Children's Hospital of Pittsburgh

Annual Progress Report: 2010 Formula Grant

Reporting Period

January 1, 2011 – June 30, 2011

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.

Anticipated 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 current project is aimed at identifying proteasomal E3 ligases that influence the longevity of the nematode model organism *Caenorhabditis elegans*. In *C. elegans*, lifespan is regulated by signals emanating from reproductive tissues. Removal of reproductive stem cells called Germline Stem Cells (GSCs) causes lifespan extension. This study aims to identify the proteasomal E3 ligases that mediate the longevity brought about by GSC loss by employing large-scale RNAi screening. Once the E3 ligases are known, these will be used in turn to identify the substrates whose proteasomal modification controls the long lifespan of GSC-ablated worms. In the past six months, we have standardized the conditions for performing the large-scale RNAi screen for E3 ligase identification (Specific Aim 1.i). These are some of the first experiments that have been conducted in the Ghazi lab, since the Primary Investigator has recently started her independent lab (January 2011). Consequently, many of the experiments involved standardizing experimental protocols and conditions, and establishing assays for this project in a new location. Following are the steps accomplished towards the overall goal of this study:

1. Confirmed longevity of *glp-1(-)* mutants: Temperature sensitive *glp-1(e2141ts)* mutants lack germ cells (at non-permissive temperature), are sterile, long-lived and have proven to be a valuable surrogate for longevity produced by GSC removal. We first standardized protocols for making media and solutions for proper growth of our worm strains, especially *glp-1* mutants. We then examined the lifespan of *glp-1* mutants under the new lab conditions. *glp-1(-)* mutants displayed an extended mean lifespan (~30 days, as compared to ~18 days which is the mean lifespan of wild-type N2 strain) reliably. This lifespan extension relied on the presence of the transcription factor DAF-16/FOXO , and the transcription elongation factor, TCER-1, as previously reported. We are confident this strain can now be used for the large-scale E3 ligase RNAi screen.

2. Organized and confirmed the functioning of RNAi libraries to be used for the E3 ligase screen: For our RNAi screen, we plan to use clones isolated from two commercially available RNAi libraries that together target >90% of the worm genome. One of these has been made by Julie Ahringer's laboratory and has 16,757 bacterial strains (made from PCR amplified genomic fragments targeting ~86% of predicted worm genes). The second library has been made from cDNA fragments of worm genes by Marc Vidal's laboratory and has 10,953 clones that target ~55% of the worm genome. The RNAi clones targeting E3 ligases to be used in our screen will be derived from these two libraries. As a first step towards this process, a copy of each of these libraries (made previously at UCSF) was shipped to the Ghazi lab at Pittsburgh. The libraries (totaling 353 96well plates) were organized in their new location. We tested the integrity of the both the libraries by (a) isolating random clones and testing their growth, and (b) making a small sub-library of clones targeting nuclear hormone receptors (NHRs). The NHR experiments are part of another project and will not be described here, but this sub-library creation helped us test and confirm the integrity and functioning of both the large-scale libraries. We can now use these libraries to make a sub-library of E3 ligases that will be used to screen for suppressors of *glp-1(-)* extended lifespan.

3. Established conditions for secondary assays for screen suppressors: We standardized the protocols for some of the secondary assays that will be used to screen the suppressors isolated in

the E3 ligase screen, and are working towards standardizing others. One of the most important tests that potential suppressor E3 ligase clones will undergo is to examine their effect on DAF-16/FOXO nuclear localization. In worms, when GSCs are eliminated, DAF-16/FOXO translocates to intestinal nuclei. This nuclear localization is critical for and underlies the transcriptional up-regulation of a spectrum of genes that together promote longevity. Previous studies have suggested that this nuclear localization is most noticeable in adult worms. We undertook a systematic expression analysis of DAF-16/FOXO nuclear localization at different stages of larval and adult life. Our results suggest that Day 2 of adulthood is the ideal stage for observing nuclear localization. Surprisingly, we have also found no nuclear relocation of DAF-16/FOXO in larval stages (although GSCs are eliminated during early larval development). This raises very interesting questions about the temporal control of expression of longevity genes. For the purpose of our RNAi screen, we will focus on Day 2 of adulthood, and will ask if any of the *glp-1(-)* suppressor clones interfere with DAF-16/FOXO nuclear relocation at this stage.
The transcription elongation factor, TCER-1, is up-regulated in intestinal and neuronal cells of GSC(-) worms and this up-regulation is essential for their longevity. Another important secondary test for E3-ligase clones we identify will be to document their effect on TCER-1 expression, and its up-regulation in *glp-1(-)* mutants. We had previously used a GFP-tagged transgenic strain to examine TCER-1 expression. But this strain is *extragenic* (only a portion of the population carries the GFP reporter) and hence could not be used for large-scale experiments. To be able to use the strain for our experiments, we had previously integrated the construct (using a standard worm protocol). Following integration, we have now outcrossed the strain to the wild-type N2 worms six times (to ensure that any background mutations induced by the integration were eliminated). We then examined the expression of TCER-1 and found it to be nuclear and widely-expressed, as expected. We are now in the process of introducing this construct into a *glp-1* mutant background, so the strain can be used for the secondary assays of our suppressors. Any *glp-1(-)* suppressors that either target TCER-1 up-regulation or DAF-16/FOXO nuclear localization will be extremely interesting for future study. We have also commenced experiments to optimize the conditions for other secondary assays including stress response tests. Once these secondary assays are established, we will generate the E3 ligase sub-library and perform the screen to identify ligase sub-units that are essential for the longevity of *glp-1(-)* mutants.

Research Project 2: Project Title and Purpose

Defining the Role of microRNAs in the Regulation of Podocyte Structure and Function – 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, a leading cause 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.

Anticipated Duration of Project

1/1/2011 - 12/31/2013

Project Overview

Glomerular disease is a leading cause of chronic kidney disease and can progress to endstage renal disease necessitating renal transplant or dialysis in children and adults. The glomerulus acts as the filtering unit of the kidney, and understanding the mechanisms that regulate the filtration barrier are critical to making an impact on these diseases. One component of the barrier is the podocyte, a highly specialized epithelial cell that is often injured or lost in glomerular diseases. Our long-term goal is to characterize the molecular pathways that regulate the structure and function of the podocyte, which is key in the formation and maintenance of the glomerular filtration barrier.

Our laboratory has preliminary data addressing a novel microRNA (miRNA)-mediated mechanism for regulating podocyte structure and function. miRNAs are a novel class of small RNAs that control gene expression through the post-transcriptional repression of specific target mRNAs. Our preliminary work demonstrates that miRNA function in the podocyte is critical in maintaining this filtration barrier. *We hypothesize that miRNAs regulate key mRNA transcripts that are required for podocyte structure and function.* To test this hypothesis, we propose the following aims:

Specific Aim 1: To identify podocyte-specific miRNA-mRNA target interactions.
Bioinformatic approaches will be utilized to identify miRNA-mRNA target interactions that will subsequently be validated using an *in vitro* luciferase reporter assay and an *in vivo* transgenic mouse model in which there is a loss of miRNAs in the podocyte.

Specific Aim 2: To define the function of miRNAs in podocytes using complementary *in vitro* and *in vivo* approaches.
The role of specific miRNAs in determining podocyte structure and function will be evaluated using miRNA knockdown in conditionally immortalized podocyte cell lines and through the generation of a transgenic gain-of-function mouse model.

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Other Participating Researchers

Andrew J Bodnar - employed by University of Pittsburgh

Expected Research Outcomes and Benefits

In our previous work, we demonstrated 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 control the complex cellular architecture needed for podocyte function and impact our fundamental understanding of the pathophysiology behind proteinuric kidney diseases. We expect that we will identify novel biologically important miRNA-mRNA interactions in podocytes. Furthermore, we anticipate that this project will provide mechanistic insights into the role of specific miRNAs in regulating the structure, and hence the function, of podocytes using complementary *in vitro* and *in vivo* models.

The project is intended to form the basis for an ongoing research program to define the function of miRNAs in podocytes in healthy individuals and in disease states. Ultimately, this will have significant implications for patients with chronic kidney disease due to 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 initial six month period of funding were to complete a bioinformatics analysis to identify putative miRNA-mRNA target interactions in the podocyte (Specific Aim 1), and to characterize an immortalized podocyte cell line for miRNA expression (Specific Aim 2).

Progress on Specific Aim 1:
We showed in our original application that we could define the expression of miRNAs in the glomerulus using miRNA microarrays, followed by subsequent confirmation with Northern blot and locked nucleic acid (LNA) in situ hybridization. The top twenty glomerular miRNAs as measured by miRNA microarray were selected for the bioinformatics analysis (in descending order: mmu-let7a-e, mmu-let-7c, mmu-let-7b, mmu-miR-143, mmu-miR-23b, mmu-miR-23a, mmu-miR-26a, mmu-miR-126-3p, mmu-miR-30c, mmu-miR-744, mmu-let-7i, mmu-miR-10a, mmu-miR-125a, mmu-miR-214, mmu-miR-24, mmu-miR-30b, mmu-miR-503, mmu-miR-16, mmu-miR-26b, mmu-miR-191). In collaboration with Dr. Priyanka Pandey, potential mRNA

targets were individually analyzed using four publicly available bioinformatics miRNA target prediction tools: TargetScan, miRBase/miRanda, microT and MAMI. Custom-written Perl scripts were used to generate lists of predicted miRNA targets based on their identification by two, three or all four target prediction algorithms. These targets were then mapped against a recently published 'meta-analysis' of previous glomerular transcriptome profiling experiments (He et al, 2008, JASN, 19: 260-268). This bioinformatics analysis identified 85 miRNA-mRNA target pairs that were predicted by all four algorithms and 929 target pairs that were predicted by three or more tools (data not shown).

The list of possible targets was narrowed to a subset of miRNA-mRNA target interactions for further study based on the following characteristics: the bioinformatics scores for the predicted interactions, the predicted and/or known function(s) of the encoded proteins and the expression of the miRNAs in the glomerulus. Initially, we focused on potential targets of mmu-miR-23b, mmu-miR-24 and mmu-miR-26a (Table 1), since we had previously defined their glomerular expression by LNA in situ hybridization. We anticipate that biologically important targets of these miRNAs would also be expressed in glomeruli, and are specifically interested in those that may be implicated in the regulation of podocyte structure and function. Thus, we confirmed the expression of nine out of ten candidate miRNA target genes using reverse-transcriptase polymerase chain reaction (RT-PCR) on total RNA from wild-type mouse glomeruli (Fig. 1). Semaphorin-6D was the only transcript that was not identified in glomeruli. To exclude those transcripts that were expressed by other cell types present in glomeruli such as endothelial or mesangial cells, RT-PCR was performed on total RNA isolated from a conditionally immortalized podocyte cell line that was grown under conditions that would permit differentiation (37^oC) or ongoing replication (33^oC). Both Sema-6D and Mrc-1 were not expressed in the podocyte cell line.

The putative miRNA targets include transcripts that have been implicated in integrin-mediated cell-adhesion (Rap1a), cell-cell junctions (Pkp4), axon branching (Ulk1), collagen synthesis (Plod2), polycystic kidney disease (Tmem2), cytokinesis (Pitpnc1), mesangial cell function (Rap1b), and podocyte injury (Adm). These studies provide support for potential biologically relevant miRNA targets in podocytes. We are currently evaluating these targets using the *NPHS2-cre; Dicer*$^{Flx/Flx}$ mouse model and an *in vitro* luciferase reporter assay.

Progress on Specific Aim 2:

We have obtained a conditionally immortalized murine podocyte cell line that was recently characterized by Dr. Valerie Schumacher at Children's Hospital of Boston, Harvard Medical School. When these cells are grown under nonpermissive conditions (37^oC), the podocytes undergo cell cycle arrest, express numerous podocyte-specific proteins and form cellular processes with an ordered array of actin fibers and microtubules, reminiscent of podocyte foot processes *in vivo*. The expression of glomerular miRNAs in this cell line was confirmed by Northern blot (Figure 2).Total RNA was isolated from podocytes grown at 33^oC and 37^oC, run on a polyacrylamide gel, transferred to a nylon membrane, and hybridized overnight with P^{32}-labeled oligonucleotides complementary to the mature miRNA sequence. Interestingly, expression of all three glomerular miRNAs of interest (mmu-miR-23b, mmu-miR-24 and mmu-miR-26a) was up-regulated following differentiation of the podocyte cell line. Although mmu-let-7c and mmu-miR-10a were also induced in differentiated podocytes, mmu-let-7c is expressed

ubiquitously and mmu-miR-10a in tubular elements of the adult kidney based on our previous LNA in situ hybridization data. Several miRNAs that are expressed in the developing mouse kidney were either not detected by Northern (mmu-miR-335; mmu-miR-30c) or down-regulated in differentiated podocytes (mmu-miR-130a). We are currently optimizing techniques for miRNA knockdown in the podocyte cell line to evaluate the requirement for these miRNAs in podocyte structure *in vitro*.

Table 1: Potential glomerular targets of mmu-miR-23b, mmu-miR-24 and mmu-miR-26a. Targets were predicted using the following bioinformatic algorithms: TargetScan, miRBase/miRanda, microT and MAMI. Predicted targets were mapped against a 'meta-analysis' of previous glomerular transcriptome profiling experiments (He et al, 2008, JASN, 19:260-268). TS, Target Scan; MI, miRBase. The context score refers to the score assigned by TargetScan (the higher the score, the more likely the target represents a true biologic target).

Gene ID	Gene	Expression data	Target Prediction	No of miRNA sites	Context Score	Biological Relevance
RAP1A	small GTPase RAP	Affymetrix	TS, MI	2 23a; 1 24; 1 26a	-0.35	integrin-med cell adhesion
MRC1	mannose receptor, C type 1	EST, GlomChip	TS, MI	3 miR23b	-0.58	dendritic cells (APCs)
MRC1L1	mannose receptor, C type 1-like 1	EST, GlomChip	TS, MI	3 miR23b	-0.58	
ULK1	unc-51-like kinase	Affymetrix	TS, MI	2 miR26a; 1 miR24	-0.49	axon branching
SEMA6D	semaphorin 6D	Affymetrix, RT	TS, MI	2 miR23b; 1 miR26a	-0.41	axon guidance
PLOD2	procoll-lys, 2-oxoglut 5-dioxygen 2	Affymetrix	TS	1 miR24;2 miR26a	-0.37	collagen synthesis
TMEM2	transmembrane protein 2	Affymetrix	TS, MI	1 miR23b; 1miR26a	-0.52	deafness/PKD
PITPNC1	phosphatidylinositol transfer prot	Affymetrix	TS, MI	1 miR23b; 1 miR26a	-0.41	retinal neurons; cytokinesis
RAP1B	small GTPase RAP	EST	TS	1 miR24; 1 miR23b	-0.33	mesangial expression
ADM	adrenomedullin	Affymetrix, T Podo	TS	1 miR26a, 1 miR-24	-0.30	podocyte injury (anti-O)
PKP4	plakophilin 4	SAGE	TS, MI	1 miR23b	-0.50	cell-cell junctions
TOB1	transducer of ERBB2, 1	EST	TS, MI	1 miR26a	-0.49	tumor suppressor
ZDHHC6	zinc finger, DHHC type	SAGE, array	TS, MI	1 miR26a	-0.47	novel
PTP4A2	protein tyrosine phosphatase 4A2	EST; ISH	MI	1 miR23b	-0.37	tumorigenesis
PTPN13	protein tyrosine phosphatase	EST	TS, MI	1 mir26a	-0.35	apoptosis; axon branching
ZNF608	zinc finger 608	Glomchip	TS, MI	1 mIR26a	-0.35	DNA damage
DLG5	discs, large homolog 5	Affymetrix; ISH	TS, MI	1 miR26a	-0.33	cysts in KO; CD
CD200	CD200	GlomChip; Affy; IHC	TS	1 miR26a	-0.32	immune modulation
EPHA2	EPH receptor A2	GlomChip	TS, MI	1 mir26a	-0.30	axon guidance

Figure 1. RT-PCR for putative targets of mmu-miR-23b, mmu-miR-24 and mmu-miR-26a using total RNA isolated from mouse glomeruli (top), undifferentiated podocyte cell line (middle) and differentiated podocyte cell line (bottom). For each sample, a no RT control was run to the left of the transcript of interest. GAPDH was used as the positive control.

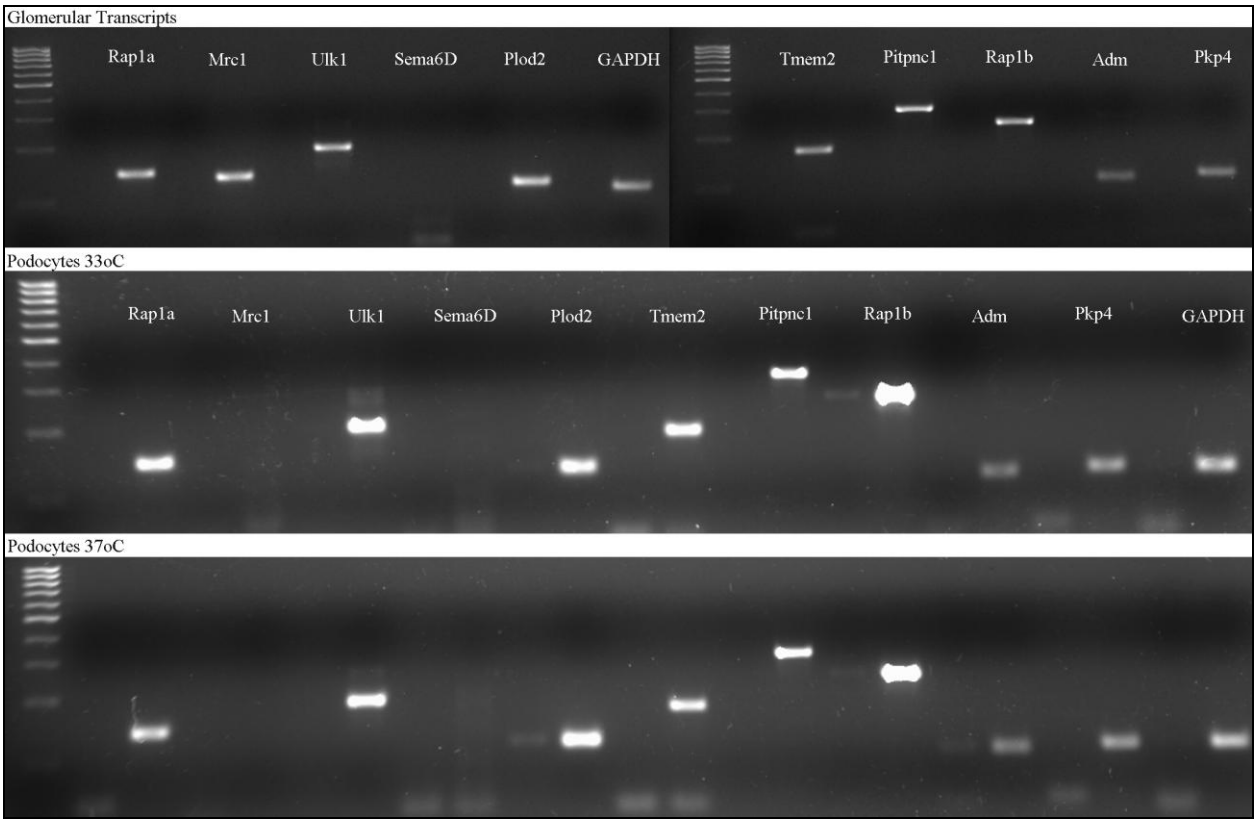

Figure 2: Northern blot analysis for miRNA expression in podocytes grown under permissive (33°C) or nonpermissive conditions (37°C). 5S rRNA was used as a loading control.