ARTICLE

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Improving the Long-Term Storage of a Mammalian Biosensor Cell Line Via Genetic Engineering

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ABSTRACT: The unique properties of mammalian cells make them valuable for a variety of applications in medicine, industry, and diagnostics. However, the utility of such cells is restricted due to the difficulty in storing them non-frozen for an extended time and still maintaining their stability and responsiveness. In order to extend the active life span of a mammalian biosensor cell line at room and refrigerated temperatures, we have over expressed genes that are reported to provide protection from apoptosis, stress, or oxidation. We demonstrated that over expression of genes from the extremophile, *Artemia franciscana*, as well as GADD45β, extends room-temperature storage of fully active cells 3.5-fold, while over production of several anti-apoptotic proteins extended 4°C storage 2- to 3-fold. Methodologies like these that improve the stability of mammalian-cell-based technologies in the absence of freezers may enable widespread use of these tools in applications that have been considered impractical based solely on limited storage characteristics.

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KEYWORDS: apoptosis; anti-apoptosis; mammalian cells; cell engineering; extremophiles; cell-based assays

Introduction

Long-term storage of active, viable mammalian cells is a critical problem for applications such as tissue engineering and medical treatments; for example, platelets have a shelf life of only 5 days at 22°C, but lose their effectiveness if stored at lower temperatures. In addition, cell-based assays are valuable for both research and as sensors because they provide a level of complexity, specificity, and sensitivity that cannot be achieved with ex vivo cellular components. Although most cell types can be stored frozen and thawed with success, this method requires ultra-low temperature freezers or liquid nitrogen as well as some post-thaw handling to remove cryopreservatives.

There is still a need for effective methods for long-term stabilization of active mammalian cells that exclude cryopreservation. One example where this capability is critical is the field of transplantation, where cryopreservation is not an option and tissues must be refrigerated in order to preserve their structure and activity. Research in this area has shown that damage to cells during hypothermic storage can be attributed to several events, including the release of intracellular iron (Rauen and de Groot, 2002; Rauen et al., 2000), the production of reactive oxygen species (Peters et al., 1998; Rauen and de Groot, 1998), and destabilization of the cell membrane (Drobnis et al., 1993; Stefanovich et al., 1995). Desiccation of mammalian cells has been explored using trehalose, a disaccharide believed to contribute to the survival of many organisms, which are dehydration tolerant. However, attempts to preserve non-frozen, nucleated mammalian cells using trehalose have only been successful in the absence of storage, or with very short-term storage (Ma et al., 2003; Puhlev et al., 2001; Zhu et al., 2006).

The purpose of the current study was to elucidate the pathways that affect the activity of mammalian cells during storage at room and refrigerated temperatures and to extend the active shelf life of the CANARY cells, a biosensor cell line developed in this laboratory, under these conditions. The cells are derived from a murine B-cell line that is genetically modified to produce both aequorin as well as antibodies that recognize certain bacteria, viruses, and toxins, and are the sensing element in a sensitive identification assay that can provide an answer in a matter of minutes (Rider et al., 2003).

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Without any modification, CANARY cells remain fully functional (defined as detection of 50 colony-forming units (cfu) of bacteria $\geq$50% of the time) as a ready-to-use reagent after 2 days of storage at room temperature and 2 weeks of storage at 4°C. They can also be stored frozen at −80°C or lower, but some manipulation is required before they can be used in the assay. There are certain applications for which a rapid identification assay would be valuable but where the use of freezers or liquid nitrogen, and subsequent manipulation, is not practical. For example, the CANARY assay could serve at ports of entry to rapidly screen imported produce or plants for human or plant pathogens, respectively, but its usefulness is restricted by limited storage characteristics. Extending the shelf life of the cell reagent at more convenient temperatures would enable the use of this rapid assay in certain critical applications, and may broaden the use of other valuable cell-based assays as well.

Materials and Methods

Cloning, Transfection, and Screening of Transfected Cells

Artemia p26 and artemin cDNA were provided in pcDNA3.1. Cells transfected with these plasmids were selected in 500 μg/mL Hygromycin (Invitrogen, Carlsbad, CA). Murine GADD45β, Mcl-1, and human Bcl-w were cloned by RT-PCR and inserted in pEF6/V5/His-TOPO (Invitrogen). Human Bcl2 was purchased from Invivogen (San Diego, CA) amplified by PCR and inserted into pEF6/V5/His-TOPO. Cells transfected with this plasmid were selected in 5 μg/mL Blasticidin (Invitrogen). Restriction sites were added to the human Bcl-XL gene (Invivogen) by PCR and it was inserted into the *Nhe*I and *Xho*I sites of pcDNA3.1 (Invitrogen). The correct sequence of all PCR products was confirmed.

Cells were transfected by electroporation (BioRad, Hercules, CA) at 270 V, 950 μF and cloned by limiting dilution in the appropriate antibiotic. Ten to 12 clones from each transfection were analyzed for expression by quantitative RT-PCR. RNA was extracted from cells using Qiagen kits QIAshredder and RNeasy according to the manufacturer's instructions. Two micrograms of RNA were used for reverse transcription, performed with the Ambion Retroscript kit, using random decamer primers. Quantitative PCR was performed using 100 ng cDNA from each clone, the appropriate TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA: artemin and p26 custom orders; Bcl-w #Hs00187848_m1; Bcl-XL #Hs99999146_m1; Bcl2 #Hs00608023_m1; Mcl-1 #Mm01257351_g1), and compared to a standard curve to estimate expression levels.

Storage and Testing of Transfected Cells

Three to five clones with relative expression levels ranging from low to high were chosen for subsequent storage experiments. Cells were prepared for the luminescence assay as follows: Priming: incubation for 24 h at 37°C with 5% CO_2 at a concentration of 5×10^5 cells/mL in growth medium with the addition of 2% DMSO. Loading: Cells were incubated for 2 h in the dark at room temperature in assay medium [CO_2-Independent medium, 10% fetal bovine serum, 50 μg/mL streptomycin, 50 U/mL penicillin, and 250 ng/mL amphotericin B (Invitrogen)] with 50 μM coelenterazine (Molecular Probes, Eugene, OR). The cells were then washed twice, resuspended in assay medium at 5×10^5 cells/mL in 1.5 mL microcentrifuge tubes, and incubated, with rotation, overnight at room temperature. For each independent trial, prepared cells were stored at 5×10^5 cells per mL in assay medium in 0.6 mL, 1.5 mL (Axygen, Union City, CA, homo-polymer, boil-proof) or 15 mL (VWR) polypropylene tubes. Cells were stored under normal atmospheric conditions in a standard laboratory refrigerator at 4°C, or in a drawer at ambient (room) temperature. The response to each amount of antigen was tested in triplicate at weekly intervals, with the probability of detecting a given amount of antigen reported as the percentage of positive responses. Cells were counted manually and viability was determined by Trypan Blue exclusion.

Microarray/Transcriptional Profiling

RNA Isolation

RNA was isolated using the Qiagen (Valencia, CA) RNeasy isolation kit, following the manufacturer's instructions. The samples of extracted RNA were combined in equal quantity from each of five separate RNA extractions at each time point. Each extraction was from 3×10^6 cells for the control and 6×10^6 cells for the experimental conditions evaluated. RNA quality was assessed by spectral analysis (Supplementary Table II) and with the Bioanalyzer 2100 (Agilent, Santa Clara, CA, Supplementary Fig. 2) prior to transcriptional profiling.

Transcriptional Microarray

Three Affymetrix *Mus musculus* 430 2.0 chips were used for each condition. The chip has 45,000 probe sets to analyze the expression level of over 39,000 transcripts and variants from over 34,000 mouse genes. Sample preparation and analysis were performed in triplicate. Analyses were performed at the Virginia Bioinformatics Institute, with data processing utilizing ArrayAssist (Stratagene, La Jolla, CA). Data processing was performed as follows:

Filter: Eliminated genes with signals from <80% of the probes
Statistical test: *F*-Test, Multiclass (ANOVA)
Error stabilization: Variance inflation
P-Value correction: Benjamini–Hochberg (FDR)

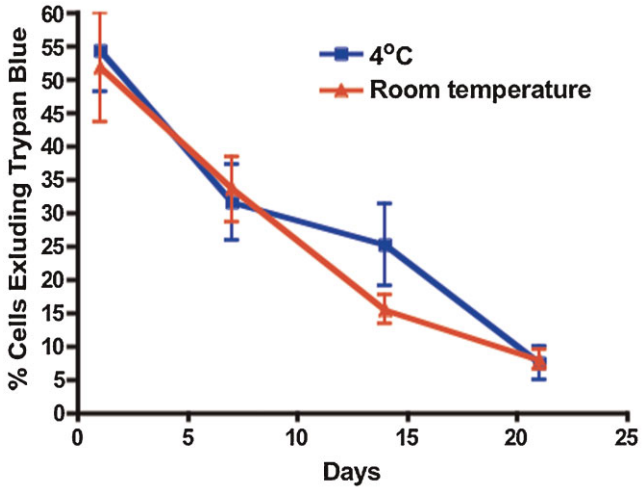

Figure 1. The percentage of Trypan-blue-excluding cells stored over time at room temperature and at 4°C. The mean ± SE are from at least eight independent trials.

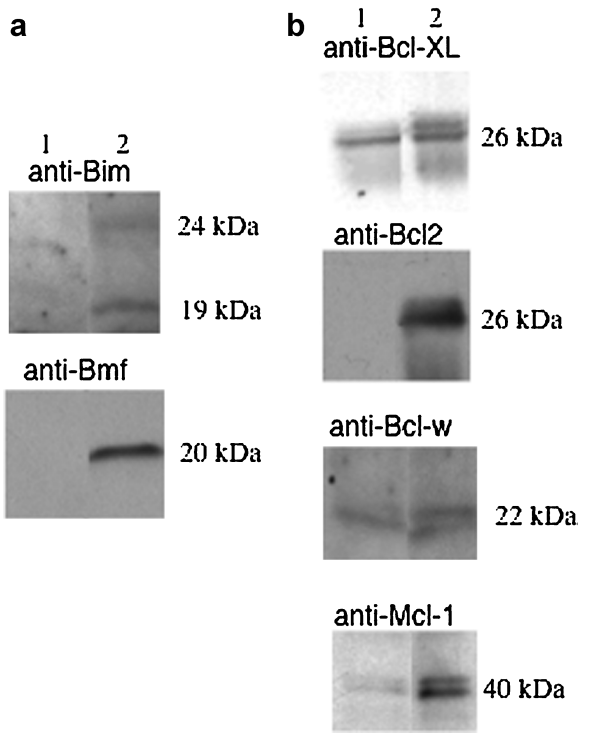

Figure 2. Immunodetection of proteins associated with apoptosis after sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting. **a**: Expression of pro-apoptotic proteins Bim and Bmf in the parental line before (**lane 1**) and after (**lane 2**) assay preparation. **b**: Expression of Bcl-XL, Bcl2, Bcl-w, and Mcl-1 in extracts from parental (lane 1) and stably transfected cell lines (lane 2).

Immunoblot Analysis

Cells were collected at the indicated times by centrifugation (200*g* for 5 min at 23°C) and were washed twice with PBS (Invitrogen). Cell lysis was performed by the addition of lysis buffer (20 mM HEPES, 2 mM EGTA, 5 mM EDTA, 0.5% Triton X-100 containing phosphatase (Pierce, Thermo Fisher, Rockville, IL) and protease (Sigma, St. Louis, MO) inhibitors). The lysates were centrifuged (30,000*g*, 30 min, 4°C) and aliquots of 15 μg protein were loaded on 12% polyacrylamide gels (BioRad) and submitted to electrophoresis (Tris-glycine buffer, 50 mA). Proteins were then transferred to PVDF membranes (Millipore, Billerica, MA) in CAPS buffer (400 mA, 105 min). The membranes were incubated in TBST (20 mM Tris, 0.5 M NaCl, 0.05% Tween-20) for 20 min (one exchange after 10 min), blocked with 1% BSA (Sigma), and subsequently washed with TBST (3×, 5 min each). Primary antibodies recognizing Bcl-XL and Bmf, (Cell Signaling Technology, Danvers, MA), Bcl-w, Bcl2, Mcl-1, Noxa, and Bim (Santa Cruz Biotechnology, Santa Cruz, CA) were prepared in 1% BSA and incubated with membranes at 4°C overnight. The membranes were then washed three times with TBST (5 min each). The secondary antibody, goat anti-rabbit IgG (H + L) alkaline phosphatase (BioRad), was prepared by dilution (1:1,000) in TBST and incubated with membranes for 1 h at room temperature. The antibody to phosophotyrosine (Sigma) was detected with goat anti-mouse-HRP conjugate (Upstate/Millipore, Billerica, MA) at a 1:5,000 dilution. The membranes were then washed with TBST up to six times for 5 min each. Detection was via chemiluminescence following the recommendations of the manufacturer (Roche, Basel, Switzerland).

Table I. Transcriptional analysis: genes on the affymetrix *Mus musculus* 430 2.0 microarray that exhibited at least a sixfold change in the level of RNA after priming, loading, and storage for 7 days at 4°C.

Gene ID	UniGene name	UniGene symbol	Primed	Primed and loaded	7 days 4°C
1454880_s_at	Bcl2 modifying factor	Bmf	12.3	12.2	12.2
1449773_s_at	Growth arrest and DNA-damage-inducible 45 beta	Gadd45b	4.3	9.6	13.3
1418203_at	Phorbol-12-myristate-13-acetate-induced protein 1	Pmaip1 (Noxa)	−1.0	9.2	6.9
1435449_at	BCL2-like 11 (apoptosis facilitator)	Bcl2l11 (Bim)	8.1	6.6	5.8
1456005_a_at	BCL2-like 11 (apoptosis facilitator)	Bcl2l11 (Bim)	8.1	4.1	4.2
1435448_at	BCL2-like 11 (apoptosis facilitator)	Bcl2l11 (Bim)	8.3	3.4	3.0

The numbers represent the fold increase (positive) or decrease (negative) of RNA levels relative to the control (prior to priming and loading). The conditions analyzed included cells that were treated with 2% DMSO for 24 h (primed), cells primed and loaded with coelenterazine in accordance with the assay-preparation protocol (primed and loaded), and cells that were prepared for the assay and stored for 7 days at 4°C (7 days 4°C). A complete list of the results is shown in Supplementary Table III.

Results and Discussion

Since hypothermic storage of primary cells and tissues has been improved by the formulation of special storage solutions that help to prevent storage-induced injury (Acker, 2006; Mathew et al., 2004), we investigated whether a variety of chemicals added to CANARY cells during preparation for the assay and/or storage would improve the activity or viability of the cells when stored at either 4°C or room temperature. We chose chemicals that fell into the following classes: apoptosis inhibitors, antioxidants, cell-cycle inhibitors, and protease inhibitors (full list in the Supplementary Material). Each additive was applied at a variety of concentrations, and activity was assessed before storage and at 1-week intervals during storage. Unfortunately, improvements in activity were inconsistent, occurring in 50–75% of the experiments (data not shown), so we next employed a genetic approach.

In order to identify which genetic modification had the greatest chance of success in preserving activity of the cell reagent during storage, we investigated whether the loss of function was due solely to a loss of viability or some other means. Although Trypan blue exclusion measures only membrane integrity, populations with a high percentage of Trypan-blue excluding cells could easily be propagated in culture, while the reverse was not true. Since, in the case of stored CANARY cells, the ability to exclude Trypan blue correlated with the ability to divide, we used the technique as a viability indicator. CANARY cells do not multiply at room temperature or 4°C (data not shown), and there is a decrease in the number of Trypan-blue-excluding cells during storage at both temperatures (Fig. 1). There are several pieces of evidence that indicate apoptosis is at least partly responsible for this loss. For example, treatment with two apoptosis inhibitors modestly improves Trypan blue exclusion, as well as the activity of stored cells (data not shown). In addition, a transcriptional analysis comparing unprepared cells with those prepared for the assay and stored at 4°C revealed that several facilitators of apoptosis, Bim, Noxa, and Bmf, are upregulated (Table I, complete results from the

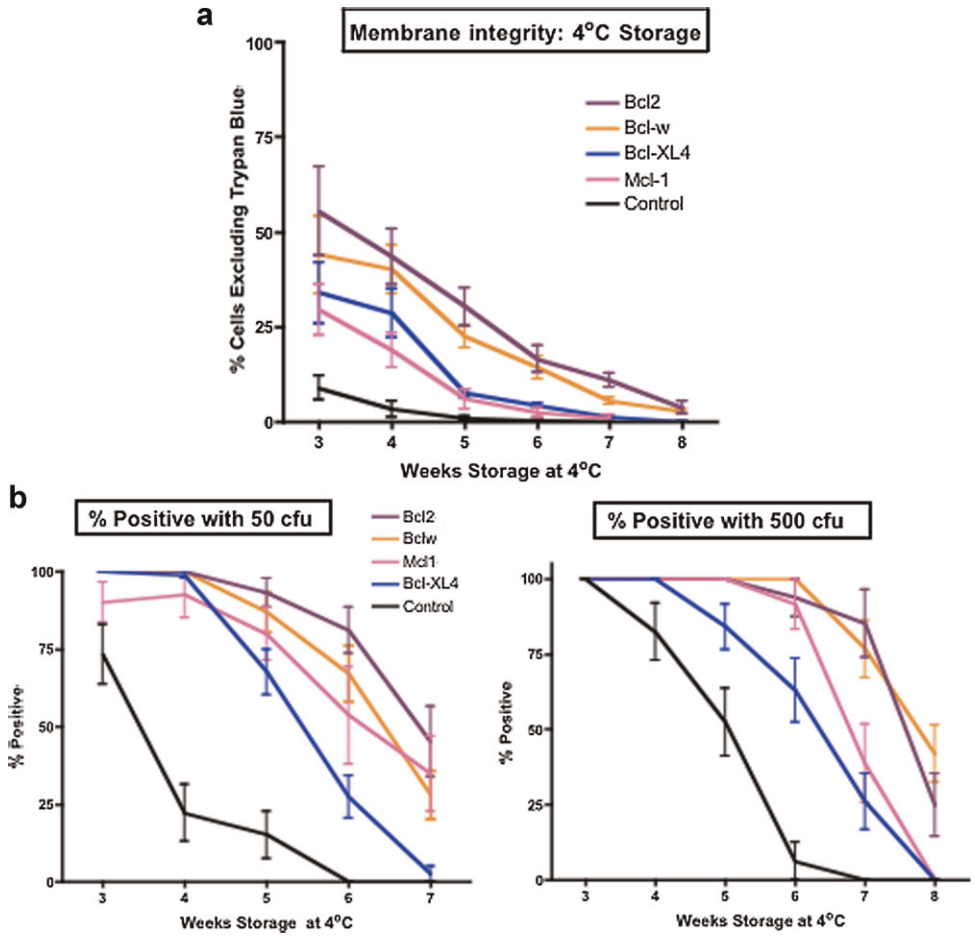

Figure 3. **a**: Over expression of cDNAs encoding anti-apoptotic proteins improves the percentage of Trypan-blue-excluding cells stored for extended periods at 4°C. Trypan blue exclusion was assessed at weekly intervals and these values are expressed as the means ± SE from at least seven independent trials. **b**: Over expression of cDNAs encoding anti-apoptotic proteins improves the performance of cells stored at 4°C. The ability to detect 50 and 500 cfu of antigen (the percent of positive responses) were determined for each cell line at weekly intervals. The results are expressed as the mean ± SE from at least 10 independent trials.

transcriptional analysis as well as RNA quality are shown in Supplementary Tables I and II, and Supplementary Fig. 1). While we were unable to confirm the upregulation of Noxa at the protein level, the immunoblots in Figure 2a demonstrate the increase of Bim and Bmf protein after assay preparation. Bmf and Bim bind to the anti-apoptotic proteins Bcl2, Bcl-w, Mcl-1, and Bcl-XL (Chen et al., 2005). Bmf is thought to inhibit the ability of these anti-apoptotic proteins to sequester and inactivate pro-apoptotic proteins, thereby de-repressing the apoptosis pathway (Kuwana et al., 2005). Furthermore, over expression of the Bmf gene in mammalian cell lines induces apoptosis, and this effect can be offset by increasing the levels of the anti-apoptotic proteins listed above (Puthalakath et al., 2001). Bim directly triggers Bax-Bak activation, leading to permeabilization of the mitochondrial membrane, the first step in mitochondrial-based apoptosis, and over production of either Bcl2, Bcl-XL, or Mcl-1 blocks apoptosis triggered by Bim (Antonsson et al., 2001; Kim et al., 2006; Wei et al., 2000).

To counter the effect of upregulation of pro-apoptotic genes, we over expressed 17 genes whose products inhibit the apoptotic pathway. (A complete list of all of the cDNAs that were transfected can be found in Supplementary Table III.) Stably transfected cells were screened by RT-PCR, and 4–8 clones encompassing a range of RNA levels were tested weekly for improvements in the number of cells with intact membranes (measured by Trypan blue exclusion) and activity after storage both at room temperature and at 4°C. Increased levels of Bcl-XL, Bcl-w, Bcl2, and Mcl-1 (confirmed by immunoblot analysis, Fig. 2b) improved both parameters after storage at 4°C. Figure 3a demonstrates that while virtually none of the control cells have intact membranes after 6 weeks of refrigerated storage, an average of 3–5% of cells over expressing Bcl-XL or Mcl-1, and 15–17% of cells over expressing Bcl-w or Bcl2 exclude Trypan blue. This improvement correlated with an improvement in activity (defined as the ability to detect a bacterial target). While the probability of detecting 50 cfu drops to 25% with the control cells after 4 weeks of storage, it remains approximately 100% with all of the transfected cell lines. Furthermore, the probability of detecting either 50 or 500 cfu after 6 weeks of storage remained higher than 70% and 95%, respectively, for cells over expressing either Bcl-w or Bcl2 (Fig. 3b). We have shown improvements in functional life span by over expressing Bcl-XL, Bcl2, Bcl-w, and Mcl-1, perhaps by out competing effects due to the increase in Bmf and Bim. In addition, higher levels of Bcl2, Mcl-1, and Bcl-w improved the activity of the cell reagent after storage at room temperature (Fig. 4 and data not shown). However, no combination of two anti-apoptotic proteins provided any additional storage benefit at either temperature.

While membrane integrity of CANARY cells decreases at the same rate when stored at room temperature or 4°C (Fig. 1), their activity degrades much more rapidly at room temperature than when stored refrigerated (compare Fig. 3b and Fig. 5). Tyrosine phosphorylation and dephosphorylation events are often associated with key cellular signaling processes. An understanding of changes in the phosphotyrosine proteome may lend insight into the underlying biochemistry and potentially identify other genes that could be exploited to extend cell reagent storage life. Therefore, in order to explore the loss of activity at room temperature, we performed a phosphotyrosine analysis to investigate changes in the levels of protein and phosphorylation status during 3 days of storage at room temperature (3d, RT), compared to unprepared cells (Control), and cells that were stored at 4°C for 7 days (7d, 4°C, Fig. 4). The immunoblot revealed differences between samples stored at room temperature versus 4°C, supporting the proposal that each condition has a separate response process. Subsequent LC-tandem mass spectral analysis of the protein bands associated with the phosphotyrosine proteome identified proteins associated with the unfolded protein response (heat shock, calreticulin, T-complex proteins), oxidative stress/DNA damage (peroxiredoxin, thioredoxin), and rearrangement of structural proteins (tubulin, actin, profilin; see Supplementary Fig. 2 and Supplementary Table IV). Interestingly, several of these proteins were recently identified as phosphotyrosine-containing proteins (Ballif et al., 2008). Although a thorough investigation of these proteins has yet to be

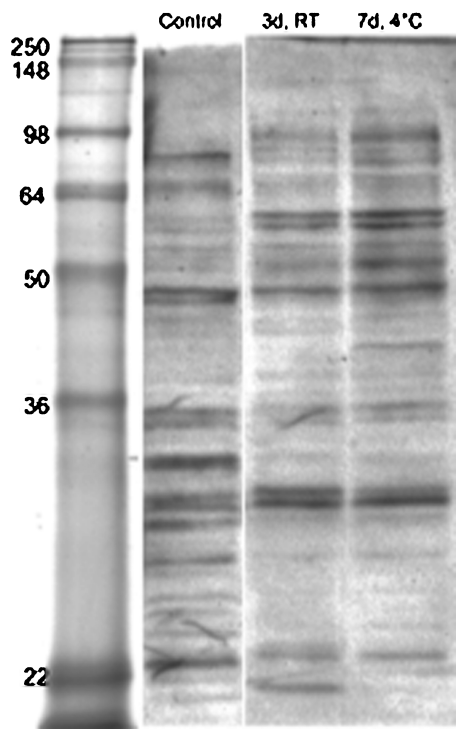

Figure 4. Phosphotyrosine analysis of CANARY cell lysates of fresh cells (Control) compared to lysates of cells stored for 3 days at room temperature (3d, RT) or 7 days at 4°C (7d, 4°C).

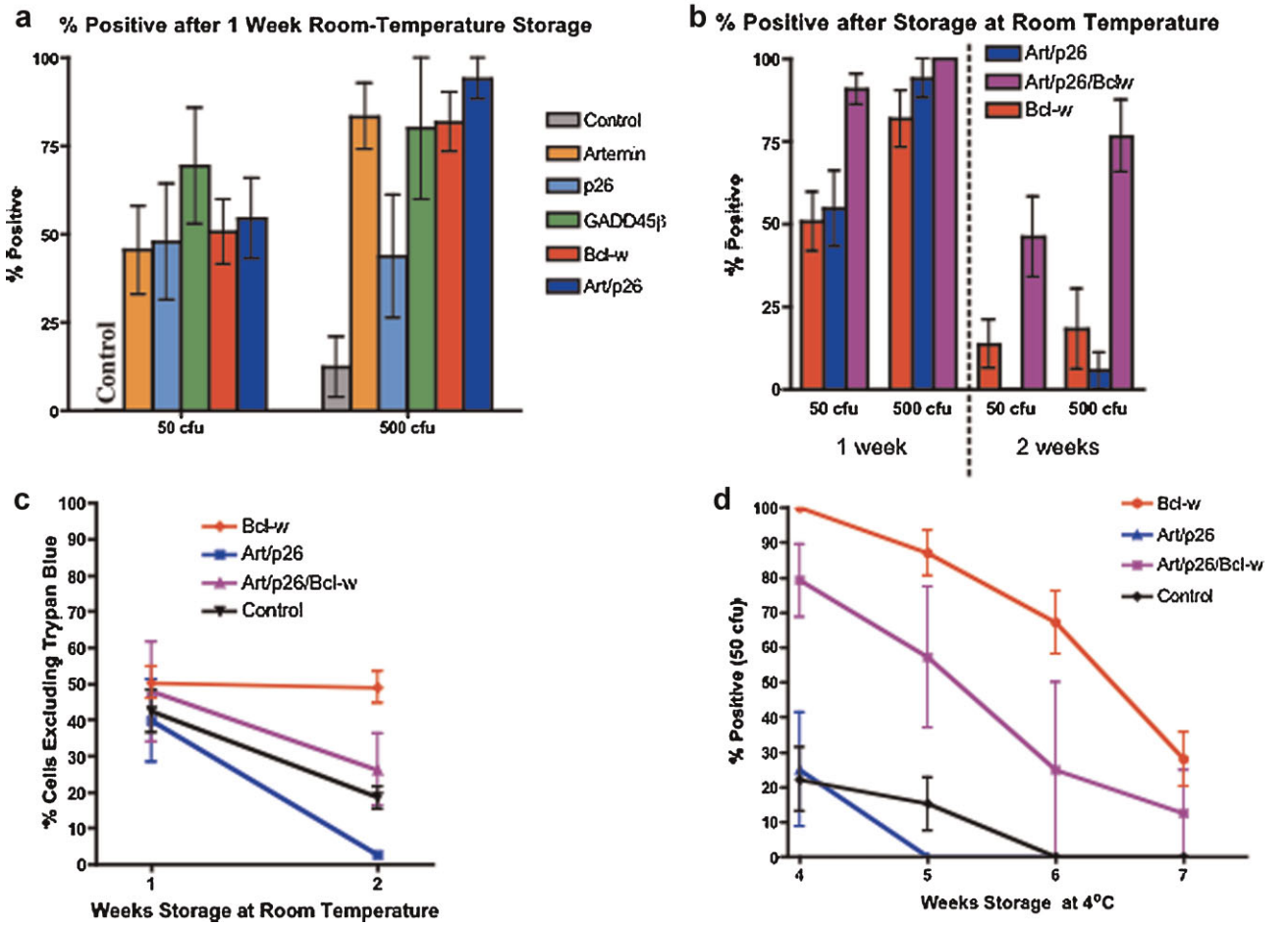

Figure 5. Over expression of *Artemia* cDNAs encoding artemin and p26, as well as GADD45β, improves the activity of cells stored at room temperature, and combining artemin, p26 and Bclw results in an additional improvement. **a**: The ability to detect 50 and 500 cfu of antigen was determined for each cell line after 1 week of storage at room temperature. The results are expressed as the mean ± SE of the probability from at least 6 independent trials. **b**: Cells stably transfected with cDNA encoding Bcl-w in addition to artemin and p26 (Art/p26/Bcl-w) demonstrate improved activity after storage at room temperature when compared to cells transfected with cDNA encoding artemin and p26 only (Art/p26). The ability to detect 50 and 500 cfu of antigen was determined after 1 and 2 weeks of storage at room temperature. The results are expressed as the mean ± SE from at least 10 independent trials. **c**: Cells stably transfected with cDNA encoding Bcl-w, artemin, and p26 demonstrate a higher percentage of Trypan blue exclusion after 2 weeks of room-temperature storage than cells transfected solely with cDNAs encoding artemin and p26. The Trypan blue exclusion of each cell line was assessed at 1 and 2 weeks. The means ± SE are from at least seven independent trials. **d**: Production of artemin and p26 in combination is detrimental to the activity of cells stored at 4°C. Detection of 50 cfu of antigen was determined for each cell line at weekly intervals. The means ± SE were determined from at least nine independent trials.

completed, the results suggested that we further evaluate genes whose products counteract the effects of oxidation/DNA damage, the proteolytic pathway, and protein misfolding.

The heat-shock response is induced under a variety of stress conditions, and is mediated by molecular chaperones and proteases, proteins which protect the cell from aggregation and precipitation of unfolded intermediates (Mosser et al., 1997; Winter and Jakob, 2004). Genes from the extremophile *Artemia franciscana* encoding artemin and p26 are expressed at very high levels in cysts, dormant life forms of the animal, which are very resistant to desiccation and temperature extremes. Artemin chaperones proteins and potentially RNA, while p26 prevents protein aggregation in vitro and in mammalian cells protects against the stresses of desiccation, heat, and oxidative damage while inhibiting apoptosis (Chen et al., 2007; Collins and Clegg, 2004; Ma et al., 2003; Qiu et al., 2006; Villeneuve et al., 2006). GADD45β mediates the suppression of Fas-induced apoptosis (Zazzeroni et al., 2003), and has been described as a stress-response gene (Smith et al., 1994); expression of GADD45β is upregulated dramatically in HEK293 cells as they enter a quiescent state that allows them to survive room-temperature storage for as long as 6 weeks (Jack et al., 2006).

In order to improve the activity of cells stored at room temperature, we transfected expression vectors containing cDNAs encoding GADD45β, artemin and p26, and screened clones from each transfection as described above. Although the RNA of the transfected genes was detected by RT-PCR,

we were unable to detect the proteins by immunoblot analysis, perhaps because the level of expression was below the sensitivity of the immunoblot assay. However, clones that possessed higher levels of mRNA encoding these proteins (compared to untransfected cells), as determined by RT-PCR, exhibited improved activity after storage at room temperature (Fig. 5a). While control cells were unresponsive to 50 cfu of bacterial agent after 1 week, cells over expressing GADD45β, artemin and p26 responded, on average, 45–70% of the time. There was no difference in membrane integrity between cells over expressing artemin, p26, or GADD45β as compared to the control cells, nor did these genetic modifications improve the length of time that the cells could be stored at 4°C. There was no additive or synergistic effect observed when these genes were combined.

An additional improvement in room-temperature storage was observed with cells over expressing the 3 cDNAs encoding artemin, p26, and Bcl-w (Art/p26/Bcl-w), where the probability of detecting 50 cfu increased to 90% after 1 week of storage, and to 45% after 2 weeks of storage (Fig. 5b). The addition of Bcl-w restored the membrane integrity of cells expressing artemin and p26 to levels similar to that of control cells after 2 weeks of room-temperature storage, but not to the level of cells overexpressing Bcl-w alone (Fig. 5c), indicating that survival is not sufficient to maintain activity during storage at room temperature. Although artemin/p26/Bcl-w cells exhibited higher activity after storage at 4°C when compared to control or artemin/p26-expressing cells stored at this temperature, they did not match the performance of cells over expressing Bcl-w alone (Fig. 5d). This contrasts with the situation at room temperature where after 2 weeks, cells expressing artemin, p26 and Bcl-w exhibited higher activity than cells transfected with Bcl-w alone. In fact, the combination of artemin and p26, while beneficial to the activity of cells stored at room temperature, appears to be detrimental during storage at 4°C.

Ambient or refrigerated storage of mammalian cells is particularly important for any application where the use of ultra-low-temperature freezers or liquid nitrogen is impractical, such as a cell-based technology that must be transported and employed outside of laboratories. In this study we have shown that manipulation of the apoptotic and stress-response pathways prolongs the active shelf life of a mammalian-cell reagent during refrigerated and room temperature storage. We have also demonstrated that proteins from extremophiles provide a storage benefit, suggesting that biomimetic approaches can improve the long-term storage characteristics of mammalian cell lines. While these improvements are encouraging, there are biochemical and metabolic events yet to be identified which continue to limit the active life of these cells. Investigation and subsequent manipulation of systems such as autophagy, necrosis, membrane integrity, calcium homeostasis, or DNA or cytoskeleton stability may extend non-frozen shelf life further (Acker, 2006; Kim et al., 2005). Methodologies like these that improve the stability of mammalian cells in the absence of freezers may enable widespread use of mammalian cells in applications that have been considered impractical based solely on limited storage characteristics.

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