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Cryoprotection and Cytotoxicity Resistance of Tardigrade Stress Resistant Genes in U87
Cells for Improved Cryopreservation

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Bioengineering

by

Melissa Pimentel

June 2023

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Committee Chairperson

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ABSTRACT OF THESIS

Cryoprotection and Cytotoxicity Resistance of Tardigrade Stress Resistant Genes in U87
Cells for Improved Cryopreservation

by

Melissa Pimentel

Master of Science, Graduate Program in Bioengineering
University of California, Riverside, June 2023
Dr. Joshua Morgan, Chairperson

Cryopreservation is an essential tool for the storage of biological material while retaining their biological processes; however, reduced cell and tissue viability persists due to ice crystal formation and cryoprotectant agent (CPA) toxicity. Further, cryopreservation of larger tissues or 3D cultures presents additional challenges due to temperature and CPA gradients. Incorporation of tardigrade stress resistance genes is an untested mechanism to enhance cell viability during cryopreservation. Here, we show the expression of tardigrade stress resistance genes (Dsup, MAHS, and RvLEAM) into U87 cells to improve resistance to cryopreservation related stresses. The study reveals that cells expressing Dsup and MAHS genes had a greater survival rate than those expressing RvLEAM when exposed to chronic and acute DMSO. Furthermore, MAHS provided increased cell viability during cryopreservation. Our results demonstrate that tardigrade stress resistance genes improve cell viability under CPA and cryogenic stressors in 2D and 3D cell cultures. These findings offer valuable insights into using tardigrade gene expression to boost cryopreservation viability and reduce the use of CPA.

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Summary and Aims

Cryopreservation is an important tool for biomedical research and clinical medicine. Currently, it is the most common form of cellular and tissue preservation, allowing for long-term storage[1]. While essential for maintaining cell and tissue banks for research and treatment, cryopreservation presents several challenges. Despite the advances in organ preservation technologies, about 70% of organs suitable for transplantation become unusable and are discarded[2]. The causes of these issues include but are not limited to ice crystal formation, cryoprotectant toxicity, oxidative and osmotic stresses which lead to reduced cell and tissue viability[3]. Increasing cell stress tolerance could allow for increased viability after cryopreservation.

The stresses of cryopreservation are separated into two broad categories, the stresses of freezing/thawing the cells and the toxicity of the cryoprotectants used to mitigate those effects. The process of freezing and thawing causes a loss of cell viability and damage to the extracellular matrix, which can potentially destroy the structural integrity of organoids, which is needed to maintain morphology and function[4]. The most commonly used cryoprotectant for mammalian cells is dimethyl sulfoxide (DMSO); while effective, DMSO can reduce cell viability and interfere with grafting procedures[3]. Increasing cellular resistance to DMSO and/or reducing the need for DMSO would expand the usefulness of cryopreservation.

Tardigrade stress resistant genes have great potential to resolve this challenge. Tardigrades ("water bears") are microscopic animals that possess remarkable stress tolerance; tolerating extreme temperatures, radiation, and desiccation[5]. While incompletely

understood, several factors in their resistance have been identified. The proteins associated with resistance are tardigrade intrinsically disordered proteins (TDPs). These proteins include cytoplasmic, secreted, and mitochondrial abundant heat soluble (CAHS, SAHS, and MAHS) proteins. They provide tolerance from desiccation, protein stabilization and shielding, organelle stabilization, and membrane integrity[6]. Additionally, two other tardigrade stress resistance genes include RvLEAM and Dsup. RvLEAM is a group 3 late embryogenesis abundant (LEA) protein, and it protects against protein aggregation due to desiccation or osmotic stresses associated with freezing[7]. Finally, Dsup ("damage suppressor") protects DNA from oxidative and radiative damage[8].

Here, we propose transducing mammalian cells to express tardigrade stress tolerance genes transgenically. We hypothesize that cells expressing the transgenes will be more resistant to the stresses of cryopreservation. Specifically, we propose to use U87 cells expressing tardigrade stress resistant genes as a proof-of-concept model and assess the degree of protection against cryopreservation and the cryoprotectant DMSO.

Aim 1: To determine whether 2D cultures of cells expressing tardigrade stress resistant genes increase cell viability when exposed to stress associated with cryopreservation

Using U87 cells expressing tardigrade resistance genes, specifically MAHS, Dsup, and RvLEAM, we will determine whether tardigrade tolerance genes in 2D cell cultures improve viability after treatment with DMSO or when cryopreserved. Assess cell viability of tardigrade stress tolerance genes expressing U87 cells compared to non-expressing cells.

Cell viability will be assessed using confocal imaging and MTT assays and compare tardigrade genes to the results of a control gene, AcGFP.

Aim 2: To determine whether 3D cultures of cells expressing tardigrade stress resistant genes increase cell viability when exposed to stress associated with cryopreservation

Using U87 cells expressing tardigrade resistance genes, specifically MAHS, Dsup, and RvLEAM, we will determine whether tardigrade tolerance genes in 3D gelatin-transglutaminase cultures improve viability after treatment with DMSO or when cryopreserved. Assess cell viability of tardigrade stress tolerance genes expressing U87 cells in comparison to non-expressing cells. Cell viability will be assessed using confocal imaging and live dead assays and compare tardigrade genes to the results of a control gene, AcGFP.

Introduction

Cryopreservation

Cryopreservation is the process of freezing biological samples, such as cells, tissues, and whole organisms at cryogenic temperatures, typically between −80°C (standard ultralow freezer) and −196 °C (liquid nitrogen storage)[9]; storage in vapor phase above liquid nitrogen is typically between −135 and −196 °C. Cryopreservation allows to store biological material for long periods while retaining its normal chemical, biological, and physical processes after thawing. It prevents the degradation of biological material, allowing them to remain usable for future use. Cell cryopreservation has become an

essential supporting technology for various cell-based applications such as stem cell therapy, tissue engineering, assisted human reproduction, and transfusion medicine[10]. While routinely used, cryopreservation still presents challenges, including decreased cell viability or low recovery efficiency.

Cryopreservation Stresses

While cryopreservation is a valuable tool, it is often linked to the induction of cell stress. During the freezing and thawing process, cells can experience physical and chemical injury. The primary source of damage is the formation of ice crystals which form during freezing, puncturing the cell and causing damage to the cell membrane and other structures[11]. The freezing processes can also lead to osmotic stress caused by the increased extracellular osmolarity as ice crystals form. The cooling rate during freezing impacts which type of damage is caused to the cells. When freezing rates are slow, extracellular ice formation causes dehydration of the cells resulting in hyperosmotic stress[12]. At rapid cooling rates, cells do not dehydrate, keeping osmolarity more balanced, but increased intracellular ice formation will damage cellular membranes and structures[13].

Cryopreservation can cause the generation of reactive oxygen species (ROS). Cells suffer from oxidative stress by ROS formed during the thawing process[14]. Slow cooling can reduce the possibility of intracellular ice formation but can lead to oxidative stress through ROS, resulting in molecular damage to DNA, proteins, and lipids inside cells, disrupting organelle function[15]. Oxidative stress can result in the release of apoptogenic

factors, which can lead to cell death. During thawing, reoxygenation of cells and tissue can also cause ROS production, exacerbating the damage and reducing cell viability.

Additionally, the thawing process can cause thermal stress due to uneven heating, especially in larger organoids and in thick or large tissues, which can cause proteins to denature and disrupt cellular processes. The freezing of 3D tissues poses many complications due to heat and mass transfer limitations. Non-uniform cooling and warming rates create significant thermal gradients between the surface and interior of the cell system, causing harmful osmotic stresses and intracellular and extracellular ice formation[4].

The stresses and damages caused by cryopreservation can result in the loss of viability, reduced cell growth, and altered gene expression. Although the severity of the stresses depends on several factors, including the cell or tissue type, cooling and warming rates, and the type of cryoprotectant used. Some of the techniques used to reduce the stress on cells during cryopreservation are the use of cryoprotectants to mitigate and prevent intracellular ice formation, optimizing cooling and warming rates, and proper storage conditions.

Cryoprotectant Toxicity

Cryoprotective agents (CPAs) have been used to reduce cryoinjury and improve cell survival during cryopreservation by preventing the formation of ice crystals and therefore reducing cryoprotective stresses. CPAs can be classified as cell membrane permeable or non-permeable[16]. Permeable CPAs mainly include organic solvents, such as glycerol and dimethyl sulfoxide (DMSO). They permeate the cell membrane and primarily provide cell protection against hyperosmotic stress. Non-permeable CPAs provide

extracellular protection by mitigating ice formation, they include non-toxic carbohydrates such as trehalose and sucrose[17]. Permeable and non-permeable CPAs can be used together to provide intracellular and extracellular protection. Permeable CPAs lack biocompatibility. DMSO is associated with side effects in patients like neurotoxicity, cardiovascular failure, respiratory arrest, fatal arrhythmias, and others[18].

Cells, tissues, and organs have boosted viability during cryopreservation with the use of CPAs, but their toxicity limits the amount which can be used[19]. The toxicity of CPAs can be due to the high concentrations, causing osmotic and metabolic injuries. Some interfere with cellular metabolism and function, leading to cell death. High levels of CPAs within the cell lead to cell viability loss and damage to the extracellular matrix during freezing and thawing, which can compromise the structural integrity of organoids, which is essential for maintaining morphology and function[20]. The appropriate amount of CPAs depends on balancing CPA toxicity and cryoprotective effect. In some cases, vitrification is used to prevent this. CPAs become increasingly toxic as concentration increases. There have been attempts at optimizing cooling and warming rates to minimize toxicity. Strategies remain inadequate, therefore, CPA toxicity remains an obstacle to cryopreservation[21].

Cryopreservation becomes a more difficult and complex problem with 3D organoids or large tissues. CPAs do not evenly distribute throughout the tissue, and freezing and rewarming is non-uniform[4]. The application of conventional slow freezing methods to cultures of 3D organoids of stem cells is limited by their size[22]. There are

continuing efforts to improve post-thaw cell viability and reduce or potentially eliminate the use of CPAs[23].

Tardigrade Stress Tolerance Genes

Tardigrades are microscopic animals that are known for their tolerance to extreme environments. A genome and transcriptome analysis of the tardigrade *Ramazottius varieornatus* revealed high expression of proteins in their active and anhydrobiotic states not found in other organisms[24], including intrinsically disordered proteins. Intrinsically disordered proteins help tolerate abiotic stresses such as freezing, osmotic stress, high temperatures, and desiccation in different organisms, including tardigrades [6].

Three families of intrinsically disordered proteins in tardigrades are cytoplasmic, secreted, and mitochondrial abundant heat soluble (CAHS, SAHS, and MAHS) proteins. The expression of each family is constitutively or significantly enriched in response to desiccation [6]. Additional to these proteins, tardigrades also express group 3 late embryogenesis abundant protein mitochondrial (RvLEAM) and damage suppressor proteins (Dsup).

MAHS and RvLEAM enhance the tolerance to osmotic stress when expressed in human culture cells[7]. Both proteins are found in the mitochondria and protect the cells during anhydrobiosis. MAHS is hypothesized to maintain the structural integrity of the tardigrade's mitochondrial membrane during dehydration. RvLEAM protects the cell from protein aggregation due to desiccation or osmotic stresses[25]. Oxidative stress also occurs during desiccation, and protection from oxidative stress associated with mitochondrial oxidative phosphorylation is crucial for desiccation tolerance[26]. Another tardigrade unique

protein is the damage suppressor protein (Dsup). Unlike MAHS and RvLEAM, Dsup localizes to the nucleus and directly interacts with DNA. Dsup expression in human culture cells nearly doubled X-ray radiation tolerance levels, and the amount of DNA double-strand breaks was significantly reduced[24]. Dsup wraps around chromatin which protects the DNA from ROS-mediated damage[27]. The specific role of Dsup in DNA protection also suggests that the defense mechanism in anhydrobiosis is a highly complex, broadly protecting multiple subcellular localizations and multiple forms of damage[24].

Prior work has shown that some stress tolerance proteins described above preserve function in mammalian and human cells with transient expression. However, they have not been tested with constitutive expression or explicitly applied to cryopreservation. In these studies, lentiviral transduction of tardigrade transgenes is tested as a potential mechanism to increase the viability of human cells during cryopreservation.

Materials and Methods

Cells and Reagents

Human glioblastoma cells (U87; ATCC, Manassas, VA) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin (P/S) Penicillin/Streptomycin, and 0.5 µg/mL amphotericin B and maintained in a 5% CO_2 incubator at 37 °C. Cells were previously modified using lentivirus to express tardigrade transgenes constitutively. Briefly, tardigrade transgenes were cloned into the pCDH-CMV-MCS (SBI, San Jose, CA) lentiviral transfer plasmid from existing and readily available plasmids: CAHS (pAcGFP1-N1-CAHS3; Addgene

#90031), MAHS (pAcGFP1-N1-MAHS; Addgene #90034), RvLEAM (pAcGFP1-N1-RvLEAM; Addgene #90035), Dsup (pAcGFP1-N1-Dsup; Addgene #90020), and control (AcGFP1-N1; Addgene #54705) using routine methods. A virus was generated in HEK293TN using triple transfection of a 2nd generation lentiviral system (Mirus Bio, Madison, WI) with plasmids for viral packaging (psPAX2, Addgene #12260), viral envelope (pMD2.G, Addgene #12259), and transfer plasmid containing the gene of interest. Viral media was collected and used at 72 h, cells were selected using 2 µg/mL puromycin.

Fixation and Imaging

At the end of the experiment, cells were fixed using 4% paraformaldehyde and 0.5% Triton X-100 in PBS. After fixation, the cells were stained with DAPI (nuclei) and DyLight-555 conjugated phalloidin (filamentous actin) and mounted on slides. For each condition, 10 random fields were selected and imaged using a Leica DMI8 microscope with DFC9000 GT sCMOS camera (Leica Microsystems, Deerfield, IL). Images were analyzed using custom scripts implemented in MATLAB (2022a; Mathworks, Natick, MA).

Live/Dead Assay

Cells are stained using Ethidium Homodimer III (4 µM; dead stain) and Calcein-AM (2 µM; live stain) in PBS according to manufacturer instructions (Biotium, Fremont, CA). They were incubated at 37 °C for 15 minutes before imaging.

Chronic DMSO Experiments

The cells were dissociated from the 90 mm culture dishes after reaching a minimum of 60% confluency using 0.25% trypsin and subcultured into 48-well plates at 10,000 cells/well. After reaching about 60% confluency, the wells were treated with DMSO at concentrations of 0-7% for 72 hours. The cells were fixed and treated with DAPI and phalloidin before imaging. Imaging was conducted as described above.

MTT Experiments

Cells were transferred into a 48-well plate and treated with various concentrations of DMSO. After 72 hours, the DMEM-DMSO solution was aspirated from each well, and new DMEM was added to 10% of the MTT (4 mg/mL) solution and incubated at 37 °C for 5 hours. The MTT solution was removed, and 500 µL of DMSO was added. The plate was mixed for 20 minutes at 150 rpm at 37 °C. Absorbance was measured using a microplate spectrophotometer at a wavelength of 570 nm.

Acute DMSO Toxicity Experiments

Cells were transferred into a 48-well plate at a concentration of 20,000 cells/well and treated with 10% to 30% DMSO in increments of 5%, with a control of 0%. The plate was put in ice for 30 minutes. The DMEM and DMSO solution in each well was aspirated before performing the live/dead assay, as described above.

2D Cell Culture Cryopreservation

One million cells were transferred into cryovials suspended in 1 mL of complete media (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The cryovials were stored at -80°C for 72 hours in a CoolCell (Corning, Corning, New York). The cryovials were thawed at room temperature, and 400 µL were transferred into 24-well plates and were left to settle for 20 minutes. The live/dead assay was performed as described above.

3D Cell Culture Gelatin-Transglutaminase Hydrogels

Gelatin-transglutaminase hydrogels were produced by using porcine skin gelatin (40%, Type A, 300 g bloom, Sigma Aldrich, St. Louis, MO), it was diluted in a solution of DMEM containing cells and 1M NaOH (to neutralize the gelatin's pH) to create a solution of 6% gelatin. Transglutaminase (Modernist Pantry, Eliot, ME) was dissolved in PBS to form a 4% solution. The 4% transglutaminase was mixed into the gelatin-DMEM solution and pipetted into PDMS molds. The hydrogels were allowed to set in the incubator at 37 °C and suspended in DMEM.

3D Cell Culture Cytotoxicity

Gelatin-TG hydrogels were incubated at 37°C for 5 days, and a live/dead assay was performed as described above.

Gelatin-TG hydrogels were left to set in a 37°C incubator for 24 hours. The hydrogels were transferred into cryovials and stored at -80°C in a CoolCell® for various days. The vials were then left to thaw at room temperature. Once completely thawed, the hydrogels were transferred into 48-well plates, and a live/dead assay was performed.

Experimental Design and Statistical Analysis

All experiments were performed in triplicate. Statistical significance was assessed using ANOVA followed by Tukey's HSD post-hoc test.

Results

Tardigrade Transgene Expression Improves Viability After Chronic DMSO Exposure

U87 is a cell line isolated from a malignant glioma and is both immortal and easy to handle, making it ideal for highly reproducible human cell studies[28]. U87 cells expressing three tardigrade stress resistance genes (Dsup, MAHS, and RvLEAM) tagged with AcGFP, and an AcGFP control, are used to determine whether tardigrade stress resistance genes in 2D and 3D cell cultures enhance viability following treatment with DMSO and cryopreservation. Cell viability is assessed using confocal imaging and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MTT assay is a colorimetric assay for assessing cell metabolic activity and proliferation[29].

U87 cell cultures expressing three tardigrade genes (Dsup, MAHS, and RvLEAM) or AcGFP were exposed to chronic DMSO (72 h treatment) to determine its effect on cell

viability and metabolic activity. DMSO concentrations of 0-7% were used, exceeding 7% leads to no cell survival (data not shown). As one measure of viability, we performed nuclei counts of fixed cells after treatment (**Figure 1**). There is an apparent loss of cell density in control cells at 3% and nearly complete ablation at 7%. Nuclei counts were quantified, data shown in **Figure 2**. For control cells, the LD_{50} was approximately 3%, with 4%-7% resulting in almost complete cell loss. MAHS and Dsup had increased survival, with a significant effect observed for both genes compared to control in two-way ANOVA (Tukey's HSD post-hoc). RvLEAM provided a significant reduced benefit, and apparent loss of viability compared to control. The results show that under chronic DMSO exposure, cells expressing Dsup and MAHS exhibit significantly higher viability compared to those expressing AcGFP.
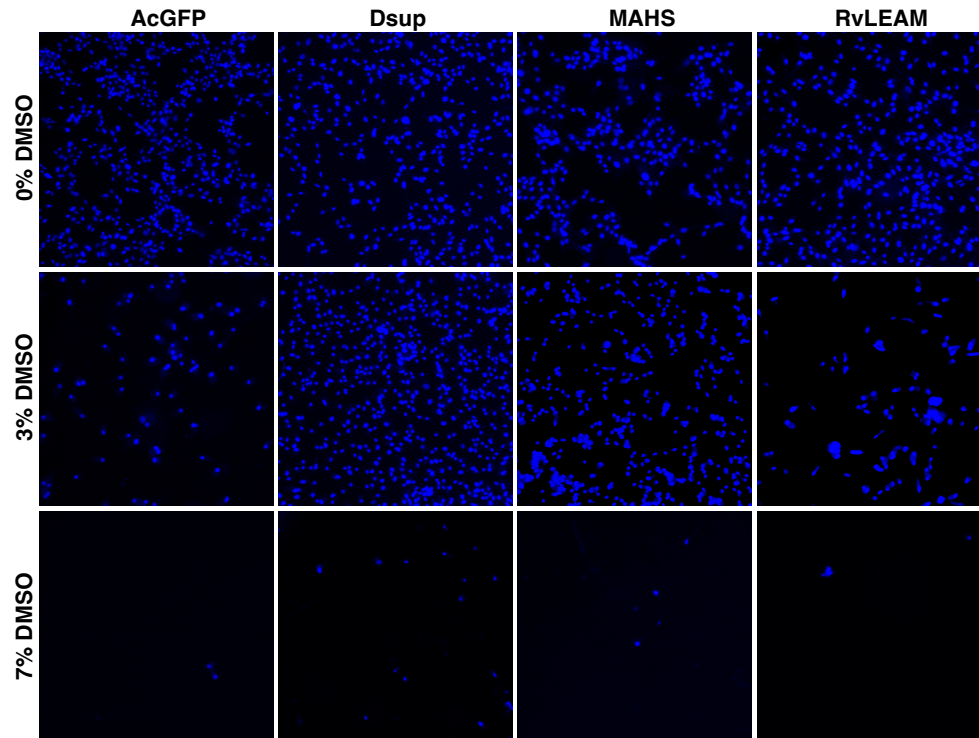
Figure 1 Panel of images of DAPI stained nuclei for nucleus count of cells expressing tardigrade stress resistance genes and AcGFP for 72-hour chronic DMSO exposure.
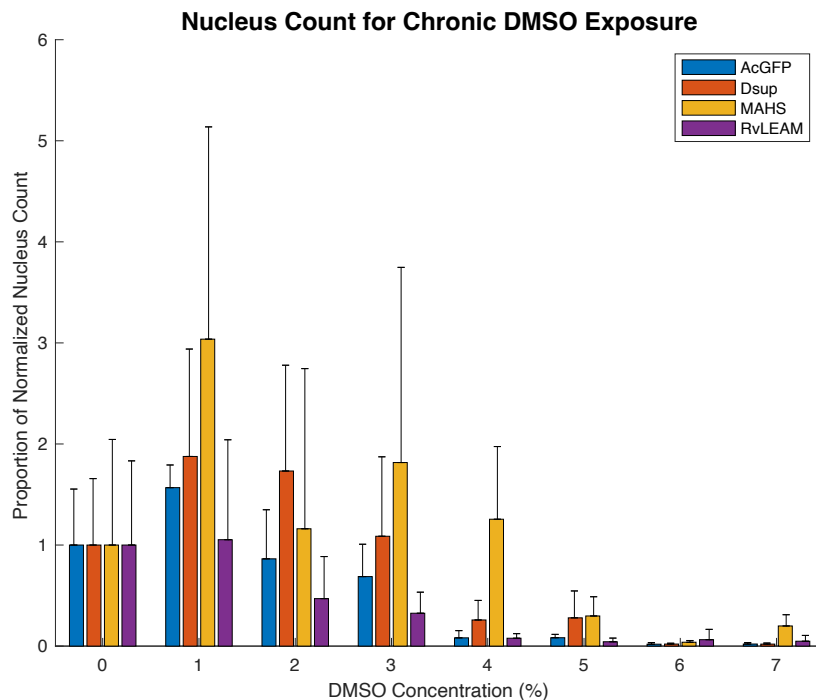
Figure 2 Normalized nucleus count of DMSO used during a 72-hour exposure for AcGFP and the three tardigrade genes. The nuclei were stained with DAPI and fixed prior to imaging using confocal microscopy. Statistical analysis using two-way ANOVA revealed a significant difference ($p < 0.05$) between the total nucleus count at different concentrations of DMSO. The post-hoc test shows that 2 and 3% DMSO concentrations are not significantly different compared to 0% DMSO. The two-way ANOVA test also showed a significant difference ($p < 0.05$) between the genes in comparison to AcGFP.

The MTT assay measures the effects of DMSO on mitochondrial function. Similar to cell count measurements, increasing DMSO concentration reduced mitochondrial function (**Figure 3**). U87 cells expressing Dsup consistently exhibited a higher metabolic rate compared to all other genes tested across all DMSO concentrations. In contrast, cells expressing MAHS and RvLEAM demonstrated similar or lower metabolic rates than cells expressing AcGFP. Dsup-expressing cells have the potential for improving mitochondrial function in the presence of DMSO, while cells expressing MAHS and RvLEAM may not provide any significant advantages over AcGFP under the conditions tested.
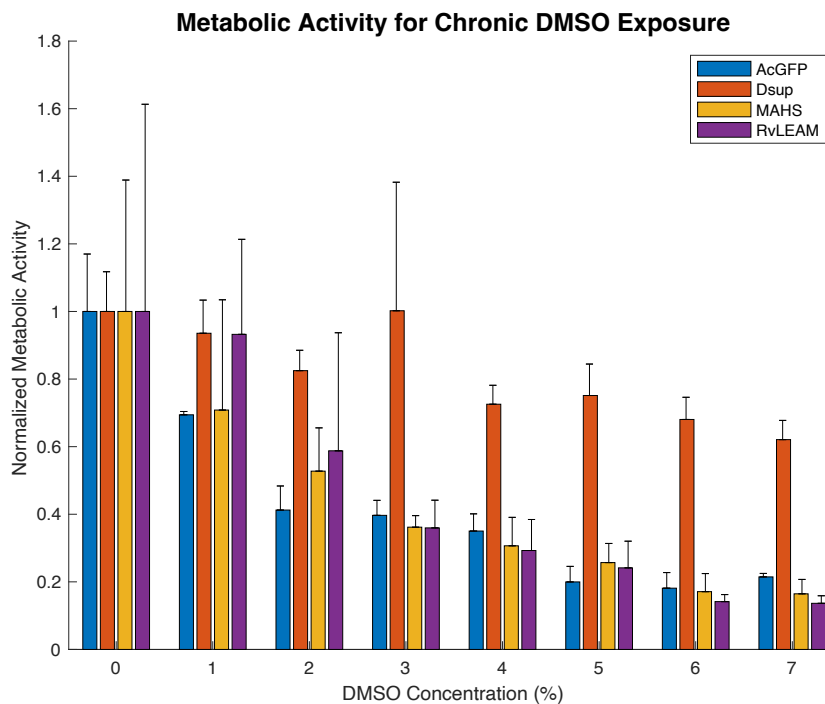
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Metabolic Activity for Chronic DMSO Exposure

Figure 3 Normalized cell metabolic activity of DMSO used during the 72-hour exposure for cells expressing AcGFP, Dsup, MAHS, and RvLEAM. The results indicate that cells expressing tardigrade stress resistance genes had higher metabolic activity than AcGFP-expressing cells in DMSO concentrations ranging from 0% to 2%. Statistical analysis using two-way ANOVA revealed a significant difference ($p<0.05$) of all DMSO concentrations in comparison to the 0% DMSO and a significant difference ($p<0.05$) between the genes. The post-hoc test shows a significant difference between all DMSO concentrations when compared to 0% DMSO, and all genes when compared to cells expressing AcGFP.

Tardigrade Transgene Expression Improves Viability After Acute DMSO Exposure

U87 cells were treated with concentrations of 0-30% DMSO for 30 minutes on ice to evaluate the impact of acute DMSO exposure in a format more consistent with standard cryopreservation practices. The results indicate that cells expressing Dsup exhibit greater viability than those expressing the other proteins. Cells expressing Dsup had a greater percentage of live cells compared to all other genes across all DMSO concentrations. Cells

expressing MAHS and RvLEAM show similar or lower viability compared to cells expressing AcGFP (**Figure 5**).
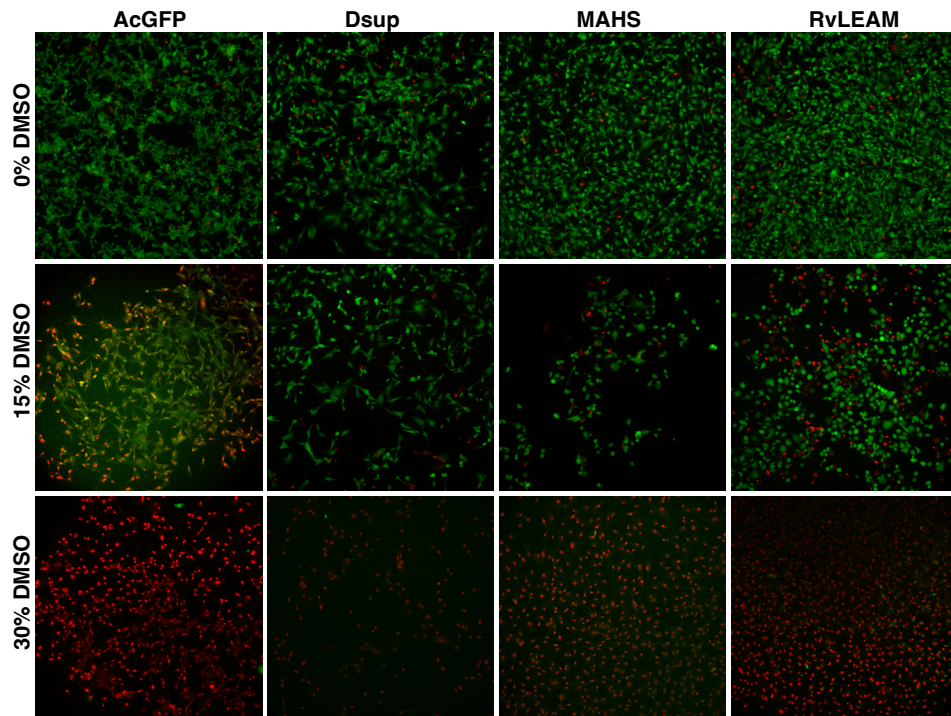

Figure 4 Panel of images after live-dead assay for all genes for acute exposure of DMSO concentrations of 0%, 15%, and 30% after 30 minutes. Live cells are stained green, and dead cells are stained red.
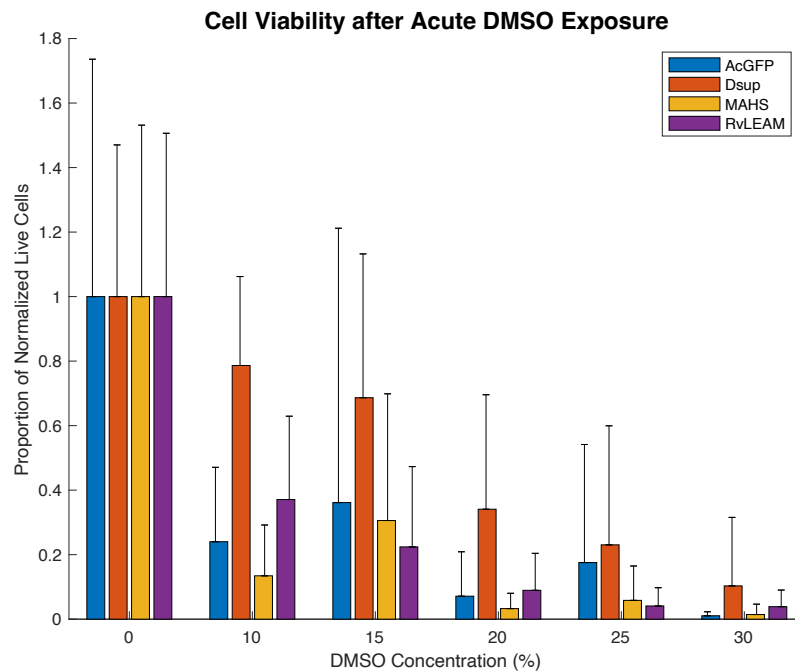
Figure 5 Normalized live cells after cells expressing AcGFP, Dsup, MAHS, and RvLEAM are exposed to DMSO for 30 minutes. The graph shows that as the percentage of DMSO increases, there is a corresponding decrease in living cells for all four proteins. Statistical analysis using two-way ANOVA revealed a significant difference ($p<0.05$) between the nucleus count at different concentrations of DMSO and the different genes. The post-hoc test shows a significant difference between all DMSO concentrations in comparison to 0% DMSO, and cells expressing RvLEAM had significant results in comparison to cells expressing AcGFP.

Tardigrade Transgene Expression Improves Viability After Cryopreservation

U87 cells in 2D cultures were cryopreserved without DMSO, using standard cooling rates of -1 °C/min. After thawing, cell viability was assessed (**Figure 6**). The results demonstrate 13% of AcGFP-expressing cells were viable after cryopreservation, closely followed by RvLEAM-expressing cells with 9% viability. In contrast, Dsup and MAHS demonstrated higher levels of surviving cells, with 39% and 41% viability, respectively (**Figure 7**). A one-way ANOVA test shows all genes have significant results ($p<0.05$). The post-hoc test demonstrates all genes are significantly different than AcGFP. Cells expressing Dsup and MAHS provide some level of cryoprotection to cells during slow cryopreservation without CPA, while cells expressing RvLEAM do not provide sufficient protection against cryopreservation under these conditions.
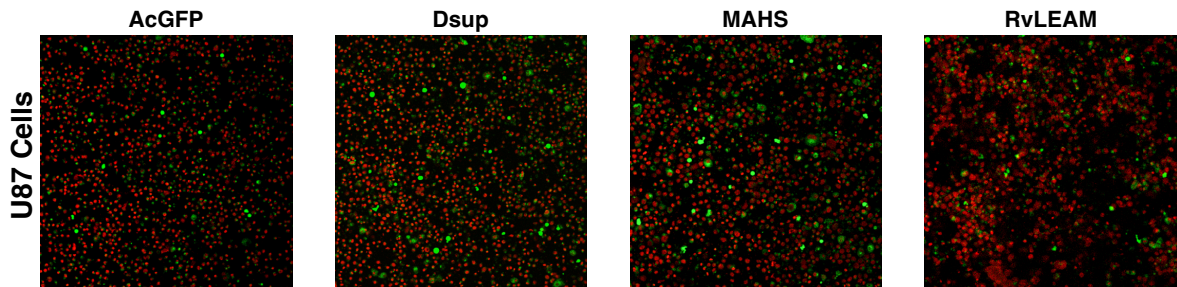

Figure 6 Panel of images after live-dead staining used to determine cell viability for cells expressing AcGFP and tardigrade stress resistance for 2D cell cultures after cryopreservation.
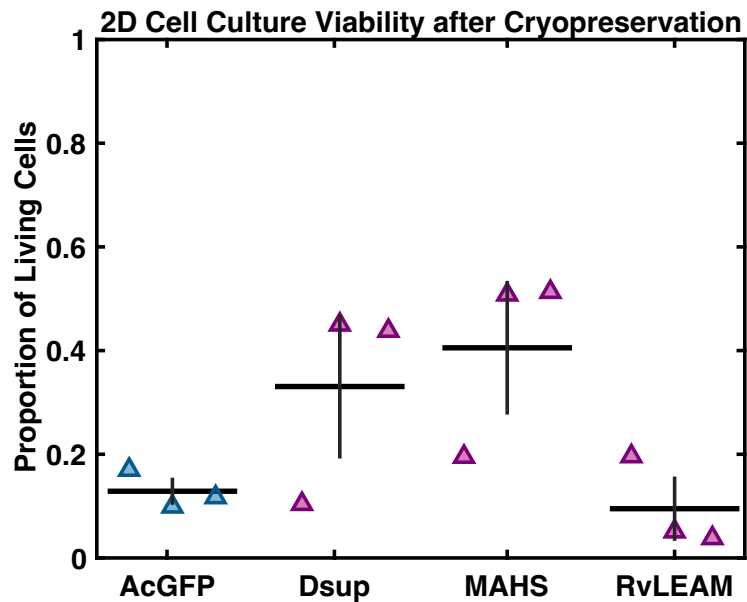
Figure 7 The proportion of living cells in 2D cell cultures after cryopreservation at -80 ℃ and thawed at room temperature.

U87 Cells Are Viable in 3D Gelatin-Transglutaminase Culture

   Cell cultures were suspended in 6% gelatin and 4% TG hydrogels and incubated for 5 days to assess the viability in 3D culture (**Figure 8**). Cells expressing AcGFP have survival of 64%, Dsup 56%, MAHS 74%, and RvLEAM 67% (**Figure 9**). The one-way ANOVA test showed that the results were significant ($p<0.05$). The post-hoc test showed cells expressing Dsup and MAHS had a significant difference compared to AcGFP.
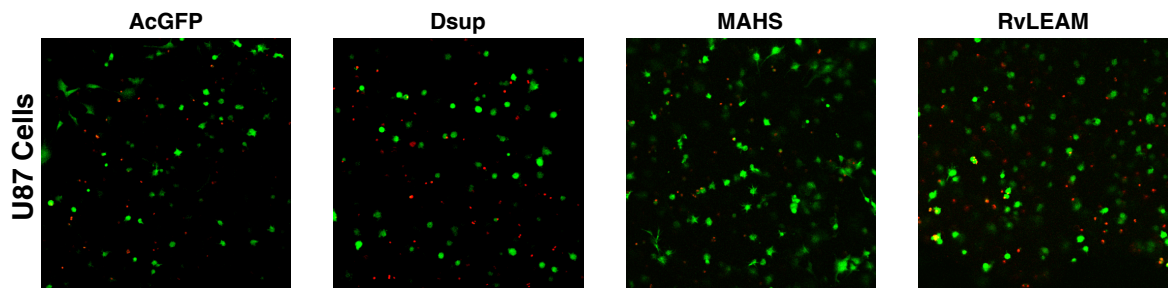
Figure 8 Panel of images after live-dead staining to determine the cytotoxicity of 3D cultures in gelatin-TG hydrogels for cells expressing AcGFP and tardigrade stress resistance genes.
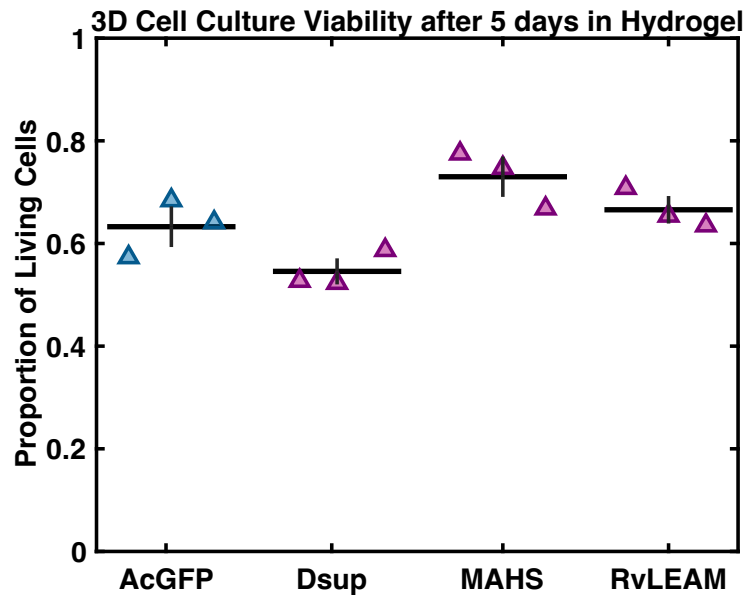

Figure 9 Proportion of living cells in gelatin-TG hydrogel 3D cell cultures after incubating at 37°C for 5 days without cryopreservation.

Tardigrade Transgene Expression Improves Viability After Cryopreservation in 3D

Culture

3D cell cultures in gelatin-TG hydrogels were frozen in the absence of DMSO to determine cell viability through 3D cryopreservation but using standard cooling rates of -1 °C/min (**Figure 10**). U87 cells expressing AcGFP exhibited 58% viability, Dsup showed

53% viability, MAHS showed 66% viability, and RvLEAM showed 52% (**Figure 11**). One-way ANOVA tests showed a significant difference between the cell viability of the genes (p<0.05). The post-hoc test showed a significant difference between cells expressing Dsup and RvLEAM compared to AcGFP.
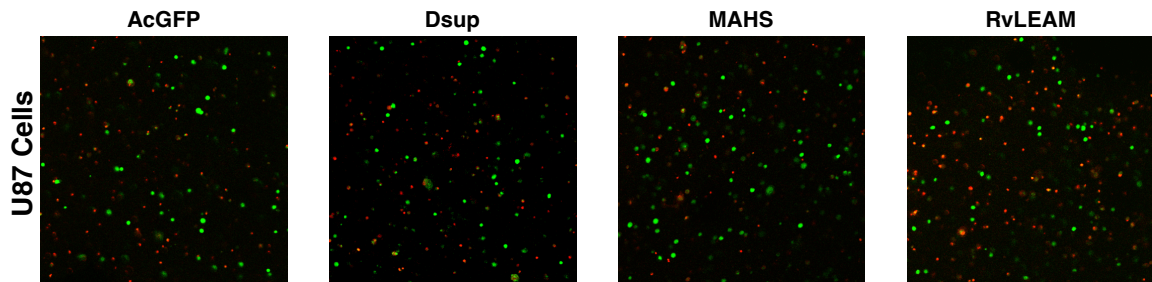

Figure 10 Panel of images taken after live-dead staining to determine cell viability of 3D cultures in gelatin-TG hydrogels after cryopreservation for AcGFP and cells expressing tardigrade stress resistance genes.
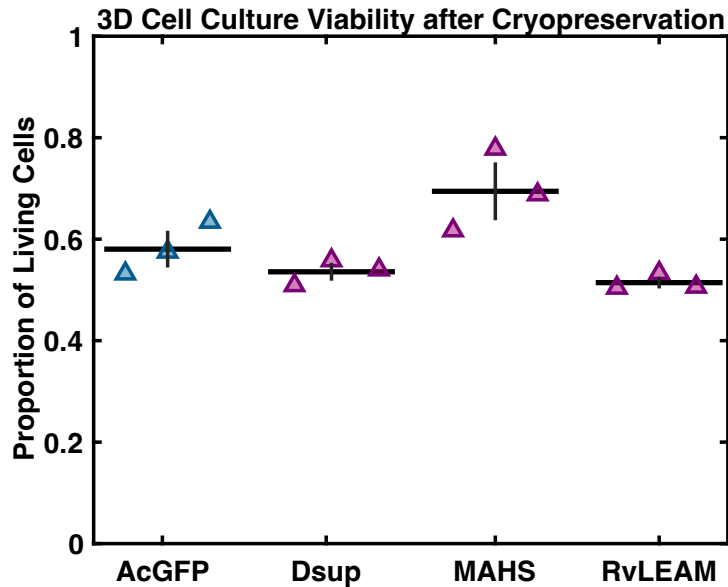

Figure 11 Proportions of living cells in gelatin-TG hydrogel 3D cell cultures after cryopreservation and thawing at room temperature.

Discussion

Effects of tardigrade genes on CPA toxicity

This study investigated the potential of tardigrade stress resistance genes in enhancing cell viability in U87 cells under chronic and acute DMSO exposure and their impact on suspension and embedded cryopreservation. During chronic exposure to the cryoprotectant DMSO, the expression of Dsup and MAHS provided protective benefits to the cells, as shown by increased cell counts after treatment (**Figures 1-2**). MTT showed similar results for Dsup, with expressing cells maintaining mitochondrial capacity compared to the other tested genes during chronic DMSO exposure (**Figure 3**).

While DMSO toxicity is a component of cryopreservation, cells are ideally only exposed to DMSO for short periods of time while chilled, immediately prior, and immediately after freezing. Further, the DMSO concentrations tend to be higher for cryopreservation, 5-20%. To this end, we also tested cells during short duration DMSO treatment. Again, cells expressing Dsup exhibited greater viability, demonstrating protection against acute DMSO exposure (**Figures 4-5**). Cells expressing MAHS and RvLEAM had a lower proportion of surviving cells than cells expressing AcGFP for all DMSO concentrations. The statistical analysis confirmed the difference in cell viability between all genes is significant. This suggests that these MAHS and RvLEAM proteins are not as effective as Dsup in promoting cell viability and protecting cells against the detrimental effects of DMSO.

The use of DMSO had toxic effects on U87 cells, regardless of which tardigrade stress resistance genes the cells were expressing. There was diminished cell viability and

cell metabolic activity. Several types of cryoprotectants are available, each with different properties that make them suitable for different types of cells. DMSO treatment of human embryonic stem cells (hES) increases $O_2^{\bullet-}$ by two-fold, after cryopreservation, it leads to a five-fold increase in the $O_2^{\bullet-}$ [33]. In iPSCs, DMSO was the most toxic cryoprotective agent, and glycerol was the least toxic to the cells, ethylene glycerol and propylene glycerol were of moderate toxicity. The cell recovery rate was the highest when DMSO or ethylene glycerol was used[34]. Glycerol is a non-toxic cryoprotectant however, it has a lower permeability across cell membranes than DMSO, making it less effective. Both glycerol and DMSO lack biocompatibility, making their use difficult in clinical applications [35].

There are non-toxic cryoprotective agents, such as trehalose. Trehalose has low toxicity to cells and can protect cells from damage caused by freezing and thawing by stabilizing cell membranes and preventing ice crystal formation. But due to trehalose's lack of membrane permeability, cell recovery is low. For ADSCs, the use of trehalose was not as efficient as 10% DMSO due to its lack of membrane permeability[36]. DMSO continues to be effective despite its toxicity. In contrast, trehalose-based polymers used as cryoprotectants and 3D cell scaffolds for skin fibroblasts, HeLA cells, and PC3 cancer cells improved cell survival after freezing and thawing by reducing cell osmotic stress[37].

Consistent with these results, Dsup has protective effects on different types of cells. Studies demonstrated that the Dsup protein could protect the DNA from ROS. In HEK293 cells treated with hydrogen peroxide, Dsup protects DNA and activates several detoxification pathways that remove intracellular free radicals[31]. In our results, it protects against DMSO exposure, but not during cryopreservation. This could be due to Dsup not

being able to provide sufficient protection for mechanical or osmotic stress caused by cryopreservation while providing protection against ROS induced by DMSO treatment. Although tardigrade stress resistance genes have a high potential for improving cell viability, their effects differ by cell type. In cortical neurons, Dsup promotes neurotoxicity, leading to neurodegeneration [32]. Therefore, it is essential to test the function of tardigrade stress tolerance genes in a context that matches the intended application to avoid adverse effects.

Current study findings, along with our results, provide important insights into the potential of tardigrade stress resistance genes in enhancing cell viability under various conditions. The transfection of MAHS and RvLEAM genes to human HEp-2 cells significantly improved osmotic tolerance and metabolic activity level[7]. Tardigrade stress resistance genes could be a therapeutic agent by enhancing tolerance to hyperosmotic stress, one of many stresses caused to cells during cryopreservation. Alternatively, after the knockdown of group 1 late embryogenesis abundant (LEA) proteins in *Artemia franciscana,* embryos lacking group 1 LEA proteins showed significantly lower survival after desiccation and freezing. But when exposed to hydrogen peroxide, there was a similar response between the cells expressing group 1 LEA proteins and those that did not [30]. Therefore, RvLEAM could provide protection against the desiccation occurring during cryopreservation but would not provide sufficient protection against ROS production induced by DMSO. In our results, RvLEAM often performs similarly to the control group, potentially, the primary cause of death for U87 cells is ROS production, and RvLEAM does not provide enough protection for the cells to survive.

Effects of tardigrade genes on freezing in the absence of CPA

In the absence of CPAs, cells typically suffer low viability due to ice crystal formation and membrane damage. Cryopreservation of suspension cells without CPA demonstrated a benefit for Dsup and MAHS expression compared to AcGFP (**Figure 7**); cells expressing RvLEAM had equivalent viability to those expressing AcGFP. Dsup and MAHS provide triple or greater protection against 2D cell culture cryopreservation than cells expressing RvLEAM or AcGFP.

An alternative to the use of cryoprotective agents are hydrogels. The different hydrogel cryopreservation methods can confine ice crystal growth and decrease the change rates of osmotic shock in cell encapsulation systems, minimizing cell damage[38]. Hydrogels also provide physical support to cells and tissues during cryopreservation, which helps prevent damage due to mechanical stress. Prior work using transglutaminase to crosslink bovine collagen showed high viability of the cells[39]; in contrast, our results showed a viability of about 65% with transglutaminase and gelatin (**Figure 9**). A possible source of toxicity was heating the gelatin during mixing, which may have stressed the cells. However, cells expressing MAHS and RvLEAM demonstrate greater resilience to the 3D culture method. After cryopreservation of the 3D cell cultures, we observed no significant increase in viability provided by the tardigrade genes. However, viability after thaw (**Figure 11**) was similar to the overall viability of the unfrozen cultures (**Figure 9**), suggesting the hydrogels provide protection against cryopreservation stress. Indeed, when compared to 2D cell culture cryopreservation, the proportion of living cells in all groups was double or greater.

Conclusion

The results demonstrated that cells expressing Dsup and MAHS have improved tolerance for the stresses of cryopreservation while RvLEAM-expressing cells performed similarly or worse than the control group. Our findings are in agreement with previous research on the potential of tardigrade genes for improving cryopreservation outcomes. Overall, our study contributes to the growing knowledge of tardigrade genes and their potential for improving stress tolerance in mammalian cells, highlighting the need for continuing research in this area. Future research will investigate the molecular mechanisms underlying the potential benefits of Dsup and MAHS expression in enhancing human cell viability during cryopreservation and further extend these studies to more clinically relevant models such as primary cells and stem cells. Overall, our study highlights the potential of tardigrade stress resistance genes in enhancing cell viability during cryopreservation, providing a foundation for further research in this area.

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