis, MI, USA) supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries USA, Cromwell, CT, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin.

2.4. Assays for cell proliferation

MT-1 and MT-2 cells (5×10^4 cells) were seeded in triplicates into 96-well plates. Cells were treated with different concentrations of Luseogliflozin or Tofogliflozin for 24, 48, and 72 h. Cell proliferation was determined by use of Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) [28]. The relative ratio of cell proliferation, calculated by dividing by the number of untreated cells without reagents, was shown as the mean ± standard error of the mean of triplicate experiments.

ATL cells obtained from peripheral blood of patients (1×10^5 cells) were seeded with RPMI-1640 medium containing 20 U/ml of recombinant human IL-2 (Peprotech, Rockey Hill, NJ, USA) as a robust mitogen. After 1 day-incubation, all proliferated cells were collected and the population rate of $CD4^+CADM1^+$ (cell adhesion molecule 1) double-positive cells was assessed by the flow cytometry. CADM1 is commonly utilized as a valid cell surface marker for HTLV-1-infected cells. It has been shown that $CD4^+CADM1^+$ double-positive cells are significantly correlated with pro-viral load (PVL) in both ATLs and HTLV-1 asymptomatic carriers [29]. The relative ratio of cell proliferation was also calculated.

2.5. Assays for colony formation

For colony formation assay, anchorage-independent tumor growth was assessed in a soft agar. It is well known that malignantly transformed cells require less extracellular growth factors, are not inhibited by cell-cell contact, and are often immortalized. Importantly, malignantly transformed cells exhibit scaffold-independent proliferation. The colony formation assay is therefore essential to accurately evaluate malignantly transformed cells *in vitro*. Fifty microliters of base agar matrix (CytoSelect 96-well In Vitro Tumor Sensitivity Assay kit; Cell Biolabs, Inc., CA, USA) were dispensed into each well of a 96-well plate. When the agar had solidified on the bottom surface of each well, 75 μl of a cell suspension / soft agar matrix containing 5×10^4 cells were layered on top of the agar, following by the addition of 50 μl of medium with different concentrations of each SGLT2 inhibitor (0, 5, 10, 25, 50, 100 μM). After 6 days of incubation, the agar matrix was solubilized and 3-(4, 5-dimethylthiazol-2-yl)− 2, 5-diphenyl tetrazolium bromide was replenished to each well. The absorbance produced by the formation of insoluble formazan product by viable cells was recorded at 570 nm.

2.6. Analyses of flow cytometry

Flow cytometry was performed with the BD FACSCalibur cell analyzer and data were analyzed with CellQuest Pro software (BD Biosciences, San Jose, CA, USA). Cryopreserved PBMCs were retrieved by rapid thawing of cryotubes in a 37 ℃-water bath. Cell surface staining was performed on ice for 30 min using the following antibody combinations: fluorescein isothiocyanate-conjugated rabbit anti- Synaptic Cell Adhesion Molecule (SynCAM), tumor suppressor in lung cancer-1 (TLSC1)/ CADM1 monoclonal antibody (MBL Co., Ltd, Nagoya, Japan), PE-cyanine7 mouse anti-human CD7 (BD Biosciences), Alexa Fluor 647 anti-human CD4 antibody (Biolegend, San Diego, CA, USA). It is well known that the expression of CADM1 is a valid marker for aggressive ATL and stepwise downregulation of CD7 is associated with clonal expansion of HTLV-1-infected cells [29].

2.7. Assays for glucose uptake

Cells were seeded 3×10^3 MT-2 cells/well into white-walled 96-well culture plate, and treated them with 0, 50, 100 μM concentrations of Luseogliflozin or Tofogliflozin for 24 h, respectively. To evaluate 2DG uptake, cells were incubated with 0.4 mM 2DG for 20 min at the room temperature. Luminescence from accumulated 2DG 6-phosphate was detected by use of the Glucose Uptake-Glo Assay (Promega Corporation, Madison, WI, USA).

2.8. Assays for NADPH

To evaluate the involvement of PPPs within cells after the treatment of SGLT2 inhibitors, the content of NADPH in MT-2 cells was measured. Cells were seeded at a density of 2.5×10^5 cells / well into 12-well plate, and treated in triplicate with 50 μM concentration of Luseogliflozin or Tofogliflozin for 24 h. Intracellular NADPH level was measured by use of NADP / NADPH Assay Kit-WST (Dojindo Molecular Technologies).

2.9. Measurement of intracellular ATP concentration

MT-2 cells were seeded at a density of 3.9×10^2 cells/well into white-walled 96-well plates and treated in triplicate with 0, 50, 100 μM concentrations of Luseogliflozin or Tofogliflozin for 24 h. Cellular ATP level was measured using ATP luminescence assay, Cell Titer-Glo® 2.0 assay (Promega Corporation). We detected the luminescence using 0.3–1 s integration on a luminometer.

2.10. Assays for mitochondrial activity

To evaluate mitochondrial function after the 1 day-treatment of SGLT2 inhibitors, mitochondrial membrane potential was analysed. MT-2 cells were seeded at a density of 1.0×10^5 cells / well into 12-well plates and treated with 50 μM concentration of Luseogliflozin or Tofogliflozin for 24 h. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP, a mitochondrial uncoupling agent, Sigma-Aldrich) at 50 μM concentration was used as a positive control for membrane potential. Cells were loaded with the ratiometric indicator for mitochondrial potential, JC-1 at 5 μM for 30 min at 37°C. Cells were illuminated at 488 nm and the emission was collected between 515 / 545 nm and 575 / 625 nm by using the flow cytometer.

2.11. Quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific Inc., Walthtam, MA, USA) and cDNA was synthesized using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was performed using a StepOnePlus™ Real-Time PCR System, and Fast SYBR Green Master Mix (Thermo Fisher Scientific Inc). The mRNA levels were normalized by β-actin. As a control for human SGLT2 mRNA expression, kidney-derived mRNA from a healthy subject was used (Bio Chain Institute Inc., Newark, CA, USA). As a control for human SGLT1 mRNA expression, small intestine-derived mRNA from a healthy subject was analysed (Bio Chain Institute Inc.). All primer sequences are shown in the Table 1 below.

2.12. Small interfering RNA

To specifically repress the mRNA expression of SGLT2, predesigned single-stranded small interfering RNAs (siRNA; Silencer Select siRNAs, Thermo Fisher Scientific Inc.) were used. As a negative control, scrambled siRNA (Thermo Fisher Scientific Inc.) was used. All siRNA transfections were performed by using a Microporator MP-100 (Digital Bio Technology, Seoul, Korea), pulsed twice at 1140 V (pulse voltage) for 30 msec (pulse width). For cell proliferation assay, transfected cells were seeded (5×10^4 cells) and treated with 50 μM concentration of Luseogliflozin or Tofogliflozin for 48 h, respectively. Similarly, transfected cells were seeded 3.9×10^2 cells / well into white-walled 96-well plates, and treated in triplicate with 50 μM concentration of Luseogliflozin or Tofogliflozin for 24 h before assessment of the 2DG uptake.

Table 1
Human PCR primer sequences.

Gene	Sequences (5′ → 3′)	Source
SGLT2	Forward, TCTACTTCGCCATTGTGCTG; Reverse, ATCTCCATGGCACTCTCTGG	NM_003041
SGLT1	Forward, GCCCAACACTCTGATTTGCATTTA; Reverse, CTGGTTCTACTTCACCCTGAGCAC	NM_000343
CADM1	Forward, GTCCCACCACGTAATCTGATG; Reverse, CCACCTCCGATTTGCCTTTTA	NM_014333
GLUT1	Forward, GCTAACTTTTTCCGCGTGTT; Reverse, GGTGGAAGGTGAGATCCAGA	NM_006516
GLUT3	Forward, GCACGCTTGCGTATGTCTGTTA; Reverse, TGTGGGCTACACTGCACATTGA	NM_006931
Hexokinase 1 (HK1)	Forward, ATCAGGACACACTACGACAGACTGG; Reverse, TTAAGATGTTGCGGACGATTTCAC	NM_000188
Hexokinase 2 (HK2)	Forward, GGCCGAGCCAATAGACTGAAGA; Reverse, GCCATGAGTCCATTTGAAACGA	NM_000189
Phosphofructokinase (PFK)	Forward, CCATGACTACTACACCACGGAGTTC; Reverse, TGGTCCCATAGTTCCGGTCA	M64784
Pyruvate kinase (PK)	Forward, GCTTTACCGTGAACCTCCAGAA; Reverse, TGTCACCACAATCACCAGGTCTC	NM_000298
beta-actin	Forward, ACTGTGTTTGCTGACGCAAC; Reverse, CAACACCACGGAATTGTCAG	NM_001101.3

2.13. Assays for apoptosis

To evaluate the cellular apoptosis, the TdT-mediated dUTP nick end labeling (TUNEL) assay was performed using Apoptotic DNA Fragments Extraction Kit, ApopLadder Ex™ (TaKaRa Bio Inc., Shiga, Japan). After the treatment with 50 μM Luseogliflozin, 50 μM Tofogliflozin or 0.4 μM Gemcitabine, one of representative anti-cancer drugs acting as a potent nucleoside analogue, respectively, 1×10^6 cells were collected by centrifugation, and re-suspended in the lysis buffer in the kit. Fragmented DNAs from lysate were then purified and were analyzed using agarose gel electrophoresis.

2.14. Assays for caspase-3 activity

Activity of caspase-3 was measured by the absorbance at 405 nm after the cleavage of the synthetic substrate Ac-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD pNA) *via* the colorimetric assay kit (TaKaRa Bio USA Inc., MountainView, CA, USA). Cells were plated at a density of 2×10^6 cells / well. One hour after the treatment with each SGLT2 inhibitor (100 μM), cells were collected. The enzyme activity was calculated on the basis of a standard curve prepared using p-nitroaniline (pNA). The relative levels of pNA were normalized by the protein concentration of each extract.

2.15. Assays for cell cycle

The phase of cell cycle in MT-2 was analyzed by staining DNA with the flow cytometer. Cells were seeded at a density of 5×10^5 cells / well into 6-well plates and treated in triplicate with 50 μM Luseogliflozin or Tofogliflozin for 24 h. One day after the treatment of SGLT2 inhibitors,

cells were stained by using Cell Cycle Assay Solution Blue at 37ºC for 15 min (Dojindo Molecular Technologies Inc.) and detected blue fluorescence (Excitation length at 405 nm and Emission length at 450 nm) to determine the ration of the G0/1 - S phase and G2/M phase.

2.16. Statistical analyses

Data are expressed as the mean ± standard error of the mean (SEM) in independent experiments. One-way analysis of variance (ANOVA), Two-way ANOVA followed by multiple comparison tests (Tukey's post-hoc method) and Kruskal-Wallis analysis were used where applicable.

3. Results

3.1. Levels of SGLT2 and CADM1 mRNAs were substantially elevated in leukemic cells from patients with acute-type ATL and ATL cell lines

As shown in Fig. 1A left, upper panel, comparing to PBMCs from healthy subjects, mRNA level of SGLT2 was substantially elevated in normal human kidney as well as in ATL cells from patients with acute-type and MT-2 cells. There were no appreciable differences among HTLV-1 carriers, chronic-type, MT-1 cells as compared to PBMC from healthy subjects. No significant differences were observed in the mRNA level of SGLT1 among PBMCs from healthy subjects, ATL, MT-1 and MT-2 cells except HTLV-1 carriers (Fig. 1A, right, upper panel). As previously reported [21], level of SGLT1 mRNA in normal human intestine was substantially elevated.

It has been shown that CADM1 was ectopically and highly expressed in HTLV-1 infected T cells and ATL cells [30]. CADM1 has been used as one of the representative molecular markers in ATL and other T-cell lymphoma [31]. As reported, mRNA level of CADM1 was markedly elevated in ATL cells of acute-type and MT-2 cells (Fig. 1A, lower panel).

3.2. Luseogliflozin substantially suppressed the growth of leukemic cells from patients with acute-type ATL

To examine whether pharmacological inhibition of SGLT2 would suppress the proliferation of ATL cells from patients with chronic-type or acute-type of ATL as well as HTLV-1 infected T cells from clinically occult HTLV-1 carriers, Luseogliflozin was replenished in the culture media with 20 U/ml of recombinant human IL-2. Proliferation was assessed at 24 h after the replenishment by the flow cytometry. Consequently, Luseogliflozin markedly suppressed the proliferation of ATL cells from patients with acute-type as compared to chronic-type ($p = 0.022$, Fig. 1B) at the concentration of 100 μM.

3.3. SGLT2 inhibitors substantially suppressed the growth of ATL cell lines

To test whether pharmacological inhibition of SGLT2 would suppress the proliferation and colony formation of ATL cell lines, Luseogliflozin and Tofogliflozin, were replenished at various pharmacological concentrations in the media of cells. Proliferation was assessed at 24, 48 and 72 h after the replenishment of each inhibitor. Colony formation was assessed at 6 days after the replenishment of each inhibitor. Consequently, both of inhibitors significantly suppressed the proliferation of MT-1 and MT-2 cells in a dose-dependent manner as well as in a time-dependent manner (Figs. 1C, 1D). In a similar fashion, Tofogliflozin significantly suppressed the colony forming ability of MT-1 in a dose-dependent manner, and Luseoglifrozin also showed a noticeable trend to suppress the colony forming abilities in both MT-1 and MT-2 (Fig. 1E).

3.4. MT-2 cells grow more rapidly in high glucose media

To evaluate the glucose requirement for cell proliferation in ATL cells, we compared the cell growth of MT-2 between standard glucose medium (11 mM) and high glucose medium (25 mM). It was reported that inoculation of SCID mice with MT-2 cells elicited considerably high mortality, whereas that of MT-1 cells provoked subtle tumorigenesis [32]. We therefore preferentially utilized MT-2 cells henceforth in the present study. Expectedly, the cell growth of MT-2 was markedly exaggerated in a high glucose medium compared to standard one in a time dependent fashion (Fig. 2A).

3.5. SGLT2 inhibitors substantially attenuated glucose uptake, intracellular level of ATP and NADPH in MT-2 cells

We examined whether glucose uptake as well as intracellular level of ATP and NADPH level would be suppressed by SGLT2 inhibitors in MT-2 cells. There was no appreciable difference in glucose uptake between normal and high glucose media for 24 h (Fig. 2B, left). Notably, Luseogliflozin dose-dependently attenuated glucose uptake in a standard glucose medium as well as high glucose medium for 24 h (Fig. 2B, left). Based on our finding that there was no statistical difference in glucose uptake between standard and high glucose media with Luseogliflozin (Fig. 2B, left), we next performed glucose uptake assays with Tofogliflozin only in a standard glucose medium. Expectedly, Tofogliflozin also dose-dependently attenuated glucose uptake (Fig. 2C, left). Importantly, both inhibitors dose-dependently reduced intracellular ATP concentration (Fig. 2B, right and 2 C, right).

Of note, value of glucose uptake as well as intracellular level of ATP and NADPH was substantially elevated in MT-2 cells compared to PBMCs, respectively (Fig. 2D, E, F and G). On the other hand, Luseogliflozin significantly attenuated each value as compared to vehicle (Figs. 2D and 2F). Tofogliflozin also significantly attenuated intracellular ATP concentration as compared to vehicle (Fig. 2E, right), and showed a noticeable trend to reduce glucose uptake and NADPH level ($p = 0.054$, Fig. 2E left and $p = 0.126$, Fig. 2G).

3.6. Expression profile of mRNAs for glycolysis-related genes and GLUTs in ATL cell lines

To gain further insight into the facet of glucose metabolism within ATL cells, we assessed mRNA levels for glycolysis-related genes and glucose transporters (GLUTs) in MT-1 and MT-2 cells. Regarding genes involved in glycolytic metabolic pathways, the level of a rate-limiting enzyme, phosphofructokinase (PFK) was significantly elevated in MT-2 cells compared to PBMCs (Fig. 3A upper, left). The level of pyruvate kinase (PK), another rate-limiting enzyme in glycolytic metabolic pathways also showed a trend to increase in MT-2 cells ($p = 0.380$, Fig. 3A lower, left). The value of hexokinase (HK) 1 was significantly higher in MT-2 cells as compared to PBMCs, but not of HK2 ($p = 0.020$, Fig. 3A upper, middle and $p = 0.792$, right). Regarding the glucose transporters responsible for glucose uptake independent of insulin, mRNA levels for GLUT1 in MT-1 and MT-2 cells were markedly elevated compared to that of PBMCs (Fig. 3A lower, middle), whereas that of GLUT3 was inexplicably reduced in both MT-1 and MT-2 cells (Fig. 3A lower, right).

3.7. SGLT2 inhibitors did not affect the membrane potential of mitochondria in MT-2 cells

To evaluate mitochondrial function after the 1 day-treatment of SGLT2 inhibitors, we assessed the mitochondrial membrane potential by the flow cytometer. One day after the treatment with SGLT2 inhibitors, MT-2 cells were loaded with the ratiometric indicator for mitochondrial potential, JC-1. JC-1 is a fluorescent cationic carbocyanine dye and represents potential-dependent accumulation in mitochondria which form J aggregates (red), and then diffuses across mitochondria to form a monomeric state (green) upon depolarization. Subsequently, JC-1 red-colored cells containing high mitochondrial membrane potential were detected in normally-functioned mitochondria. In our assays among

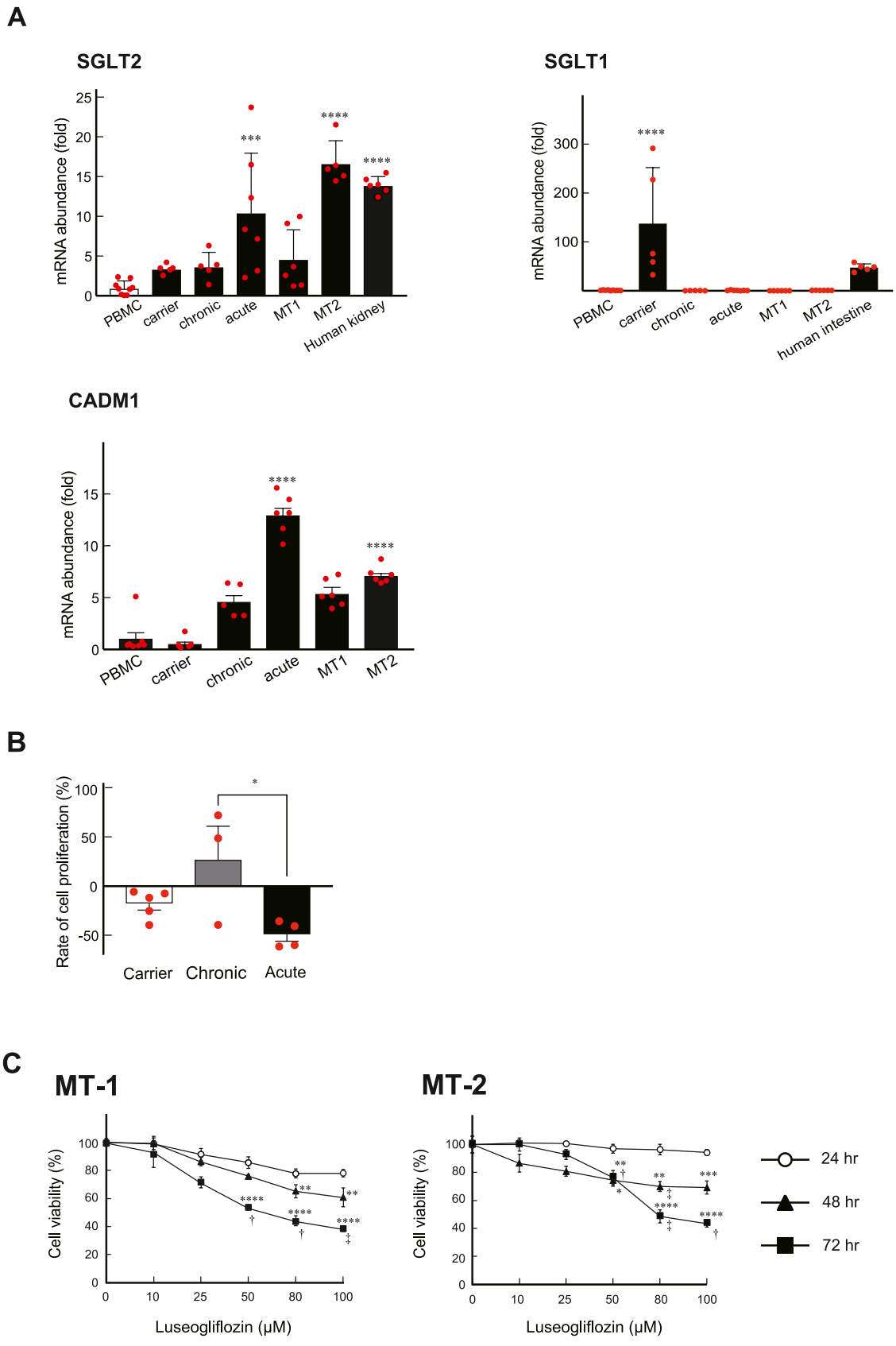

Fig. 1. SGLT2 inhibitors substantially suppressed the growth of ATL cell lines and leukemic cells from peripheral blood in patients with acute-type ATL. **(A)** Level of mRNAs for SGLT2, SGLT1 and CADM1 in peripheral blood mononuclear cells (PBMCs) from healthy subjects (n = 9), HTLV-1 infected T-cells from clinically occult carriers (n = 5), malignant cells from peripheral blood of ATL patients (chronic-type: n = 5, acute-type: n = 7), ATL cell lines (MT-1 and MT-2) (n = 5–6 in each group), and human kidney or intestine from healthy subjects (n = 5–6 in each), respectively. Data are expressed as the mean ± SEM, * ** : $p < 0.001$, * ** *: $p < 0.0001$ *vs.* PBMCs by Kruskal-Wallis analysis and Tukey's post-hoc analyses. **(B)** Luseogliflozin substantially suppressed the growth of

malignant cells in peripheral blood from patients with acute-type ATL. Proliferation of ATL cells from patients with chronic-type or acute-type and HTLV-1 infected T-cells from clinically occult carriers under the treatment with Luseogliflozin for 24 h at a dose of 100 μM. After 1 day-incubation, all proliferated cells were collected and the population rate of $CD4^{+}CADM1^{+}$ double-positive cells was assessed by the flow cytometry. The relative ratio of cell proliferation was calculated. Data are expressed as mean ± SEM (n = 3–5/group). * : $p < 0.05$ *vs.* between line-connected values by One-way ANOVA and Tukey's post-hoc analyses. **(C)(D)** SGLT2 inhibitors suppressed the growth of ATL cell lines in adherent conditions. Proliferation of MT-1 or MT-2 cells treated with (**C**) Luseogliflozin or (**D**) Tofogliflozin, respectively. Data are expressed as mean ± SEM (n = 8). * : $p < 0.05$, * *: $p < 0.01$, * ** : $p < 0.001$, * ** *: $p < 0.0001$ *vs.* 0 μM at the same time. †: $p < 0.05$, ‡: $p < 0.001$ *vs.* 24 h in the same concentration of each inhibitor by Two-way ANOVA and Tukey's post-hoc analyses. **(E)** SGLT2 inhibitors suppressed the anchorage-independent growth of ATL cell lines. The growth of MT-1and MT-2 cells treated with Luseogliflozin or Tofogliflozin for 5 or 6 days at a dose of 0, 5, 10, 25, 50, 100 μM, respectively. Data are expressed as mean ± SEM (n = 3). * *: $p < 0.01$, * ** : $p < 0.001$, * ** *: $p < 0.0001$ *vs.* 0 μM in the same inhibitor by Two-way ANOVA and Tukey's post-hoc analyses.

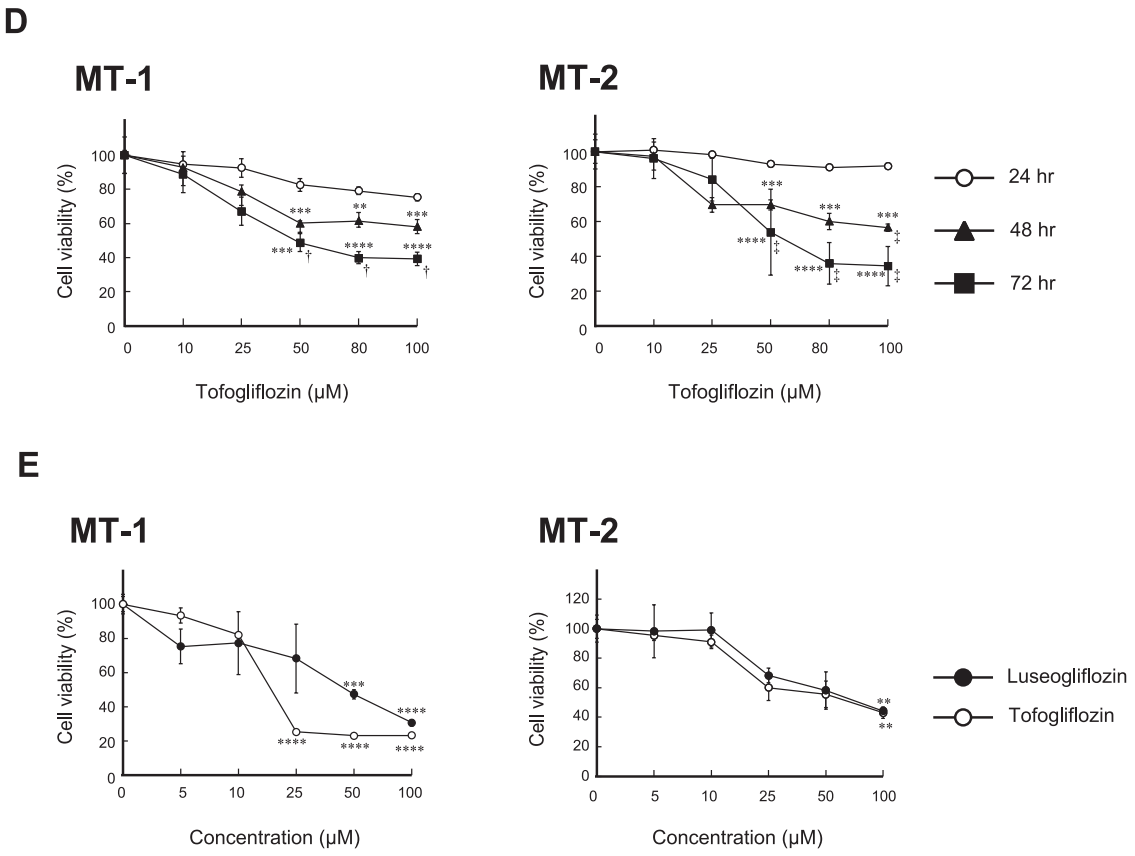

Fig. 1. (*continued*).

vehicle and two of SGLT2 inhibitors, there was no statistical difference in mitochondrial depolarization rate as demonstrated by the change of color after JC-1 staining (Fig. 3B, upper panel). The decrease in the red / green fluorescence intensity ratio indicates the depolarization of the mitochondrial membrane, resulting in apoptosis due to mitochondrial collapse. Fig. 3B, lower panel shows representative examples of color sorting of typical JC-1 positive cells after the addition of SGLT2 inhibitors. No significant increase in JC-1 green cells was detected after the replenishment of Luseogliflozin or Tofogliflozin. In contrast, most of cells turned a green color when CCCP, a potently depolarizer in the mitochondrial membrane potential, was replenished (Fig. 3B, lower panel).

3.8. Suppression of SGLT2 via the specific RNA interference completely abolished the effect of SGLT2 inhibitors on glucose uptake and proliferation of ATL cells

To clarify whether the function of SGLT2 would directly influence the proliferation of ATL cells, specific RNA interference against SGLT2 was introduced. Because mRNA level of SGLT2 in MT-2 cells was apparently higher than that of MT-1 cells (Fig. 1A, left), we preferentially utilized MT-2 cells in pertinent experiments. MT-2 cells were transfected with either siRNA against SGLT2 or scramble siRNA *via* the electroporation methods. Consequently, replenishment of specific siRNA against SGLT2 markedly decreased the mRNA level of SGLT2 (Fig. 4A), where cell proliferation was considerably attenuated compared to those treated with negative-control (Scrambled) siRNA (Fig. 4B, left and 4C, left, respectively). Of note, effects of both of SGLT2 inhibitors on glucose uptake and the cell proliferation were disappeared in cells under the treatment of specific siRNA (Fig. 4B, 4C), further supporting a crucial role of SGLT2 for growth of MT-2 cells.

3.9. Decrease in growth of MT-2 cells by SGLT2 inhibitors was independent of apoptosis

To further explore mechanisms of SGLT2 inhibitors on growth suppression of MT-2 cells, we examined whether SGLT2 inhibitors would augment apoptosis. Contrary to the apparent DNA ladder in gel electrophoresis by the treatment with Gemcitabine [33], no appreciable changes were observed in MT-2 cells treated with either Luseogliflozin or Tofogliflozin (Fig. 4D). In contrast to the augmented activity of caspase-3 in MT-2 cells treated with Gemcitabine, no appreciable changes were also observed in MT-2 cells treated with either Luseogliflozin or Tofogliflozin (Fig. 4E). These data suggest that decrease in the growth of MT-2 cells by SGLT2 inhibitors is unlikely to relate directly with apoptosis.

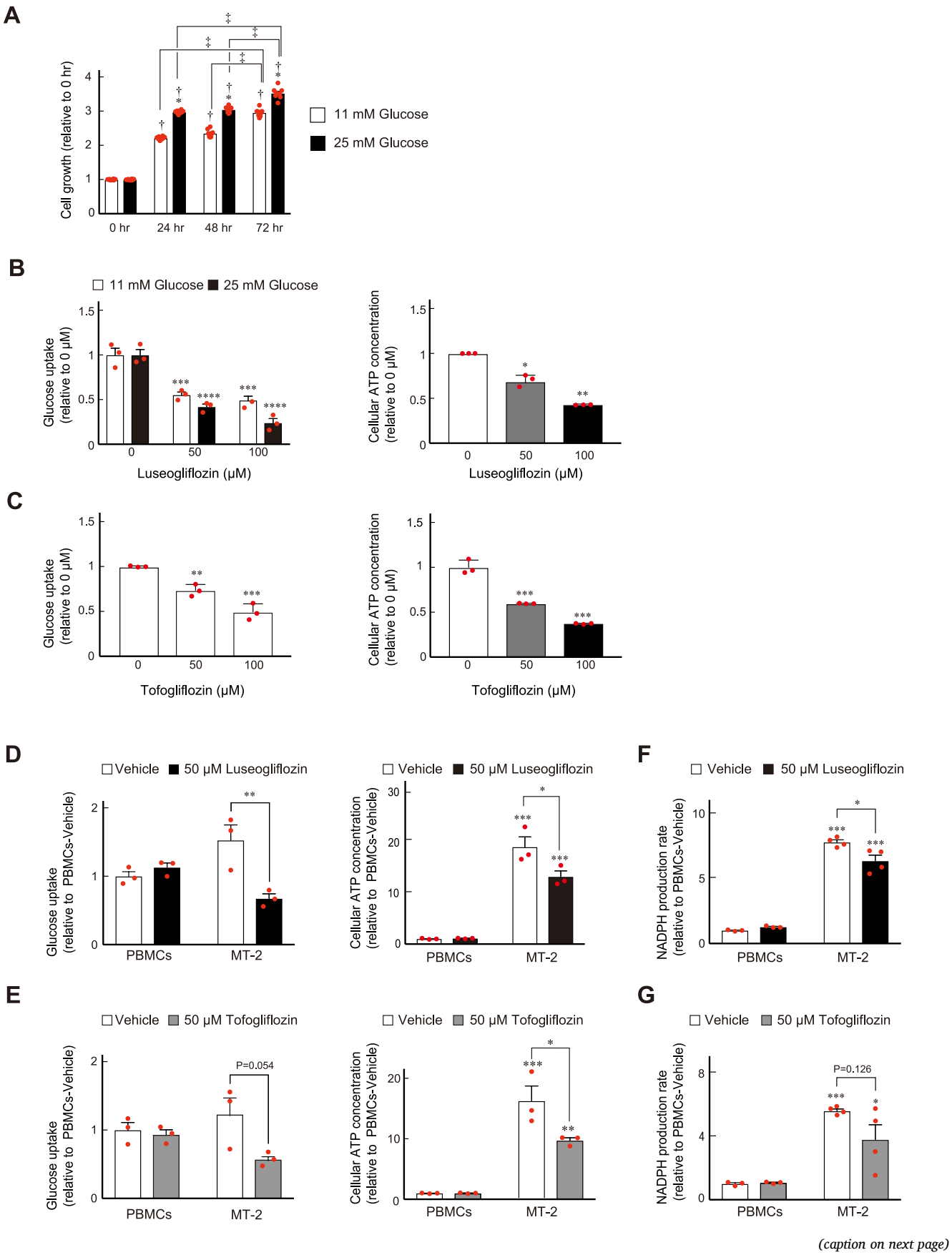

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Fig. 2. SGLT2 inhibitors substantially attenuated glucose uptake, intracellular level of ATP and NADPH in MT-2 cells.(**A**) MT-2 cells grow more rapidly in a high glucose medium as compared to regular glucose medium. Cells were cultured in a regular glucose medium (11 mM, open column) or high glucose medium containing 25 mM glucose (closed column). * : $p < 0.05$ *vs.* 11 mM glucose at the same time. †: $p < 0.05$ *vs.* 0 h (hr) at the same glucose concentration. ‡: $p < 0.001$. (n = 8/group).(**B, C**) SGLT2 inhibitors attenuated glucose uptake and intracellular ATP concentration in MT-2 cells. Effects of (**B**) Luseogliflozin and (**C**) Tofogliflozin on glucose uptake for 24 h in MT-2 cells (left). MT-2 cells were cultured in a regular glucose medium (11 mM) under the treatment of Tofogliflozin. * *: $p < 0.01$, * **: $p < 0.001$, * ** *: $p < 0.0001$ *vs.* 0 μM at the same concentrations of glucose. (n = 3/group). Effects of SGLT2 inhibitors on intracellular ATP concentration for 24 h in MT-2 cells (right).* : $p < 0.05$, * *: $p < 0.01$, * **: $p < 0.001$ *vs.* 0 μM. (n = 3/group).(**D,** E) Glucose uptake and intracellular ATP concentration in MT-2 cells were considerably elevated as compared to PBMCs, and SGLT2 inhibitors substantially attenuated glucose uptake and intracellular ATP concentration in MT-2 cells. Effects of (**D**) Luseogliflozin and (**E**) Tofogliflozin on glucose uptake and intracellular ATP concentration for 24 h in MT-2 cells. * : $p < 0.05$, * *: $p < 0.01$, * **: $p < 0.001$, *vs.* the same treatment in PBMCs. (n = 3/group).
(**F, G**) NADPH level in MT-2 cells was markedly elevated as compared to PBMCs, and SGLT2 inhibitors significantly attenuated NAPDH level in MT-2 cells. Effects of (**F**) Luseogliflozin and (**G**) Tofogliflozin on NADPH level for 1 day-treatment in MT-2 cells. * : $p < 0.05$, * **: $p < 0.001$ *vs.* the same treatment in PBMCs. (n = 3 in PBMCs, n = 4 in MT-2 cells). Statistical significance was calculated using Two-way ANOVA and Tukey's post-hoc analyses (A), (B left), (D), (E), (F) and (G) or One-way ANOVA and Tukey's post-hoc analyses (B right) and (C). Data are expressed as the mean ± SEM.

3.10. SGLT2 inhibitors considerably modulated the cell cycle of MT-2 cells

To evaluate the effect of SGLT2 inhibitors on the proliferation of MT-2 cells, after the treatment with 50 μM concentration of Luseogliflozin or Tofogliflozin for 24 h, the cell cycle was analyzed by the flow cytometry. Treatment with Luseogliflozin or Tofogliflozin significantly increased the ratio of cells at G0/G1 phase ($p = 2.23e\text{-}4$ and $p = 1.81e\text{-}5$ *vs.* corresponding value for same phase of vehicle treatment), suggesting that both of compounds induce the G0/G1 arrest in MT-2 cells (Fig. 5, upper panel).

As shown in representative examples (Fig. 5, lower panel), the ratio of cells at the G2/M phase showed an apparent decrease in cells treated with Luseogliflozin (12.23% *vs.* corresponding value for the same phase of vehicle treatment, 18.78%) or a remarkable decrease in cells treated with Tofogliflozin (10.40% *vs.* corresponding value for the same phase of vehicle treatment, 18.78%), in accordance with the notion that G0/G1 arrest is exaggerated in MT-2 cells treated with SGLT2 inhibitors.

4. Discussion

To our knowledge, the present study is the first to demonstrate that SGLT2 inhibitors exert potent tumor-suppressive effects in both ATL cells from patients and ATL cell lines by attenuating cellular glucose uptake. Recent works in mice suggested that pharmacological inhibition of SGLT2 may provide cancer-reducing impact on solid tumors [20–23]. However, therapeutic potential of SGLT2 inhibitors on hematological malignancies has not yet been elucidated. In this context, the present study provides the first convincing evidence that SGLT2 inhibitors would be beneficial as an adjunctive for hematological malignancies in humans.

It is noteworthy that mRNA level of SGLT2 in both ATL cells from patients and ATL cell lines was substantially elevated, while that of SGLT1 was not increased except healthy carrier patients (Fig. 1A). On the other hand, the mRNA level of CADM1 was also substantially elevated in ATL cells of acute-type and MT-2 cells (Fig. 1A, lower panel). CADM1 was originally identified as a tumor suppressor gene in lung cancer, playing a pivotal role in the cell-cell adhesion among endothelial cells. In line with our results, it has been reported that the mRNA level of CADM1 was significantly elevated in patints with both chronic and acute type ATL as compared to healthy volunteers [30]. In this context, comparison of expression profile between SGLT2 and CADM1 may provide a clue to estimate the metabolic status of intractable hematological malignancy such as ATL.

The reason for the unexpectedly higher level of SGLT1 mRNA in carrier patients still remains unclear. Since it has been reported that mRNA expression of SGLT1 was upregulated predominantly in activated T cells [34], it would be possible to speculate that the mRNA level of SGLT1 in caririer patients is substantiated in activated T cells under the chronic infection of HTLV-1. Furthermore, Madunic et al. and Ishikawa et al. recently reported that the level of SGLT1 mRNA was comparable in primary lung cancer and metastatic lesions, while that of SGLT2 was markedly elevated in metastatic lesions as compared to primary lesions in lung cancer [35,36]. These findings tempt us to imagine that SGLT2 would be further involved in glucose uptake in glucose-requiring metastatic regions. In fact, SGLT1 is a high-affinity, but low-capacity Na+ /glucose cotransporter for glucose, while SGLT2 is a low-affinity, but high-capacity Na+ /glucose cotransporter [35]. In this context, such a functional property may be a facet explaining the differential expression of SGLT isoforms in a respective stage of cancer cells. Importantly, MT-2 cells grew more rapidly in a high glucose medium compared to a standard medium (Fig. 2A), consistent with recent reports that SGLT2 is highly expressed in a line of progressive cancer cells with robust uptake of glucose [21,37–39], further supporting a pivotal role of SGLT2 in a glucose-dependent growth of ATL cells.

The present study demonstrated that suppression of glucose uptake *via* the SGLT2 was linked with the decreased proliferation of ATL cells (Fig. 1B-E). Luseogliflozin substantially suppressed the proliferation of ATL cells from patients with acute-type as compared to those with chronic-type ($p = 0.022$, Fig. 1B) at the concentration of 100 μM. Our results suggest that Luseogliflozin has a potential to suppress aggressive ATL representing high level of SGLT2 expression. We also demonstrated that glucose uptake as well as intracellular level of ATP and NADPH were substantially elevated in MT-2 cells, and that SGLT2 inhibitors significantly attenuated a line of value (Fig. 2 D-G). Furthermore, RNA interference against SGLT2 resulted in a marked decrease in the mRNA level of SGLT2 (Fig. 4A) accompanied by a considerable decrease in cell proliferation and glucose uptake (Figs. 4B and 4C, open columns). Under the interference, no statistical significance was observed in cell proliferation (Fig. 4B, left and 4 C, left) and glucose uptake (Fig. 4B, right and 4 C, right) among vehicle, Luseogliflozin and Tofogliflozin, highly suggesting that SGLT2 contributes to the growth of MT-2 mainly through the supply of glucose into cancer cells. Furthermore, we demonstrated that the decrease in cell proliferation of MT-2 by SGLT2 inhibitors was unrelated with apoptosis (Figs. 4D and 4E), further supporting that growth suppression of MT-2 is largely attributed to the attenuation of glucose uptake by inhibiting SGLT2.

The mRNA level for the rate limiting enzymes in glycolytic metabolic pathways, PFK and PK, showed a trend to elevate in MT-2 cells (Fig. 3A, left), in agreement with previous reports that glycolytic metabolic pathways were augmented in glucose-requiring malignant cells [40,41]. Furthermore, mRNA level for the first-stage enzymes in glycolytic metabolic pathways, HK1 and HK2, also showed a trend to elevate in MT-2 cells as previous reports (Fig. 3A, upper) [42,43]. In contrast, it has been reported that GLUT3 plays a major role for glucose uptake in human B cell lymphomas [41]. Basically, the mRNA level for GLUT1 and GLUT3, high affinity glucose transporters, was reported to elevate in lymphocytes in healthy humans [44,45]. However, little has been known about the expression of SGLTs and GLUTs exclusively in T cell lymphoma in humans. In this context, we would speculate that there seems a cancer-specific and stage-dependent manner of glucose uptake. For example, a recent work by Scafoglio et al. demonstrated that SGLT2

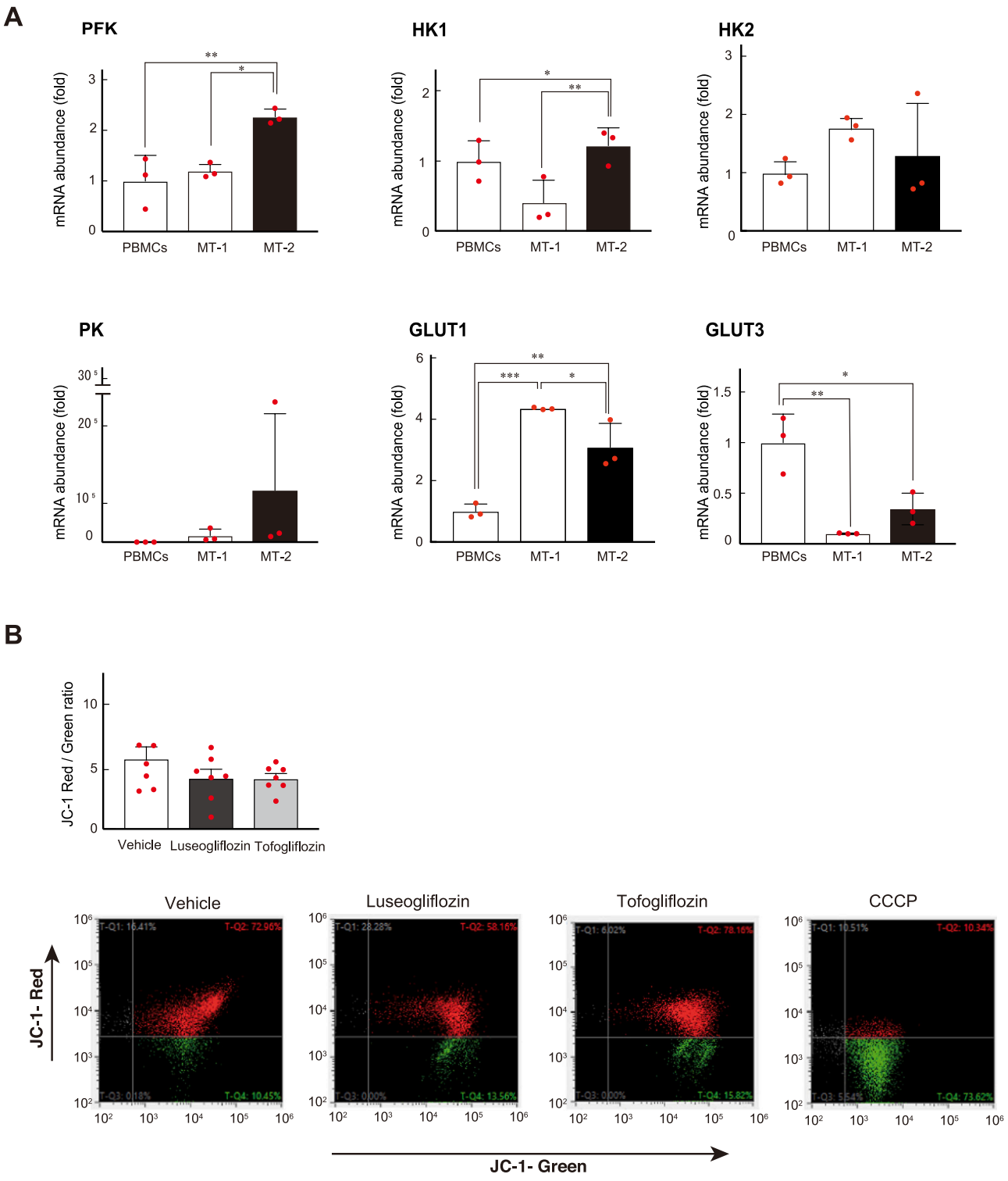

Fig. 3. SGLT2 inhibitors showed no effect on mitochondrial activity in ATL cell lines with enhanced expression of glycolysis-related genes.(**A**) Level of mRNAs for a line of genes involved in glycolytic metabolic pathways (PFK, PK, HK1 and HK2) and glucose transporters (GLUT1 and GLUT3) in MT-1 and MT-2 cells. Relative values against PBMCs are expressed as the means ± SEM (n = 3/group), * : $p < 0.05$, * *: $p < 0.01$, * ** : $p < 0.001$ by One-way ANOVA and Tukey's post-hoc analyses. PFK: phosphofructokinase, PK: pyruvate kinase, HK1: hexokinase 1,HK2: hexokinase 2, GLUT1: glucose transporter 1, GLUT3: glucose transporter 3. (**B**) Mitochondrial viability and function as evaluated by JC-1 Red / Green ratio were unaffected by SGLT2 inhibitors in MT-2 cells as shown in the upper panel. After cells were treated with Luseogliflozin or Tofogliflozin for 24 h, cells were loaded with the ratiometric indicator for mitochondrial potential, JC-1. Cells were illuminated at 488 nm and the emission was collected between 515 / 545 nm and 575 / 625 nm by use of the flow cytometer. Data are expressed as the mean ± SEM, P values were calculated by One-way ANOVA and Tukey's post-hoc analyses (n = 7/group). Representative data were shown in the lower panels. As compared to vehicle and two of SGLT2 inhibitors, the ratio of JC-1 Green cells was apparently elevated in the treatment of CCCP at 50 μM concentration. CCCP: Carbonyl cyanide m-chlorophenyl hydrazone.

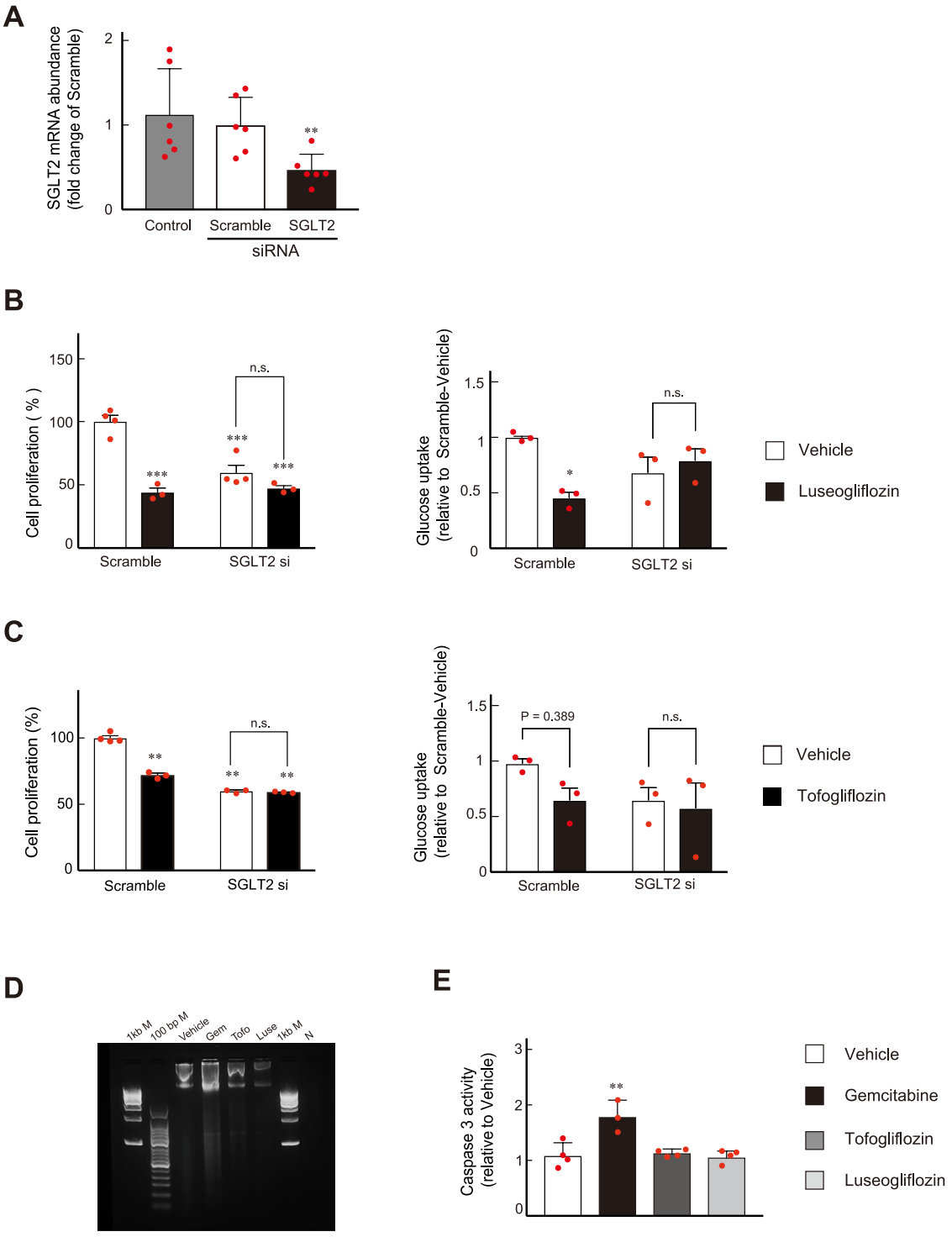

Fig. 4. SGLT2 inhibitors suppressed the proliferation of MT-2 cells mainly *via* the blockade of glucose uptake without inducing apoptosis.(**A**) MT-2 cells were treated with SGLT2 siRNA. Level of mRNA for SGLT2 was normalized against that of β-actin. Control means cells without transfection of siRNAs. Data are expressed as mean ± SEM (n = 6). * *: $p < 0.01$ *vs.* scrambled siRNA-transfected cells by One-way ANOVA and Tukey's post-hoc analyses.(**B, C**) After treating with SGLT2 siRNA, proliferation and glucose uptake of cells replenished with (**B**) Luseogliflozin or (**C**) Tofogliflozin in a dose of 50 μM for 48 h were analyzed. Data are expressed as mean ± SEM (n = 3–4). n.s.: no significant difference, * : $p < 0.05$, * *: $p < 0.01$, * ** : $p < 0.001$ *vs.* scrambled vehicle by Two-way ANOVA and Tukey's post-hoc analyses. (**D, E**) Decrease in growth of MT-2 cells by SGLT2 inhibitors was unrelated with apoptosis. DNA ladders of MT-2 cells treated with 50 μM Luseogliflozin, 50 μM Tofogliflozin or 0.4 μM Gemcitabine by a conventional phenol-chloroform method (**D**). After treating with 100 μM concentration of SGLT2 inhibitors or 0.4 μM Gemcitabine for 1 h, caspase-3 activity was measured *via* the colorimetric assay kit (**E**). Data are expressed as mean ± SEM (n = 4). * *: $p < 0.01$ *vs.* vehicle by One-way ANOVA and Tukey's post-hoc analyses. Gem: Gemcitabine; Tofo: Tofogliflozin; Luse: Luseogliflozin; 100 bp M: 100 bp DNA ladder, Roche, 11721933001; 1 kb M: 1 kb DNA ladder, NEB, N0468S; N: no loading lane.

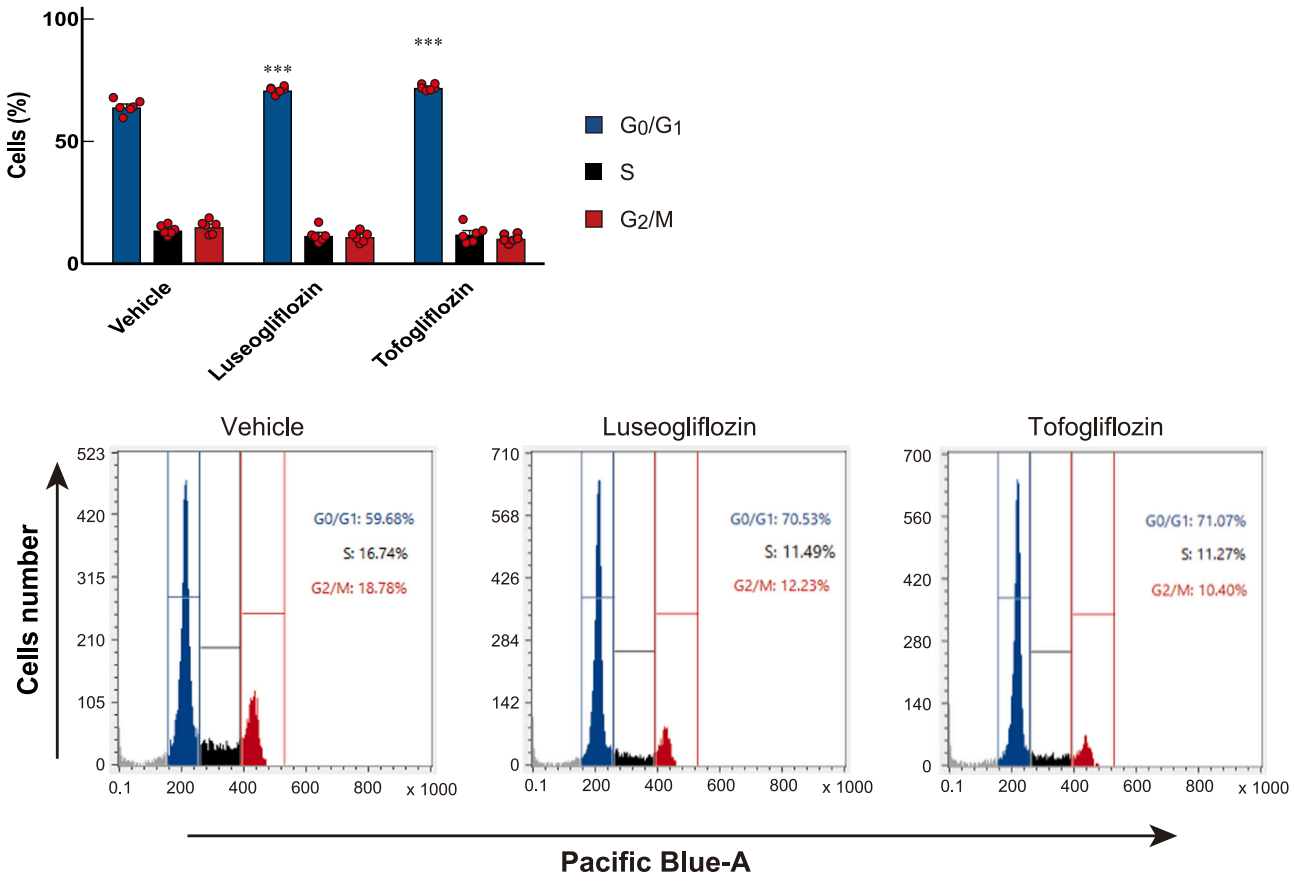

Fig. 5. SGLT2 inhibitors potentiated the cell cycle arrest at the G0/G1 phase in MT-2 cells.Effects of SGLT2 inhibitors on the cell cycle and DNA replication in MT-2 cells.The cell cycle was analyzed by staining DNA by the flow cytometer. One day after the treatment with SGLT2 inhibitors, cells were stained by use of Cell Cycle Assay Solution Blue, and blue fluorescence (Pacific Blue-A: Excitation length at 405 nm and Emission length at 450 nm, respectively) was detected to determine the ratio of the G0/G1, S and G2/M phase. Data are expressed as mean ± SEM (n = 6). * ** : $p < 0.001$ *vs.* corresponding value for same phase of vehicle treatment by Two-way ANOVA and Tukey's post-hoc analyses. Representative data are shown in the lower panels. After 24 h of SGLT2 inhibitor treatment, double-stranded DNA was stained with Pacific Blue-A, and the cell ratios of G0/G1 phase, S phase, and G2/M phase were calculated from the gating by the difference of fluorescence intensity using the flow cytometer.

preferentially expressed in the early stage of human lung adenocarcinoma and GLUT1 mainly expressed in the late stage, suggesting that SGLT2 and GLUT1 may play a stage-related role for glucose uptake in lung adenocarcinoma [23]. Similarly, the mRNA level for GLUT1 showed a trend to elevate in both cell lines (MT-1 and MT-2) as compared to that for GLUT3, suggesting the expression of glucose transporter differ according to cell types or malignant grade (Fig. 3A, lower).

In the present study, we demonstrated that NADPH level in MT-2 cells was remarkably increased as compared to that of PBMCs, and significantly decreased under the treatment of Luseogliflozin (Fig. 2 F) and showed a trend to decrease under the treatment of Tofogliflozin (Fig. 2G). These results suggest that SGLT2 inhibitors may suppress PPPs in MT-2 cells. Since the PPPs synthesize both nucleic acids and NADPH serving as fatty acid synthesis, it is reasonable to hypothesize that inhibition for PPPs by SGLT2 inhibitors also contribute to growth inhibition in MT-2. Furthermore, cell cycle analyses demonstrated that both Luseogliflozin and Tofogliflozin facilitated the G0/G1 arrest in MT-2 cells (Fig. 5), which is also likely to suppress DNA synthesis in cancer cells. In this context, further studies are required to clarify the stage-dependent contribution of SGLT2 to glycolytic metabolic pathways and PPPs in hematological malignancies in humans.

We do recognize that the present study has a couple of limitations. In *in vitro* experiments, we used only two kinds of cell lines for ATL. Studies using a variety of cell lines are required to strengthen our findings. Second, we could not fully address the mechanism of discordance between the suppression of cell proliferation and the inhibition of glucose uptake by SGLT2 inhibitors. Because simultaneous assessment of both phenomena in the same *in vitro* system seems technically a herculean task, further studies are warranted to clarify this issue.

5. Conclusion

Collectively, the present study is the first to demonstrate that pharmacological inhibition of SGLT2 has a potent ability to suppress the growth of ATL cells by attenuating glucose uptake and resultantly decreasing intracellular levels of ATP and NADPH, all of which are likely to potentiate cell cycle arrest at G0/G1 phase. Our findings may shed light on the SGLT2 inhibitors-based novel adjunctive therapies against intractable hematological malignancies such as ATL.

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Conflict of interest disclosure

The authors declare no competing interests.

Author contributions

SN, SO, IN, YN, KT and MT contributed to the research design, acquisition, assembly, analysis and interpretation of data, and drafting the manuscript. TF, YT, MI, SM, MT, MM and SM contributed to the conceptual design of experiments and discussion. HM conceived the idea, contributed to the overall experimental design, data analysis and interpretation, and writing and critical revision of the manuscript. All authors read and approved the final manuscript. HM and SO are responsible for the integrity of the present study as a whole.

Conflict of interest statement

The authors declare no competing interests.

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