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

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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 CHO-K1 cells (CHO-TRET1) and the CHO-K1 cells transfected with an empty vector (CHO-vector). The experiments involve freezing under various trehalose concentrations in an extracellular medium. The freeze-thawing viabilities of CHO-TRET1 cells are higher than those of CHO-vector cells for most freezing conditions. This result differs from control experiments with a trans-membrane type cryoprotectant, dimethyl sulfoxide (Me_2SO), which had similar viabilities in each condition for both cell types. We conclude that the trehalose loaded into the cells with TRET1 significantly improves the cryoprotective effect. The higher viabilities occurred when the extracellular trehalose concentration exceeded 200 mM, with 250–500 mM being optimal, and a cooling rate below 30 K/min, with 5–20 K/min being optimal.

(158 words)

<Abbreviations>

TRET1, trehalose transporter 1; Me_2SO, dimethyl sulfoxide; SD, standard deviation; calcein-AM, acetoxymethylated calcein; PI, propidium iodide

＜Key words＞

trehalose, trehalose transporter 1, cryoprotectant, freezing rate, 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 [2, 3, 12, 15, 19–21, 25]. In addition, trehalose inhibits ice-crystal growth [7, 22, 24]. However, sugars such as sucrose and trehalose 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 [8], engineered pores [5, 6, 18], activation of native channels [4], microinjection [5, 6], electropermeabilization [19], and endocytosis [9, 17].

Recent studies show that anhydrobiotic insect larvae use specific trehalose transporters during desiccation stress. The trehalose transporter (TRET1) from the anhydrobiotic larvae of the African chironomid, *Polypedilum vanderplanki*, has been isolated and characterized [10, 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 [1].

Here, we investigate the cryoprotective effect of trehalose on mammalian cells expressing TRET1. To show the effectiveness of TRET1 on the simple cryopreservation process, we measured the

freeze-thawing viabilities of the CHO-K1 mammalian cells with two treatments: 1) those expressing TRET1 and 2) those transfected with an empty vector. The viabilities are measured under various trehalose concentrations in extracellular medium and various temperature conditions. The viabilities of CHO-K1 cells in which enough trehalose was expected to be loaded are found to be significantly higher than those without trehalose loading. To examine the cryoprotective role of trehalose loaded in the cells, we compare this finding with the results of control experiments with 10 vol% Me_2SO as a trans-membrane-type cryoprotectant instead of trehalose. As the Me_2SO experiments show almost no difference between the two kinds of CHO-K1 cells, it appears that the trehalose loads into the cell and significantly increases the cryoprotective effect. Finally, we discuss the role of the cooling rate on the optimum conditions for the cryoprotective effect.

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 in the CO_2 incubator (MCO-18AIC, Sanyo, Osaka, Japan) at 310 K under 100% humidity condition, 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 similar to those in a previous study [1].

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 and CHO-TRET1 cells reached more than 80% confluence during their cultivation, 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 (about 10^5 cells/mL) was put into a 1.8-mL cryotube vial (Thermo Fischer Scientific) and incubated for 6 h in the CO_2 incubator for loading trehalose.

Cryopreservation protocol

Following trehalose loading, both CHO-vector and CHO-TRET1 cells in solution were put into a freezer set to cool down to the setting temperature T_{set} = 198 K. Under this setting, the average rate of temperature decrease for the samples (including 1-mL medium) V_f, was about 13 K/min. Here we calculated V_f as the maximum temperature gradient after the freezing starts (see supplementary). For investigating the setting-temperature dependence on the freeze-thawing viabilities, the samples were

put in the freezer set on T_{set} = 258, 253, 243, 233, 213, and 198 K. To investigate T_{set} 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 was immersed into the liquid nitrogen (77 K). The other four vials were clipped in upper parts of the holder and thus held in the cold vapor above the liquid nitrogen. Their temperatures were approximately 100, 140, 170, and 200 K. Under these T_{set} conditions, V_f was estimated as ranging between 1 and 200 K/min (see supplementary Fig. S4). 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 was stored either at its T_{set} condition or in the deep freezer at 198 K if the T_{set} was below 198 K. As a preliminary survey showed no significant differences in the viabilities whether stored at 198 K for one day or for two weeks, we fixed the storage period at 7 ± 2 days.

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

Viability and growth assays

After being stored for a week, the individual vials were then thawed in a 310-K water bath until all extracellular ice was observed to melt (about 5 min). During this process, the temperature increased by about 50 K/min. The freezing solution in the thawed cell suspension was then rinsed with the culture medium twice by centrifugation and the supernatant solution removed. In this way, the cryoprotectants were largely removed from the culture medium.

The survival rate of CHO-K1 cells was assessed using fluorescence dyes, calcein-AM (acetoxymethylated calcein) and PI (propidium iodide) solutions (Cellstain; Dojindo, Kumamoto, Japan). Soon after the solution replacement (approximately 0.5-h after thawing), the cell suspension with fluorescence dyes was incubated for 20 min in a CO_2 incubator. Then 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 were considered as alive, whereas bright red were scored as dead. The viabilities were calculated as the number of living cells (stained by calcein-AM), normalized by the total cell population.

To determine the survival and growth efficiency, each CHO-K1 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 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 during this period, and qualitatively comparing to the control CHO-K1 cells.

Uncertainties of viability measurements and statistical analysis.

Considering the sample variation, all freeze-thawing tests and 5-day growth experiments were repeated at least three times for the independent cell lines. The resulting viability here was averaged at the indicated experimental conditions ± the standard deviation (SD) of all measured data. The cells displaying no color were treated as a measurement uncertainty, but this uncertainty was much less than 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 ($p < 0.01$).

Results

Effect of trehalose concentration on cryopreservation of CHO-K1 cells

We measured the effect of trehalose concentration in the medium on the CHO-TRET1 cells viability and growth after thawing. Figure 1 shows the fluorescence image of CHO-K1 cells after a freeze–thawing test at various trehalose concentrations. Without trehalose, nearly all cells of either kind die after undergoing freezing and storing for 1 week at 193 K (Figs. 1a, b); that is, the viability is very low without trehalose. For the same conditions, the presence of extracellular trehalose only slightly improves the viability of CHO-vector cells (Figs. 1c, e), but significantly improves the viability of the CHO-TRET1 cells (Figs. 1d, f). 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 5-days and observed their adhesion-proliferation processes. Figure 2 shows micrographs of cryopreserved CHO-K1 cells under 500-mM trehalose concentration for one-day and three-day cultures after thawing. Adhesion of the surviving cells occurs after a one-day culture in both types of cells (the left column in Fig. 2), although only a few CHO-vector cells survive. After two more days in 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 their proliferation rates are almost the same as those of normally cultivated cells, it appears that the cryopreserved cells keep their normal functions after a freeze–thaw cycle.

Concerning the effect of trehalose in the cells on the health of the cells during the culture in normal medium, we argue that the effect is likely negligible. Our argument is based on a related experiment on oocyte. In that experiment, Kikawada et al. [11] incubated oocyte in 105-mM trehalose buffer for three hours, then found that it took only 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, which is only a small part of the 5-day

cultivation. Hence, the cultivation conditions of 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 after a freeze–thaw to 193 K. Figure 3 shows the resulting 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 0.099 ± 0.077 at 250 mM for the CHO-vector, but 0.806 ± 0.051 at 400 mM for the CHO-TRET1 cells, showing that the viability is nearly 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 trehalose concentrations exceeding 200 mM. Consequently, we confirm that the trehalose loading into the cell with TRET1 significantly improves the cryopreservation process.

Effect of setting temperature on cryopreservation of CHO-K1 cells

Here we examine the setting-temperature dependence of the viability of the cryopreserved cells. Two concentrations are considered: trehalose-free (0 mM) and the near-optimum concentration (500 mM). As a control experiment, we also used the most widely used trans-membrane type cryoprotectant instead of trehalose, in this case a 10 vol% of Me_2SO.

For CHO-vector cells, the viabilities in both 0-mM and 500-mM trehalose concentrations are much lower than the control case. For example, Fig. 4a shows them at most being 0.072 ± 0.060 for 0-mM trehalose at 145 K, whereas the control case always exceeds 0.6 above about 140 K. Conversely, at temperatures above 140 K and for the 500-mM extracellular trehalose condition ($p < 0.01$), the viabilities of CHO-TRET1 cells are comparable to the control case (Fig. 4b). The maximum viability is 0.740 ± 0.071 at 213 K with a 500-mM concentration. Based on the good agreement of the setting-temperature dependence of the viabilities between CHO-TRET1 cells and Me_2SO, the behavior

of the 500 mM-trehalose cases appear to be a result of the trehalose loaded into the CHO-K1 cells.

Discussion

In our freeze-thaw experiments, CHO-vector cells have only small viabilities under all cryopreservation conditions. This result is consistent with that obtained for neurons with extracellular trehalose [16]. Conversely, for the CHO-K1 cells loaded with trehalose through TRET1, the trehalose significantly increases the cryosurvival. Therefore, we consider that the viability difference between these cases results mainly from the difference of the intracellular trehalose concentrations. Here we consider the optimum experimental conditions when trehalose is loaded through TRET1.

At first, we consider the effect of extracellular trehalose concentration on 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 [1] and of cryopreservation of 3T3 fibroblasts and human keratinocytes [5], 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 and the loading time [1, 10, 11], this result suggests the existence of an optimum concentration of trehalose loaded into the cell as a cryoprotectant.

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 before freezing (6 h), the resulting intracellular-trehalose concentration should be in equilibrium with the extracellular conditions. Hence, we argue that the minimum extracellular trehalose concentration to

show a sufficient cryoprotective effect is about 200 mM. By extrapolating trehalose uptake data for CHO-TRET1 cells [1] to this condition, we estimate the minimum intracellular trehalose concentration for sufficient cryoprotection to be about 20 mM, or about 1.4×10^{-14} mol/cell (assuming the average volume of CHO cells [1]). This value is 14 times larger than the intracellular trehalose concentration loaded without TRET1 in CHO-vector cells [1].

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 trehalose concentration condition might weaken the cells. One possible source of weakening is osmotic shock on CHO-TRET1 cells under a large trehalose concentration gradient. A large gradient occurs during the trehalose loading before freezing and also during the trehalose removal after thawing. A hyper- and hypo-osmotic shock would be serious when the large concentration gradient has continued for a long time. To reduce the likelihood of these osmotic shocks, we used a high-capacity type of trehalose transporter (PvTRET1)[10], which acts relatively quickly to reduce trehalose gradients across cell membranes.

Another possible source of weakening of CHO-TRET1 cells is excessive dehydration during the trehalose loading process before freezing. To estimate this dehydration effect, consider that the Michaelis-Menten constant K_m for CHO-TRET1 cells is 137 ± 87 mM [1], which indicates a maximum trehalose transport of TRET1 about 500 mM. Hence, even when the extracellular trehalose concentration goes above 500 mM, the intracellular trehalose concentration should hardly increase. Thus, a trehalose concentration in the extracellular medium above about 500 mM may cause excessive dehydration of CHO-TRET1 cells during the pre-freezing incubation.

In addition, consider the setting-temperature dependence on the freeze-thawing viability of CHO-K1 cells. Here we used a simple cryopreservation method in which the cooling rate V_f was not controlled. But Mazur [13] showed that the viability is sensitive to V_f. So, we use our relation between T_{set} and V_f

(Fig. S4) to replot in Fig. 5 the setting-temperature dependence diagram in terms of V_f. The control experiments with 10-vol% Me_2SO show that the freeze–thawing viabilities are high for V_f values of 1–30 K/min in both CHO-cells. This range with high viabilities is roughly equal to that for CHO-TRET1 cells with 500-mM trehalose. Conversely, the viabilities of CHO-vector cells are low in any conditions. Clearly, the trehalose loaded into the CHO-K1 cells through TRET1 works as an effective cryoprotectant.

The viability of CHO-TRET1 with 500-mM trehalose starts decreasing at V_f > 20 K/min, nearly vanishing at V_f > 50 K/min. As the viabilities of both CHO-cells with Me_2SO are also zero when V_f > 50 K/min, the low viabilities at T_{set} below 140 K in Fig. 4 are considered a result of the higher cooling rate. This finding suggests that the cryopreservation protocol with trehalose loading through TRET1 requires a cooling rate V_f less than 20 K/min, even at T_{set} below 140 K.

The viabilities of 500-mM trehalose CHO-TRET1 cells at V_f < 5 K/min (or T_{set} > 230 K) are lower ($p < 0.01$) than those under optimum conditions. This difference might result from a characteristic of trehalose in which the trehalose inhibits the crystal growth of ice I_h and forces the H_2O solid phase into a glassy state. X-ray diffraction analysis on trehalose solution [23] indicates that the structure of ice changes from amorphous and ice I_c to ice I_h above the glass-transition temperature when enough trehalose is present. Although the glass-transition temperatures for the freezing solutions used in the present study are not measured precisely, we speculate that the slight decrease in the viabilities of 500-mM trehalose CHO-TRET1 cells at T_{set} above 230 K may be caused by injuries of the cells from growing ice I_h crystals.

Conclusion

We investigated the effect of intracellular trehalose transferred through TRET1 on the cryopreservation of CHO-K1 cells. Our cryopreservation method involved changing either the trehalose

concentration in the medium or the freeze-storage temperature. We found that the trehalose-loaded CHO-TRET1 cells had freeze-thawing viabilities significantly higher than those of CHO-vector cells. The cultivation of the cryopreserved cells indicated that the surviving cells retain their functions. Conversely, control experiments with the trans-membrane type cryoprotectant Me_2SO affected the viabilities of both CHO-TRET1 and CHO-vector cells equally. Therefore, the trehalose loading into the cell with TRET1 is found to improve the cryopreservation process significantly. 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 cooling rate V_f below 30 K/min, with V_f = 5–20 K/min being optimal.

Acknowledgments

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352 **Figure captions**

353

354 **Fig. 1.** Effect of trehalose concentration in the medium on the viability of cryopreserved cells. (a), (c),
355 and (e) show CHO-vector cells and (b), (d), and (f) show CHO-TRET1 cells. The trehalose concentration
356 is 0, 200, and 400 mM for left, middle, and right columns, respectively. Samples stored at 193 K for 1
357 week. Red stain is PI, indicating dead cells, and green stain is calcein-AM, indicating live cells.
358 Horizontal distance across bottom is 2.2 mm.

359

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

366

367 **Fig. 3.** Viabilities of cryopreserved CHO-vector cells (solid diamonds) and CHO-TRET1 cells (solid
368 circles) at various extracellular trehalose concentrations. The cells were incubated for six hours at 310
369 K prior to the sample setting in the deep freezer (193 K). After 1-week storage, the frozen sample was
370 thawed in the 310 K bath. Soon after the thawing, the suspended cells were rinsed twice with the
371 phosphate-buffered saline (PBS) solution and incubated with fluorescence dyes. The viability of each
372 condition is the mean ± SD of epifluorescence microscopic measurements. The asterisks mark cases
373 with significant difference of viabilities between CHO-vector cells and CHO-TRET1 cells at the same
374 freeze-thaw conditions ($p < 0.01$).

375

376 **Fig. 4.** Effect of setting temperature and trehalose transporter (TRET1) on the viability of cryopreserved cells. (a)
377 CHO-vector cells. (b) CHO-TRET1 cells. The CHO-K1 cells were suspended in the medium with trehalose at the
378 indicated concentration of either 0 mM or 500 mM, then set at the indicated temperature condition (77 – 263 K) after

379 a six-hour incubation at 310 K. The temperature dependence on viabilities with 10-vol% Me_2SO solution is plotted as
380 a control. The viability of each condition is the mean ± SD of the epifluorescence microscopic measurements.
381
382 **Fig. 5.** Effect of cooling rate V_f and trehalose transporter (TRET1) on the viability of cryopreserved cells. (a)
383 CHO-vector cells. (b) CHO-TRET1 cells. The data and symbols are the same as those in Fig. 4.

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

(Figure files)

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Fig. 1

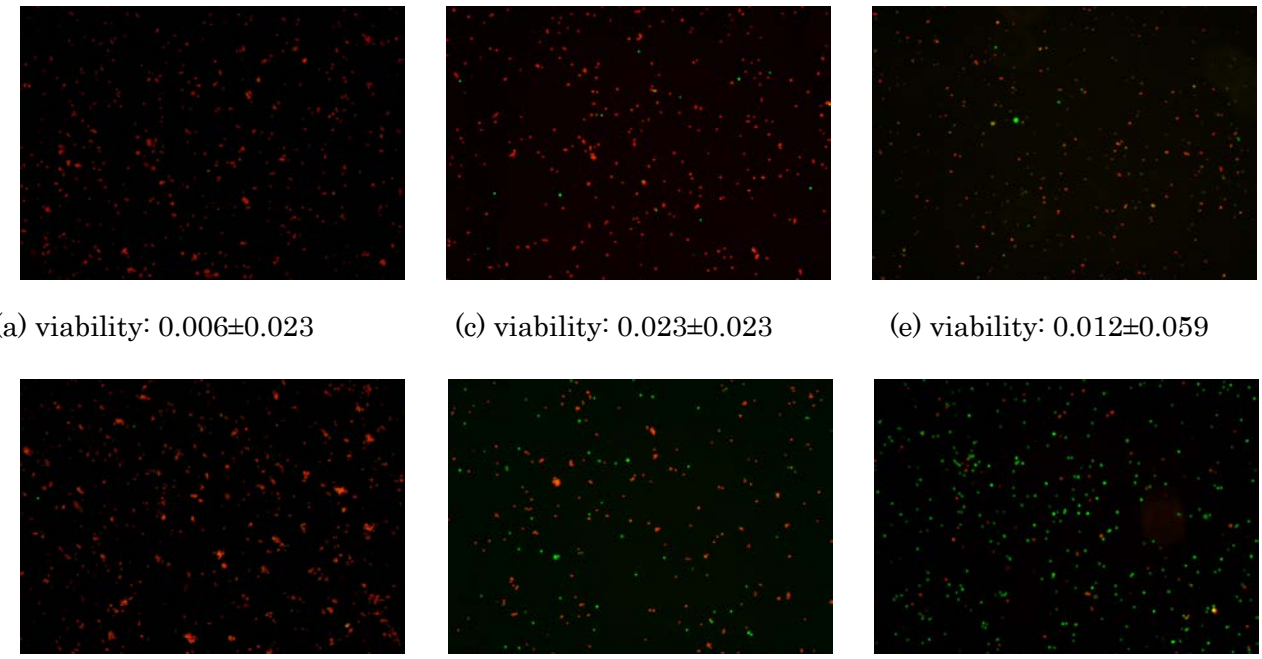

Fig. 1. Effect of trehalose concentration in the medium on the viability of cryopreserved cells. (a), (c), and (e) show CHO-vector cells and (b), (d), and (f) show CHO-TRET1 cells. The trehalose concentration is 0, 200, and 400 mM for left, middle, and right columns, respectively. Samples stored at 193 K for 1 week. Red stain is PI, indicating dead cells, and green stain is calcein-AM, indicating live cells. Horizontal distance across bottom is 2.2 mm.

Fig. 2

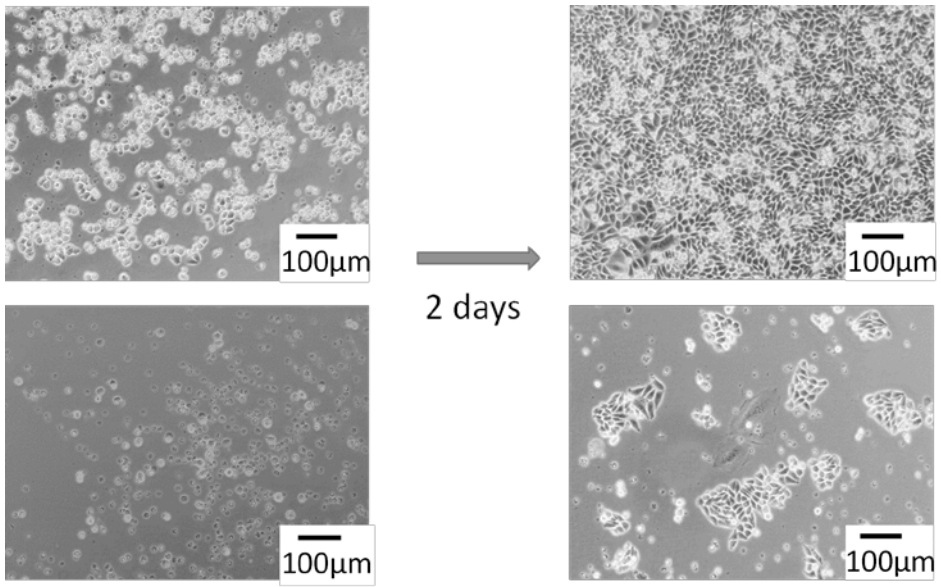

Fig. 2. Micrographs of cells from the medium with 500-mM trehalose cryopreserved at 193 K for one week. (a) CHO-TRET1 cells. (b) CHO-vector cells. After thawing in the bath at 310 K, the freezing medium was replaced with culture media twice by centrifugation and removal of the supernatant solution. Each CHO-K1 cell suspension 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 days after thawing. The left column shows a 1-day culture after thawing, and the right column shows a 3-day culture.

Fig. 3

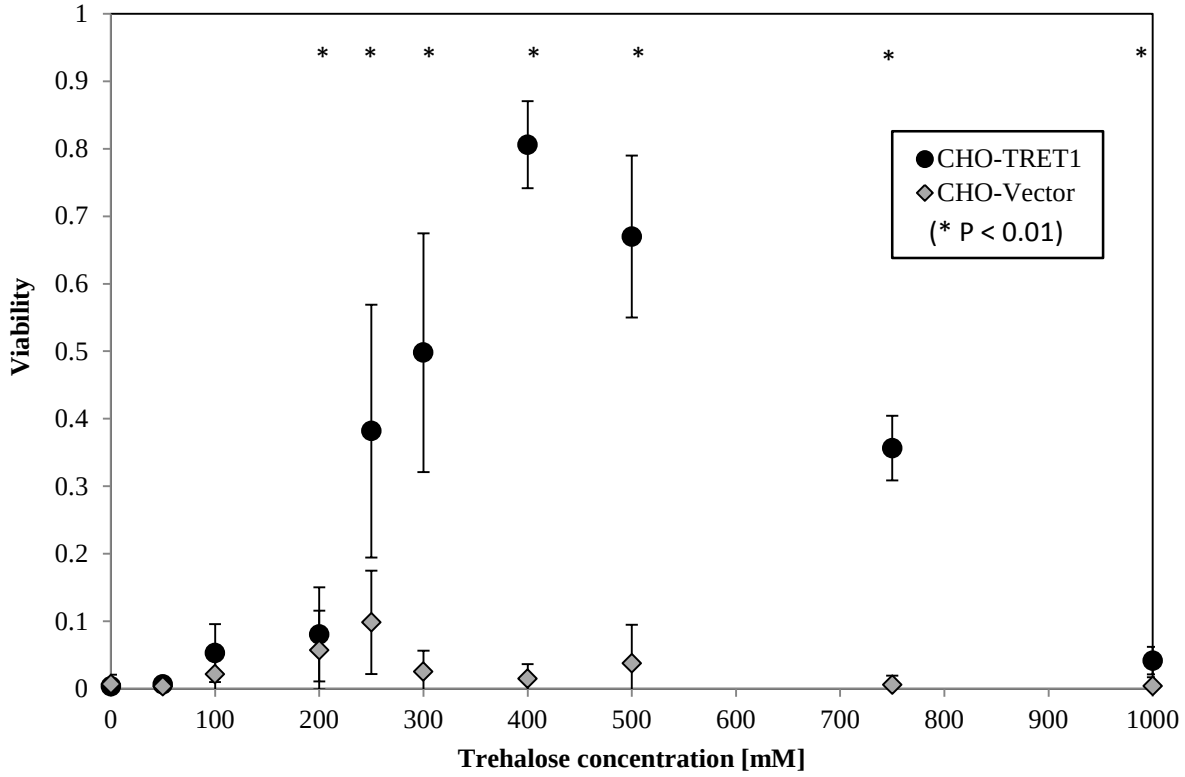

Fig. 3. Viabilities of cryopreserved CHO-vector cells (solid diamonds) and CHO-TRET1 cells (solid circles) at various extracellular trehalose concentrations. The cells were incubated for six hours at 310 K prior to the sample setting in the deep freezer (193 K). After 1-week storage, the frozen sample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

Fig. 4

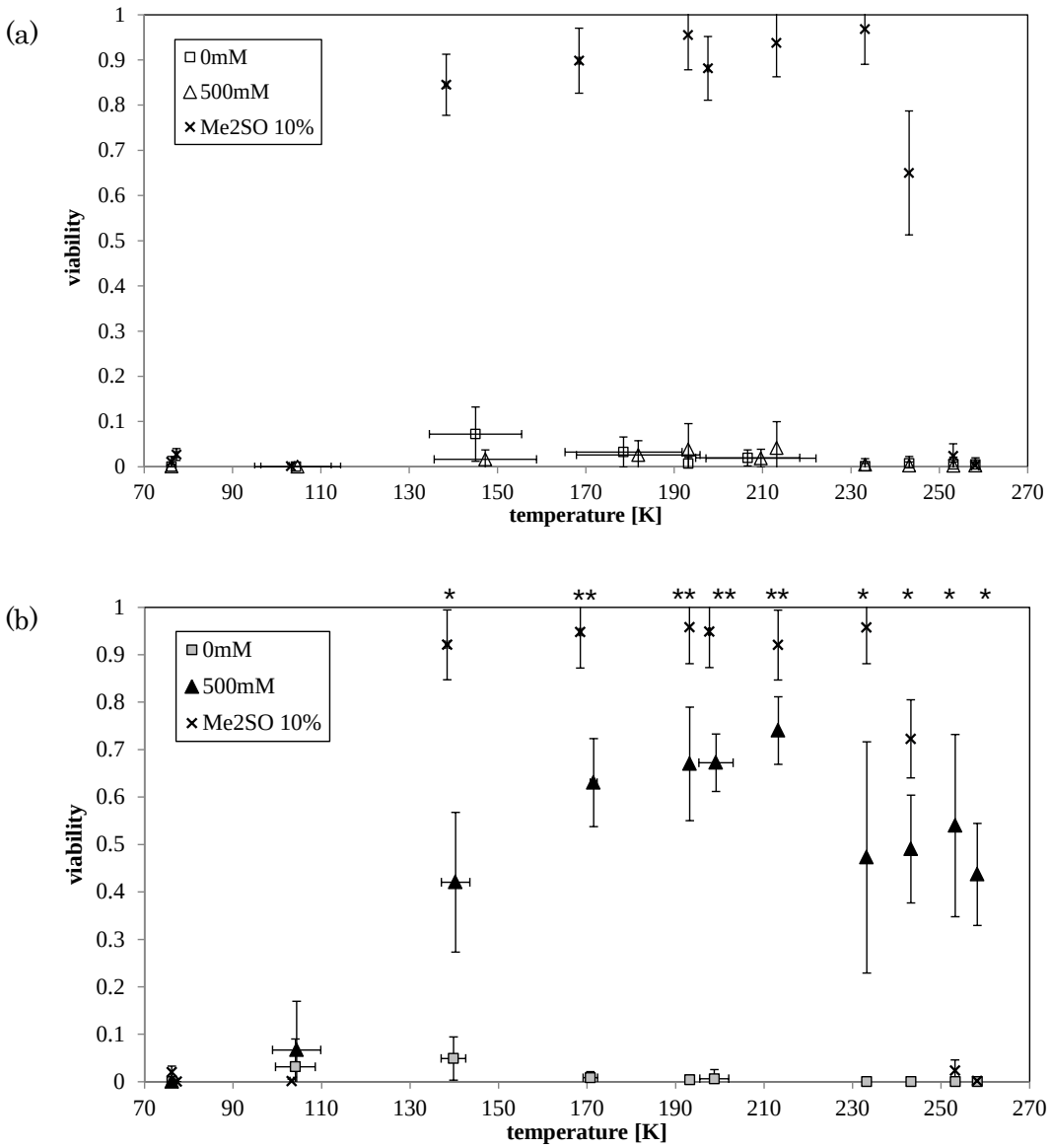

Fig. 4. Effect of setting temperature and trehalose transporter (TRET1) on the viability of cryopreserved cells. (a) CHO-vector cells. (b) CHO-TRET1 cells. The CHO-K1 cells were suspended in the medium with trehalose at the indicated concentration of either 0 mM or 500 mM (triangles), then set at the indicated temperature condition (77 – 263 K) after a six-hour incubation at 310 K. The temperature dependence on viabilities with 10-vol% Me_2SO solution is plotted as a control. The viability of each condition is the mean ± SD of the epifluorescence microscopic measurements.

Fig. 5

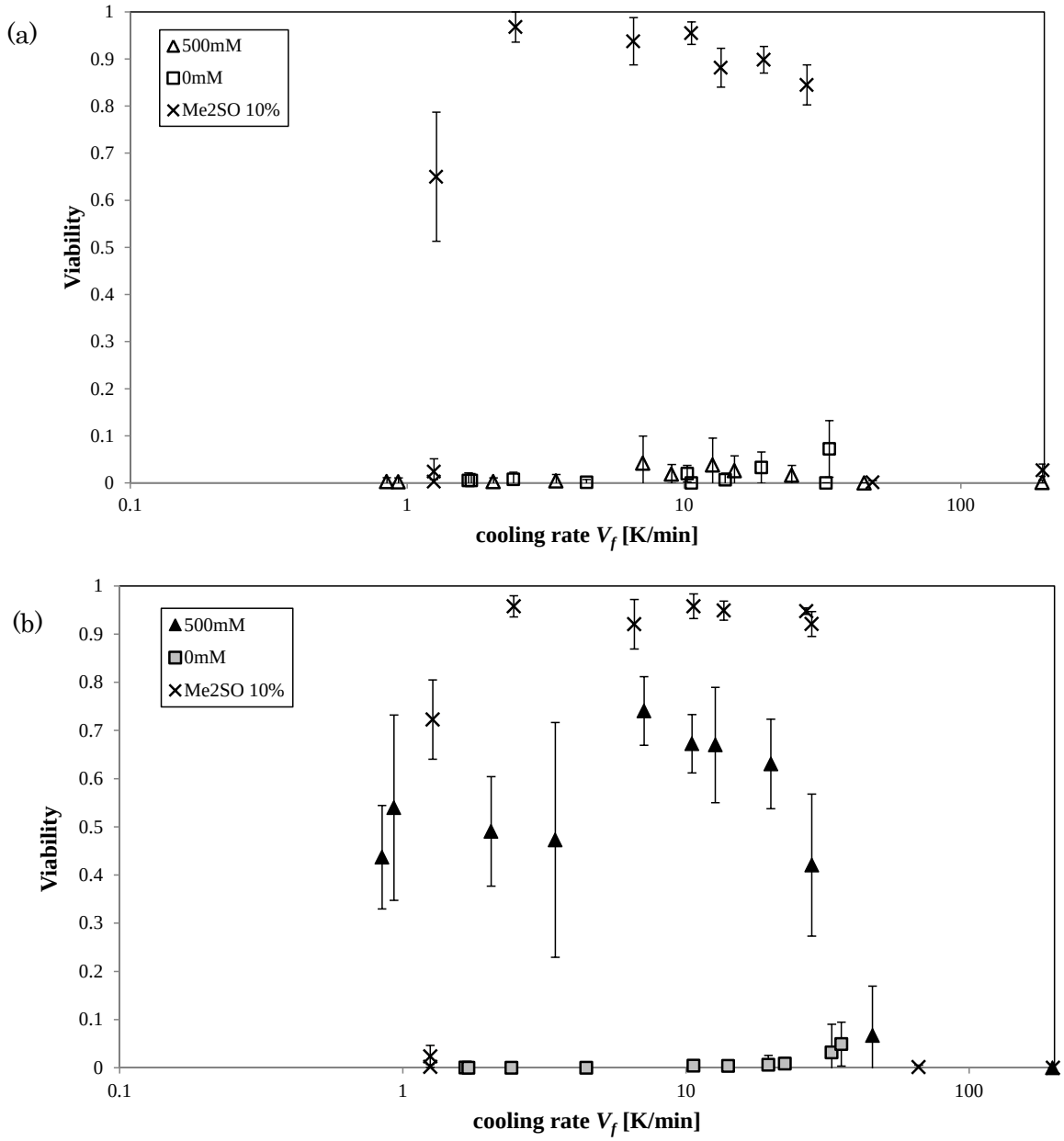

Fig. 5. Effect of cooling rate V_f and trehalose transporter (TRET1) on the viability of cryopreserved cells. (a) CHO-vector cells. (b) CHO-TRET1 cells. The data and symbols are the same as those in Fig. 4.