EXPRESSION OF TARDIGRADE STRESS TOLERANT PROTEINS IN MAMMALIAN CELLS

By

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Abstract

Tardigrades are an aquatic microscopic invertebrates with the capability to enter a state of anhydrobiosis. Within this state tardigrades are in a very low metabolic state which allows them to survive a wide range of physical stresses such as heat, UV radiation, or even desiccation. Tardigrades produce a variety of heat-soluble proteins in order to protect cellular organelles such as the cytoplasm, nucleus, or mitochondria. Two proteins demonstrated to localize to the mitochondria are MAHS and RvLEAM. RvLEAM is an LEA protein which is family of proteins within other anhydrobiotic organisms which aids in relieving protein aggregation during desiccation. MAHS is a tardigrade unique heat soluble protein similar to RvLEAM that also localizes to the mitochondria. Prior research has shown these proteins can be expressed in mammalian cells and provide transient stress tolerance, but this has not been tested with chronic stress. In order to test this, we have produced lentiviral vectors capable of inserting RvLEAM and MAHS into mammalian cell lines. Using lentiviral transduction, we have created human and canine cells that stably express MAHS and RvLEAM. This work demonstrates the successful creation of these vectors and stable cell lines along with preliminary findings of increased stress tolerance in mammalian cells.

Acknowledgements

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Introduction

Tardigrades, or water bears, are remarkable eight-legged micro-animals that exhibit extreme stress tolerance. These creatures are typically found in damp terrains such as moss, ferns, and water sediments where they feed, grow and reproduce. Tardigrades feed on a fluid diet, consuming the fluid from plant and animal cells[1]. Remarkably, tardigrades can undergo a process termed anhydrobiosis in which the metabolic processes are stopped giving the organism the ability to survive stresses in a desiccated state[2]. Though anhydrobiosis can be seen in other invertebrate species during specific life stages, tardigrades can undergo this process during any life stage. During this stage, dehydrated tardigrades can withstand temperatures for -273 °C to 100 °C[1,3], pressures of 7.5 GPa[4], the vacuum of space, and even radiation demonstrating the physical extremes these creatures can withstand[1].

While the mechanisms of stress tolerance in tardigrades are currently poorly understood, trehalose, a sugar molecule, is thought to be a protector of organelles in other species such as arthropods and nematodes. Trehalose is known to accumulate in large quantities in these species, however several studies have shown that little to no trehalose is present in tardigrades that were and weren't under desiccation. This demonstrates that other biomolecules or mechanisms are allowing for anhydrobiosis within these creatures[5,6].

Another mechanism known to mediate stress tolerance in many species are late-embryogenesis abundant (LEA) proteins, which are also expressed in tardigrades[6]. These proteins are hydrophilic with an unstructured shape allowing it to maintain its solubility during boiling water. LEA proteins have been proven to be expressed during desiccation in many anhydrobiotic species and are known to weakly interact with macromolecules[6,7,8].

A previous study found the presence of two proteins present during desiccation localized to the mitochondria[7]. MAHS and RvLEAM, a heat-soluble protein and LEA protein respectively, are novel proteins linked to tardigrade stress tolerance. MAHS is part of a family of heat-soluble proteins that are conserved in tardigrades that protect several cellular compartments.

Utilizing these identified proteins, this work demonstrates the development of lentiviral vectors for the expression of tardigrade proteins, MAHS and RvLEAM, in mammalian cells with initial testing on their stress tolerance. Current research has proven the physical extremes these creatures can survive in. To fully harness the capabilities of these creatures, our objective is to demonstrate the potential of these novel proteins within mammalian cells. Exhibiting the potential of these proteins within mammalian cells can lead to future research in the preventions of health issues such as ischemia-reperfusion, which can lead to myocardial infarctions, ischemic strokes, trauma and is common in organ transplants and general surgeries[9,10].

Materials and Methods

Cultured Cells

Human U87 glioblastoma cells, HEK293TN cells and human pancreatic cancer cells (PANC1) were cultured in Dulbecco's Modification of Eagle's Medium (DMEM; Corning, Tweksbury, MA) 1x with 4.5 g/L glucose, L-glutamine & sodium pyruvate containing 5% Fetal Bovine Serum (FBS; Corning,), 1% penicillin/streptomycin (P/S; Genesee Scientific, El Cajon, CA), and 1% ampicillin (Corning). Madin-Darby Canine Kidney (MDCK) cells were cultured similarly except in Eagle's Minimum Essential Medium (EMEM; Corning, Tweksbury, MA). Cells were kept at 37 °C and 5% CO_2. Cells were passaged are approximately 50% confluency and maintained in 90 mm culture dishes.

Lentiviral Vectors

Plasmids containing MAHS (Addgene plasmid # 90034; http://n2t.net/addgene:90034; RRID:Addgene_90034) and RvLEAM (Addgene plasmid # 90035; http://n2t.net/addgene:90035; RRID:Addgene_90035) conjugated to the fluorescent marker AcGFP were purchased from Addgene. The lentiviral transfer plasmid, pCDH-CMV-MCS-EF1-Puro was the recipient plasmid. All plasmids were grown in E. coli using 50 mL of Lysogeny broth (LB) and ampicillin then allowed to grow overnight. Plasmids were isolated using plasmid DNA mini kit 1 (OMEGA Bio-Tek) according to manufacturer's instructions. Purified DNA were double digested at restriction sites NotI and NheI, which flank the sequences of interest. DNA fragments were separated by gel electrophoresis and fragments with the appropriate molecular weight were purified using a gel extraction kid (OMEGA Bio-Tek). MAHS-AcGFP and RvLEAM-AcGFP fragments were ligated into pCDH-CMV-MCS-EF1-Puro and transformed into chemically competent E. coli (Zymo Research, Irvine, CA). Transformed E. coli were grown up and DNA was purified using a DNA

mini kit. To confirm successful ligation, Sanger sequencing (Retrogen, San Diego, CA) was performed.

Transfection

Triple transfection was performed in HEK293TN (System Biosciences, Palo Alto, CA) cells with the packaging plasmid, psPAX2 (Addgene plasmid #12259; http://n2t.net/addgene:12259; RRID:Addgene_12259), the envelope plasmid pMD2.G (Addgene plasmid #12260; http://n2t.net/addgene:12260; RRID:Addgene_12260), and the new transfer plasmids previously mentioned. Triple-transfected HEK293TN cells produce replication incompetent lentiviral vectors containing the transfer sequence. Using the Mirus TransIT-Lenti kit with proper mass ratios, Hek293TN cells at 60% confluency were transfected in 60 mm dishes for each vector. Viral supernatant was collected at 24 and 72 hours, spun down and then filtered.

Transduction

Using a ~50% confluent dish of U87 glioblastomas and MDCKs, filtered viral suspension was added at a 1:10 ratio. Cells were incubated and then checked for fluorescence at 24 and 48 hours. As pCDH-CMV-MCS-EF1-Puro provides resistance to puromycin, cells were treated with 1-3 μg/mL puromycin after 48 hours. Cells were regularly thinned to ~20% and selected daily for 2 weeks until a ~95% expressing population was present.

Heat Shock Testing

Cultured transduced U87 cells were seeded at a density of $5.0x10^4$ cells in a 24 well plate for 24 hours prior to stress exposure. For exposure to the heat, cells were wrapped with plastic wrap and foil to reduce light exposure and humidity loss. The plate was then transferred to an Incu-Shaker (MaxQ™ 4450 Benchtop Orbital Shakers) pre-set to 44 °C for 1 hour and 0% CO_2. A control plate with identically seeded wells were wrapped and placed into a 37 °C incubator

containing 0% CO_2 for 1 hour. Following the heat treatment, the fresh media was placed on the cells and the cells were allowed to recover for 96 hours.

Fixing and Staining

Once recovery time was complete, the wells were rinsed 3x with PBS containing Ca+ and Mg+. Cells were then fixed with 4% paraformaldehyde (PFA) and 0.25% Triton. Staining for Senescence-associated β-Galactosidase (SA-βgal) according to the manufacturer's instructions followed (Cell Signaling, Danvers, MA). SA-βgal allows for senescent cells in culture to be identified. Cell counts were then performed in order to determine the number of senescent cells in the population.

Organic Solvent Testing

Transduced AcGFP U87 glioblastomas were plated into 24 well plates at $2.5x10^4$ cells per well. Cells were allowed to adhere to the plate for 24 hours prior to treatment. A solution was made utilizing mixtures of media with either methanol, ethanol, 1-propanol, or 1-butanol. To determine proper ranges, each solution began with 1,3,5,7 or 10% of the solvent combined with DMEM media for a 1 ml solution. This media was administered to the cells post the 24-hour plating. Cells were treated with this solution for 24 hours and then given fresh DMEM media for a 24-hour recovery.

Results

Confirmation of Vector Sequence

To confirm successful ligation of these plasmids, sanger sequencing was performed then compared to the genes of interest sequence. 10 µL of each purified DNA was packed and sequenced by Retrogen. Returned results were then analyzed against the original sequences using NIH Basic Local Alignment Search Tool (BLAST). Using a nucleotide BLAST, we were able to compare the returned sequences. After analysis, the returned results expressed 100% homology for the tardigrade genes, MAHS-AcGFP and RvLEAM-AcGFP while the GFP homology was not directly verified.

Score	Expect	Identities	Gaps	Strand
1779 bits(963)	0.0	992/1015(98%)	4/1015(0%)	Plus/Plus

```
Query  96    GCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACC  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3134  GCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACC  3193

Query  156   ATGTCCAGATACCTGCTGCGCGATGTCCAGGCTGTATTACGCGGAGTTCGCAAAGTGGCC  215
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3194  ATGTCCAGATACCTGCTGCGCGATGTCCAGGCTGTATTACGCGGAGTTCGCAAAGTGGCC  3253

Query  216   GAGAGTAGCTTAAAGCTGGAGACGGAGAAAGTCAGTCTGCGGCTTGGTGACTTTCGGTCA  275
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3254  GAGAGTAGCTTAAAGCTGGAGACGGAGAAAGTCAGTCTGCGGCTTGGTGACTTTCGGTCA  3313

Query  276   CAGCCTTCCCTTCGCAGTGTGCCTGCTTCCCTCACAAGTCGATCACAGGCATTTAGCCTA  335
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3314  CAGCCTTCCCTTCGCAGTGTGCCTGCTTCCCTCACAAGTCGATCACAGGCATTTAGCCTA  3373

Query  336   CAGGAGATAGCTGCTCGTGCCGGAGTTGTTCTGCGAGGAGTGCAACAACAGTTCCGAAAC  395
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3374  CAGGAGATAGCTGCTCGTGCCGGAGTTGTTCTGCGAGGAGTGCAACAACAGTTCCGAAAC  3433

Query  396   GTCACTGGAGTGAATGCCGCTCCTGTTGTAGCCTTTGATAATGGATCAGTTCTATACAGT  455
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3434  GTCACTGGAGTGAATGCCGCTCCTGTTGTAGCCTTTGATAATGGATCAGTTCTATACAGT  3493

Query  456   GAAAGAATCCACTCGCAGAGTTCGCAGAAGCAGGCCCCGACTACAGTACCAACAGGATCC  515
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3494  GAAAGAATCCACTCGCAGAGTTCGCAGAAGCAGGCCCCGACTACAGTACCAACAGGATCC  3553
```

Fig. 1. Query of Retrogen sequence. Subject is MAHS sequence, and vertical pipes indicate homology.

Score	Expect	Identities	Gaps	Strand
1911 bits(994)	0.0	1016/1031(99%)	1/1031(0%)	Plus/Plus

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Query  3192  GAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTCGAGCTCAA  3251
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Sbjct  20    GAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTCGAGCTCAA  79

Query  3252  GCTTCGAATTCTGCAGTCGACGGTACCATGTTTCTCGCCCGAAACGCTGGACGCGCTGGA  3311
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Sbjct  80    GCTTCGAATTCTGCAGTCGACGGTACCATGTTTCTCGCCCGAAACGCTGGACGCGCTGGA  139

Query  3312  TATCGAGGTGTTGTAGCTTACCAGCAAGCAGCCTCTTTCTCTGTTTCATCAGCTAAGGCT  3371
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  140   TATCGAGGTGTTGTAGCTTACCAGCAAGCAGCCTCTTTCTCTGTTTCATCAGCTAAGGCT  199

Query  3372  GCTGGATCACGGTCAAGCGGTGGCTCCGACGCTGGCGATTATGCTAGGGAGGCCGCGGAA  3431
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  200   GCTGGATCACGGTCAAGCGGTGGCTCCGACGCTGGCGATTATGCTAGGGAGGCCGCGGAA  259

Query  3432  CATGCCAAAGCTGGATTGAAGGATCTCAAGAATGAAGCCTCGTGGAAAGCCAAAGGTGTC  3491
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  260   CATGCCAAAGCTGGATTGAAGGATCTCAAGAATGAAGCCTCGTGGAAAGCCAAAGGTGTC  319

Query  3492  GCTAACCAAGCCGCTGGAGCATTCGAACGAGCGAAGGATACAGTGAAGGAAGGTGTCCAT  3551
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Sbjct  320   GCTAACCAAGCCGCTGGAGCATTCGAACGAGCGAAGGATACAGTGAAGGAAGGTGTCCAT  379

Query  3552  GACATGAAGCGCAGTGGAAGCCGCGTCTTCGAACAAGGACAGGAGGAAGTAGAGGCTGGT  3611
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  380   GACATGAAGCGCAGTGGAAGCCGCGTCTTCGAACAAGGACAGGAGGAAGTAGAGGCTGGT  439
```

Fig. 2. Query of Retrogen sequence. Subject is RvLEAM sequence, and vertical pipes indicate homology.

Fluorescent Protein Expression

In order to visualize the protein expression within the cell lines, we utilized confocal microscopy. Using Leica TCS SPEII confocal microscope, we were able to determine the expression levels and measure AcGFP fluorescence. Examples of confocal images (Fig. 1-3) demonstrate the fluorescence in each cell line. Our control group, AcGFP (Fig 1A, 2A, 3A), demonstrates complete fluorescence throughout the cell without increased cell stress tolerance. Our test groups of MAHS and RvLEAM demonstrate localized fluorescence to the cytosolic

puncta, consistent with mitochondrial localization expected of these proteins[7]. Within the test group images, dark spots can be seen on each cell demonstrating the lack of fluorescence in the nucleus which contains no mitochondria.

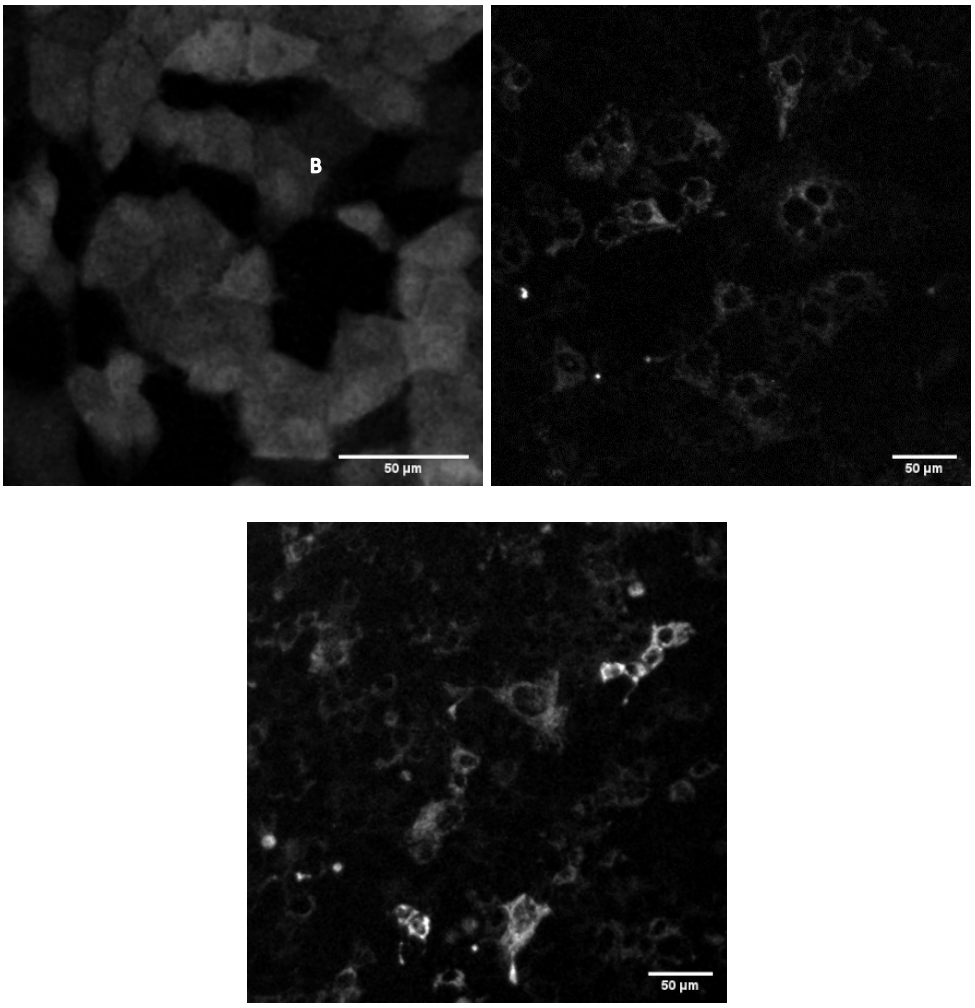

Fig. 3. Transduced MDCK cells. (A) Control group AcGFP. (B) RvLEAM AcGFP. (C) MAHS AcGFP

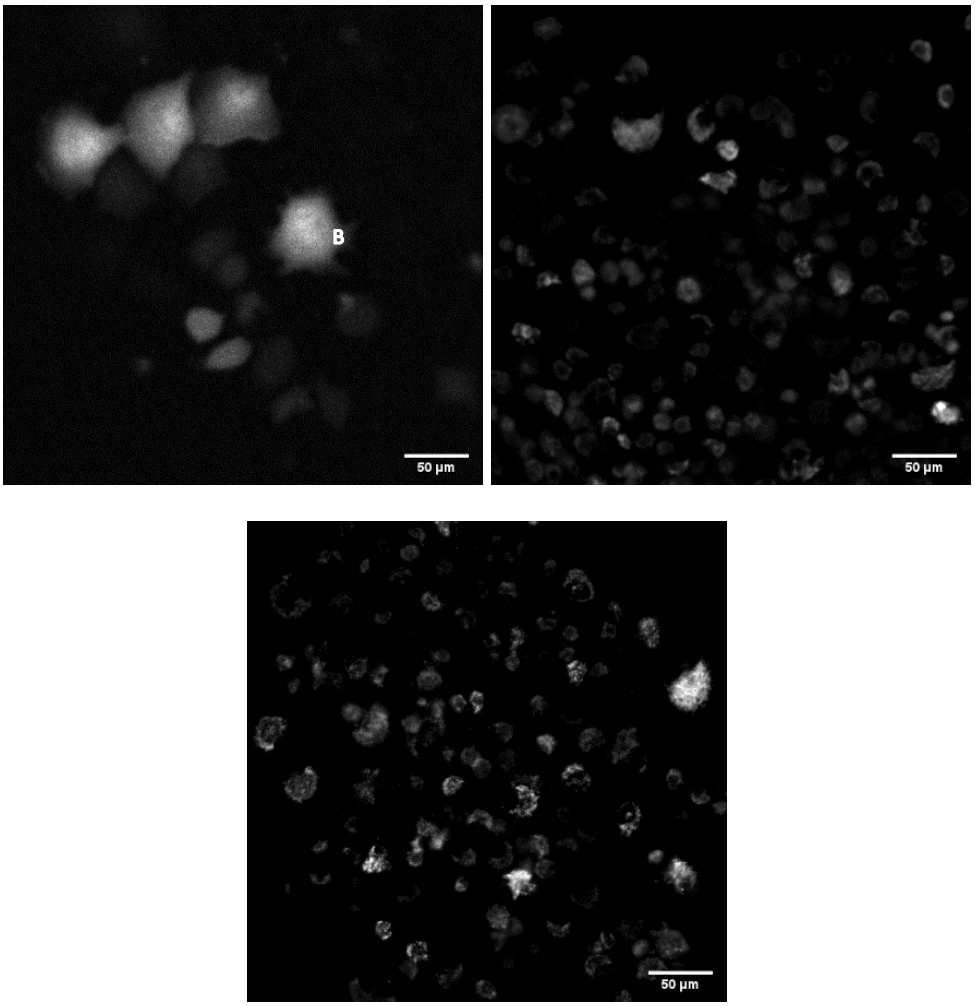

Fig. 4. Transduced human pancreatic cancer (PANC-1) cells. (A) Control group AcGFP. (B) RvLEAM AcGFP. (C) MAHS AcGFP

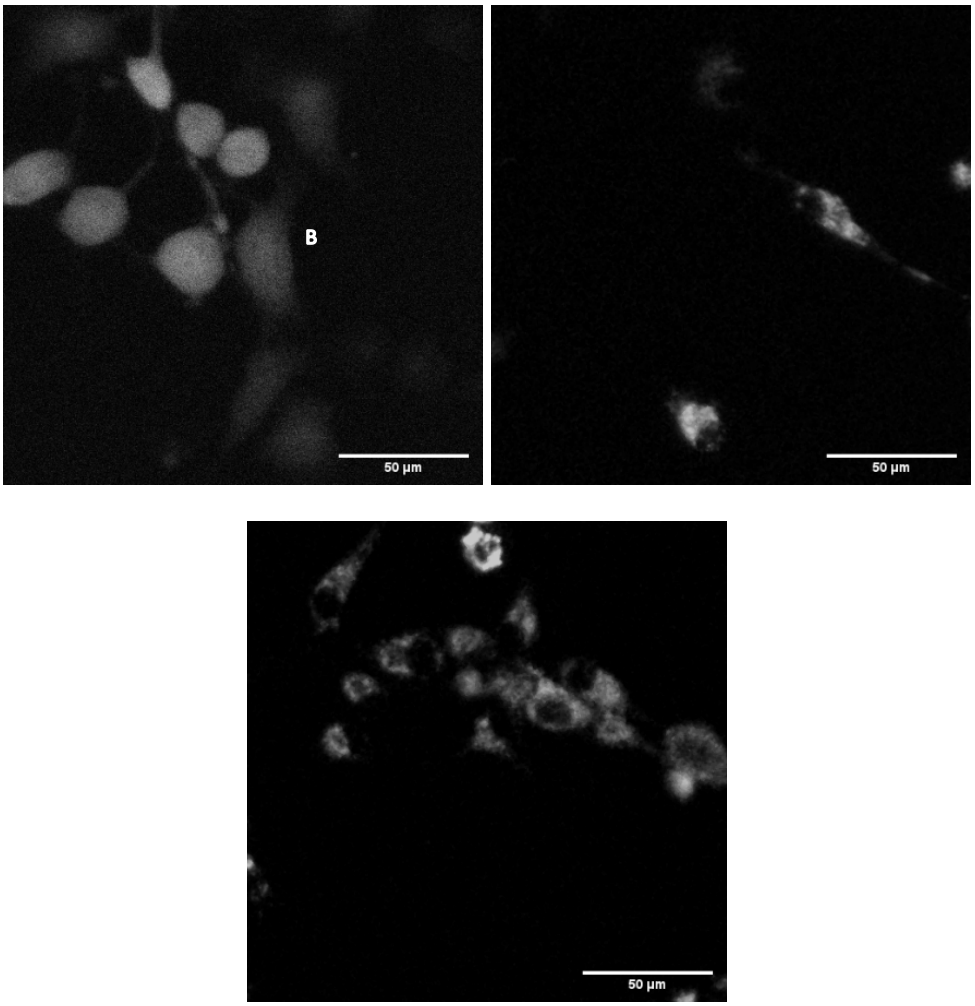

Fig. 5. Transduced Human Glioblastoma (U87) cells. (A) Control group AcGFP. (B) RvLEAM AcGFP. (C) MAHS AcGFP

Heat Shock

Preliminary stress tests began on our U87 cell line. Multiple tests were run in order to determine temperature and length of experiment. Initial tests determined 1-hour tests at 44° C. In order visualize cells that entered senescence, cells were fixed then stained with SA-βgal. Images were taken of each quadrant of a glass slide fixed with our tested cells. Senescent cells are stained blue while quiescent cells are not. Initial results can be seen in Fig. 4. Control group and experimental group for the control AcGFP demonstrates expected results of higher senescence in

the experimental. Visually MAHS-AcGFP and RvLEAM-AcGFP demonstrates much lower levels of blue staining compared to their control group and to the AcGFP control. Cell counts can be performed in order to find the number of senescent cells to quiescent cells.

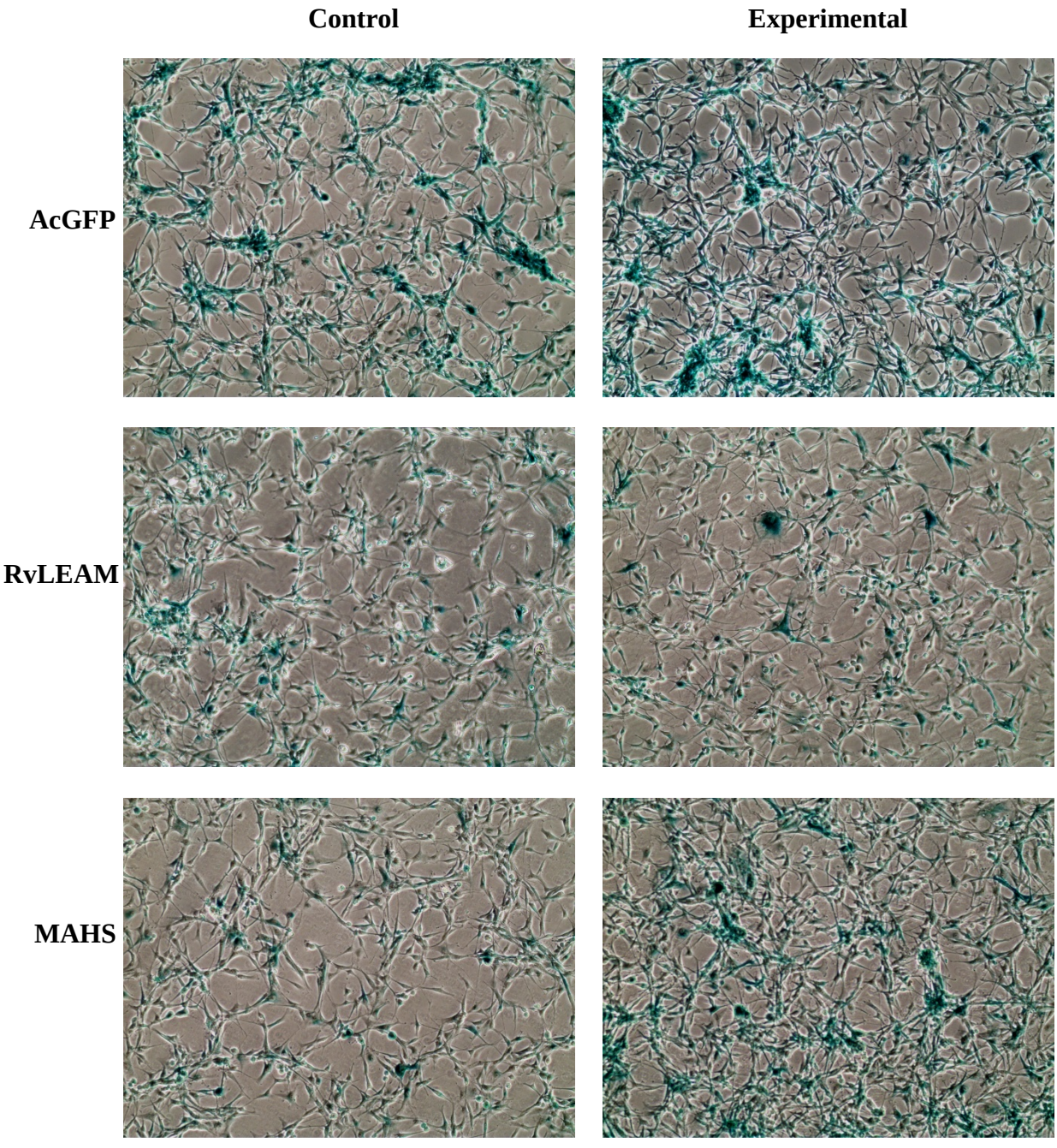

Fig. 6. Heat Shocked U87 cells stained with SA-βgal.

Organic Solvent Testing

Treatments were analyzed on a brightfield microscope post 24-hour recovery. Images of a ethanol treatment showed normal morphology amongst the cell population with minimal cell death in the 1 and 3% wells. All wells above 3% showed increased cell death and stressed morphology. U87 cells typically show elongated and attached morphology while U87 cells treated with high concentration solvents showed round and detached morphologies. U87s containing RvLEAM and MAHS, an increase in resistance can be observed at 3% ethanol. Imaging of transgenic cells could not be completed prior to shut down.

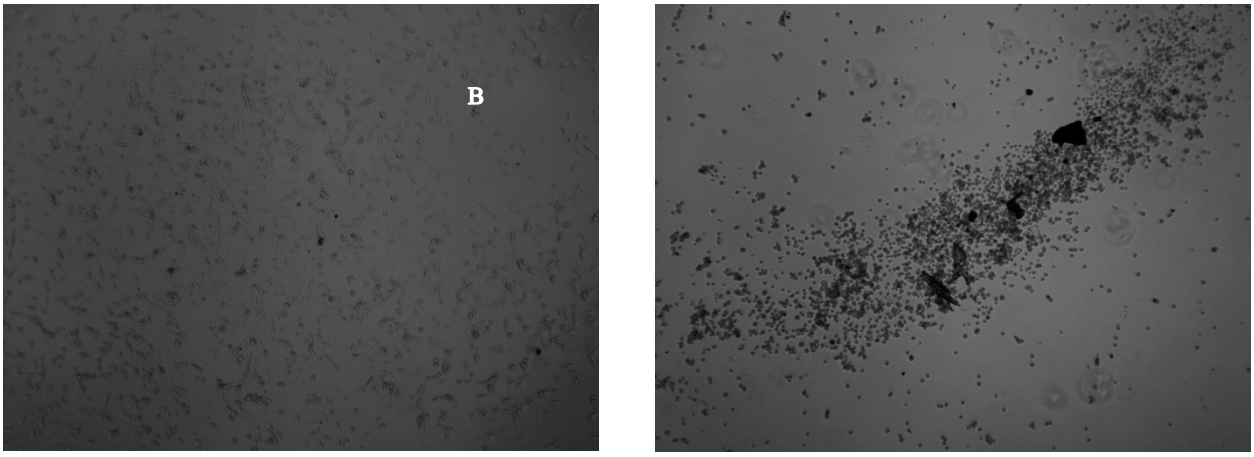

Fig. 7. Ethanol Treated U87-AcGFP Cells. (A) U87 cells in 3% ethanol media showing minimal cell death and normal morphology. (B) U87 cells in 7% ethanol media showing complete cell death.

Discussion

We have designed and produced original lentiviral vectors for the development of stable expressing mammalian cell lines. The development of these lentiviral vectors allows for retroviral transduction of the transgene into the host genome in a wide range of cell types. Previous plasmid constructs fuse the tardigrade gene sequences to the C-terminus of pAcGFP1-N1 vectors. This allows for MAHS and RvLEAM to express GFP in cell lines via transfection. However, transfection doesn't provide high efficacy incorporation into the genome required for the creation of stable cell lines. In this work, we provide an efficient way of producing stable cell lines for the expression of fluorescent tardigrade proteins.

Through the development of these lentiviral vectors, the creation of 3 stable mammalian cell lines was achieved. Transducing MDCKs, U87 glioblastomas, and human pancreatic cells were stabilized using a puromycin resistance selectable marker. This provides advantages such as testing under multiple morphologies and physiological properties. Currently, only one transient cell line has been produced with these proteins, Hep-2 cells. With the production of these cell lines, we can provide a lasting line for reproducible data.

Though nothing is conclusive, early testing via heat shock and solvent has demonstrated the possibility of enhanced stress tolerance in human U87 glioblastomas. This is an early visual marker showing the ability of MAHS and RvLEAM for mammalian cells. In order to conduct this test from beginning to end, the test took over 4 days. An experiment like this could not be replicated in transient cell lines as they could only express these genes for a maximum of 2-3 days. This demonstrates the importance of the designed cell lines as tests can be run for longer and can be run from the same population producing more accurate data.

With access to these cell lines, we can develop multiple stress tests in order to test the tolerance of the cell lines. Stressors can be applied such as UV exposure, osmotic testing, or even desiccation. Testing can be applied to individual cell lines containing only one transgene, however progression past these tests will include combining multiple individual tardigrade protein sequences into one large construct. This will allow us to find the tolerance and symbiotic functions between the multiple proteins. Lastly, future work will focus on scaling these proteins in larger functioning systems such as tissues.

In conclusion, we have shown the creation of lentiviral vectors that have allowed the development of 3 stable mammalian cell lines. Each cell line individually expresses the tardigrade proteins, RvLEAM and MAHS which are known to aid in stress tolerance of the mitochondria. Images show proteins localized to mitochondria, demonstrating the functionality of our lentiviral vectors. This has allowed for longer lasting experiment versus the commonly used transient cell lines, enabling future work to be done in complex tissues.

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