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A PROPOSED STUDY TO EXPLORE THE RELATIONSHIPS OF METHIONINE RESTRICTION, THE MITOCHONDRIAL UPR, AND ANIMAL LIFESPAN DETERMINATION

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ABSTRACT

A PROPOSED STUDY TO EXPLORE THE RELATIONSHIPS OF METHIONINE RESTRICTION, THE MITOCHONDRIAL UPR, AND ANIMAL LIFESPAN DETERMINATION

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The determination of chronological age is complex and can be influenced by both genetic and environmental factors. Mitochondria, essential organelles that generate the vast amount of cellular energy, are closely associated with the aging process. While many studies have been performed, we still lack a complete understanding of how animal lifespan is determined. In the following research proposal, a strategy is outlined to uncover the mechanism of the mitochondrial unfolded protein response (UPR^{mt}) and extended lifespan resulting from restriction of the amino acid methionine. The model organism *Caenorhabditis elegans* and a forward genetic screening strategy will be used to identify the genetic basis of methionine restriction-induced UPR^{mt}. We predict that

our findings will help explain the relationship of mitochondrial functional status to the aging process as it relates to the activation of stress responses such as the UPR^{mt}.

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CHAPTER 1

AN INTRODUCTION TO AGING

The concept of aging is defined as the progressive loss of physiological functions leading to an increased risk of disease and death. Previous research has found that the rate of aging is affected by genetic pathways as well as biochemical processes [1]. While the accumulation of cellular damage is considered to be the general cause of aging, numerous hallmarks have been identified to represent the common characteristics observed specifically in mammalian aging. These attributes include telomere attrition, loss of proteostasis, mitochondrial dysfunction, and stem cell exhaustion [1]. Each of these factors play a role in the accumulation of genetic damage throughout an organism's life and can lead to premature aging diseases.

Chromosomal regions, such as telomeres, are known for being vulnerable to age-related deterioration. These regions of DNA are found at the ends of each chromosome and serve as protection to preserve genetic material. During normal cellular processes, small segments of these telomeres are lost in the process of cell division. Over time, telomeres shorten with age leading to senescence, apoptosis, or oncogenic transformation of cells [2]. This is known as telomere attrition, which is often associated with development of diseases stemming from the loss of tissue regenerative capacity [3]. Another hallmark of aging involves the impairment of proteostasis. This mechanism ensures correct protein folding by chaperones as well as the degradation of misfolded proteins by the proteasome or lysosome in order to minimize proteotoxicity [4]. Studies have shown that chronic presence

of damaged proteins can result in the development of age-related pathologies including Parkinson's disease and Alzheimer's disease [5].

Mitochondrial dysfunction is often correlated with the aging process. Notably, the mitochondrial free radical theory of aging suggests that aging-related mitochondrial dysfunction is the result of increased production of reactive oxygen species [6]. Paradoxically, mild impairment to mitochondria may trigger mitochondrial defensive responses that extends lifespan; a phenomenon known as mitohormesis. This can result in the improvement in cellular fitness over time in organisms [7]. Furthermore, stem cell exhaustion is another hallmark of the aging process, observed with the decrease in the regenerative potential of tissues as a hallmark of aging. Also, there is an overall decrease in cell cycle activity, specifically in hematopoietic stem cells, which results in reduction of adaptive immune cell production [8]. Immunosenescence and the gradual deterioration of the immune system can result in the reduced ability to respond to new antigens and lingering inflammation [9].

Overall, these are some of the very many hallmarks of aging. The cellular insults that are associated with the aging are diverse but may have beneficial effects depending on their intensity. Indeed, lower levels could potentially trigger compensatory responses that can be used to protect the organism. Conversely, elevated levels may cause greater damage and accelerate the aging process [1]. Understanding the mechanisms associated with each will give us a greater understanding of how animal lifespan is governed.

CHAPTER 2

LITERATURE REVIEW

2.1 Methionine Restriction and Aging

Methionine is an essential amino acid that is typically consumed through eating meat, fish, and dairy products. It is necessary for normal developmental processes and is most known for its role as an initiator of protein synthesis [10]. However, methionine's impact exceeds its role in aiding in the translation of proteins. This amino acid heavily influences other cellular functions including epigenetic regulation, phospholipid homeostasis, and redox balance [11].

While dietary restriction is known to extend animal lifespan through caloric restriction, this method may serve little practical purpose due to the likelihood of malnutrition. Alternatively, one can refine a diet's nutritional composition to mimic the effects of caloric restriction but without the ill effects of completely limiting all dietary intake. One such approach involves decreasing the level of essential amino acids found in proteins such as methionine. Current findings have shown that restriction of the methionine amino acid increases organism longevity similar to dietary restriction and calorie restriction, but without risking malnutrition [12]. The pro-longevity effect of methionine restriction is thought to be related to the induction of autophagy, a cellular process that degrades and recycles unwanted cellular material [13]. Methionine restriction has also been shown to decrease reactive oxygen species, which can damage proteins, DNA, and lipids in the cell [10], as well as increasing the production of hydrogen sulfide that promotes the

reduction of oxidative stress [10]. These mechanisms ultimately result an increase in an organism's lifespan and longevity [10]. Oxidative stress is a disturbance in the balance of reactive oxygen species and antioxidant defenses. It is considered one of the proposed mechanisms involved in the aging process especially in the impairment of metabolic health in organs such as the brain, heart, liver, and kidneys. The beneficial effects of methionine restriction have been documented in organisms including yeast, *Drosophila*, *Caenorhabditis elegans* (*C. elegans*), mice, and rats [10]. In these model organisms, a lack of the methionine amino acid has resulted in extended longevity and increases stress tolerance [14].

2.2 Mitochondrial Stress

Mitochondria are double membrane-bound organelles of endosymbiotic origin and are commonly known as the "powerhouse of the cell". The function of this organelle is to generate the majority of the chemical energy that is crucial for powering the cell's biochemical reactions. Energy is generated through a process called cellular respiration in which mitochondria mediate a set of metabolic reactions to convert chemical energy from food nutrients into usable energy for the cell in the form of adenosine triphosphate (ATP). Mitochondria are also involved in other cellular including innate immunity, maintaining calcium homeostasis, synthesizing cofactors, regulation of apoptosis, urea cycle, β-oxidation, and lipid synthesis [15]. Mitochondria are challenged by a variety of different stresses and thus rely on cell recovery mechanisms to function properly. One of these stresses is caused by localized reactive oxygen species (ROS) which, as stated before, can be damaging to cellular organelles causing widespread disruptions to normal cell physiology. Many of the stresses that mitochondria encounter result in disruptions to the

mitochondrial protein folding environment. Elevated levels of misfolded or unfolded mitochondrial proteins is detrimental to the organelle and has ramifications for the entire cell. Mitochondria employ molecular chaperones and proteases to tend to this proteotoxic stress, which assist in protein folding or are involved in degraded misfolded proteins, respectively. However, there may be pathological consequences if the accumulation of misfolded proteins exceeds the capacity of molecular chaperones and proteases [15].

2.3 Mitochondrial Unfolded Protein Response

Even though mitochondria are able to detect disturbances in their proteomes, the organelle itself cannot express stress response genes such mitochondrial chaperones and protease. Mitochondria have thus evolved a means of communicating with the cell nucleus to induce the transcription of mitochondrial chaperones and proteases when proteotoxic levels have peaked. This type of retrograde signaling is known as the Mitochondria Unfolded Protein Response (UPR^{mt}). In nematodes such as *C. elegans*, stress signals such as mitochondrial dysfunction, translation disturbance, impairment of oxidative phosphorylation, and misfolding of proteins can trigger the activation of the UPR^{mt}, which helps restore homeostasis [16]. The core regulator of the UPR^{mt} is the bZIP transcription factor known as ATFS-1. A peculiar feature of ATFS-1 is the presence of two cellular targeting signals: one that targets ATFS-1 to mitochondria and the other to the nucleus. The localization of ATFS-1 depends on the level of stress to mitochondria: under healthy conditions, ATFS-1 will localize in mitochondria where it is proteolytically degraded. However, during stress, ATFS-1 is unable to be imported into mitochondria and instead transits to the nucleus where it will aid in the transcription of mitochondrial chaperones, proteases and other protective genes which help recover the damaged organelle. Therefore,

mitochondrial protein import efficiency acts as the stress signal to induce this protective response. When the level of damage to mitochondria exceeds recovery efforts, cells eliminate the damaged organelles through a specialize form of autophagy known as mitophagy [17]. The functional capacity of both the UPR^{mt} and mitophagy decline with age as organisms tend to lose the ability to turn on stress signaling pathways [18].

The connection between mild mitochondrial stress and increased animal lifespan is believed to be related, at least in part, to the activation of the UPR^{mt}. Presumably, mild mitochondrial stress primes the organism for more effective mitochondrial repair later in life. The extension in animal lifespan observed with methionine restriction was previously found to be linked with the activation of the UPR^{mt} [19]. Here, methionine restriction resulting from genetic loss of methionine synthase (gene *metr-1*), induced the UPR^{mt} [19]. Importantly, the UPR^{mt} regulator ATFS-1 was required for this activation as well as the lifespan extension resulting from methionine restriction [19]. However, the mechanism that connects the UPR^{mt} to methionine restriction and animal lifespan is still unresolved. In the following, a strategy is outlined to determine the mechanistic basis of methionine restriction-induced animal lifespan via the UPR^{mt}.

CHAPTER 3

METHODOLOGY

3.1 *Caenorhabditis elegans* and the Study of the UPR^{mt}

Much of what is known about the regulation of the UPR^{mt} and its diverse functions has been derived from the model organism *C. elegans*. This microscopic nematode offers numerous advantages for the study of biological questions. This includes a rapid life cycle of 3-5 days, a completely sequenced and annotated genome, and a plethora of genetic manipulations that are available to interrogate a cell pathway of interest including double stranded-RNA interference (RNAi), forward and reverse genetic screens, and CRISPR/Cas9 genome editing [20]. Importantly, a considerable homology exists between the proteome of *C. elegans* with that of higher eukaryotes, including humans. Therefore, it is highly possible that the findings derived from a *C. elegans* study may have implications for understanding human biology and disease.

Another significant advantage of using *C. elegans* as a model organism is its transparent nature. Therefore, one can visualize all organs and cells of *C. elegans* using basic light microscopy. Furthermore, one can introduce fluorescent reporters to visualize gene expression or protein localization in vivo, without the need of complicated fixation techniques. Fluorescent reporters often employ green fluorescent protein (GFP), a protein that originated from the jelly fish (*Aequorea victoria*) [21]. Two types of GFP reporters are possible. First, transcriptional reporters which consist of the promoter of a gene interest fused to the coding sequence of GFP, can be used to detect expression patterns of specific

genes. Second, translational GFP reporters, which introduce the GFP coding sequence in frame with the coding sequence of a protein interest, can be used to examine protein localization.

A major breakthrough in our understanding of the UPR^{mt} was made possible through the generation of a transcriptional reporter that specifically detects the activation status of this stress response. The UPR^{mt} reporter, *hsp-6*::GFP, contains the promoter for the gene *hsp-6*, a mitochondrial chaperone that is a direct transcriptional target of the main regulator of the UPR^{mt}, ATFS-1. The *hsp-6* promoter is fused with the coding sequence of GFP. Under homeostatic conditions when mitochondria are not undergoing stress, the transcription factor ATFS-1 does not bind to the promoter of the *hsp-6*::GFP reporter and thus the nematode does not fluoresce. However under conditions of mitochondrial stress, ATFS-1 binds to the *hsp-6*::GFP reporter, resulting in transcription of the GFP gene and the animal fluorescing bright green. Therefore, this reporter serves as a valuable tool to detect the activation of the UPR^{mt}. Indeed, there have been many hallmark studies using this UPR^{mt} reporter that have uncovered various regulators of the UPR^{mt} when used in combination with genetic techniques such as RNAi and forward genetic screens. This includes Clpp-1 protease, the ubiquitin-like protein UBL-5, several chromatin remodelers, and other factors that act activate the UPR^{mt} both self-autonomously and non-autonomously [22].

3.2 *Caenorhabditis elegans* Maintenance

C. elegans are maintained on nematode growth medium (NGM) as previously described [23]. The various strains of *C. elegans* are obtained from the Caenorhabditis

Genetics Center (CGC) and were cultured at 20℃. The mutant stains are backcrossed at least four times prior to being used [19].

3.3 Genetic Screening Methods

There are many types of approaches that *C. elegans* researchers can use to identify and explore cellular pathways and processes. These include forward and reverse genetic screens, functional genomic approaches, and system biology approaches [24] [25]. Mutations in specific genes can be very informative when trying to understand how certain pathways function. Mutations can be classified as either germline mutations, somatic mutations, chromosomal alterations, point mutations, and frameshift mutations. Germline mutations occur in gamete cells and can be inherited from a parent. Somatic mutations are found in cells outside of the germline and are not heritable. Chromosomal alterations occur either numerically or structurally. Numeric chromosomal alterations involve the number of chromosomes and can result in monosomies or trisomies. Structural chromosomal alterations can involve translocation, deletion, or duplication. Translocation is when a portion of a chromosome breaks off from the original chromosome and attaches to a different chromosome. Deletion occurs when a portion of the chromosome breaks off and does not attach anywhere else resulting in the loss of genetic material. Duplication is when a portion of a chromosome is copied resulting in the addition of genetic material. Point mutations occur when one nucleotide of a genetic sequence is altered. These point mutations can be characterized as silent, nonsense or missense. In a silent mutation, the change of nucleotide in the genome does not influence the amino acid identity and therefore the protein synthesized is not affected. Missense mutations result in a change in amino acid composition which can lead to a reduction or loss of protein function. Nonsense mutations

change the amino acid to a "stop" codon in which the resulting protein is premature and will not be able to carry out its correct function. Frameshift mutations involve additions and deletions of a nucleotides that result in a shift in the reading frame [26].

Forward genetic screens are particularly useful since they are an unbiased means to identify gene players for specific pathway. During this type of screen, *C. elegans* are exposed to a powerful mutagen, typically the chemical ethyl methane sulfonate (EMS), which introduces point mutations throughout the genome of the animal [30]. This process begins when *C. elegans* are harvested from NGM plates with S-basal and washed to remove bacteria. EMS solution is then added to the worm suspension and placed on a rocker. Following mutagenesis, the worms are washed with S-basal, resuspended in S-basal, and then placed onto seeded NGM plates [19]. After overnight incubation, mutagenized animals can then be screened for specific phenotypes either in the F1 or F2 generation [24]. Mutants that are recovered in the F1 generation are genetically dominant, whereas those in the F2 generation are typically recessive.

3.4 Genetic Mapping Techniques

Once the mutagenized *C. elegans* have been isolated in the forward genetics screen, the causative mutated gene of interest must then be identified. Genetic mapping techniques are used to narrow down a set of genes depending on their location on the chromosome. Broadly, the mutant of interest is crossed with various other mutants with known genetic locations and display obvious phenotypes when mutated (e.g., physically short, inability to move etc.). The location of your gene of interest can be approximated based on the recombination efficiency of these defined genetic markers. Once a refined region with a manageable number of genes has been determined, other validation techniques can then be

employed to precisely pinpoint the exact causative gene responsible for the desired phenotype of interest.

3.5 Mutant Validation Strategies

After identifying a specific location containing the gene of interest, validation approaches then follow. One method of validation includes reintroducing a wild-type copy of the gene of interest into the mutagenized worm. A wild-type gene is simply a gene that is naturally present in the animal's genome. Typically, the gene of interest in amplified from the *C. elegans* genome using polymerase chain reaction (PCR). The wild-type gene PCR product is then microinjected into the germline of *C. elegans* using standard microinjection rescue techniques [19]. Double-stranded RNA can be microinjected into the worm and RNAi will spread throughout the body into the germline [27]. The wild-type PCR product is then incorporated into the next generation as an extra-chromosomal array. An assessment can then be made as to assess whether transgenic animals carrying the wild-type gene product effectively restores the normal function of your phenotype of interest.

Another validation method would be to obtain an independently-derived mutant of the suspected gene of interest from a public repository such as the Caenorhabditis Genetics Center [28]. This organization houses *C. elegans* with individual mutations in most genes, making it possible for one to obtain a nematode with a mutation in the same gene as the gene of interest [29]. The use of an independently-derived mutant will validate or invalidate that the isolated point mutation of interest is the causative agent.

Lastly, reconstitution of the same missense point mutation in the nematode using CRISPR/Cas9 genome editing can be used to introduce the same mutation obtained in the unbiased forward genetics screen. CRISPR is used as a defense mechanism against viral

infection in bacteria and archaea and was characterized as a bacterial genome region in which foreign DNA fragments are embedded between repetitive sequences of DNA. Cas9 is an endonuclease that can be programmed to cleave any desired sequence which makes it ideal for genomic editing [30]. This system allows the making of targeted mutations in order to investigate relationships between gene function and phenotype. For example, the co-CRISPR strategy allows the researcher to make genome substitutions that result in the production of point mutants [31]. Using this approach, one can observe whether the mutation created by CRISPR/Cas9 genome editing mimics the phenotype of the mutant that was originally isolated in the forward genetics screen.

CHAPTER 4

CONCLUSION

4.1 Research Significance

The significance of this research is to investigate the mechanisms behind how organisms age and the determinants of the aging process. Through this, we are able to explore the influence of diet and metabolism and their effects on an organism's lifespan. Mitochondrial dysfunction is intimately associated with organismal aging. Mild dysfunction to mitochondria prolongs animal lifespan presumably through the activation of stress responses that function to repair damage experienced by this organelle, including the UPR^{mt}. Many conditions exist that activate the UPR^{mt} and extend longevity, including nutritional alterations such as reduced methionine availability. The mechanism of how methionine restriction activates the UPR^{mt} remains elusive. This proposal seeks to uncover the genetic basis of this relationship. The methodology proposed using an unbiased forward genetic screening strategy will provide a solid framework of the genes and related pathways that act downstream of methionine restriction to activate the UPR^{mt} and extend lifespan. Ideally, the findings from this proposed study using an invertebrate model organism will provide a solid framework to understand how animal aging is controlled, including in humans.

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BIOGRAPHICAL INFORMATION

Joanne Mai is graduating Fall 2021 with an Honors Bachelor of Science in Biology. During her time at the University of Texas at Arlington, she was involved in multiple organizations. Joanne is a member of the Honors College, secretary of the Pre-Optometry Professional Society, and a Pre-Optometry Peer Mentor through the university's Pre-Health department. She initially became interested in mitochondrial research through her faculty mentor, Dr. Pellegrino, after taking his Cell Physiology course. She became fascinated with how cellular pathways could influence the aging process and neurodegenerative diseases such as cancer and Alzheimer's disease. Outside of the university, she works at a private optometry practice where she is a receptionist, technician, and a scribe. Joanne plans to further her education at the University of Houston College of Optometry and is matriculating fall 2022. She hopes to one day start her own private optometry practice after completing a residency in vision therapy or ocular disease.