Glucosamine decreases the stemness of human ALDH$^+$ breast cancer stem cells by inactivating STAT3

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Abstract. Cancer stem cells (CSCs) are a subpopulation of cancer cells responsible for tumor maintenance and relapse due to their ability to resist various anticancer effects. Owing to the resistance of CSCs to the effects of targeted therapy, an alternative strategy that targets post-translational glycosylation may be an improved approach to treat cancer as it disrupts multiple coordinated signaling that maintains the stemness of CSCs. Glucosamine acts as an anticancer agent possibly by inhibiting N-linked glycosylation. The aim of the present study was to investigate the effect of glucosamine on the stemness of breast CSCs, which is regulated by signal transducer and activator of transcription 3 (STAT3) signaling. Human aldehyde dehydrogenase-positive (ALDH$^+$) breast CSCs and MCF7 cells were treated with various concentrations (0.25, 1 or 4 mM) of glucosamine for 24 h. Subsequently, cell viability was determined by performing a trypan blue exclusion assay, pluripotency gene [ALDH 1 family member A1 (*ALDH1A1*), octamer-binding transcription factor 4 (*OCT-4*), and Krüppel-like factor 4 (*KLF4*)] expression was determined using the reverse transcription-quantitative polymerase chain reaction, and STAT3 and phosphorylated STAT3 (pSTAT3) levels were determined by performing western blot analysis. Furthermore, the number of mammosphere-forming units (MFUs) in ALDH$^+$ breast CSCs and MCF7 cells was determined. It was determined that glucosamine treatment decreased the viability of ALDH$^+$ breast CSCs. Glucosamine treatment also decreased the stemness of ALDH$^+$ breast CSCs and MCF7 cells, as indicated by decreased *ALDH1A1*, *OCT-4* and *KLF4* expression level, and a decreased number of MFUs. This effect of glucosamine may be associated with a decreased pSTAT3/STAT3 ratio, indicating that glucosamine inhibited STAT3 activation; therefore, the results of the present study indicated that glucosamine treatment may be an improved approach to target the stemness of CSCs.

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Introduction

Cancer stem cells (CSCs) are a subpopulation of cancer cells that exhibit self-renewal and pluripotency (1). These traits of CSCs are associated with their ability to divide asymmetrically and produce an increased proportion of differentiated progeny cells, respectively, which enables them to seed tumors (2). Furthermore, CSCs possess distinctive characteristics, including high tumor-initiating potential, resistance to therapies and tumor recurrence (3). The stemness of a population of cancer cells corresponds to the proportion and tumorigenicity of CSCs present in this population (1). Furthermore, stemness is associated with distinct changes in pluripotency gene expression (3). The expression of transcription factors, including octamer-binding transcription factor 4 (OCT-4), sex-determining region Y-related high mobility group box gene 2 (SOX2) and Krüppel-like factor 4 (KLF4), is increased in CSCs, compared with non-CSCs, and confers the ability of self-renewal on CSCs (4-6).

CSCs have been successfully isolated from various tumor types, with breast CSCs being the first solid tumor-derived CSCs (7). Expression of cell-surface markers, including $CD24^{low}CD44^{high}$, may be used to identify breast CSCs (8). Alternatively, breast CSCs may be identified on the basis of aldehyde dehydrogenase (ALDH) activity (9). High pluripotency gene expression has also been observed in $CD24^{low}CD44^{high}$ and ALDH$^+$ CSCs (10).

In breast CSCs, pluripotency gene expression is regulated by complex signal transduction pathways, including the signal transducer and activator of transcription 3 (STAT3) signaling pathway (11,12). Previous studies have identified that the transcription factor STAT3 serves a significant function in the expression of pluripotency genes, including *OCT-4*, *SOX2*, *KLF4* and ALDH 1 family member A1 (*ALDH1A1*) (13,14). Phosphorylation of STAT3 at Tyr^{705} induces its activation and enables it to act as a potent activator of pluripotency gene transcription. Results of *in vitro* and *in vivo* studies have demonstrated that an increased level of phosphorylated STAT3

(pSTAT3) is associated with mammosphere-forming capacity, self-renewal, increased invasiveness, tumor-generating capacity and metastatic potential, and that targeted STAT3 inhibition suppresses relapse and metastasis in an animal model (15).

Post-translational modification (PTM) is an important step in cellular protein maturation and involves the chemical modification of the protein structure, resulting in the generation of various modified forms of a protein. Global inhibition of protein N-glycosylation inhibits the Janus kinase (JAK)-STAT signaling pathway and other signaling pathways in cancer cells (16). Owing to targeted therapy being resisted by CSCs, PTM inhibition is preferred over targeted therapy for cancer treatment; however, PTM inhibition is undermined by the problem of toxicity, as it induces considerable damage by affecting multiple signaling pathways. A previous study identified that the toxicity was associated with tunicamycin, an N-glycosylation inhibitor (17).

Glucosamine is a naturally occurring amino monosaccharide that is primarily located in connective and cartilage tissues, where it serves as an essential component for maintaining flexibility and elasticity; therefore, glucosamine is frequently used for treating osteoarthritis in humans (18). Glucosamine has been indicated to be a candidate N-glycosylation inhibitor due to its anticancer activity (16,18). Chesnokov *et al* (16) indicated that glucosamine decreased the N-linked glycosylation of gp130, a highly glycosylated interleukin 6 (IL-6) receptor subunit, resulting in the inhibition of the IL-6-STAT3 signaling pathway. Currently, to the best of our knowledge, it has not been examined whether glucosamine is able to modify CSC stemness; therefore, in the present study, the effect of glucosamine on the stemness of ALDH+ breast CSCs was investigated.

Materials and methods

Reagents. D-glucosamine hydrochloride was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Dulbecco's modified Eagle's medium/Ham's F12 (DMEM-F12) and high-glucose DMEM were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Penicillin/streptomycin/amphotericin B mixture was purchased from Lonza Group, Ltd. (Basel, Switzerland). An ALDEFLUOR™ kit was purchased from Stemcell Technologies, Inc. (Vancouver, BC, Canada). Recombinant human fibroblast growth factor (cat. no. 064-05381) was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). The antibodies used in the present study were as follows: Mouse anti-human STAT3 antibody (124H6; cat. no. 9139; Cell Signaling Technology, Inc., Danvers, MA, USA); mouse anti-human pSTAT3 (Tyr^{705}) antibody (3E2; cat. no. 9138; Cell Signaling Technology, Inc.); mouse anti-human GAPDH antibody (cat. no. sc-47724; Santa Cruz Biotechnology Inc., Dallas, TX, USA); and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.).

Cell culture and glucosamine treatment. ALDH+ breast CSCs isolated from pleural effusion of a patient with metastatic breast cancer were provided by Professor Osamu Ohneda (Laboratory of Regenerative Medicine and Stem Cell Biology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan). Additionally, these cells have been established as a cell line, as described previously (19,20). To retain their stemness, ALDH+ breast CSCs were cultured in serum-free DMEM-F12 supplemented with 1% penicillin/streptomycin/amphotericin B at 37˚C in an atmosphere containing 5% CO_2, as described previously (21,22). Our preliminary experiments performed using the ALDH+ breast CSCs confirmed that treatment with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) induced differentiation of these CSCs, with notable changes in their morphology from floating and sphere-like cells to attached and epithelial-like cells (data not shown).

Human adherent epithelial adenocarcinoma cell line MCF7 was purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in high-glucose DMEM supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin/amphotericin B at 37˚C in an atmosphere containing 5% CO_2.

ALDH+ breast CSCs and MCF7 cells were seeded in a 6-well plate ($1x10^5$ cells/well) and were cultured under aforementioned conditions. After 24 h, DMEM-F12 was replaced with serum-free medium containing D-glucosamine hydrochloride. The concentrations of D-glucosamine used for ALDH+ breast CSCs were 0.25, 1, 4, 10 or 16 mM, whereas for MCF7 cells the concentrations used were 0.25, 1 or 4 mM. The control was ALDH+ breast CSCs or MCF7 cells without D-glucosamine treatment. Following 24 h treatment at 37˚C, the cells were harvested for further analysis.

Cell viability assay. Cell viability was determined by performing a trypan blue exclusion assay. Cell suspension was stained with 0.4% trypan blue solution (1:1 mixture) and was allowed to stand for 2 min at room temperature. Viable and dead cells were counted using a Luna™ automated cell counter (Logos Biosystems, Anyang, Gyeonggi, Korea). Relative viability was calculated using the following formula: Viability (%)=(number of viable cells/number of total cells) x100.

Mammosphere formation assay. Breast CSCs were seeded at a density of 100 cells/well in an ultra-low attachment 96-well plate (Corning Incorporated, New York, NY, USA) and grown in DMEM-F12 supplemented with 0, 0.25, 1, 4, 10 or 16 mM glucosamine at 37˚C in an atmosphere containing 5% CO_2. Formation of mammospheres from the breast CSCs was determined after 3 days. MCF7 cells were seeded at a density of 200 cells/well in an ultra-low attachment 96-well plate and grown in high-glucose DMEM supplemented with 20 ng/ml basic fibroblast growth factor at 37˚C in an atmosphere containing 5% CO_2. Formation of mammospheres from MCF7 cells was determined after 7 days.

The formation of mammosphere was observed under inverted microscope at x100 magnification (model no. IM-3; OPTIKA Srl, Ponteranica, Italy). Spheres ≥60 μm in diameter were counted as mammosphere-forming units (MFUs) (23) using OPTIKA Srl software (version 2.7; OPTIKA Srl). Diameters of irregularly shaped spheres were determined using the shortest diameter.

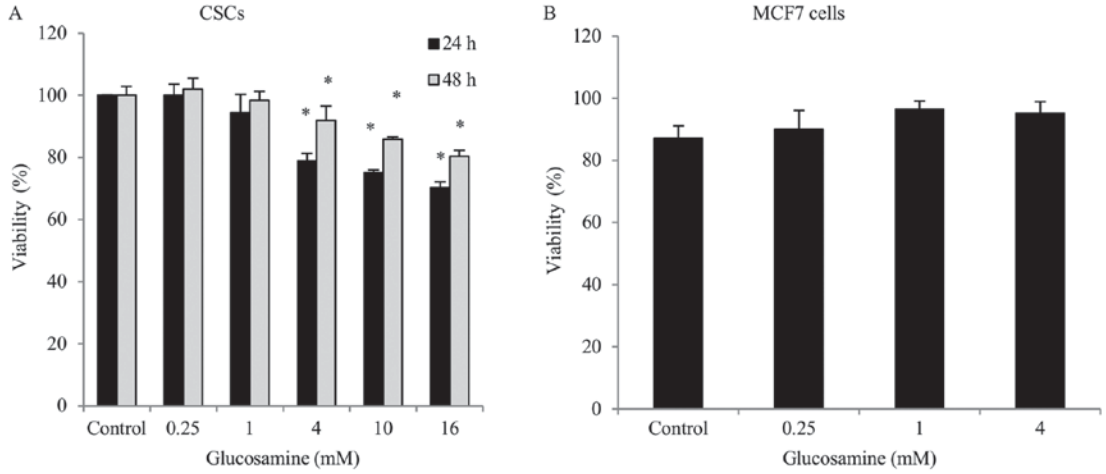

Figure 1. Viability of (A) aldehyde dehydrogenase-positive breast CSCs and (B) MCF7 cells. The CSCs were treated with 0.25, 1, 4, 10 or 16 mM glucosamine, whereas MCF7 cells were treated with 0.25, 1 or 4 mM glucosamine. Cell viability was determined by performing a trypan blue exclusion assay. Data are presented as the mean ± standard deviation of at least three independent experiments. *P<0.05 vs. corresponding control cells. CSCs, cancer stem cells.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For performing RT-qPCR, total RNA was extracted from the cells using the TriPure Isolation Reagent (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. RNA concentration was measured spectrophotometrically by using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Inc.) and the isolated RNA was stored at -80˚C. RT-qPCR was performed using a KAPA™ SYBR® FAST One-Step qRT-PCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and Exicycler™ 96 thermal block (Bioneer Corporation, Daejeon, Korea). PCR was performed using the following primers: *OCT-4* forward, 5'-GAGGAG TCCCAGGACATCAAA-3' and reverse, 5'-AGCTTCCTC CACCCACTTCT-3'; *ALDH1A1* forward, 5'-GGAGGAAAC CCTGCCTCTTTT-3' and reverse, 5'-TTGGAAGATAGG GCCTGCAC-3'; *KLF4* forward, 5'-CCGCTCCATTACCAA GAG-3' and reverse, 5'-TTTCTCACCTGTGTGGGTTC-3'; and 18S rRNA gene forward, 5'-AAACGGCTACCACATCCA AG-3' and reverse, 5'-CCTCCAATGGATCCTCGTTA-3'. The conditions for PCR are as follows: Initial denaturation at 42˚C for 5 min and 95˚C for 5 min; followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at optimal temperature (57˚C, 59˚C, 55˚C and 60˚C for *OCT-4*, *ALDH1A1*, *KLF4*, and 18S rRNA, respectively) for 20 sec and extension at 72˚C for 20 sec. Amplicon levels of the target genes are expressed relative to those of the 18S rRNA gene, which was used as an internal control, using the $\Delta\Delta C_q$ method (24).

Western blot analysis. Following treatment with glucosamine, the cells were washed twice with PBS and were lysed for 10 min in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Inc.) containing an anti-protease mixture. Protein concentration was determined using the Bradford method (25). Protein fractions were suspended in a Laemmli sample buffer (Bio-Rad Laboratories, Inc.) and were denatured at 100˚C for 5 min. Total protein (20 μg/lane) were separated by SDS-PAGE (12% gel) and were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked by incubation with 5% bovine serum albumin (Nacalai Tesque, Inc., Kyoto, Japan) in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature and were incubated overnight at 4˚C with mouse anti-human monoclonal antibodies against STAT3 (1:2,000 dilution), pSTAT3 (1:1,000 dilution) and GAPDH (1:200 dilution). The blots were visualized using HRP-conjugated anti-mouse IgG (1:2,000 dilution) and enhanced chemiluminescence reagent (cat. no. ab133406; Abcam, Cambridge, UK). Intensities of bands representing pSTAT3 and STAT3 expression levels were calculated using ImageJ software (Version 1.50i; National Institutes of Health, Bethesda, MD, USA), and the pSTAT3/STAT3 ratio was calculated using the formula: pSTAT3/STAT3 ratio=(pSTAT3 intensity/GAPDH intensity)/(STAT3 intensity/GAPDH intensity).

Statistical analysis. All results are expressed as mean ± standard deviation of at least three independent experiments. Statistical analysis was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Statistical differences among the groups were determined using one-way analysis of variance and Duncan post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Glucosamine decreases ALDH$^+$ CSC viability. The effects of various concentrations of glucosamine on the viability of ALDH$^+$ breast CSCs and MCF7 cells was determined using a trypan blue exclusion assay. Glucosamine treatment (0.25, 1, 4, 10 or 16 mM) gradually decreased ALDH$^+$ breast CSC viability in a dose-dependent manner. Treatment with glucosamine in ALDH$^+$ breast CSCs for different durations demonstrated that the shorter duration (24 h) of treatment resulted in a greater decrease in cell viability, compared with the longer duration (48 h) of treatment (Fig. 1); however, MCF7 cell viability did not significantly alter following glucosamine treatment (0.25, 1 or 4 mM) (Fig. 1).

Glucosamine downregulates the expression of stemness genes in ALDH$^+$ breast CSCs and MCF7 cells. ALDH1A1 is a detoxifying enzyme in the aldehyde metabolic pathway (9). Furthermore, ALDH1A1 has been proposed as a marker

for identifying and isolating CSCs from various cancer cells, including breast cancer cells (9). *ALDH1A1* expression was increased 1.8-fold in untreated ALDH+ breast CSCs, compared with in MCF7 cells (Fig. 2A). Compared with the control, treatment with 4 mM glucosamine significantly downregulated *ALDH1A1* expression in ALDH+ breast CSCs (0.7-fold; P<0.05) and MCF7 cells (0.49-fold; P<0.01) (Fig. 2A). OCT-4 and KLF4 are transcription factors expressed in embryonic and adult human stem cells. They are termed Yamanaka factors due to their major functions in induced pluripotent stem cells (26). *OCT-4* and *KLF4* expression was increased 1.8- and 2-fold, respectively, in untreated CSCs, compared with untreated MCF7 cells (Fig. 2B and C). Glucosamine treatment (4 mM) significantly downregulated *OCT-4* (0.46-fold; P<0.01) and *KLF4* expression (0.42-fold; P<0.01) in ALDH+ breast CSCs, compared with their respective control. The downregulation of *OCT-4* expression following glucosamine treatment demonstrated a similar pattern in ALDH+ breast CSCs and MCF7 cells (Fig. 2B); however, *KLF4* expression was upregulated in MCF7 cells (Fig. 2C), compared with the control.

Glucosamine decreases mammosphere formation in ALDH+ breast CSCs and MCF7 cells. To examine whether glucosamine treatment affected the self-renewal capacity of CSCs, an *in vitro* mammosphere formation assay was performed. Results of our preliminary experiments demonstrated that mammospheres of ≥60 μm in diameter were formed in ALDH+ breast CSCs on the third day, whereas in MCF7 cells these formed on the seventh day following seeding, indicating increased tumorigenicity of ALDH+ breast CSCs, compared with MCF7 cells. Furthermore, this result demonstrated that CSCs were more enriched in ALDH+ breast cancer cells, compared with MCF7 cells. The number of mammospheres formed by untreated ALDH+ breast CSCs was markedly increased (5.02-fold), compared with MCF7 cells (Fig. 3). Treatment with 4 mM glucosamine significantly decreased the MFUs in ALDH+ breast CSCs and MCF7 cells. Notably, the decrease in MFUs in glucosamine-treated ALDH+ breast CSCs was significantly increased, compared with glucosamine-treated MCF7 cells (Fig. 3).

Glucosamine suppresses STAT3 signaling in ALDH+ breast CSCs and MCF7 cells. Subsequently, the activation of the STAT3 pathway, a key signaling pathway, was examined in ALDH+ breast CSCs. STAT3 is activated through phosphorylation, and activated STAT3 induces the expression of target genes, including *OCT-4*, *SOX2*, *KLF4* and *ALDH1A1* (13,14). STAT3 and pSTAT3 levels were determined by western blot analysis. Activation of the STAT3 signaling pathway was determined by calculating the pSTAT3/STAT3 ratio. Glucosamine treatment inhibited STAT3 phosphorylation in ALDH+ breast CSCs and MCF7 cells (Fig. 4); however, 4 mM glucosamine-induced inhibition of STAT3 phosphorylation in ALDH+ breast CSCs was less significant (P<0.05), compared with that in MCF7 cells (P<0.01).

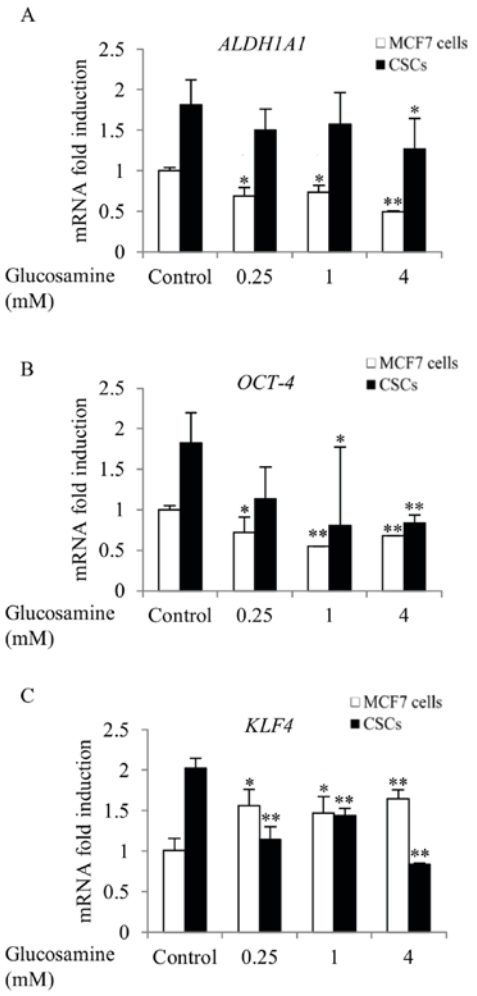

Figure 2. Expression of stemness genes in ALDH+ breast CSCs and MCF7 cells following glucosamine treatment. The cells were treated with various concentrations of glucosamine for 24 h, as indicated. Total RNA was isolated, reverse-transcribed and analyzed using the quantitative polymerase chain reaction for determining the expression of (A) *ALDH1A1*, (B) *OCT-4* and (C) *KLF4*. Data are presented as the mean ± standard deviation of at least three independent experiments. *P<0.05 and **P<0.01 vs. corresponding control cells. ALDH+, aldehyde dehydrogenase-positive; *ALDH1A1*, aldehyde dehydrogenase 1 family member A1; *OCT-4*, octamer-binding transcription factor 4; *KLF4*, Krüppel-like factor 4; CSCs, cancer stem cells.

Discussion

Eradication of cancer cells following therapy has always been difficult due to their resistance to the anticancer effects of the therapy. In chemotherapy, cancer cells resist eradication by pumping out drugs or by preventing the induction of apoptotic cascades (27). In targeted therapy, cancer cells resist eradication by compensating for the decreased or missing activity of a particular protein by activating other pathways, including IL-6/STAT3 and Notch3, to sustain their oncogenic state and/or stemness (28).

In the present study, the decision to inhibit PTM as an approach for non-targeted anticancer therapy was primarily based on tumor heterogeneity, which is one of the causes of the failure of cancer treatment. Theoretically, a highly heterogeneous population of cancer cells can survive targeted therapy due to them having a high possibility of containing a resistant clone (29). This assumption was confirmed by reports on the enrichment of CSCs following chemotherapy (30). Residual CSCs adopt numerous mechanisms to withstand various therapies, including targeted therapy (27); therefore, to prevent any form of 'bounce back' following targeted

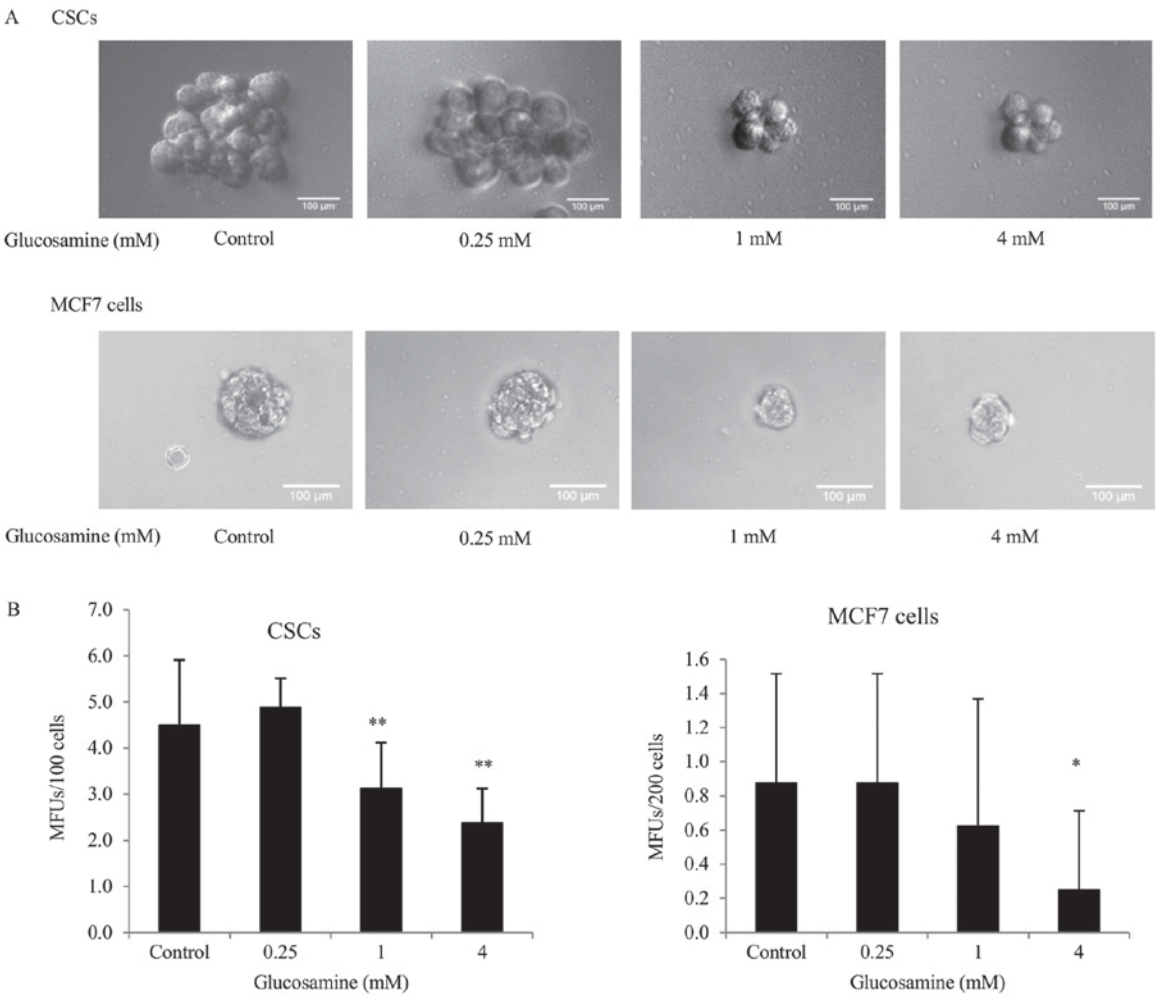

Figure 3. Effects of glucosamine on mammosphere formation by ALDH+ breast CSCs and MCF7 cells. (A) Representative photographs of aldehyde dehydrogenase-positive breast CSC and MCF7 cell mammospheres were acquired on the third and seventh day, respectively, following seeding. Scale bar, 100 μm. (B) The number of mammospheres was counted under an inverted microscope, and MFUs were calculated. Spheres ≥60 μm in diameter were counted as a single MFU. Diameters of irregularly shaped spheres were determined using the shortest diameter. Data are presented as the mean ± standard deviation of at least three independent experiments. *P<0.05 and **P<0.01 vs. control cells. CSCs, cancer stem cells; MFUs, mammosphere-forming units.

therapy, it was considered that a reasonable approach was to target the most pathways possible without endangering normal cells. As reported previously, CSCs have difficulty rewiring important pathways due to PTM inhibition affecting multiple pathways (16). Furthermore, due to drug efflux transporters, including multidrug resistance protein 1 (MDR1), requiring appropriate glycosylation, cancer cells may not be able to resist the effects of chemotherapy following PTM inhibition (31); therefore, it was indicated that PTM inhibition is preferable to targeted therapy for eradicating CSCs.

As described previously, toxicity is the primary drawback of the global inhibition of N-glycosylation. This indicates the requirement for developing a strategy, in which cancer cells intake increased amount of PTM inhibitor, compared with normal cells. A possible way to address this issue is to utilize the concept of cancer cell metabolism, known as the Warburg effect. To survive under relatively hypoxic conditions, cancer cells adjust their metabolism to a glycolytic state, thus increasing lactate production; therefore, cancer cells exhibit high glucose uptake (32). Glucosamine was used to inhibit the protein N-glycosylation in the present study due to its structure mimicking the structure of glucose and it exhibiting low toxicity (16,33).

The anticancer activity of glucosamine has been known for >50 years; however, the mechanism underlying the anticancer activity of glucosamine remains unclear (34). A number of mechanisms, including autophagy induction, proteasomal activity inhibition, cell cycle arrest, nuclear factor-κB inhibition and N-glycosylation inhibition, have been proposed (18). Glucosamine is indicated to inhibit the activity of JAK/STAT signaling proteins, including STAT3, by inhibiting the N-glycosylation of gp130, a subunit of the IL-6 receptor complex (16); however, to the best of our knowledge, it has not been examined whether glucosamine affects stemness. In the present study, it was observed that glucosamine decreased *ALDH1A1*, *OCT-4* and *KLF4* expression and the mammosphere-forming ability of ALDH+ breast CSCs and MCF7 cells, indicating that glucosamine decreased the stemness of these cells. These changes are consistent with the decreased pSTAT3/STAT3 ratio in these cells. On the basis of results of previous studies (13,15), we hypothesized that the downregulation of stemness gene expression in ALDH+ breast CSCs and MCF7 cells following glucosamine treatment may be a functional consequence of STAT3 inactivation.

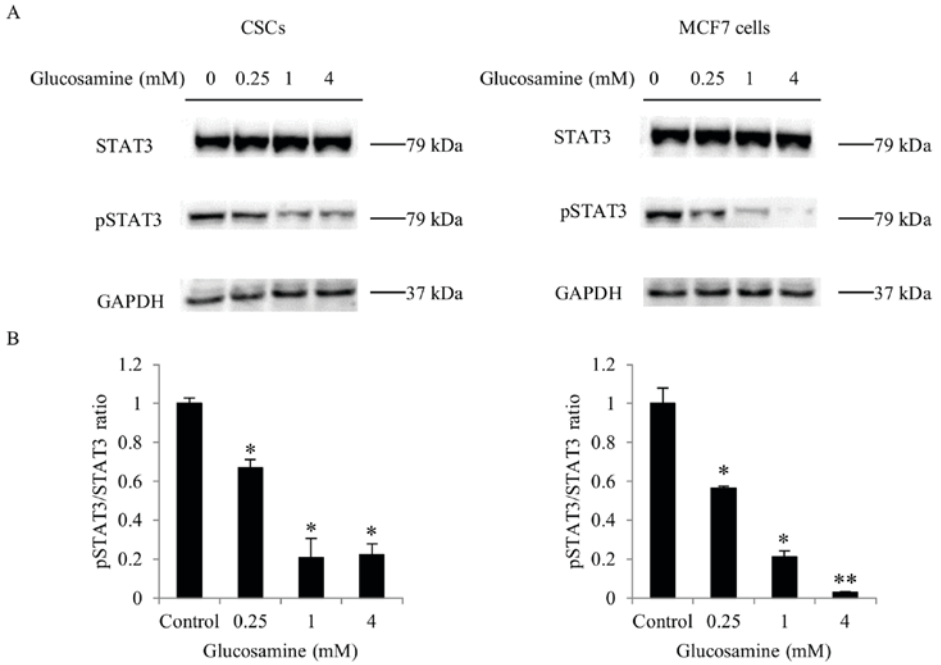

Figure 4. Glucosamine treatment decreased STAT3 phosphorylation in aldehyde dehydrogenase-positive breast CSCs and MCF7 cells. (A) STAT3 and pSTAT3 levels were examined by performing western blot analysis, with GAPDH as a loading control. (B) The pSTAT3/STAT3 ratio was calculated based on densitometric quantification of the blots compared with GAPDH. Data are presented as the mean ± standard deviation of at least three independent experiments. *P<0.05 and **P<0.01 vs. control cells. STAT3, signal transducer and activator of transcription 3; pSTAT3, phosphorylated STAT3; CSCs, cancer stem cells.

The results from the western blot analysis confirmed that glucosamine significantly inhibited STAT3 phosphorylation of CSCs and MCF7 cells to a similar degree. The less significant inhibitory effect of 4 mM glucosamine in ALDH$^+$ breast cancer cells may be due to the increased abundance of CSCs in ALDH$^+$ breast cancer cells, compared with MCF7 cells, which exerted a density-dependent effect on chemoresistance, as indicated by He *et al* (35). CSCs may also attempt to maintain their stemness by activating other pathways that contribute to STAT3 activation, including overactivation of the G-protein-coupled receptor signaling pathway or overexpression of gp130 (36).

Furthermore, it was observed that the reduction of the pSTAT3/STAT3 ratio was consistent with the decrease in the expression of stemness genes *OCT-4* and *ALDH1A1*. These results confirmed the results of previous studies that reported that *OCT-4* and *ALDH1A1* were regulated by the transcription factor STAT3 (13,14); however, a STAT3 inhibitor was not used in the present experiments, which is a limitation of the present study. Lin *et al* (13) demonstrated that inhibition of STAT3 phosphorylation using Stattic and LLL12 decreased the tumorigenicity and viability of ALDH$^+$ breast CSCs; therefore, we hypothesized that the downregulation of stemness gene expression observed in the present study was a functional consequence of STAT3 inactivation.

Consistent with previous studies (16,37) involving various prostate cancer and non-small cell lung cancer cells, the results of the present study demonstrated that treatment with ≥4 mM glucosamine significantly decreased the viability of human ALDH$^+$ breast CSCs. This confirmed the requirement of a high glucosamine concentration for *in vitro* study. In the present study, glucosamine treatment in a xenograft mice model was not investigated owing to circumstances and limitations in available animal laboratory facilities. In order to reach an effective concentration *in vivo*, it is considered that there will be difficulties. In the study reported by Song *et al* (37), using a xenograft mouse lung tumor model, glucosamine was required to be introduced at a concentration as high as 500 mg/kg body weight in order to achieve a significant benefit of glucosamine. Furthermore, Weimer *et al* (38) reported that the plasma level of glucosamine in a mouse model may be increased up to ~2 μM. Notably, it was observed that the effects of glucosamine on ALDH$^+$ breast CSC viability are increased after 24 h of treatment, compared with after 48 h; however, the glucosamine treatments for longer durations were not performed in the present study due to MCF7 cells not surviving in serum-free treatment medium for ≥48 h. If the effect of stemness suppression is only short-term, it may indicate that glucosamine treatment should be repeated as necessary to replenish the effect.

In contrast with ALDH$^+$ breast CSCs, a significant effect of glucosamine on the viability of MCF7 cells was not observed. Distinct metabolic pathways in CSCs and non-CSCs may be responsible for different responses to glucosamine treatment. For example, CD44$^+$CD117$^+$ ovarian CSCs exhibit increased glucose uptake, compared with CD44$^+$CD117$^-$ ovarian non-CSCs (39). Additionally, the differential effects of glucosamine on the viability of ALDH$^+$ breast CSCs and MCF7 cells may depend on the density of CSCs in ALDH$^+$ breast cancer cells, compared with MCF7 cells, due to STAT3 inactivation as aforementioned. Owing to the notably lower density of CSCs in MCF7 cells, compared with ALDH$^+$ breast cancer cells, we hypothesized that STAT3 inactivation following glucosamine treatment may be a mediator of the antiproliferative effect of glucosamine, which specifically targets CSCs.

Although it was determined that glucosamine treatment significantly decreased CSC viability, the extent of the decrease in cell viability was less than that induced by Stattic- and LLL12-targeted therapy in $ALDH^+$ breast CSCs (13,15); therefore, we hypothesized that glucosamine treatment may be improved when applied as an adjuvant therapy. There are two previous studies that identified that an N-glycosylation inhibitor acts as a chemosensitizer by affecting transporter proteins involved in multidrug resistance, including MDR1 (31). Furthermore, the global inhibition of PTM may affect the CSC niche (40), thus overcoming problems associated with CSC targeting, particularly problems associated with plasticity and epithelial-mesenchymal transition (41).

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that glucosamine affected the stemness of human $ALDH^+$ breast CSCs, thus decreasing their viability. This effect of glucosamine may be associated with the inhibition of STAT3 phosphorylation; however, further investigations with xenograft animal models are required to verify the effects of glucosamine observed in the present study.

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Availability of data and material

The datasets used and/or analyzed in the present study can be made available by the corresponding author upon reasonable request.

Authors' contributions

RH and SIW designed all the experiments, and analyzed and interpreted the study data. RH performed the experiments and prepared the manuscript. NSH and OO assisted in conducting cell sorting. SIW and RH edited the manuscript prior to submission. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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