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Abstract

Trehalose is a promising natural cryoprotectant, but its cryoprotective effect is limited due to difficulties in trans-membrane transport. Thus, expressing the trehalose transporter TRET1 on various mammalian cells may yield more trehalose applications. In this study, we ran comparative cryopreservation experiments between the TRET1-expressing cells and the wild-type cells under various trehalose concentrations in an extracellular medium and under various freezing temperatures. We show here that the intracellular trehalose significantly improves the viability of freeze-thawing cells. The optimum trehalose concentration is about 400 mM, and the optimum freezing temperature ranges between 140 and 240 K. We find a qualitative coincidence of the optimum freezing temperature with that from including 10 vol% dimethyl sulfoxide, a result that indicates that both intra- and extra-cellular trehalose principally work to inhibit ice crystal growth and thus protect the membrane and proteins from ice crystals during relatively slow freezing.

(144 words)

<Abbreviations>

TERT1, trehalose transporter 1; Me_2SO, dimethyl sulfoxide; SD, standard deviation

＜Key words＞

trehalose, trehalose transporter 1, cryoprotectant, freezing temperature, trehalose concentration, CHO-K1 cell

Introduction

Cultured cells from animals are widely used as alternative samples for pharmacological tests and as in-vitro models in studies of principal biological mechanisms. The cells are usually incubated with a suitable culture medium immediately after collection. However, due to an increased demand for such in-vitro models in tissue engineering, cell transplantation, and genetic technologies, we need better long-term storage techniques for living cells.

The traditional approach to such cell storage relies on cryopreservation methods that involve cryoprotectants such as dimethyl sulfoxide (Me_2SO), glycerol, and ethylene glycol [13]. Recently, small carbohydrate sugars, such as trehalose and sucrose, have been found to have an exceptional ability to stabilize and preserve cellular proteins and membranes [3, 4, 12, 15, 22-24, 30]. In addition, trehalose inhibits ice-crystal growth [8, 25, 28]. However, sugars such as glucose do not easily penetrate mammalian cells unless specific proteins are present in the cell membrane to facilitate transport. Consequently, a number of methods have been explored to introduce non-native sugars such as trehalose into mammalian cells. These include transfection [9], engineered pores [6, 7, 21], activation of native channels [5], microinjection [6, 7], electropermeabilization [22], and endocytosis [10, 18].

Recent studies show that anhydrobiotic insect larvae use specific trehalose transporters during desiccation stress. The trehalose transporter (TRET1) from the anhydrobiotic larvae of African chironomid, *Polypedilum vanderplanki*, has been isolated and characterized [11, 14]. By stably expressing the TRET1 in mammalian cells, extracellular trehalose was introduced into the cells with smaller stresses, and such cells significantly increased in viability under partial desiccation [2].

Here, we investigate the cryopreserving effect of trehalose on mammalian cells expressing TRET1. Under various trehalose concentrations in extracellular media and freeze temperature conditions, we measure the viabilities of mammalian cells and obtain the optimum conditions. Comparing these results with those of Me_2SO-including media, and with the physico–chemical properties of trehalose solution [28], we discuss the roles of trehalose as a cellular cryoprotectant.

Materials and methods

Cell culture and transfection of CHO-K1 cells with trehalose transporters

Gene-modified Chinese hamster ovary (Flp-In™-CHO) cells were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) and cultured in Nutrient Mixture F-12 Ham medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 1% penicillin–streptomycin (100 U/mL penicillin G and 100 μg/mL streptomycin, Thermo Fisher Scientific), 1% gluta max (100x, Thermo Fisher Scientific), and 0.8% Hygromycin B solution (Nacalai Tesque, Kyoto, Japan). Cultures were maintained at 310 K, and equilibrated with 5% CO_2–95% air in 75-cm^2 tissue culture flasks (AS ONE, Osaka, Japan). Further details of the culture preparation are in a previous study [2].

The trehalose transporter (TRET1) expression vector (pcDNA5/FRT-PvTret1-AcGFP1; supplementary Fig. S1) for mammalian-cell expression was prepared and the TRET1 sequence was fused with a green fluorescent protein (GFP) tag at the C-terminus by subcloning into the vector to generate a stable cell line. Stable cell lines were generated using the Flip-in system (Thermo Fisher Scientific). Briefly, intact Flp-In™-CHO cells were transfected with pcDNA5/FRT-PvTret1-AcGFP1 and pOG44 Flp-recombinase expression vector (Thermo Fisher Scientific) using Fugene6 (Roche, Basel, Switzerland) and stably transfected cells were selected with hygromycin B, and checked by the GFP expression on the cultured cell membrane with an epifluorescence microscope (IX71; Olympus, Tokyo, Japan). We refer to this stable line as CHO-TRET1 cells. As a negative control, the pcDNA5/FRT vector was transfected to Flp-In™-CHO cells, which is designated as CHO-vector cells.

Preparation of cell suspension for freezing

When CHO-vector cell and CHO-TRET1 cells reached more than 80% confluence, the cells were dissociated from the tissue culture flasks by trypsinization (0.05% trypsin-EDTA solution, Thermo Fisher Scientific) and collected by centrifugation at 1000 rpm for 3 min. Then the cell-culture medium in the tissue culture flask was removed completely using a Pasteur pipette with vacuum pump and immediately replaced with the culture media that was supplemented with 0 ~ 1000 mM trehalose (purity 99.9%, endotoxin free, donated by Hayashibara). One milliliter of the cell suspension (10^5 cells/mL) was put into a 1.8-mL cryotube vial (Thermo Fischer Scientific) and incubated for 6 h under the conditions described above for loading trehalose.

Cryopreservation protocol

Following trehalose loading, both CHO-vector and CHO-TRET1 cells in solution were put into a freezer at temperature settings of 263, 253, 243, 233, 213, and 198 K. Under these temperatures, the average rate of temperature decrease for the samples (including 1-mL medium) V_f, is about 5 K/min. Here we calculate V_f as the temperature difference from room temperature to the freezing-state temperature divided by the time period prior to the freezing start. To investigate temperatures down to below 198 K, we used the temperature gradient in a partly filled liquid nitrogen dewar (Taylor-Wharton, Theodore, AL, USA). When about 770-mL liquid nitrogen was put in the 5-L container, we inserted the vials. One vial was clipped at the lowest part of the vial holder (Shur-Bend, St. Paul, MN, USA), which contacts the liquid nitrogen (77 K). The other four vials were clipped in upper parts of the holder and thus held above the liquid nitrogen. Their temperatures were approximately 100, 140, 170, and 200 K. Under these temperatures, V_f is estimated as ranging between 20 and 200 K/min. Each temperature was measured by a thermocouple (T-type) on the side of each vial and recorded with a data logger (Graphtec, Yokohama, Japan; type GL200A). After the temperature stabilizes to the environmental one, the sample is stored at either the same temperature or in the deep freezer (198 K). As a preliminary survey showed no differences in the viabilities whether stored at 198 K for one day or for two weeks, we fixed the storage period at one week (±2 days).

To investigate the cryopreserving characteristics of trehalose, we also ran a similar test using 10 vol% Me_2SO (Sigma), a popular trans-membrane type cryoprotectant. In this case, the sample was set in the dewar immediately after replacing with the solution. We skipped the incubation procedure to inhibit the toxic effect of Me_2SO on the cell activities.

Viability and growth assays

The individual vials from each temperature conditions are then thawed in a 310-K water bath until all extracellular ice is completely melted (about 5 min). During this process, the temperature increases by about 50 K/min. The solution in the thawed cell suspension is then rinsed with culture media twice by centrifugation and the supernatant solution removed. In this way, the cryoprotectants are largely removed from the culture media.

The survival rate of CHO-K1 cells was assessed using fluorescence dyes, calcein-AM and PI solutions (Cellstain; Dojindo, Kumamoto, Japan), soon after the solution replacement (approximately 0.5-h after thawing). After incubating for 20 min, we extracted 10 μL from the cell suspension of live and dead cells and measured their emissions at 515 and 620 nm on a hemocytometer (Waken counter, Waken B-tech, Kyoto, Japan). Fluorescence images were captured using the epifluorescence microscope (IX71; Olympus) through an Olympus LCPLFL 20× objective and collected with a charge-coupled device (DP70; Olympus). Cells of bright green fluorescence are considered as alive, whereas bright red are scored as dead. Those displaying either both colors or no color were used for estimating measurement uncertainties.

To determine the survival and growth efficiency, each CHO-cell suspension was replaced with the culture medium and plated in a 24-well cell-culture plate (Nippon Genetics, Tokyo, Japan). The plated cells were then incubated in a CO_2-incubator as described above for five days after thawing. The normality of the functions for the living CHO-K1 cells was checked by observing the adhesion and growth of the cell, and qualitatively comparing to the control CHO-K1 cells.

Uncertainties of viability and statistical analysis.

The viabilities are calculated as the number of living cells (stained by calsein-AM) normalized by total cell populations (more than 500 cells). Considering the sampling variation, the staining error, and the human error of counting the cells on a hemocytometer, the total uncertainty of any given data point is at most 8%. At least three experiments were run per condition, so the resulting viability here is averaged at the indicated experimental conditions ± the standard deviation (SD) of all measured data. For the uncertainty of the data, we selected the larger value between 8% of the mean value and the SD value.

For experiments on CHO-K1 cells, data were analyzed using two-way ANOVA with a Dunnett post-hoc test (Prism, GraphPad Software) for at least 99% confidence ($p < 0.01$). All cryopreservation tests and 5-day growth experiments were repeated at least three times for the independent cell lines.

Results

Effect of trehalose concentration on cryopreservation of CHO-K1 cells

We measured the effect of trehalose concentration in the medium on the TRET1-expressing CHO-K1 cells viability and growth after freezing. Figure 1 shows that after freezing and storing for 1 week at 193 K only a very small fraction of both kinds of CHO-K1 cells survived in the absence of trehalose. The presence of extracellular trehalose only slightly improves the viability of CHO-vector cells, but significantly improves the viability of CHO-TRET1 cells. Moreover, the viability of the CHO-TRET1 increases with trehalose concentration.

To check the health of the cryopreserved cells, we cultured them at 310 K for one week and observed their adhesion-proliferation processes. Figure 2 shows micrographs of cryopreserved CHO-K1 cells under 500-mM trehalose concentration for three-day culture. Adhesion of the surviving cells occurs after a one-day culture in both types of cells (the left column of Fig. 2), although only few CHO-vector cells survive. After a two-day culture, the CHO-TRET1 cells become almost confluent. Although the viability of CHO-vector cells is small, those that survive can adhere and proliferate as normal cells. As these proliferation rates are almost the same as the control, we consider that the surviving cells keep their normal functions after a freeze-thaw cycle.

As the cryopreserved cells were cultured in normal medium, the effect of intracellular trehalose on the health of the cells is likely negligible. Our argument is based on a related experiment on oocyte: Kikawada et al. [11] incubated oocyte in 105-mM trehalose buffer for three hours, then found that it took about two hours to eliminate the intracellular trehalose from the oocyte. Given that CHO-K1 cells are smaller than oocytes, most of the intracellular trehalose in our CHO-TRET1 cells should be removed within several hours. Hence, the cultivation condition between CHO-vector and CHO-TRET1 cells are considered to be similar.

Now consider the effect of trehalose concentration on the viability of CHO-K1 cells. Figure 3 shows the viability of CHO-vector cells (gray diamonds) and CHO-TRET1 cells (black circles). The data roughly follow bell-shaped curves, having relatively low viability at the lowest and highest trehalose concentrations. The maximum viabilities are at 250 mM (0.099 ±0.077) for CHO-vector and at 400 mM (0.806 ±0.051) for CHO-TRET1 cells, which indicates the viability is ten times larger in the latter case. Statistical analysis indicates a significant increase in viability of CHO-TRET1 cells to that of CHO-vector cells ($p < 0.01$) at the trehalose concentration exceeding 200

mM. Consequently, we confirm that the trehalose loading into the cell with TRET1 significantly improves the cryopreservation process.

Effect of frozen temperature on cryopreservation of CHO-K1 cells

Here we examine the temperature-dependence of the viability of the cryopreserved cells. Two concentrations are considered: trehalose-free (0 mM) and the near-optimum concentration (500 mM). To better understand the cryopreserving mechanism of the intracellular trehalose, we also used 10 vol% Me_2SO instead of trehalose.

For CHO-vector cells (Fig. 4a), the viabilities in both 0-mM and 500-mM trehalose concentrations are low (at most 0.072 ± 0.060 for 0-mM trehalose at 145 K) at all temperatures. Conversely, the viabilities of CHO-TRET1 cells (Fig. 4b) are significantly higher for the 500-mM extracellular trehalose condition than those for the 0-mM condition and those of CHO-vector cells at temperatures above 140 K ($p < 0.05$ at this temperature range). The maximum viabilities occur at 213 K with a 500-mM concentration (0.740 ± 0.071). The results also show that the intracellular trehalose improves the viability of the cryopreserved cells for temperatures at and above 140 K, although the extracellular trehalose exhibits only a small cryopreservation effect.

Now compare the viabilities of CHO-K1 cells with trehalose to that with Me_2SO. Me_2SO is the most popular trans-membrane type cryoprotectant, so we use it here as a positive control. For Me_2SO, more than 90% viability occurs at temperatures between 130 and 230 K (marked as crosses in Figs. 4). This optimum temperature range coincides well with that for CHO-TRET1 cells, which suggests that the intracellular trehalose cryoprotects via a mechanism similar to that of Me_2SO. However, as the temperature exceeds 250 K, the Me_2SO loses its cryoprotection ability for both CHO-vector and CHO-TRET1 cells, even though the intracellular trehalose retains sufficient cryoprotecting effect. Thus, near the ice melting point, the intracellular trehalose appears to have additional cryoprotecting roles than those of Me_2SO.

Discussion

In our freeze-thaw experiments, extracellular trehalose did not significantly affect cryopreservation of CHO-vector cells. This result is consistent with that obtained for neurons with extracellular trehalose [16]. Conversely,

for the CHO-K1 cells loaded through TRET1, the intracellular trehalose did significantly increase the cryopreservation. But what is the cryoprotecting mechanism of the intracellular trehalose? To address this question, we examine how the viability was affected by the trehalose concentration and by the freezing temperatures.

Consider first the cryopreservation of CHO-TRET1 cells at 193 K. The viability versus trehalose concentration in the extracellular medium shows a bell-shaped curve in Fig. 3, peaking near 400 mM. This result is qualitatively consistent with the viability of desiccation tolerance in CHO-K1 cells [2] and of cryopreservation of 3T3 fibroblasts and human keratinocytes [6], despite differences in experimental procedures and the findings of the optimum condition. As the concentration of the intracellular trehalose is expected to be proportional to that of the extracellular concentration [2, 11], this result suggests an optimum intracellular concentration for the cryoprotecting effect of trehalose.

Concerning the minimum trehalose concentrations needed for sufficient cryoprotection, note that the viability of CHO-TRET1 cells essentially vanishes for extracellular trehalose concentration below 200 mM. This behavior is likely caused by having an insufficient amount of intracellular trehalose to prevent intracellular ice formation and growth. Due to the relatively long loading time prior to freezing (6 hours), this intracellular-trehalose concentration should be in equilibrium with the extracellular conditions. Hence, we argue that the minimum extracellular trehalose concentration to show a sufficient cryoprotection effect is about 200 mM. By extrapolating trehalose uptake data for CHO-TRET1 cells [2], this suggests a minimum intracellular trehalose concentration for sufficient cryopreservation of about 27 nM/cell.

At the other extreme, the viability of the cryopreserved CHO-TRET1 cells decreases when the extracellular trehalose concentration exceeds 500 mM. This result suggests that, even though trehalose is nontoxic for CHO-K1, a higher intracellular trehalose concentration might weaken the cells. A possible reason for this result is a weakening of CHO-TRET1 cells from hyperosmotic shock. As the Michaelis-Menten constant *Km* for CHO-TRET1 cells is 137 ± 87 mM [2], the maximum trehalose transport of TRET1 would be about 500 mM. Hence, even when the extracellular trehalose concentration goes above 500 mM, the intracellular trehalose concentration does not increase so much. If this effect occurs during incubation for the trehalose loading, the large amount of extracellular trehalose would harm the cells via excessive dehydration. Another possible reason is that, as

trehalose has a high hydration effect, an oversupplied trehalose would reduce the intracellular water activities [28, 30], which may, for example, inhibit the intracellular material transport.

Second, consider the freezing-temperature dependence on the freeze-thawing viability of CHO-K1 cells. In Fig. 4, we compare this viability for cells cryopreserved under two trehalose concentrations and one Me_2SO concentration. High viabilities occur in both 500-mM-trehalose CHO-TRET1 cells and in the 10-vol%-Me_2SO CHO-K1 (both vector and TRET1) cells at freezing temperatures between 140 and 240 K. This result suggests that intracellular trehalose, in the optimum quantities, cryoprotects via a similar mechanism as Me_2SO.

About the mechanism for Me_2SO, we consider the solid-phase structures in Me_2SO solution. For such solution, the glass transition temperature T_g' is 143 K, and the 3:1 compound, referred to here as the 'Me_2SO hydrate', forms at temperatures between 143 and 210 K [17, 19]. In the 10 vol% (about 1.3 M) Me_2SO solution, the ice melting point is about 260 K [17, 30]. This temperature is equivalent to the ice melting point in the culture medium (without any additional cryoprotectant) [30]. Therefore, the Me_2SO hydrate, not the vitrification state, is expected to form at the optimum cryoprotection temperature range (140 ~ 240 K). According to Rall et al. [19], heterogeneous ice nucleation is inhibited under this optimum temperature condition and under a 10-vol% Me_2SO concentration. Therefore, we argue that the optimum cryopreservation conditions in the present study are caused by forming the Me_2SO hydrate, which then has a sufficient inhibition effect on ice nucleation and growth in the cell. On the other hand, the glass-transition temperature *Tg*' of 500-mM trehalose solution should be a little lower than 238 K, which is *Tg'* in 50 wt% (about 1.46 M) trehalose solution [1]. Thus, the high viabilities of 500-mM-trehalose CHO-TRET1 cells at temperatures between 140 and 220 K may be caused by the vitrification of intracellular solution, or by a sufficient inhibition effect of the intracellular trehalose on ice nucleation and growth.

Above 240 K, the viabilities of 10 vol%- Me_2SO CHO-K1 (both vector and TRET1) cells decrease drastically. This temperature range is considered to be insufficient for the ice formation because of the reduction of the ice freezing point down to about 260 K. For example, several samples with 10-vol% Me_2SO were not frozen during a 1-week-storage period at about 260 K (not shown in the plots). Therefore, the low viabilities of this temperature range may occur either because the intracellular Me_2SO cannot inhibit the ice-crystal growth in the cell, or because the cells are weakened by a toxic effect of Me_2SO [e.g., 29, 30] prior to the freezing.

241 The viability of 500 mM-trehalose CHO-TRET1 cells are as high as 0.5 at temperatures above 230 K. This high
242 viability in the trehalose-including conditions may be caused by a different mechanism than that for Me_2SO. As
243 the melting point of the medium including 500-mM trehalose is much higher than 230 K, the intracellular
244 trehalose would inhibit intracellular ice formation by reducing the activities of intracellular H_2O molecules [27,
245 28]. However, the cryoprotecting effect of trehalose occurs not only through inhibition of ice crystal growth [8, 25,
246 28] but also from a protection effect of cell membranes and the stabilization of proteins [3, 4, 12, 15, 22-24, 30]. If
247 the trehalose exists in both extra- and intra-cellular circumstances, the cell membrane may be sufficiently
248 protected both ways from the injury of ice crystal growth. The intracellular trehalose is expected not only to
249 protect the cell membrane and several organelles, but also to stabilize proteins in cytosol.
250
251 The slight decrease of viabilities near and above 230 K may be caused by the following mechanism. As the *Tg*' of
252 500-mM trehalose solution is just above 230 K, some of the intracellular water should be ice I_h [26] with further
253 growth inhibited by trehalose. The remaining intracellular water would include an amount of trehalose over the
254 optimum concentration, which reduces the activities of the intracellular H_2O molecules. This reduction would
255 reduce the viabilities of the cells rather than contribute to the cryoprotecting effect. The decrease in the viability at
256 high extracellular trehalose concentrations for CHO-TRET1 cells at 193 K (Fig. 3) supports this speculation.
257
258 However, below 140 K, the viabilities for both 500-mM trehalose CHO-TRET1 cells and 10 vol%- Me_2SO CHO-
259 K1 cells are low. This decrease is particularly sudden in the Me_2SO-administrated cells. As this critical
260 temperature coincides with the T_g' of 10 vol%- Me_2SO solution, the induction of a glassy state may be the cause.
261 This result differs from our expectation given that the cryoprotecting effect is caused by the vitrification of
262 intracellular condition. Rasmussen and MacKenzie [20] showed that vitrification required a 40 ~ 80 wt% Me_2SO
263 concentration for this particular cooling rate, which is much higher concentration than that in the present study.
264 Therefore, we suggest that the lower viabilities of 10 vol%- Me_2SO CHO-K1 cells at temperatures below 140 K
265 may be caused by intracellular freezing because the intracellular Me_2SO can neither induce vitrification nor form
266 the Me_2SO hydrate.
267
268 Consider the role of the freezing rate for the case of 500-mM trehalose CHO-TRET1 cells. The viabilities of 500-
269 mM trehalose CHO-TRET1 cells gradually decrease with temperature below 170 K. As the T_g' of 500-mM
270 trehalose solution is well above 140 K, no drastic changes, such as a phase change, could produce this decrease.

So, we consider instead the cooling rate V_f, which in these experiments varied with the freezing temperature. At freezing temperatures above 180 K, V_f is below 10 K/min; At 140 K, it is about 20 K/min; and at 77 K, it is about 100 K/min. Based on these estimates, the viability of 500-mM trehalose CHO-TRET1 cells is high at V_f below 10 K/min, but decreases with increasing V_f above 10 K/min, and essentially vanishes when V_f exceeds 20 K/min.

The relation between V_f and the viability may be explained as follows: when V_f is large, the supercooling is large (the ice nucleation occurs at lower temperatures). The larger the supercooling, the faster the ice-crystal growth, and the greater difficulty for intracellular trehalose to inhibit the growth. According to Uchida et al. [25], ice crystals formed in the 500-mM (~ 17 wt%) trehalose solution by quenching in liquid nitrogen were of order several-microns across. This crystal size roughly equals that of CHO-K1 cells. When such an ice crystal forms in the cell, the cell dies. To test this speculation, we plan to run additional experiments in which we control the cooling rate.

We should also examine the melting rate. Uchida et al. [27, 28] showed that the existence of 10-vol% Me_2SO in the culture medium reduced the melting point of ice below 265 K. As this temperature is below that of maximum ice-grain growth, the frozen sample with Me_2SO should not have rapid ice recrystallization during melting. Conversely, the ice melting point in the culture media with trehalose is about 270 K for a 500-mM trehalose concentration [27, 28]. Thus, to understand the difference in viabilities of CHO-K1 cells with Me_2SO versus those with trehalose, we should consider a possible effect of the rapid ice recrystallization during melting in the frozen sample with trehalose. Future experimental studies including several additional parameterizations are needed.

Conclusion

We investigated the effect of intracellular trehalose transferred through TRET1 on the cryopreservation of CHO-K1 cells. Our method involved changing either the trehalose concentration in the medium or the freeze-storage temperature. We found that the intracellular trehalose improves the viability significantly. Cultivation of the cryopreserved cells indicated that the surviving cells retain their functions, and therefore, that the trehalose transferred via TRET1 minimizes the stresses on the cells. The optimum conditions for the cryopreservation of CHO-TRET1 cells are (1) an extracellular trehalose concentration that exceeds 200 mM, with 250–500 mM being optimal, and (2) a freezing temperature that exceeds 140 K, with 170–210 K being optimal.

To examine the cryoprotecting mechanism, we compared the temperature dependence of viabilities between the optimum trehalose concentration and the most popular trans-membrane type cryoprotectant, Me_2SO. The results indicated that the intracellular trehalose would not only inhibit the intracellular ice crystal growth, but also protect the cell membrane and organelles as well as stabilize the proteins in cytosol. The latter effect becomes significant at higher temperatures. An insufficient amount of intracellular trehalose, arising from a low extracellular trehalose concentration, cannot provide cryoprotection. At the other extreme, too much intracellular trehalose may have reduced the activities of intracellular water, thus lowering the measured viability. Even when the intracellular trehalose concentration was near-optimal for cryopreservation, we found that the cooling rate should be below 10 K/min to prevent large supercooling conditions.

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387

388 **Figure captions**

389

390 **Fig. 1.** Effect of trehalose concentration in the medium on the viability of cryopreserved cells. (a) CHO-vector
391 cells. (b) CHO-TRET1 cells. Samples stored at 193 K for 1 week. Red stain is PI, indicating dead cells, and green
392 stain is calcein-AM, indicating live cells. Horizontal distance across bottom is 2.2 mm.

393

394 **Fig. 2.** Micrographs of cells from the medium with 500-mM trehalose cryopreserved at 193 K for one week. (a)
395 CHO-TRET1 cells. (b) CHO-vector cells. After thawing in the bath at 310 K, the freezing medium was replaced
396 with culture media twice by centrifugation and removal of the supernatant solution. Each CHO-K1 cell suspension
397 was plated in a 24-well cell-culture plate. The plated cells were then incubated in a CO_2-incubator at 310 K for 5
398 days after thawing. The left column shows a 1-day culture after thawing, and the right column shows a 3-day
399 culture.

400

401 **Fig. 3.** Viabilities of cryopreserved CHO-vector cells (solid diamonds) and CHO-TRET1 cells (solid circles) at
402 various extracellular trehalose concentrations. The cells were incubated for six hours at 310 K prior to the sample
403 setting in the deep freezer (193 K). After 1-week storage, the frozen sample was thawed in the 310-K bath. Soon
404 after the thawing, the suspended cells were rinsed twice with PBS and incubated with fluorescence dyes. The
405 viability of each condition is the mean ± SD (or measurement error) of epifluorescence microscopic measurements.
406 The asterisks mark cases with sufficient difference of viabilities between CHO-vector cells and CHO-TRET1 cells
407 at the same freeze-thaw conditions ($p < 0.01$).

408

409 **Fig. 4.** Effect of freezing temperature and trehalose transporter (TRET1) on the viability of cryopreserved cells.
410 (a) CHO-vector cells. (b) CHO-TRET1 cells. CHO-K1 cells suspended in the medium with trehalose at a
411 concentration (0 mM: squares, 500 mM: triangles) were set at the indicated temperature condition (77 – 263 K)
412 after a six-hour incubation at 310 K. The temperature dependence on viabilities with 10-vol% Me_2SO solution is
413 also plotted (marked as crosses). The viability of each condition is the mean ± SD (or measurement error) of the
414 epifluorescence microscopic measurements.

Intracellular trehalose via transporter TRET1 as a method to cryoprotect CHO-K1 cells

(Figure files)

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Fig. 1

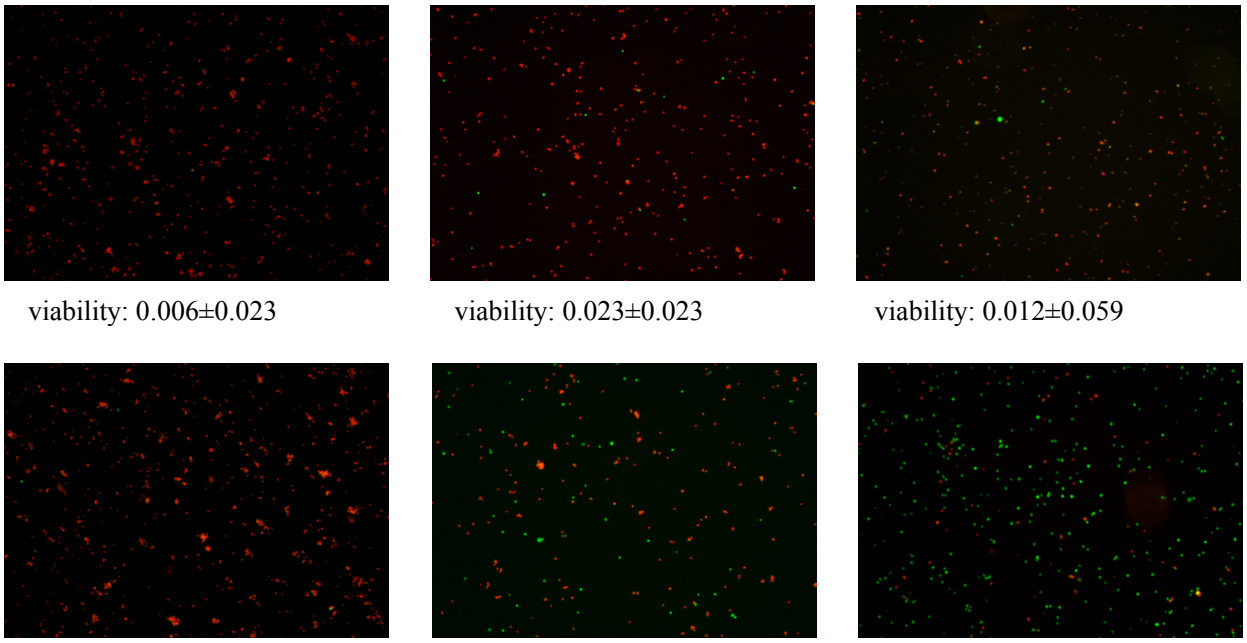

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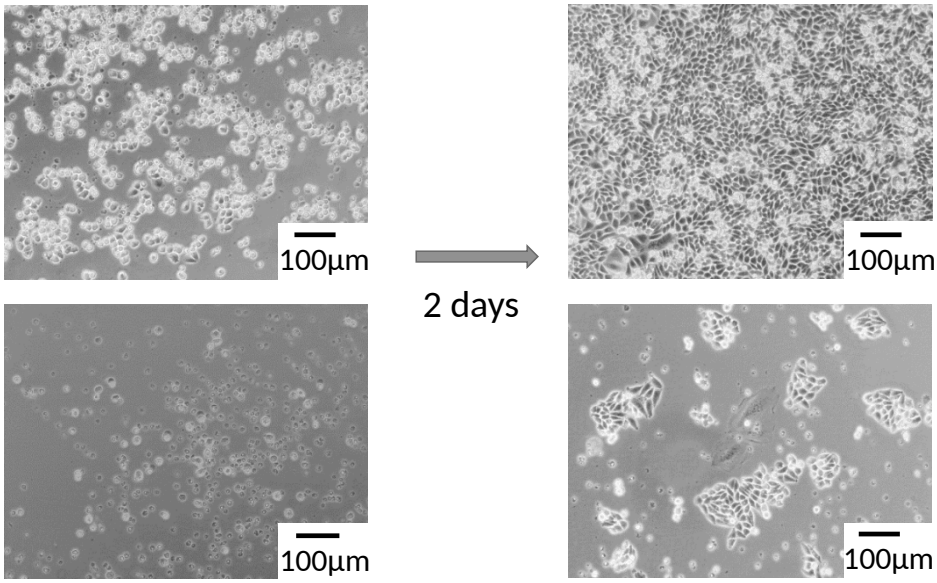
Fig. 2



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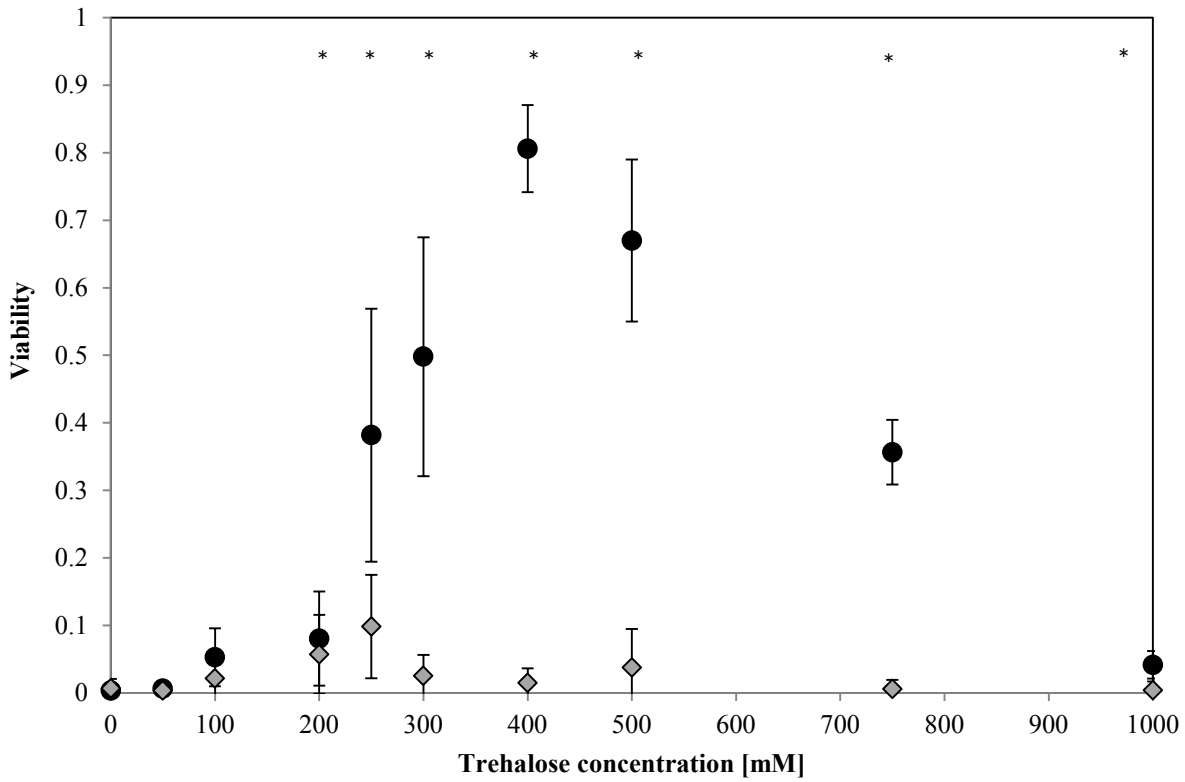

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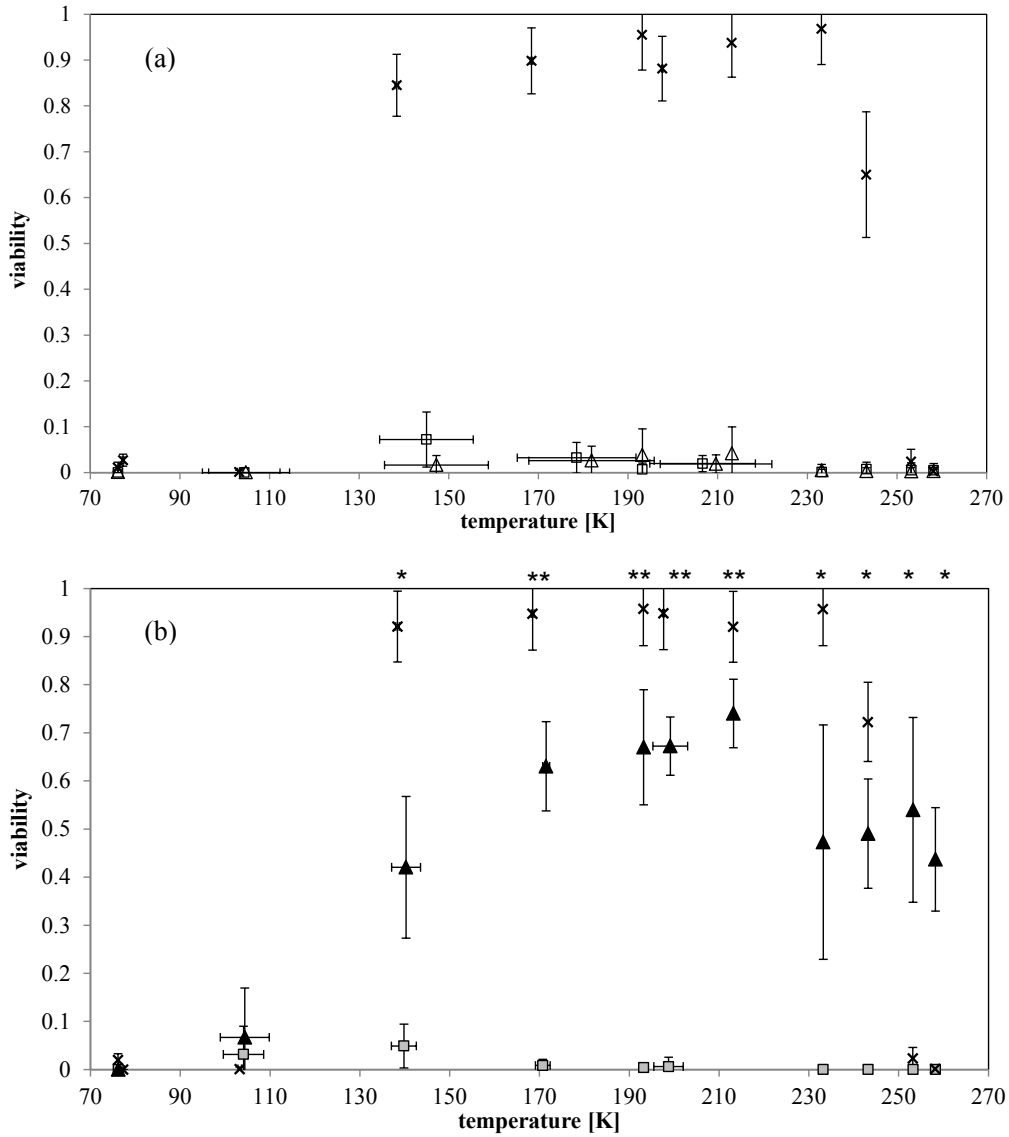
Fig. 4



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