Children's Hospital of Pittsburgh

Annual Progress Report: 2010 Formula Grant

Reporting Period

July 1, 2013 – December 31, 2013

Formula Grant Overview

The Children's Hospital of Pittsburgh received $527,174 in formula funds for the grant award period January 1, 2011 through December 31, 2013. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Regulation of Aging by the Proteasomal Pathway – Aging is a universally relevant phenomenon and a fascinating biological process. Increasing age is the largest cause for pre-disposition to a spectrum of age-related diseases, including cancer and neurodegenerative diseases such as Alzheimer's disease. An understanding of the genetic mechanisms that determine the rate of aging can lead to preventive and therapeutic measures that simultaneously target multiple age-associated ailments. This study is aimed at understanding how a molecular pathway that controls the degradation of proteins in the body helps determine the rate of aging in response to reproductive signals. The studies described here can eventually help design preventive and therapeutic measures for age-related ailments.

Duration of Project

1/1/2011 - 12/31/2013

Project Overview

Aging and reproduction are universally relevant biological phenomena and fundamental aspects of any animal's life history. The long-term objective of this project is to unravel the genetic mechanisms that govern aging, particularly to understand how signals from reproductive tissues influence lifespan. As part of my postdoctoral research, I discovered that the proteasomal pathway of protein degradation regulates the aging of the model nematode worm *Caenorhabditis elegans*. In mutants of the insulin/IGF-1 receptor *daf-2*, that live twice as long as normal worms, inactivation of specific components of the CUL-1/SKR-1,-2/F-Box proteasomal E3 ligase complex abolishes the extended lifespan. E3 ligases determine the specificity of substrate proteins targeted for degradation, and utilize the F-Box adaptor proteins for identification of degradation targets. This data was one of the first evidences that the proteasome pathway influences the longevity of a metazoan organism. It suggested that the targeted proteasomal modification of specific substrate protein(s) is crucial for the extension of lifespan in *C. elegans*, by mutations in the *daf-2* pathway. Besides the insulin/IGF-1 signaling (IIS) pathway, *C. elegans*

lifespan is also regulated by signals emanating from reproductive tissues. In worms, germline removal causes lifespan extension. In the current project, we plan to identify the proteasomal E3 ligases that mediate the longevity brought about by germline loss by performing large-scale RNAi screens. Once the E3 ligases are known, we will use these to identify the substrates whose proteasomal modification controls the long lifespan of germline-ablated worms. To this end, we will utilize genetic mutants in the genes encoding the E3 ligases, as well as molecular protein-protein interaction screens. The identified targets will undergo extensive cellular, molecular and functional analyses to understand how they allow signals from reproductive tissues to alter the rate of aging of the entire organism. The elaboration of these molecular details can provide a platform to design preventive and therapeutic strategies to deal with a multitude of age-related debilitations and diseases.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Aging is a public-health issue of remarkable significance as it is the biggest risk factor associated with debilitating diseases such as many cancers and neurodegenerative diseases. Research into the genetic mechanisms that underlie the causes of normal aging promises to be a comprehensive and possibly least expensive means of targeting many pathologies simultaneously. One of the important processes that influence the rate of aging is reproduction. While we know much about how aging affects reproduction, very little is known in any system about how reproduction alters aging of animals. This project aims to identify genes of the proteasomal pathway that act at the intersection of reproduction and aging. Based on previous work we have performed, we now know that the proteasomal pathway influences the lifespan of worms. We will now ask how this pathway modulates the genes that are used by reproductive tissues to alter lifespan. Once we identify such genes, they can provide valuable molecular information about the genetic mechanisms that determine longevity. Since a significant number of genes are conserved between worms and humans, these candidates can lead to the identification of human aging genes. Besides, such lifespan-regulatory candidates can be used in large-scale chemical and drug screens to identify therapeutics that have the potential of retarding age-associated pathologies in humans.

Summary of Research Completed

The complex relationship between aging and reproduction is clearly manifested in the nematode *C. elegans* wherein removal of the germline-stem cells (GSCs) produces a striking increase in the length of life of the sterile adults. This lifespan extension is regulated by a precise genetic circuit that includes two conserved transcription factors, DAF-16 and TCER-1. Although the details are likely to vary, the reproductive control of aging is widely prevalent in the natural world as changes in longevity following alterations to reproductive status have been observed in many other species. A key determinant of length and quality of life is the protein homeostasis of the animal. How pathways that ensure protein quality control are influenced by reproductive signals, and in turn how they influence aging is the focus of this project. Specifically, we aim to identify proteasomal E3 ligases (enzymes that determine and catalyze the degradation of specific cellular proteins) that allow the animal to translate a loss of reproductive stem cells into lengthened lifespan. Our previous observations suggested that a DAF-16 and TCER-1 target, NHR-49 (the worm functional homolog of PPARα, the vertebrate transcription factor) is a more useful read-out for the proposed screen. In the past six months, we have integrated a GFP-tagged NHR-49 DNA construct into the genome of *C. elegans* to complete the generation of a transgenic worm strains that will facilitate the identification of these E3 ligases. We have standardized the experimental conditions of the screen in small-scale pilot efforts with different RNAi clones, and have established the healthspan assays that will be used to perform crucial secondary tests to distinguish the bonafide aging-regulatory E3 ligases from those with non-specific phenotypes. In addition, we have confirmed our previous observations that stress-resistance and longevity can be uncoupled by the action of proteins such as TCER-1 and the E3-ligase LIN-23/βTRCP.

Genomic Integration of NHR-49::GFP Construct for RNAi Screen

Our previous experiments led us to the conclusion that the nuclear hormone receptors (NHR) encoded by the gene *nhr-49* is a more suitable candidate to identify proteasomal E3 ligases that are activated by reproductive signals to ubiquitinate and likely destroy proteins that inhibit longevity. The NHR-49::GFP transgene that we introduced into worms showed a robust, high expression that is localized to all somatic cells in both nuclear and cytoplasmic compartments. The GFP is expressed at a high-enough level that a fluorescence-stereomicroscope is sufficient to visualize the signal. In addition, we found that in germline-ablated adults the levels of NHR-49::GFP increase significantly, so using the change in GFP levels would be an expedient assay. However, the transgene was not integrated in the worm genome permanently (but is carried in the cells as an extra-chromosomal array made up of variable concatemers of the DNA-construct that can be lost during cell divisions) and this resulted in two issues:

(a) Heterogeneity of expression levels: since the number of DNA arrays could not be controlled within different cells, tissues and animals, there was a wide variation in the levels of GFP. As a result, the effects of an RNAi gene-inactivation on GFP levels were difficult to distinguish from the normal variation exhibited by a population.

(b) Transgene transmission frequency: the extragenic nature of the array results in only a fraction of progeny of any transgenic worm carrying the GFP construct. Consequently, the strain needs to be maintained by manually picking fluorescent worms in each generation. Although our NHR-49::GFP transgenic strain showed a high-frequency of transmission (>50%), we found that for

the purpose of a screen the scaling up required is arduous with an un-integrated strain.
To circumvent the above two issues, we integrated the NHR-49::GFP construct into the genome. This was performed using a standard technique that relies on the use of UV or γ rays to irradiate the parents. The radiation introduces DNA breaks that can undergo recombination with the extragenic array to cause integration of the transgene at relatively random genomic locations, usually in single copies. We used both UV and γ radiation for our experiment. Irradiated parents were allowed to lay eggs and their GFP+ve progeny were picked and put onto individual plates. In the next generation, about 600 lines were set up from the progeny of these fluorescent worms and scored for plates where all the worms were green. We isolated a single integrant from screening 600+ lines. The integrant was outcrossed to the lab wild-type stock (to eliminate background mutations that may have arisen from the radiation) and tested for authenticity of the NHR-49::GFP construct by multiple tests.

RNAi Screen Paradigm on 24-well Petridishes
While the integration of the NHR-49::GFP was in progress, we focused on developing a screen pipeline in which 24-well tissue-culture petri-dishes can be used to culture worms instead of regular 6cm nematode plates. This reduces the space and material requirements of the experiment and makes it possible to test much larger number of RNAi clones in a shorter duration. We determined that about 20-25 worms could be grown per well of a 24-well plate for 3-4 days without causing starvation or hypoxia or cross-contamination between wells. We found that the increase in NHR-49::GFP upon loss of germline is most striking on Day 2 of adulthood (~4 days of nematode culture) and this will be used as the major time-point for conducting the screen. We are now conducting additional pilot tests (with RNAi sub-libraries targeting chromatin factors) to fine-tune the screen conditions, while we compile afresh the gene annotation data for E3 ligases in worms. The new virtual library will help create an updated RNAi sub-library of E3 ligase clones that will be used in our screen.

The Healthspan Assay Toolkit for E3-ligase Clones Influence Longevity:
Since protein homeostasis is such a fundamental aspect of survival, reduced function of most proteasomal genes is highly deleterious to animal health and causes death rapidly. Consequently, it is difficult to distinguish the direct longevity functions of proteasome genes as compared to their role in cellular maintenance. We have focused on this issue extensively, as tests that will help us identify E3 ligases whose RNAi-knockdown accelerates aging (as compared to treatments that just cause sickness and general dysfunction) will be the most crucial aspect of this project. In addition to lifespan, a series of healthspan assays and stress-response tests can be used that reflect the overall health of the animal. Besides stress-resistance that declines with age, worms also display age-related physical and physiological dysfunction that can be quantified precisely. In a previous report, we have described molecular tests (DAF-16 and TCER-1 GFP assays) and a combination of biotic (pathogen attack) and abiotic (oxidative stress, heat) stress assays that will be used on screen candidates to identify genuine aging regulators. We have now established healthspan tests that will be used to address other aspects of age-related dysfunction. RNAi clones that not only reduce GFP expression but also negatively impact these measures of overall health will be the most attractive candidates that will be studied molecularly. The healthspan assays are listed here in brief.
1. Morphometric Age-Associated Changes: Worms exhibit a series of well-characterized

morphological changes that reflect the aging of tissues, similar to humans. RNAi clones that influence NHR-49::GFP will be evaluated for their effect on the rate of aging by examining the anatomic age-related declines.
(a) Sarcopenia: The deterioration undergone by muscle cells and nuclear architecture.
(b) Aging pigment accumulation: Lipofuscin and Advanced Glycation End products (AGEs) together constitute the age-related increase in intestinal autofluorescence.
2. Functional Age-related Decline: Similar to humans and other animals, aging nematodes also experience declining functionality. We have found that the following measures of functionality are easy and reliable markers of age-related decline that can be used on our screen candidates.
(a) Loss of mobility: Closely associated with sarcopenia, reduced mobility can be directly correlated with the rate of aging, independent of the effect of a gene on length of life.
(b) Pharyngeal Pumping: Worms use a muscular pharynx to grind the bacteria they consume before it is transferred to the digestive tract. The rate of pumping undergoes a sharp decline with age. The pharynx is often compared to the human heart due to its muscle physiology, so this is a particularly useful assay to screen E3 ligases that influence aging.

In addition to the above, we have confirmed two of the observations made in the previous year(s).
(a) The gene *tcer-1* influences the longevity of germline-ablated animals but not their stress-resistance capacity. We have confirmed that *tcer-1* is not required for the enhanced resistance to oxidative stress exhibited by long-lived, germline ablated worms. In addition, this uncoupling is applicable to heat-shock resistance as well.
(b) The E3-ligase LIN-23/βTRCP is indeed required for the longevity and enhanced stress-resilience of germline-ablated animals, and it interacts with SKN-1/NRF2 transcription factor in mediating this function.

Research Project 2: Project Title and Purpose

Defining the Role of microRNAs in Podocyte Function and the Renal Stroma - Endstage renal disease costs over 25 billion dollars annually in the United States, and individual patients with renal failure have significantly impaired quality of life and are at increased risk of mortality. The long-term objective of this project is to gain a better understanding of the molecular pathways that cause glomerular disease and congenital anomalies of the kidney, leading causes of renal failure in children and adults. Completion of these studies will have significant implications for patients with kidney disease and may lead to novel therapeutic avenues for the treatment of chronic kidney disease in patients.

Duration of Project

1/1/2011 - 12/31/2013

Project Overview

MicroRNAs (miRNAs) are a novel class of small noncoding RNAs that regulate gene expression via the post-transcriptional repression of specific target mRNAs. The long-term goal of our laboratory is to characterize the molecular pathways regulated by miRNAs in kidney

development and disease. Congenital nephron loss and glomerular disease represent the leading causes of chronic kidney disease and renal failure in children and young adults. Understanding the mechanisms that underlie nephron formation, including the glomerulus, is critical for making an impact on these diseases.

The renal stroma plays a crucial role in nephron formation, and given its close proximity to nephron progenitors, likely forms an important microenvironment or "niche" for these cells during kidney development. However, the interaction between nephron progenitors and their presumed "niche" in the determination of congenital nephron number remains poorly defined. Our laboratory has preliminary data suggesting that loss of miRNAs in the renal stroma results in an expanded nephron progenitor pool and mis-regulated nephrogenesis.

The glomerulus acts as the filtering unit of the kidney. The filtration barrier is composed of fenestrated endothelial cells, the glomerular basement membrane, and the podocyte, a highly specialized epithelial cell that is often injured or lost in glomerular diseases. Our preliminary work demonstrates that miRNA function in the podocyte is critical in maintaining this filtration barrier.

In this research project, we wish to characterize the molecular pathways that regulate the podocyte and renal stroma during kidney development. *We hypothesize that miRNAs regulate key mRNA transcripts required for podocyte structure and function, and the renal stroma.* To test this hypothesis, we propose the following aims:

Specific Aim 1: To validate and determine the function of miRNA-mediated regulation of Ulk1 in podocytes.
Specific Aim 2: To define the role of miRNAs in regulating developing nephron progenitors in the early mesenchyme and the renal stroma.

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Expected Research Outcomes and Benefits

In our previous work, we demonstrated that (1) loss of miRNAs in the renal stroma resulted in an expansion of the nephron progenitor pool and (2) that there is a requirement for miRNAs in the regulation of podocyte structure and function, which is critical in maintaining the glomerular

filtration barrier. This research project will further define the miRNA-mediated mechanisms that regulate the interactions between nephron progenitors and their "niche", as well as the complex cellular architecture needed for podocyte function during renal development. We anticipate that this will impact our fundamental understanding of the determination of congenital nephron number and the pathophysiology behind proteinuric kidney diseases. We expect that we will identify novel biologically important miRNA-mRNA interactions in podocytes and the renal stroma.

The project is intended to form the basis for an ongoing research program to define the function of miRNAs in podocytes and the renal stroma in healthy individuals and in disease states. Ultimately, this will have significant implications for patients with chronic kidney disease due to congenital nephron loss or glomerular disease, and may lead to novel therapeutic avenues through the manipulation of miRNA pathways in patients.

Summary of Research Completed

Specific Aims:
Our goals during the final 6 month period of funding were to continue defining Ulk1 as a potential miRNA target in podocytes (Specific Aim 1) and to characterize the phenotype observed in the early mesenchyme and the renal stroma of the developing kidney following loss of miRNAs (Specific Aim 2, revised).

Progress on Specific Aim 1: We continue to work on confirming the regulation of Ulk1 using an *in vitro* luciferase reporter assay. The Ulk1 antibodies that we have tested to date have not worked in adult kidney sections or Western blot, so these are ongoing experiments.

Progress on Specific Aim 2:
To define the requirement for miRNAs in the early metanephric mesenchyme (which gives rise to nephron progenitors and the renal stroma during kidney development), we generated a transgenic mouse model that lacks mature miRNAs in this lineage: *Pax3CreTg, Dicer*$^{Flx/Flx}$. Over the past 6 months, we confirmed the expression of mmu-miR-10a and mmu-miR-17 in the developing kidney at E11.5 by locked nucleic acid *in situ* hybridization, and that there was loss of these miRNAs in *Pax3CreTg, Dicer*$^{Flx/Flx}$ mutant kidneys by both LNA-ISH and quantitative real-time PCR using Taqman miRNA assays (Figure 1). Overall, these data are the first to demonstrate an early requirement for miRNAs in the metanephric mesenchyme, and suggests a crucial role for miRNAs in regulating the survival of this lineage.

To examine the requirement for miRNAs in the renal stroma, we generated mice that lack functional miRNAs in this lineage using a conditionally floxed allele of *Dicer*, which is required to form mature miRNAs, and *Foxd1cre*, which drives the loss of *Dicer* expression in renal stroma and its derivatives. Our data suggests that this results in renal hypodysplasia, expansion of the "cap" of nephron progenitors around ureteric bud tips, loss of Foxd1-positive renal stroma related to apoptosis, and disorganization of the developing nephron structures just below the renal capsule. Our data support the notion that miRNA loss in the renal stroma influences the stromal signals that normally regulate nephron progenitor proliferation, nephron differentiation, and ureteric branching. In addition, the aberrant renal stroma development results in abnormal

vascular patterning and an absence of glomerular mesangial cells in mutant kidneys. These data together provided the preliminary data for a grant submission to the March of Dimes Basil O'Connor Starter Scholar Research Award, which has subsequently been funded.

To identify miRNAs expressed in the renal stroma, we crossed *FoxD1cre; Dicer*$^{flx/+}$ mice with a Rosa-CAG-dTomato reporter mouse line, which will label renal stroma and its derivatives in fluorescent Tomato red. We performed fluorescence-activated cell sorting (FACS) to isolate control and mutant FoxD1-derived cells from dissociated kidneys in collaboration with Dr. Sunder Sims-Lucas, Department of Pediatrics, University of Pittsburgh. We plan to compare the profile of small RNAs and mRNA transcripts in control and *FoxD1cre; Dicer*$^{flx/flx}$ CAG-positive renal stroma to identify miRNAs expressed in the renal stroma, and define changes in the renal stromal transcriptome of mutant kidneys. Thus, we have prepared total RNA for high-throughput RNA sequencing on three independent sets of pooled total RNA from CAG-positive stromal cells isolated from control and mutant kidneys, and sent these samples BGI Americas. For mRNA deep sequencing, they are generating cDNA libraries following amplification. They will then perform 50 base pair single-end sequencing reads for small RNAs, or 100 base pair paired-end sequencing reads for mRNA transcripts (with a goal of 10 million reads/sample for small RNAs and 30-40 million reads/sample for mRNA transcripts to achieve adequate depth of sequencing coverage) using the Illumina HiSeq 2000 sequencing system. We are currently awaiting the sequencing data.

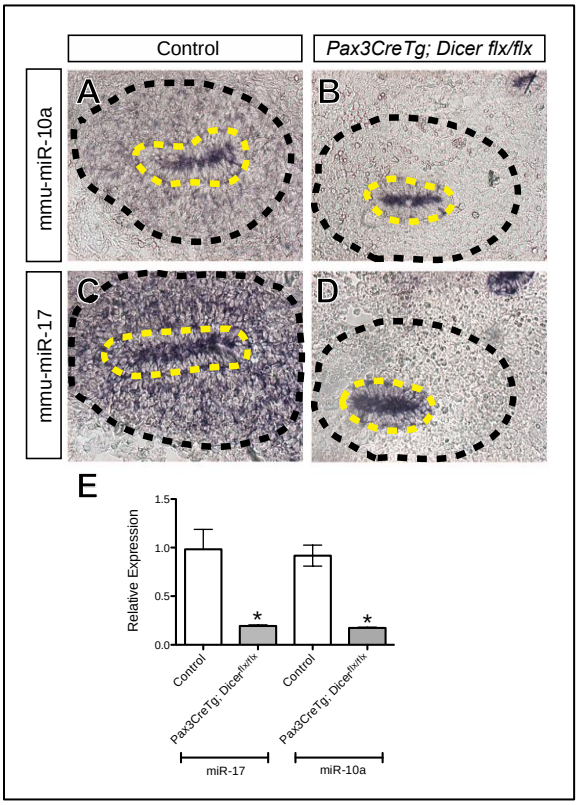

Figure 1. Cre-mediated excision of the conditionally floxed *Dicer* allele via the *Pax3Cre*^{Tg} allele results in loss of miRNA expression in the metanephric mesenchyme at E11.5. (A-D) LNA in situ hybridization demonstrates loss of mmu-miR-10a and mmu-miR-17-5p expression in the metanephric mesenchyme of *Pax3Cre*^{Tg}, *Dicer*$^{flx/flx}$ kidneys (B, D) compared to controls (A, C). Black dashed line, metanephric mesenchyme; yellow dashed line, ureteric bud. The magnification is 20X. (E). Quantitative real-time PCR performed on total RNA isolated from E11.5 kidneys confirms decreased expression of mmu-miR-10a and mmu-miR-17-5p ($p<0.05$, paired t-test, *). The relative expression of mmu-miR-10a and mmu-miR-17-5p denotes the fold change in delta CT in mutants normalized to controls. Bars represent standard errors of the mean.