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The Role of Late Embryogenesis-Abundant Proteins in Desiccation-Induced Apoptosis

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by

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ABSTRACT

Encysted embryos of the brine shrimp *Artemia franciscana* survive extreme water loss by expressing the late embryogenesis abundant (LEA) protein AfLEA1.3 and the moss *Physcomitrella patens* expresses PpDHNA under conditions of dehydration. I investigated the impact of transgenic expression of PpDHNA and AfLEA1.3 on the metabolism of mammalian cells exposed to 0.5 M sucrose. The gene *PpDHNA* was transgenically expressed in human embryonic kidney (HEK) cells and *AfLEA1.3* was cloned into human hepatocellular carcinoma (HepG2) cells. Calorimetry was utilized to measure the metabolic activity of both cell lines. HEK cells showed a metabolic output of -24.1 ± 2.04 (SD) μW per million cells, independent of the presence of the transgene (HEK-PpDHNA: -22.9 ± 2.44 μW). Metabolic heat flow was reduced by 0.5 M sucrose in HEK and HEK-PpDHNA cells to -15.4 ± 2.13 μW and -17.9 ± 1.93 μW, respectively. The average heat flows in HepG2 under control and osmotic stress conditions were -27.2 ± 1.75 μW and -23.3 ± 1.13 μW, respectively. HepG2-AfLEA1.3 cells showed an average heat output of -35.1 ± 4.27 μW in control medium, while HepG2-AfLEA1.3 in sucrose medium was -24.03 ± 1.14 μW. The average output for HepG2-AfLEA1.3 cells in control medium after gene induction was -24.5 ± 1.86 μW, while heat flow after exposure to 0.5 M sucrose was -24.4 ± 1.65 μW. These results show that PpDHNA did not impact performance of HEK cells under osmotic stress, while AfLEA1.3 might play a role in preservation of cell metabolism under conditions of osmotic stress.

ACKNOWLEDGMENTS

I would like to thank Dr. Michael Menze for mentoring me during my research experience and allowing me to utilize his laboratory facilities to perform my work. The many valuable skills I have learned from him will continue to benefit me as I pursue further scientific knowledge in the future. I would also like to thank the Eastern Illinois University Honors College for the Undergraduate Research, Scholarship, and Creative Activity Grant, an award that partially funded my work during the fall 2013 semester. I would also like to thank the Eastern Illinois University College of Sciences for providing me with additional funding through a Scholar in Undergraduate Research (SURE) award during the spring 2014 Semester. Lastly, I would like to thank the entire faculty of the EIU Department of Biological Sciences for imparting upon me the knowledge necessary to make sense of my research and to apply it in further circumstances.

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INTRODUCTION

One of the most crucial compounds necessary for organisms to survive on Earth is water (Shih et. al., 2008). When organisms are severely dehydrated, their bodies and structures may be heavily damaged, which may ultimately lead to their death. However, some types of organisms can survive very low water levels (Mertens, et. al., 2007). Anhydrobiosis is the ability of some organisms to survive dehydration and subsequent rehydration. The term "desiccation tolerant" refers to organisms that can survive up to 99% water removal from their systems (Shih et. al., 2008). Organisms in this state often persist until they again obtain the water content necessary for the continuation of their vital metabolic processes.

Desiccation tolerance is seen in a variety of organisms (Alpert, 2005). There are four animal phyla that contain desiccation tolerant members: Rotifera, Arthropoda, Nematoda, and Tardigrada. Desiccation tolerance in arthropods appears to be limited to the larval stage of the African chironomid *P. vanderplanki* and the encysted embryonic stage of crustacean species in the *Artemia* genus (Alpert, 2005; Clegg, 2005). Desiccation tolerance is also seen in a variety of plant groups (Alpert, 2005). Bryophytes and the seeds and spores of pteridophytes and angiosperms commonly show desiccation tolerance. Several types of bacteria, algae, lichens, and yeasts also show desiccation tolerance (Alpert, 2005). Many of the organisms that demonstrate desiccation tolerance can survive severe water loss for several years (Alpert, 2006). How do such organisms persist under these very hostile conditions? Many desiccation-tolerant organisms reduce metabolic activity greatly during desiccation and a wide variety of tolerance levels are

seen in nature (Yobi et. al., 2012). Even in bryophytes of the same genus *Selaginella*, tolerance levels for desiccation vary greatly among species (Yobi et. al., 2012).

One molecular strategy of desiccation tolerance seen in eukaryotic organisms is the accumulation of highly hydrophilic macromolecules termed late embryogenesis abundant (LEA) proteins (Menze et. al., 2009). The term "late embryogenesis abundant" refers to the plant developmental period in which the proteins are abundantly expressed (Shih et. al., 2008). LEA proteins are common in the later stages of plant embryogenesis, or when a young plant develops within the seed body. In the later stages of embryogenesis, the majority of the water in orthodox (non-recalcitrant) plant seeds is lost (Shih et. al., 2008; Campos et. al., 2013). LEA proteins have been identified throughout many plant groups (Campos et. al., 2013). In addition, LEA proteins have been found in a variety of organisms that do not belong to plants, including bacteria, arthropods, and nematodes. Seven different groups of LEA proteins have been defined based on their amino acid sequences, which have been used to predict their biophysical characteristics such as being intrinsically unstructured in the hydrated state. All LEA proteins (Group 5 being the exception) are highly hydrophilic. LEA proteins accumulate in body tissues when organisms are exposed to adverse environmental conditions like water loss through freezing, evaporation, and osmotic or salt stress. The function of LEA proteins is still largely unknown. Some possible roles include preventing protein inactivation during dehydration, gathering free oxygen radicals, and acting as a hydration buffer. Group 1 LEA proteins isolated from wheat have been shown to prevent aggregation of proteins that are easily unfolded by dehydration. Group 1 proteins have been identified in a variety of organisms, including the brine shrimp *Artemia franciscana*. Group 2 proteins

have also been shown to act in dehydration and rehydration tolerance and are found in plants such as the moss *Physcomitrella patens* (Saavedra et. al., 2006).

The brine shrimp, *A. franciscana,* is a multicellular extremophile that can survive under many unfavorable environmental conditions (Clegg, 2005). Most notably, the encysted embryonic stage is extremely tolerant to harmful radiation and can survive anoxia for years at physiological temperatures and conditions where water is present. The cysts are also capable of surviving extreme levels of desiccation. *Artemia franciscana* is able to survive such extreme conditions by utilizing both sugar solutes (e.g. trehalose) and LEA proteins [Menze et. al., 2009]. High levels of the disaccharide trehalose have been shown to play a role in desiccation tolerance (Crowe, 2008). Trehalose has been shown to preserve protein structure and cellular integrity during desiccation. High trehalose levels have been seen in *A. franciscana* cysts. In these cysts, trehalose sugar glasses at low water content are likely stabilized by LEA proteins (Menze et. al., 2009; Hengherr et. al., 2011). A highly stable glassy state in the desiccated cytoplasm at ambient temperatures might allow brine shrimp cysts to endure extremely harsh conditions until water is again available (Clegg, 2005; Hand et. al., 2011).

In plants, LEA protein expression may be triggered by dehydration and subsequent abscisic acid production (Shinde et. al., 2012). One plant that utilizes LEA proteins during desiccation is the moss *P. patens* (Saavedra et. al., 2006). This moss belongs to a group of primitive land plants (Shinde et. al., 2012). Plants in this group inhabit ecosystems with widely varying environmental conditions throughout the year or even in a single day. Therefore, dehydration tolerance is especially important for these organisms as it helps them to survive in widely fluctuating environments. When pre-

treated with abscisic acid, *P. patens* experiences a decrease in electrolytes leaked from its tissues after dehydration and subsequent rehydration, which may be caused in part by the accumulation of Group 2 LEA proteins. Group 2 LEA proteins are also known as dehydrins and have been suggested to provide protection to proteins to preserve enzymatic activity under cold or dehydration, to gather free radicals, and to provide membrane stabilization (Saavedra et. al., 2006). The gene *PpDHNA* codes for a dehydrin (PpDHNA) in *P. patens* that has shown to be crucial in the moss to survive salt and osmotic stress (Saavedra et. al., 2006; Rubial et. al., 2012).

The Group 1 LEA protein investigated in this study was named AfLEA1.3 (NCBI ACX81198; Marunde, 2013). This protein is commonly expressed in diapause cysts from *A. franciscana* (Sharon et. al., 2009; Warner et. al., 2010). Group 1 LEA proteins have a unique protein structure (Campos et. al., 2013), are high in levels of glutamine and glycine, and lack tryptophan and cysteine residues. In addition, the proteins contain a specific 20-amino acid sequence repeated multiple times within the polypeptide chain. Marunde (2013) demonstrated that AfLEA1.3 cloned into *Drosophila melanogaster* Kc167 cells accumulates in the mitochondria. The amino acid sequence of AfLEA1.3 used to stably transfect HepG2 cells in the study presented here is identical to the sequenced used by Marunde (2013):

MELSSSKLNRSIFKRSSSVLHWHFYRLFGKKYPSKMSEQGKLSRQEAGQRGGQ
ARAEQLGHEGYVEMGRKGGQARAEQLGHEGYQEMGQKGGEARAEQLGTEGY
QEMGQKGGQKRAEQLGHEGYQEMGQKGGQTRAEQLGTEGYQEMGQEMGQT
RAEQLGHEGYVQMGKMGGEARKQQMSPEDYAAMGQKGGLARQK

Figure 1. Deduced polypeptide sequence for AfLEA1.3 (NCBI ACX81198) protein isolated from *A. franciscana.*

From *P. patens,* the protein PpDHNA was examined in this study. Dehydrin proteins (DHNs) are often found in in the cytoplasm and nucleus of plant cells, but have been found to interact with chloroplasts and mitochondria as well (Saavedra et. al., 2006). The protein PpDHNA contained high levels of glycine and low tryptophan and cysteine levels. An 11-amino acid sequence repeats multiple times throughout the entire protein structure. PpDHNA (NCBI AAR13080) is localized in the cytoplasm of the plant cells and accumulate near membranes during times of stress (Rubial et. al., 2012). In this study, the following amino acid sequence of PpDHNA was used:

MNQYGREQQDTGLVGSGTGHRD
EYGNPRQEGIMDKVKNAVGGMGPSSGTGYNNQPGYDNYGNPRQEGLVDKAK
DAVGMGPSLGTGYNNQPGYDSYNREGIVDRAKDAVGMGPNSGTGYNNQPGY
DNYGDRRHEGLADRAKDAVGMGPNSGYNHQPGYDNYGNREGVVDKAKDAV
GMGPNSGTGYNNQPGYDSYGTRRQEGLVDDRAKDAVGMGPNSGTGYNNQPG
YDNYGNPRREGVVDRAKDAVGMGPNSGYNNQPGYDNYGNREGIVDKAKDA
VGVGPHSGTGYHNQPSYDNYGNPRQEGIVDRAKDAVGMGPNSGTGYNNQSD
YDSYGNPRHEGMDKAKDDFDMGPNSGTGYNNRPGYDTYGDRKHEGIGDKVR
DAIGTGPNSGYDSRTPTGTDAYVHGNHPPGMQDRITGVNEPSILGGRENVDRH
GFGHDGRQHHGLLDNVTLQSGHIPETMVGGRRVEPGYDMTKSAGHHLTDLGH
HGNDSGVTGLGHHDTDYDERRGKGFEDPIDNKTGLGSDYDTTETGSGYGATD
TGAAPHKKGIITKIKEKL

Figure 2. Polypeptide sequence for PpDHNA protein (NCBI ACX81198) from *Physcomitrella patens.* The blue region denotes the leader sequence found in the GRP78 chaperone, while the orange sequence denotes the six histidine and KDEL sequences used for ER retention.

The protein sequence was modified to change the subcellular sorting pattern of the protein to the endoplasmic reticulum.

Protein functions are extremely dependent on the protein's specific folding pattern (Chakrabarti et. al., 2011). The folding pattern of proteins gives them a defined three-

dimensional shape that is critical to the protein's role within the cell (Sun & Jiang, 2011). Mammalian cells have defense mechanisms to protect against the adverse effects of miss-folded proteins such as the unfolded protein response, or UPR, of the endoplasmic reticulum. The endoplasmic reticulum (ER) is responsible for many cellular processes, including protein folding and assembly (Suh et. al., 2012). ER membrane-associated proteins act as stress sensors and are activated when miss-folded proteins accumulate in the ER lumen (Wang & Kaufman, 2012). When miss-folded proteins start to accumulate in the ER, reactions that target changes in protein-folding become active (Suh et. al., 2012). The UPR allows the ER to assess the integrity of proteins produced by the cell (Wang & Kaufman, 2012) and the role of this pathway is to restore normal cellular function by blocking translation of mRNA, preventing more proteins from being translated and activating signaling pathways to synthesize chaperones involved in protein folding (Suh et. al., 2012). However, the ER has a limit to the level of miss-folded proteins it can tolerate. In severe instances, external stress can lead the ER to induce apoptosis, or programmed cell death, via multiple signaling pathways in the UPR (Wang & Kaufman, 2012). In addition, apoptosis can also be signaled by processes that take place in the mitochondrion (Kong et. al., 2013). Mitochondria are key organelles involved in the intrinsic pathway of apoptosis by releasing cytochrome *c* (cyt *c*). Releasing cyt *c* leads to a signaling cascade that executes apoptosis.

Osmotic stress, as seen when cells are placed within a hypertonic or hyperosmotic solution, cause the cell size to decrease due to water loss. Solutions with osmolalities higher than found in the cytoplasm cause water to move out of the cell (Saito & Posas, 2012). The average osmolality in the cytoplasm of mammalian cells is around 270 mOsm

and cells placed within solutions of osmolalities above 380-400 mOsM/kg of water have shown to induce apoptosis (Christoph et. al., 2007). Hyperosmotic solutions and the associated water loss cause internal ion imbalances within affected eukaryotic cells, which can greatly impact many cellular functions (Pastor et. al., 2009). Proper mitochondrial function has been shown to be necessary to tolerate salt and osmotic stress. Yeast mitochondria have been shown to be sources of reactive oxygen species during osmotic stress that can lead to irreversible cellular damage. In addition, hypertonic solutions have been shown to affect ER function (Lee & Linstedt, 1999). Mammalian cells have developed biochemical pathways that assist them in surviving hyperosmotic conditions, however, such as the enzyme ALDH7A1 in human cells (Brocker et. al., 2010). When transgenically expressed in mice under normal physiological conditions, ALDH7A1 has been shown to accumulate in many tissues, including hepatocytes. The human kidney is vital to whole-body osmoregulation (Buchmaier et. al., 2013). As a result, renal cells have several mechanisms in place for handling osmotic stress, including ion transport and sugar accumulation.

Subcellular sorting of proteins is important for the maintenance of cellular homeostasis (Bexiga & Simpson, 2013). In our experiment, we directed PpDHNA and AfLEA1.3 proteins to different organelles within the cell. Human embryonic kidney cells (HEK) were stably transfected to express the gene for PpDHNA (HEK-PpDHNA), while the gene encoding for AfLEA1.3 was cloned into human hepatocellular carcinoma cells (HepG2). The gene for AfLEA1.3 naturally occurs in *A. franciscana* and the protein largely accumulates in the mitochondrion during the encysted life history stage (Warner et. al., 2010).

Cell metabolism can often be used as an indirect measure of cell viability and activity (Andreu et. al., 2012) and calorimetry has proven to be an effective method of examining the metabolic activity of a biological system (Vasconcelos et. al., 2010).

The purpose of our experiment was to investigate whether HEK cells expressing PpDHNA and HepG2 cells expressing AfLEA1.3 would withstand hyperosmotic-induced dehydration better than wild-type cells. Specifically, I hypothesized that transgenic cells will maintain higher rates of metabolic activity if challenged with high concentrations of solutes due to a reduction in the severity of apoptotic signaling. As chronic ER stress, brought on by severe dehydration due to hyperosmotic conditions, can lead to cell death, a decrease in UPR signaling may allow for higher cell survival rates under such conditions (Wang & Kaufman, 2012). In the presence of LEA proteins, the UPR may continue its role in preventing the aggregation of unfolded proteins under osmotic stress. This study may offer first indications if LEA proteins can be utilized to protect mammalian cells under adverse environmental conditions.

MATERIALS AND METHODS

1. Kyte-Doolittle Hydropathy Analysis

The hydrophobicity of the AfLEA1.3 and PpDHNA proteins was analyzed for a better understanding of their overall protein structures and how they may interact with other cellular structures. Hydrophobicity is determined by the chemical properties of the side chains of the amino acid residues found in the proteins' polypeptide sequence (Kyte & Doolittle, 1982). Hydrophilic residues will be on the protein exterior, while hydrophobic residues are likely to face the protein's interior. Kyte-Doolittle hydropathy plots attempt to provide structural information about proteins based on the hydrophobicity of their amino acid side chains and the subsequent location of the residues in the protein conformation. In Kyte-Doolittle analysis, hydrophilic residues will have more negative scores, of which the maximum is -4.6, while hydrophobic residues will have positive scores that maximize at 4.6.

2. Cell Culture

The genes *AfLEA1.3 and PpDHNA* were cloned into HEK and HepG2 (no transgenes present) cells using Tet-On 3G® Inducible Expression Systems (Clontech Laboratories, Inc., Mountain View, CA). In these systems, the Tet-On® 3G transactivator protein will bind to the P_{TRE3G} promoter in the presence of doxycycline, a synthetic tetracyclic analog. The gene for PpDHNA was genetically engineered to contain N-terminal and C-terminal amino acid sequences, however, that serve as sorting and retention signals for the ER. The leader sequence of the construct PpDHNA (blue) was generated through the addition of a 36 amino acid long sequence naturally found in the GRP78 chaperone. This chaperone is located in the ER, binds to hydrophobic portions of

amino acid chains, and is involved in signaling within the UPR (Cohen et. al., 2013). Whether or not the addition of this leader sequence leads to PpDHNA accumulation in the ER has yet to be determined. To the end of the amino acid sequence, a six histidine sequence was added for Western blot detection (green) and a "KDEL" sequence was added for ER retention (orange). H and KDEL sequences in mammalian cells have been proposed to aid in recycling proteins that reside in the ER when they move outside the organelle (Triguero et. al., 2005). Induction of the system with doxycycline will lead to transcription of the target gene.

HEK or HepG2 cells were plated using culture medium two to four days in advance of each experiment to ensure a yield of at least 1.5 million cells by the initiation of each experiment. Cells were incubated at 37 ºC, 93.5% O_2, and 6.5% CO_2 until use.

3. Solution Preparation

After incubation, the culture plate was removed from the incubator and the medium present on the plate was disposed. Then, 1.5 ml of the protease trypsin was added to the plate to detach the cells from the plate. Cell samples were exposed to trypsin for five minutes in the case of HEK cells and nine minutes for HepG2 cells. Next, the trypsin solution and 3.5 ml of medium were added to a clean centrifuge tube and centrifuged at low speed for 10.0 min to ensure complete separation of the cells from the trypsin-medium solution. The solution was poured off and the cells were re-suspended in five ml of OPTIMEM medium (Life Technologies, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) and PSA (solution of 10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B/ml). Then a small sample of the solution was used to obtain a cell count. A 20.0 µl sample of the cell solution was mixed with 20.0 µl

of Trypan Blue (Sigma-Aldrich, St. Louis, MO). A hemocytometer was used to obtain an accurate cell count of the cell solution. I conducted 3-4 cell counts per solution to ensure an accurate cell count. Once a cell count was obtained, the cells were diluted to target concentrations near 0.3 x 10^6 cells/ml (300,000 cells/ml) and 0.15 x 10^6 cells/ml (150,000 cells/ml). These concentrations were chosen to produce solutions with enough cells to produce a measurable heat output and to reduce metabolic inhibition through substrate-flux.

4. Calorimetry

A 2.5 ml portion of cell solution was added to a sterile stainless-steel ampoule and the ampoules were lowered into the Thermometric Thermal Activity Monitor (TAM) 2277 (TA Instruments, New Castle, DE), a highly-sensitive calorimeter, for analysis. Prior to use, the TAM was calibrated to monitor heat output signals within a range of -30 to +30 μW. Thermal activity within the ampoules was monitored until a stable baseline was observed. The heat output signal was recorded and used in further calculations. The ampoules were then removed from the TAM and sterilized for later use. This procedure was followed until five to six measurements were recorded for each cell line and condition. The same procedure used for HEK and HepG2 cells was applied to HEK-PpDHNA and HepG2-AfLEA1.3 cells under control and experimental conditions.

The TAM calorimeter measures heat release in J/s and converts the value to μW/s, a power signal visible on the computer monitor connected to the TAM. Therefore, heat release from the ampoules in which the samples are located is measured in μW. The heat release for each sample was taken at the first instance in the TAM trace at which the signal was stable for an extended period of time. Usually, this occurred one to two hours

after the ampoules had been lowered to the measuring position. The heat signals were recorded for each sample and standardized to a value of μW/million cells to allow for comparison among experiments, cell lines, and environmental conditions.

5. Osmotic Stress Analysis

To examine heat flow of HEK and HepG2 cells in sucrose-containing medium, a 0.5 M solution of sucrose (34.23 g/200 ml) in OPTIMEM™ supplemented with 5.5% FBS and PSA was used. A sterile filter was then used to sterilize the medium and heat flow of cell samples was measured as described above. The sucrose-containing medium acted as a hyperosmotic solution to induce desiccation within the cells.

6. LEA Protein Induction

To examine metabolic activity of HEK-PpDHNA and HepG2-AfLEA1.3 cells after induction of protein expression with doxycycline in both control and sucrose mediums. 1 nM of the antibiotic was added to the cells 24 h prior to calorimetry. Induction using doxycycline took place 24 h prior to the initiation of an experiment to ensure the expression of LEA proteins in the target cell line. Procedures for control and sucrose tests were the same as complementary experiments done using non-doxycycline exposed cells.

7. Statistical Analysis

Raw signals obtained from the calorimeter were analyzed to determine the relationship between total cell number and metabolic output. Standardized heat values (heat output in μW per million cells) were calculated and averaged for each cell line and condition. Two-way analyses of variance (ANOVA) tests were utilized to examine the

effect of sucrose on the metabolic output of each cell line and subsequent differences in the responses of each cell line to sucrose. In addition, a two-way ANOVA was used to compare the response of each cell line to sucrose to the presence or absence of LEA proteins in the cell lines. If significant differences were detected, *post-hoc* pairwise multiple comparisons were performed using the Student-Newman-Keuls Method.

RESULTS

Red fluorescence microscopy of HEK-PpDHNA cells 24 h after induction with doxycycline shows that the mRNA of PpDHNA is produced (Fig. 1). This observation provides good evidence that the PpDHNA protein is expressed within the HEK cells 24 h after induction. AfLEA1.3 was inserted into HepG2 cells using the same mechanism as used in HEK cells for PpDHNA and a similar result was observed providing good evidence that AfLEA1.3 mRNA is present in HepG2 cells 24 h after induction as well (data not shown).

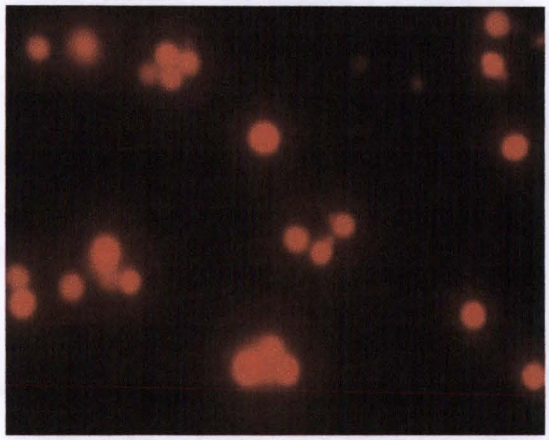

Figure 1. Human embryonic kidney cells (HEK-PpDHNA) showing red fluorescence after induction with doxycycline 24 h prior to an experiment.

To provide information about the subcellular sorting probabilities for the proteins AfLEA1.3 and PpDHNA, the WoLF PSORT program (http://wolfpsort.seq.cbrc.jp) was used. This program predicts the location of proteins within a cell based on the amino acid sequences of the proteins (Horton et. al., 2007). This program was used to elucidate how the proteins may distribute within mammalian cell lines, since neither protein originated from mammalian cells. The protein AfLEA1.3 was given a score of 24.0 for accumulation in the mitochondrion and a score of 5.0 for accumulation in the cytoplasm

(Table 1). Therefore, the protein is more likely to be located within the mitochondrion that the cytoplasm. In addition, PpDHNA, after the addition of the amino acid sequence found in GRP78, was given a score of 19.0 for accumulation in the extracellular space and a score of 11.0 for accumulation in the ER.

Table 1. A prediction of the subcellular sorting/location patterns of AfLEA1.3 and PpDHNA. The prediction, shown by a score based of the proteins' amino acid sequences, was calculated by the WoLF PSORT Subcellular Localization tool. The prediction was also based on the type of eukaryotic cell (animal or plant) that the protein is located in. AfLEA1.3 was predicted to be found in the mitochondrion and cytoplasm, while PpDHNA was predicted to be in the endoplasmic reticulum and extracellular space.

Protein	Mitochondrion	Cytoplasm	ER	Extracellular
AfLEA1.3	24.0	5.0	N/A	N/A
PpDHNA	N/A	N/A	11.0	19.0

The Kyte-Doolittle plot for AfLEA1.3 shows that the majority of the residues have a negative score, though none approaches the maximum score for a hydrophilic residue (Fig. 2). Likewise, the plot for the altered PpDHNA protein also has negative scores for the majority of its residues, with the exception being a region at the beginning of the plot that is positively-scoring. This region correlates to the leader sequence obtained from the GRP78 chaperone. The scores for PpDHNA residues are, overall, more negative than those seen in AfLEA1.3 (Fig. 3).

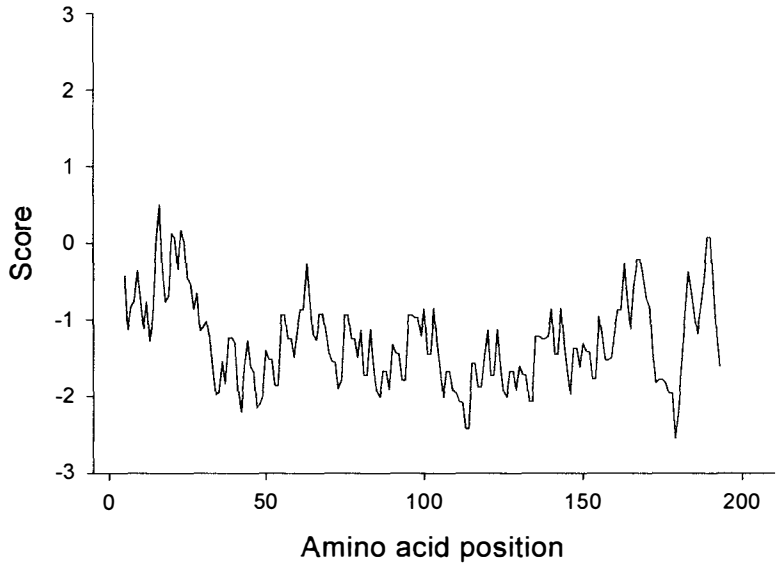

Figure 2. Kyte-Doolittle hydropathy plot for AfLEA1.3. Scores below zero for the majority of the amino acid residues indicate a highly hydrophilic protein.

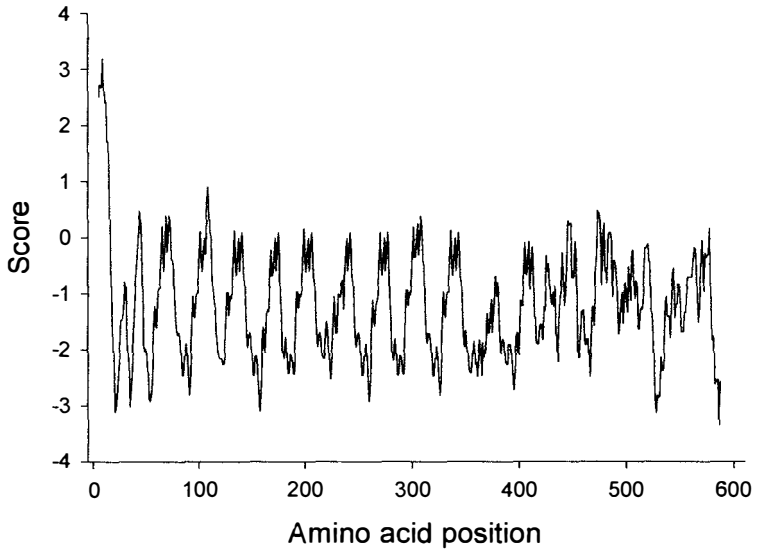

Figure 3. Kyte-Doolittle hydropathy plot for PpDHNA protein. Negative values are seen for the majority of the residues. The highly positive values at the beginning of the amino acid chain belong to the leader sequence obtained from the GRP78 chaperone.

Error in the experimental procedure led to higher and lower cell concentrations than our target concentrations of 0.3 x 10^6 cells/ml and 0.15 x 10^6 cells/ml. The raw signal, or the signal taken immediately from the TAM trace on the computer monitor, was used to examine the relationship between total cell number within each ampoule and the raw heat release of the sample. In each cell line/environmental condition combination, increasing cell number was correlated with increasing heat dissipation. Few outliers and slight variations were seen in this pattern, but this observation held constant during most experiments (Figs. 4-15).

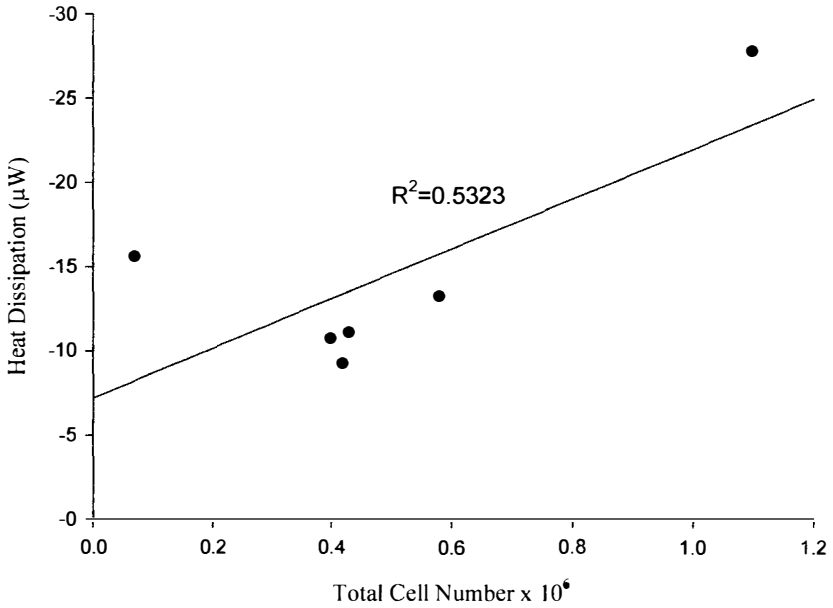

Figure 4. The metabolic heat output (μW) of HEK cells measured under control conditions.

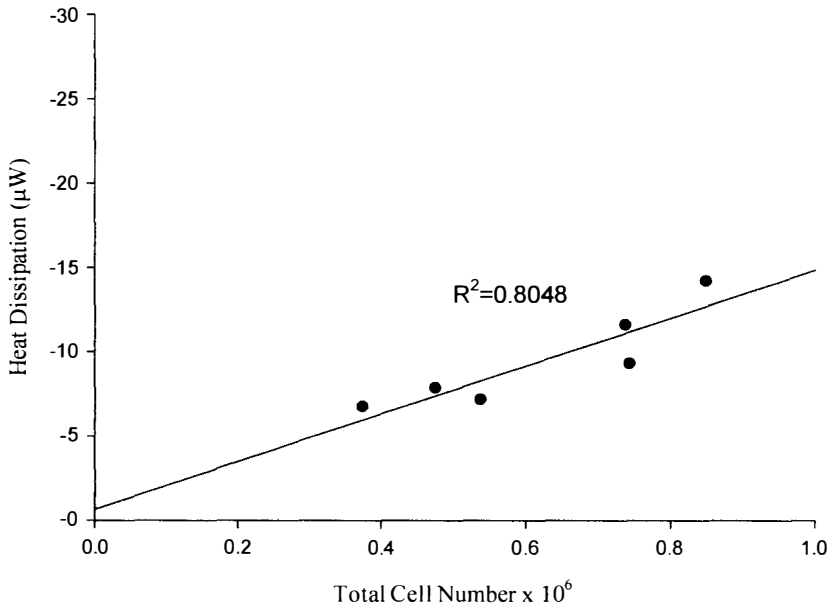

Figure 5. The metabolic heat output (μW) of HEK cells measured under osmotic stress conditions.

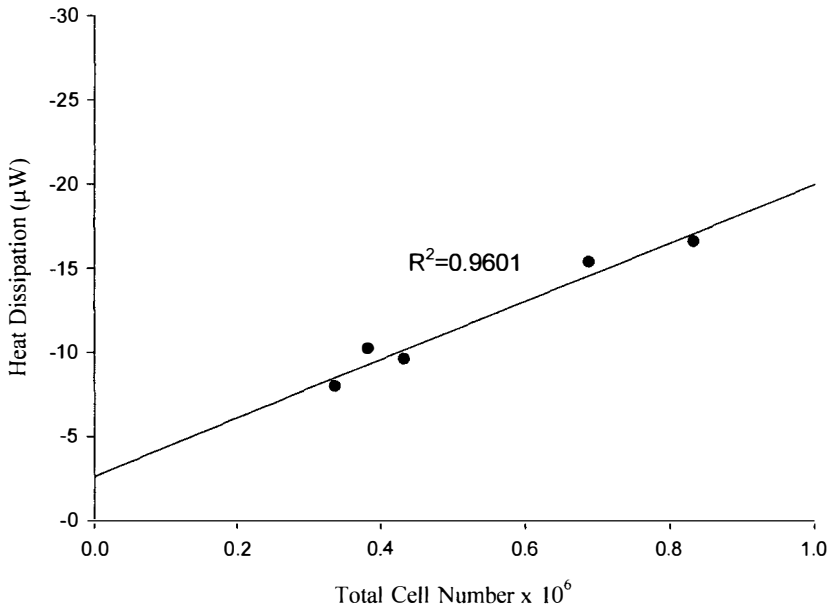

Figure 6. The metabolic heat output (μW) of HEK-PpDHNA cells measured under control conditions.

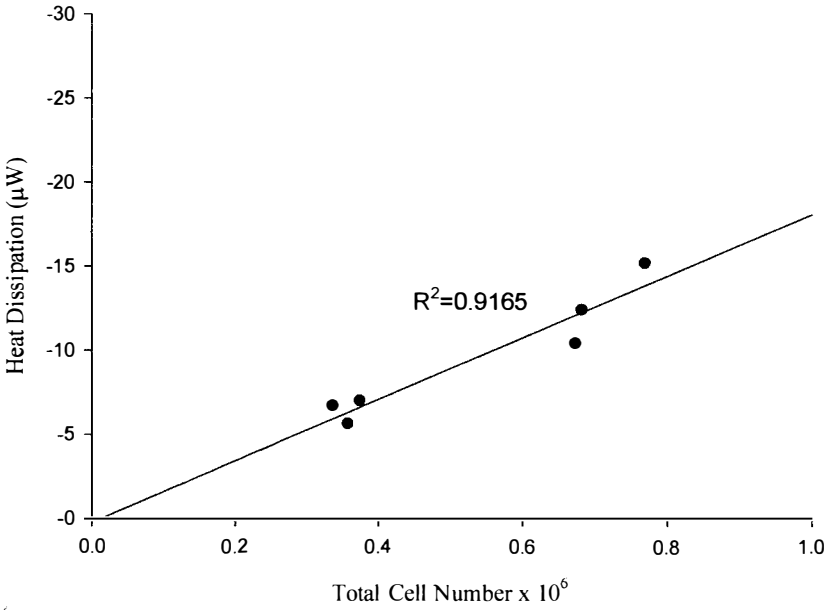

Figure 7. The metabolic heat output (µW) of HEK-PpDHNA measured under osmotic stress conditions.

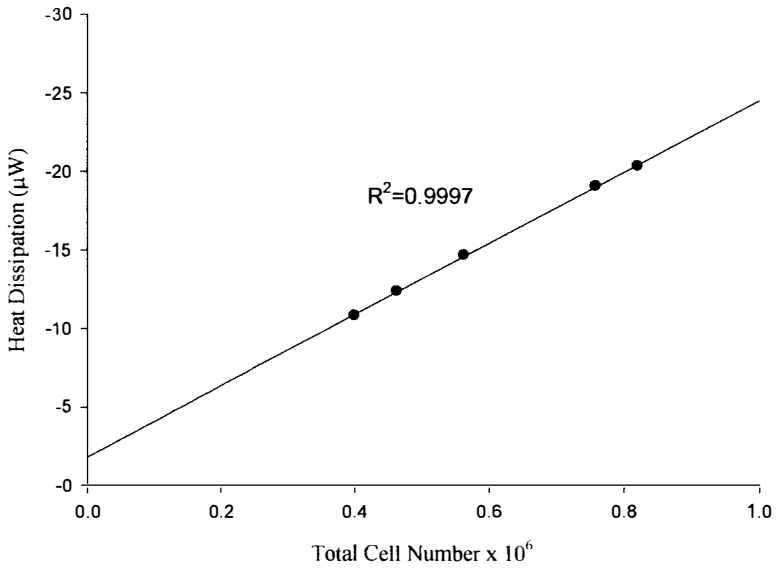

Figure 8. The metabolic heat output (µW) of HEK-PpDHNA cells after induction with doxycycline 24 h prior measured under control conditions.

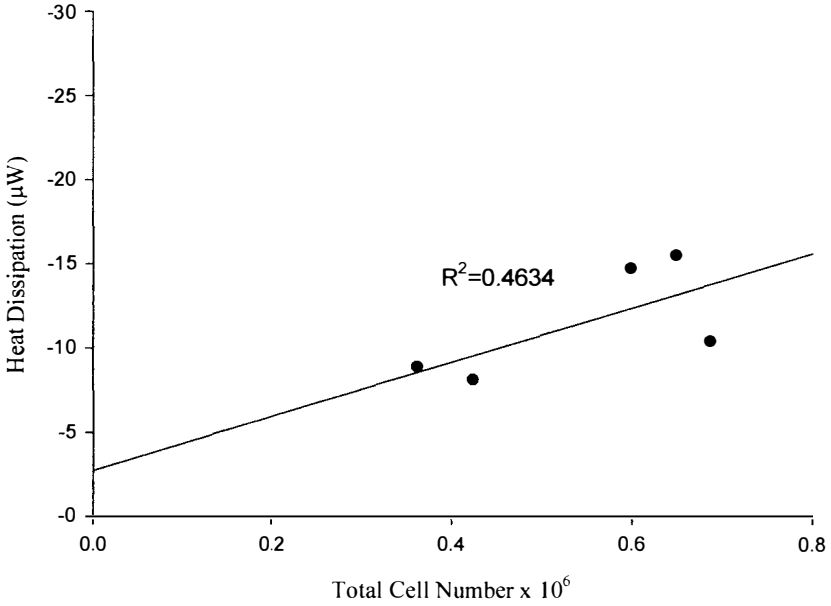

Figure 9. The metabolic heat output (μW) of HEK-PpDHNA cells after induction with doxycycline 24 h prior measured under osmotic stress conditions.

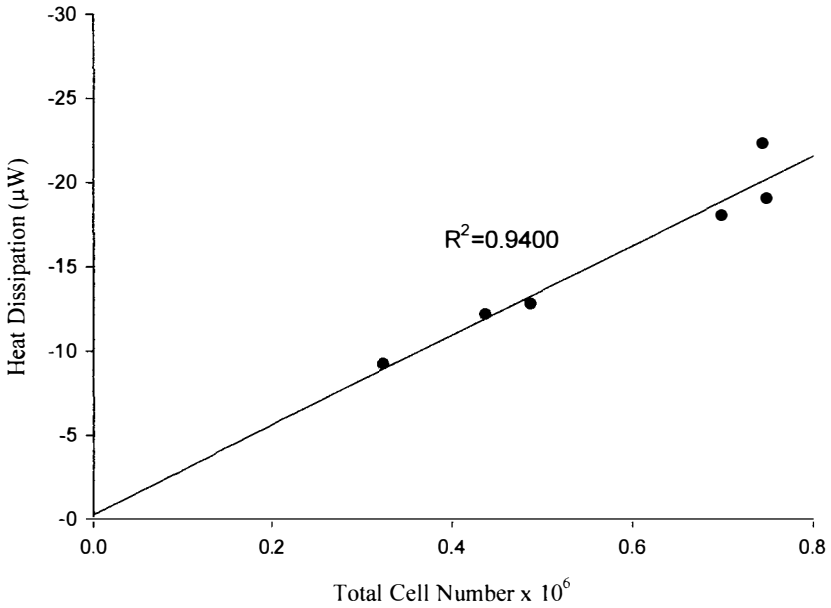

Figure 10. The metabolic heat output (μW) of HepG2 cells measured under control conditions.

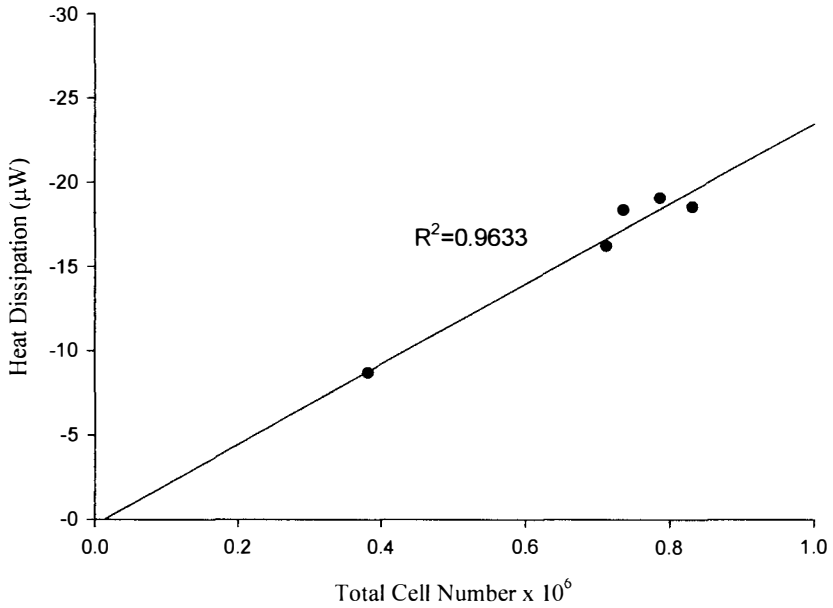

Figure 11. The metabolic heat output (μW) of HepG2 cells measured under osmotic stress conditions.

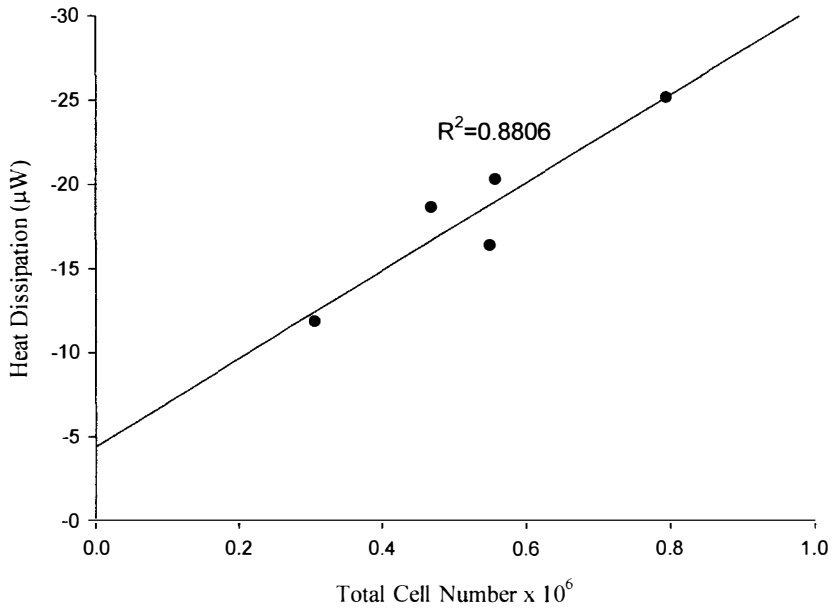

Figure 12. The metabolic output (μW) of HepG2-AfLEA1.3 cells measured under control conditions.

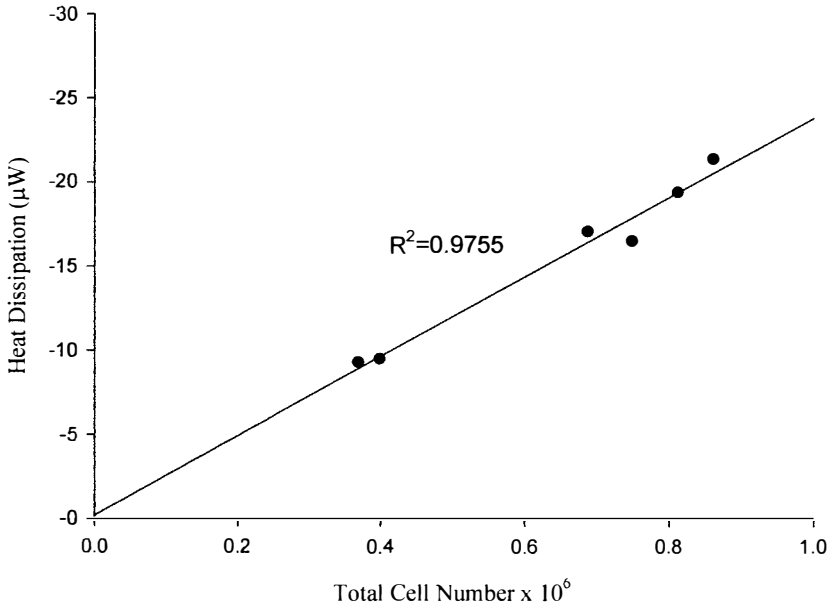

Figure 13. The metabolic output (μW) of HepG2-AfLEA1.3 cells measured under osmotic stress conditions.

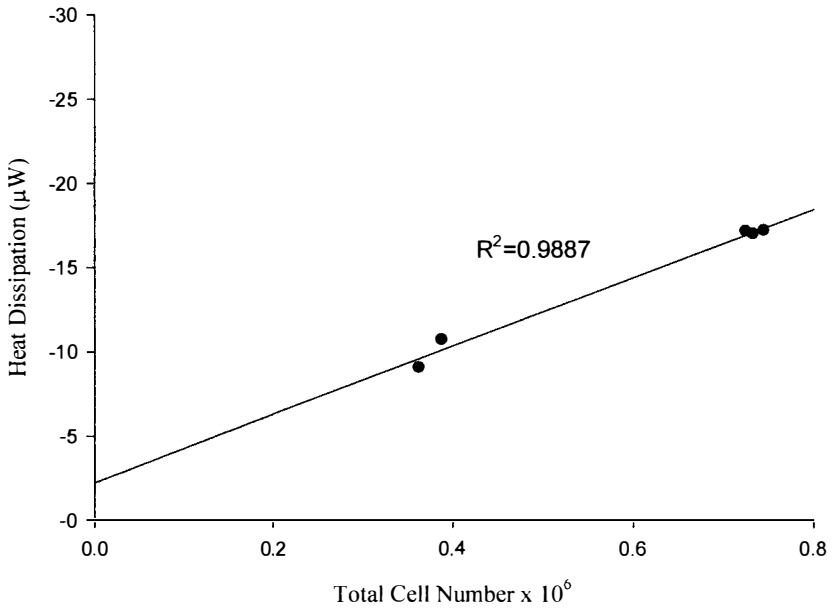

Figure 14. The metabolic heat output (μW) of HepG2-AfLEA1.3 cells after induction with doxycycline 24 h prior measured under control conditions.

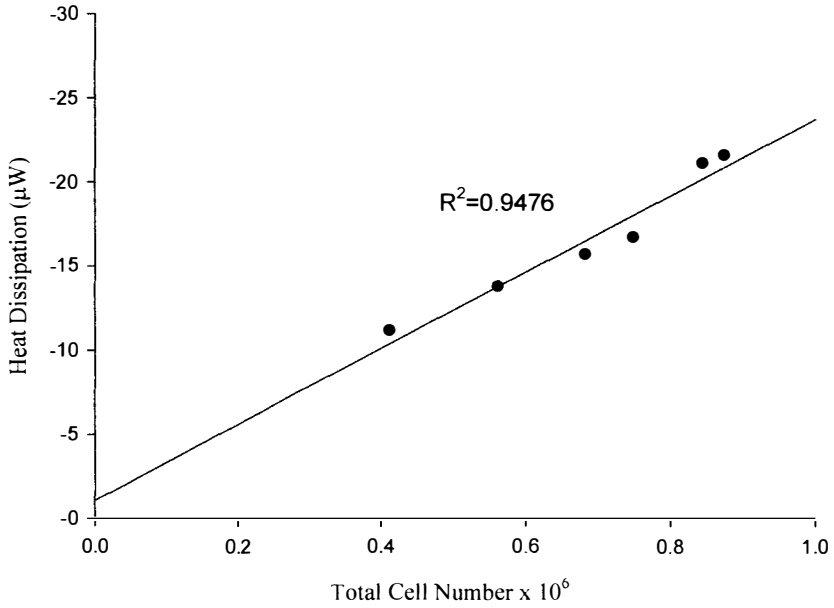

Figure 15. The metabolic heat output (μW) of HepG2-AfLEA1.3 cells after induction with doxycycline 24 h prior measured under osmotic stress conditions.

The standardized heat release (μW/million cells) values for each measurement were used to determine average metabolic output values for each cell line under experimental and control conditions. The heat output for HEK cells in control medium was -24.1 ± 2.04 (SD) μW per million cells (*n*=6), while the output for HEK-PpDHNA cells, without doxycycline treatment, in control medium was measured to be -22.9 ± 2.44 μW (*n*=6; Fig. 16). The output for HEK cells in sucrose medium was -15.4 ± 2.13 μW (*n*=6), while the average for HEK-PpDHNA without doxycycline treatment was -17.9 ± 1.93 μW (*n*=6). Both HEK and HEK-PpDHNA (without doxycycline treatment) had greatly reduced average metabolic capacities after treatment with sucrose medium when compared to their output values in control medium ($p < 0.05$). The metabolic outputs of HEK and HEK-PpDHNA (without doxycycline treatment) were similar under control or osmotic stress conditions ($p>0.05$) (Fig. 16).

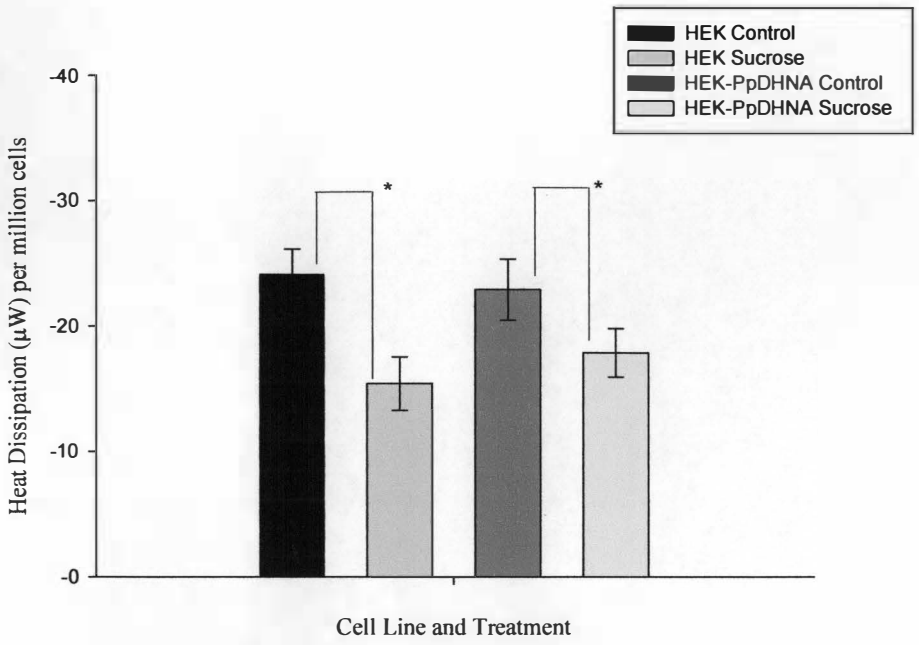

Figure 16. The average metabolic output (μW) per million cells of HEK and HEK-PpDHNA in control and respond to 0.5 M sucrose. Each bar represents mean ± SD of 5-6 separate measurements.

The average metabolic output for HEK cells under control and osmotic stress conditions were also compared to the average values seen in HEK-PpDHNA after treatment with doxycycline in control and sucrose medium (Fig. 17). The average outputs for HEK-PpDHNA after doxycycline treatment in control and sucrose mediums were -26.0 ± 0.971 μW per million cells (n=5) and -20.3 ± 4.67 μW (n=5), respectively. Sucrose treatment caused a decrease in metabolic activity in both cell lines ($p < 0.05$). The metabolic outputs of HEK and HEK-PpDHNA cells were similar under control conditions ($p > 0.05$), but a difference was seen between the cell lines under osmotic stress conditions ($p < 0.05$). HEK-PpDHNA cells prior to doxycycline treatment, when compared to cells after treatment, showed no significant difference between metabolic outputs under control or osmotic stress conditions ($p > 0.05$; Fig. 17).

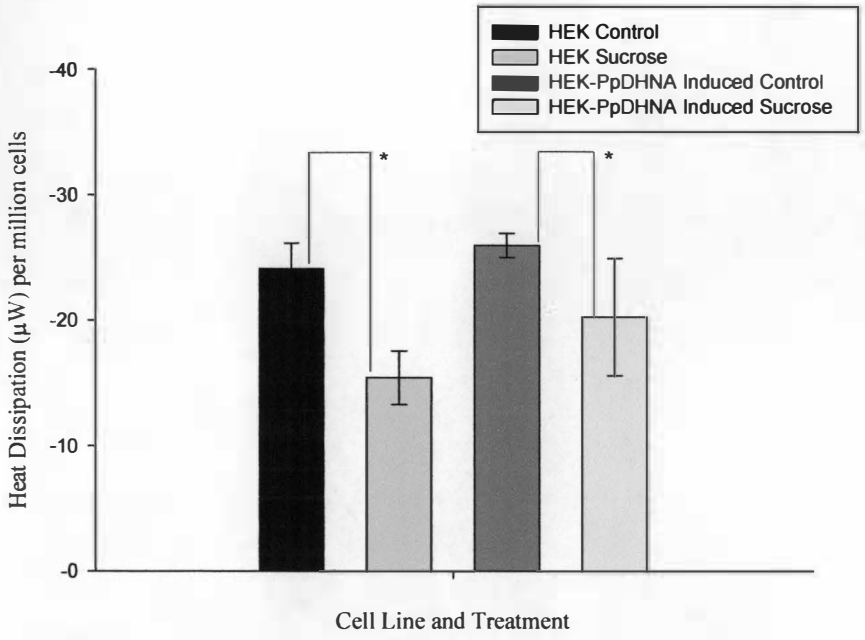

Figure 17. The average metabolic output (μW) per million cells of HEK and HEK-PpDHNA cells after exposure to doxycycline 24 h prior to experimentation in control and 0.5 M sucrose. Each bar represents mean ± SD of 5-6 separate measurements.

The average heat flows in HepG2 under control and osmotic stress conditions were -27.2 ± 1.75 μW (*n*=6) and -23.3 ± 1.13 μW (*n*=5), respectively (Fig. 18). HepG2-AfLEA1.3 cells, prior to exposure to doxycycline, showed an average output of -35.1 ± 4.27 μW (*n*=5) in control medium and in sucrose medium had an average output of -24.03 ± 1.14 μW (*n*=6). The reduction in average heat release seen for both HepG2 and HepG2-AfLEA1.3 prior to doxycycline exposure in sucrose medium was significant ($p < 0.05$). No significant difference was seen between the metabolic outputs of HepG2 and HepG2-AfLEA1.3 prior to doxycycline treatment under both osmotic stress conditions ($p > 0.05$), but a significant difference was seen between the cell lines under control conditions ($p < 0.05$; Fig. 18).

HepG2 cells were also compared to HepG2-AfLEA1.3 cells after induction with doxycycline (Fig. 19). The average output for HepG2-AfLEA1.3 control medium after induction was -24.5 ± 1.86 μW (n=5) and the average in sucrose medium after induction was -24.4 ± 1.65 μW (n=6). Again, the reduction seen in the output of HepG2 cells when placed in sucrose medium was significant ($p < 0.05$). However, the reduction seen in the output of HepG2-AfLEA1.3 cells after induction with doxycycline was not significant ($p > 0.05$). The metabolic outputs of HepG2 and HepG2-AfLEA1.3 under control and osmotic stress conditions were similar after doxycycline treatment ($p > 0.05$). The metabolic outputs of HepG2-AfLEA1.3 prior to doxycycline treatment and after treatment under control conditions were different ($p < 0.05$), but no differences were observed between the cell lines under osmotic stress ($p > 0.05$; Fig. 19).

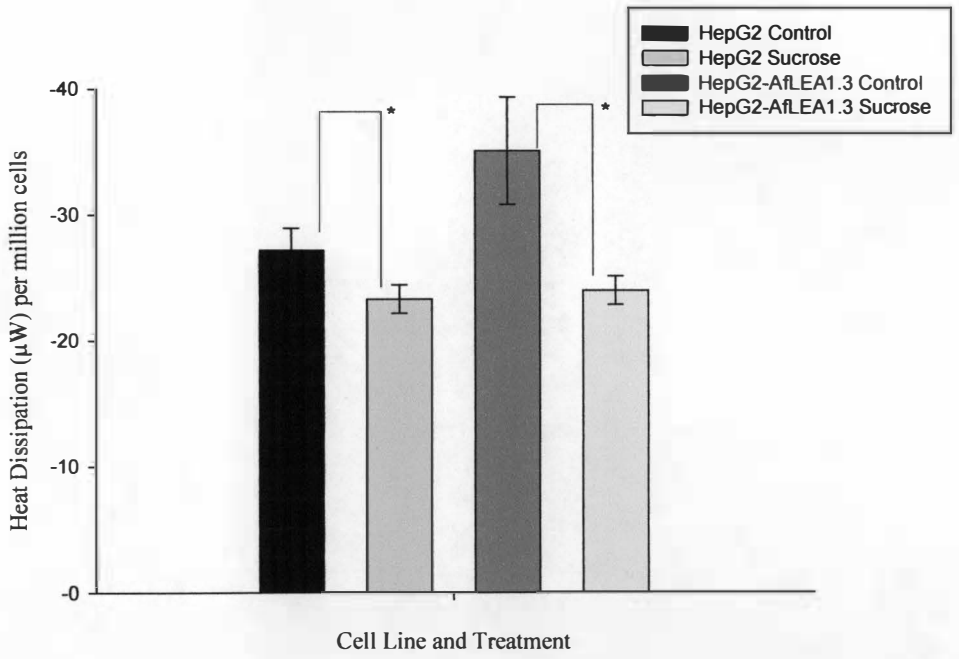

Figure 18. The average metabolic output (μW) per million cells of HepG2 and HepG2-AfLEA1.3 in control and 0.5 M sucrose conditions. Each bar represents mean ± SD of 5-6 separate measurements.

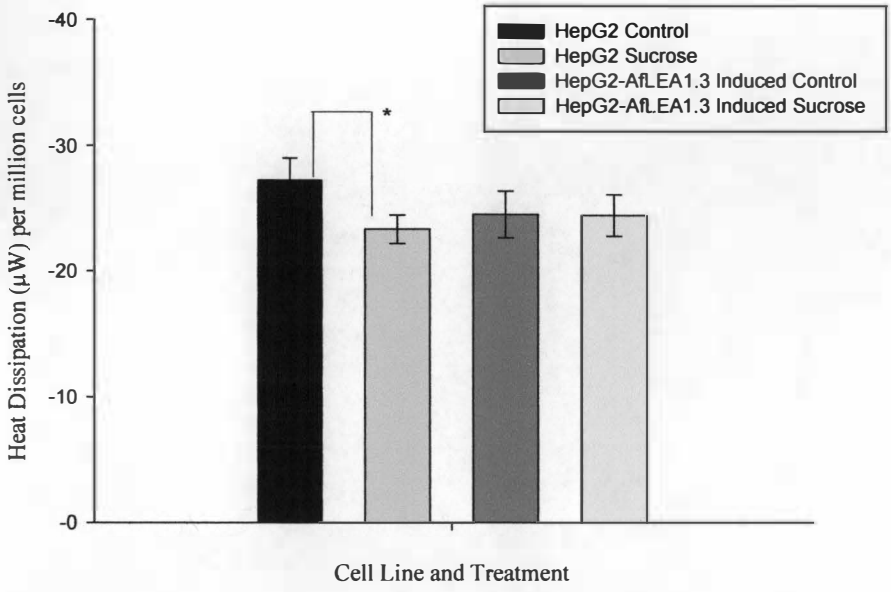

Figure 19. The average metabolic output (μW) per million cells of HepG2 and HepG2-AfLEA1.3 after exposure to doxycycline 24 h prior to experimentation in control and 0.5 M sucrose. Each bar represents mean ± SD of 5-6 separate measurements.

DISCUSSION

Metabolic activity has been demonstrated to be partially dependent on the surrounding environment of an organism (Klein et. al., 2012). In addition, calorimetric measure of heat release has shown to be a useful way to monitor an organism's metabolic capacity (Vasconcelos et. al., 2010). I used calorimetry to examine the metabolic capacity of wild type and genetically modified mammalian cell lines under control conditions and in response to hyperosmotic stress in order to determine if LEA proteins could preserve a cell line's metabolic activity during osmotic water loss. This pattern was observed in only one cell line: HepG2-AfLEA1.3 after doxycycline exposure.

The data generated by using the WoLF PSORT program indicates that AfLEA1.3 and PpDHNA would accumulate in the organelles we sought to direct them towards utilizing the cellular pathway of protein sorting. The estimates shown in the subcellular localization chart showed that AfLEA1.3 was nearly five times more likely to be incorporated into the mitochondrion than to remain in the cytoplasm and indications for sorting to other organelles were obtained. Likewise, PpDHNA had high scores for both the ER and the extracellular space. Despite the high score for the extracellular space, this data shows strong evidence that the protein is localized to the ER. Proteins sent to the extracellular space are always synthesized by the ribosomes associated with the rough ER (Boot-Handford & Briggs, 2009). The proteins the undergo modifications within the ER during the subcellular localization process. After the ER finishes its alterations, the proteins are sent to the Golgi apparatus and packaged into secretory vesicles before being secreted outside the cell (Giardina et. al., 2014). In addition, the UPR of the ER affects extracellular matrix functioning, despite being a defense mechanism for miss-folded

protein accumulation in the ER (Bateman et. al., 2009). Therefore, there is a strong correlation in protein properties for proteins that remain in the ER and those sent to the extracellular space.

The Kyte-Doolittle hydropathy plots may provide some indication of the role these proteins could play within the cells in preserving their metabolic function. Based on the amino acid sequences, both AfLEA1.3 and PpDHNA were overwhelmingly hydrophilic proteins, meaning that they will be attracted to water. If these proteins are able to interact with residual water present after dehydration, they could assist the cell in preventing complete water loss. Group 1 LEA proteins, like AfLEA1.3 isolated from wheat have been demonstrated to prevent the aggregation of proteins that are easily altered by dehydration in organisms that express them, while Group 2 proteins, like PpDHNA are suggested to have many potential preservative properties, including protection of enzymatic activity under dehydration and membrane stabilization (Campos et. al., 2013; Saavedra et. al., 2006). However, further research is needed to elucidate the exact function of both proteins and how they could interact with proteins and lipids in mammalian cells.

The linear relationship between the total cell number of each sample and the overall metabolic output shows a high level of reproducibility. In increasing the number of cells within a sample, the overall metabolic output of that sample also increased. This result being seen in all cell lines and under all experimental conditions lends credibility and accuracy to the experimental procedure. While the standard deviation is large within some experimental conditions, a sufficiently high sample size allowed for a statistically meaningful evaluation of my data. The largest experimental error often came from

mistakes made in the cell enumeration process. Oftentimes, inaccurate cell counts led to uncharacteristic heat signals from the calorimeter and unusable data. More precise cell counting techniques would also decrease the standard deviation seen in the measurements.

Overall, the average metabolic output of the HEK and HEK-PpDHNA (before and after doxycycline treatment) cells decreased when placed into sucrose medium. The average heat output of HEK cell samples in control medium decreased 36.1% compared to those cells in a sucrose medium. The average heat outputs of HEK-PpDHNA without doxycycline treatment decreased 21.8% when compared to osmotic stress conditions. After doxycycline treatment the average output of HEK-PpDHNA in control conditions decreased 20.9% when compared to those cells in osmotic stress. Although the percentage decreases were different for each cell line, each decrease was significant. Thus cells that experienced the sucrose treatment had decreased in metabolic activity, and likely, viability. This may be, in part, due to the initiation of apoptosis. The hyperosmotic sucrose solution places osmotic stress on the cells and their endoplasmic reticula in particular (Alves, et. al., 2011). In turn, stress on the ER can lead to an aggregation of unfolded proteins, overwhelming the ER and decreasing its functionality (Bicknell et. al., 2010). The unfolded protein response (UPR) might then be activated in attempt to refold the proteins (Chakrabarti et. al., 2011). If the UPR is unable to correct the problem, apoptosis may be initiated. Osmotic stress may also cause mitochondrial-dependent apoptosis pathways to be initiated (Bortner et. al., 2012). Another possible explanation is that dehydration leads to a decrease in metabolic activity without causing cellular death. A study of desiccation (severe dehydration) in wheat seedling shoots showed a metabolic

decrease within the shoots due to multiple factors, including decreased ATP levels within the organisms (Corbineau et. al., 2004). Further study is needed to examine the exact mechanism that leads to the decreased metabolic capacities seen in the cells.

The HepG2 and HepG2-AfLEA1.3 cell lines a 14.3% decrease in metabolic output compared to those cells in sucrose medium. HepG2-AfLEA1.3 prior to doxycycline exposure in control medium showed a 31.5% decrease in metabolic output compared to those cells in sucrose medium. In contrast, the metabolic outputs of HepG2-AfLEA1.3 after 24 h of doxycycline exposure were similar in control and osmotic stress conditions. The metabolic decrease seen in HepG2 and HepG2-AfLEA1.3 cells prior to doxycycline treatment during exposure to sucrose medium may be caused by the same mechanisms that are possible causes for the decrease seen in HEK and HEK-PpDHNA cells.

The presence of PpDHNA proteins in HEK cells did not increase the cells' ability to survive osmotic stress as indicated by heat dissipation. The metabolic outputs of HEK, HEK-PpDHNA without doxycycline treatment, and HEK-PpDHNA after 24 h of doxycycline exposure in control medium were similar. There was a similar decrease in the HEK and HEK-PpDHNA cell lines prior to doxycycline treatment when exposed to sucrose medium. Interestingly, the response of HEK-PpDHNA after doxycycline treatment under osmotic stress conditions compared to the response of HEK cells under osmotic stress was different, a result that contrasts to that seen for other HEK cell lines. This result indicates that HEK and HEK-PpDHNA after doxycycline treatment had a different response to the presence of sucrose. However, a reduction in metabolic output between control and osmotic stress conditions was still seen in both cell lines. Although

the response to osmotic stress between cell lines was different, there was a marked reduction in heat release within the cell lines. Therefore, the presence of PpDHNA in the HEK-PpDHNA induced cells was still seen as detrimental. The protein PpDHNA is a Group 2 LEA protein, suggested to improve cellular performance under cold stress or dehydration (Saavedra et. al., 2006). In addition, PpDHNA presence has shown to increase salt and osmotic stress tolerance in *P. patens* (Saavedra et. al., 2006, Rubial et. al., 2012). The results of the HEK and HEK-PpDHNA study might indicate that mammalian kidney cells are not able to utilize PpDHNA proteins to improve osmotic stress tolerance. Further study is needed to investigate what mechanism/process is present in bryophyte cells, but absent in mammalian kidney cells, that allows *P. patens* to utilize PpDHNA proteins in survival of such adverse conditions.

Much of the data observed for HepG2 and HepG2-AfLEA1.3 cells shows similar features to the data measured for HEK and HEK-PpDHNA cells. However, some significant differences were observed between both cell lines. The metabolic output of HepG2-AfLEA1.3 cells without doxycycline treatment in control medium was larger than the HepG2 cells. In addition, HepG2-AfLEA1.3 prior to doxycycline treatment had a higher average metabolic output than HepG2-AfLEA1.3 after doxycycline treatment in control medium as well. The data indicates that the mere presence of the transgene for the AfLEA1.3 protein provides a metabolic benefit to the HepG2 cells, allowing them to increase their metabolic activity under control conditions. I believe, however, that the larger metabolic output can be attributed to experimental error in selecting the experimental data used to calculate the metabolic output for that cell line. This conclusion seems to hold more validity because the average metabolic output of HepG2-AfLEA1.3

cells after doxycycline treatment is much closer in value to that of wild-type HepG2 cells. It is unlikely that LEA protein expression under control conditions would lead to a reduction in metabolic capacity, leading to the greater value seen before protein expression is induced most likely being experimental error.

Under osmotic stress conditions, HepG2, HepG2-AfLEA1.3 prior to doxycycline exposure, and HepG2-AfLEA1.3 after doxycycline treatment had similar metabolic outputs. This does not necessarily indicate, however, that exposure to a hyperosmotic solution impacted each cell line in the same manner. While there is a decrease in the metabolic output of each cell line in response to 0.5 M sucrose, the decrease occurs to a lower extent in the HepG2-AfLEA1.3 cells after AfLEA1.3 proteins are induced compared to un-induced cells. Recall that the metabolic output of HepG2-AfLEA1.3 cells after doxycycline treatment was similar to the value seen when the cell line is exposed to the sucrose medium. Further experimentation, including repeat trials of our experimental procedure, is needed to determine whether or not AfLEA1.3 allows for HepG2 cells to preserve their metabolic function under osmotic stress conditions.

The two-way ANOVA test found a different response to sucrose between HEK cell lines and HepG2 cell lines. Recall that each cell line, with the exception of HepG2-AfLEA1.3 induced, showed a reduction in metabolic heat output from control to osmotic stress conditions. Also, corresponding cell lines responded in the same manner, with the exception of HepG2 cell lines to control conditions and HEK versus HEK-PpDHNA after induction under osmotic stress conditions, to similar conditions. However, the data shows a difference in the overall response of HEK and HepG2 cell lines to the presence of sucrose. This result indicates that mammalian cells of different origins will respond

differently to osmotic stress. Additionally, cells of different origins could respond differently to LEA protein presence. This trend is confirmed by HEK-PpDHNA induced not being affected by PpDHNA presence under osmotic stress, but HepG2-AfLEA1.3 induced responding to AfLEA1.3 presence under osmotic stress. Therefore, cell origin will need to be considered in further research involving mammalian cell response to LEA protein presence.

Future work on this system will include completing apoptosis assays to determine if cell death is occurring in the ampoules in response to the osmotic stress. This should determine if the decrease in metabolic output of a cell sample correlates to cell death or metabolic reduction. In addition, Western blotting assays are needed to confirm LEA protein expression within the studied cell lines. Another possible direction for future research is determining the highest level of osmotic stress, in the form of a dose-response curve for sucrose. The same experimental procedures can be employed to ensure exact data is obtained and the results can be compared to those of this study. Further research will be needed before the results of this study can be used in understanding improved dehydration tolerance in human cells.

Overall, I discovered that increasing the total cell number within the examined cell solutions of HEK and HepG2 cells led to a distinct increase in the heat flow for the sample. I also witnessed a in the metabolic activity of both HEK and HepG2 cells in response to 0.5 M sucrose, indicating that osmotic stress directly impacts the metabolic activity of the cells. The LEA protein PpDHNA did not improve the metabolic activity of HEK cells under osmotic stress conditions, but the protein AfLEA1.3 preserved the metabolic function of HepG2 cells under osmotic stress.

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