Scanning the *C. elegans* genome using microarrays to identify genes that change expression in response to anoxia

Anoxia is the absence of oxygen in an environment. Some use the terms anoxia and hypoxia interchangeably, but the true definition of hypoxia is the depletion of oxygen, whereas anoxia is the complete lack of oxygen in an environment (Hajeria et al. 2005). It has been assumed that periods of anoxia and hypoxia induce a similar pathway that is dependently linked to hypoxia-inducible transcription factor I (HIF-I); however, other evidence shows that anoxia may be independent of HIF-I and that cells under this condition function differently than during a state of hypoxia (Ameri et al. 2004).

Oxygen is vital for most organisms. In humans, the absence of oxygen, the state of anoxia, for over four minutes can result in severe brain damage (Glickman-Simon 2005). Other effects in humans due to oxygen depletion include pulmonary disease and solid tumor progression, just to name a few (Hajeria et al. 2005). On a cellular level, the absence of oxygen can reduce the amount of energy produced by mitochondrial respiration and increase the amount of damaging free radicals available in the cells. This can impact a variety of cellular processes and possibly lead to apoptosis (Nystul and Roth 2004).

Cancer cells and tumors, unlike normal tissues, contain areas of both hypoxia and anoxia (Ameri et al. 2004). Expression of hypoxia-inducible transcription factor (HIF-1) in cancer cells decreases the requirement for oxygen-dependent energy sources, such as ATP (Berg et al. 2002). Analysis of these tumors show an up-regulation in hypoxia-inducible transcription factors (HIFs) (Ameri et a. 2004), which increase expression of glyocolytic enzymes and glucose transporters (Berg et al. 2002). HIFs are thought to contribute to angiogenesis and radiotherapy resistance by inhibiting apoptosis and allowing cells to survive that would normally undergo programmed cell death (Ameri et al. 2004). HIFs and hypoxia response elements have been suggested as possible targets for cancer treatments (Ameri et al. 2004). However, if tumors consist of more severe oxygen depleted areas, then alternative treatment mechanisms would be necessary since cells differ under anoxic and hypoxic conditions (Ameri et al. 2004).

The pathways that respond to anoxia are still unclear; identifying genes that change expression levels in *Caenorhabditis elegans* during an anoxic condition may help the understanding of organismal survival under stressful conditions and narrow our focus to areas

and pathways that contribute to anoxia response. These may yield genes and pathways that may be useful as future targets in cancer treatment.

C. elegans is an attractive model with a quick generation time and about 35% of its genes homologous to that of humans (Genome Sequencing Center). *C. elegans* was the first multicellular organism to have its entire genome sequenced (Consortium TCeGS 1998). Through previous studies, the hypoxia response in *C. elegans* has been recognized to cause suspended animation where cell-cycle progression and development is arrested, but after about 20 minutes of reoxygenation development continues as normal (Hajeri et al. 2005). It is thought that this reversible arrest is caused by highly condensed DNA that is untranscribable (Hajeri et al. 2005) and may be similar in response to anoxia. However, after 24 hours of anoxia arrest is not commonly reversible and the rate of *C. elegans* mortality significantly increases (Van Voorhies and Ward 2000).

I will use microarrays in order to discover genes that are significantly changing expression (up-regulation or down-regulation) in response to anoxia at 1 hour and 24 hours. Microarrays will look at the entire *C. elegans* genome and limit genes of interest. I will further filter and classify these genes and perform confirmation results using RT-PCR. The results will be confirmed and anoxia-responsive genes will be analyzed to begin to identify their role.

Summer Progress:

Throughout the summer of 2006, I analyzed an existing set of microarray data that evaluated the transcriptional expression of genes in *C. elegans* treated with a high intoxicating dose of ethanol (500mM) at 1 and 4 hours. I used the Stanford Microarray Database (SMD) and identified genes that changed expression levels at least 2-fold upon a 1 hour exposure to intoxicating levels of ethanol in *C. elegans*. A list of 81 genes was created, but only three genes were selected for further analysis based on the following criteria: 1. not a heat-shock gene, 2. gene function known or assumed, 3. mutant allele readily available. With all of the criteria applied, the three genes I decided to analyze were *cdr-4* (K01D12.11), *vem-1* (K07E3.8a and K07E3.8b), and F58E6.1.

As mentioned previously, one of the criteria to select these genes was based on the knowledge of their possible functions. *cdr-4* (cadium responsive) is known to be responsive to cadium stress and maybe involved in signal transduction mechanisms (Wormbase; other group).

vem-1 (homologous to mammalian ventral midline antigen) has been observed to be expressed in the neuron AVG and a subset of axons which project into the ventral nerve cord and possibly is involved in cell-cell surface interaction (Runko and Kaprielian 2004; Wormbase). I analyzed two variants of v*em-1*, K07E3.8a and K07E3.8b. The last gene, F58E6.1, may encode a protein that is involved in mRNA turnover and stability and is thought to be involved in apoptosis (Wormbase). All three of these genes were determined to be up-regulated in the microarray analysis, but microarrays are a more broad analysis of the entire genome at the transcriptional level. The results can depend on the purity of the isolated mRNA from the experiment and also the hybridization of the cDNA onto the array. So before asking the question "why are these genes being up-regulated," I had to verify this observation at a more specific level of these selected genes

I attempted to confirm these results through semi-quantative RT-PCR (reverse transcriptase- polymerase chain reaction). RT-PCR helps measure the amount of mRNA expressed in a treatment at a specific time point, similar to the transcription expression measured using microarrays. I treated the worms with the same dose of 500mM ethanol for the same exposure time, 1 hour, and extracted RNA using RNeasy Kit (Quiagen). Then, using the ThermoScript RT-PCR Kit (Invitrogen), I converted the RNA from the control and treated worms into cDNA. The cDNA from both the treated and control treatments were then used to amplify my four genes of interest. However, the RT-PCR was only successful for two out of the four genes, *cdr-4* and *vem-1* (K07E3.8b). The failure of the remaining two genes may have been a result of the designed primers or other PCR conditions. I continued with the verification processes with the two genes for which I had RT-PCR results. I measured the intensity of the bands on the electrophoresis gels using Image-Pro Plus software and tested the assumption that equal amounts of cDNA were loaded into the final PCR reaction using an internal control. I initially used *ama-1* as the standard control in which to compare the amplified cDNA of the RT-PCR results, but it was discovered within three different experiments that *ama-1* was in fact also being up-regulated in the treated cDNA. This sparked our interests; therefore we returned to the microarray data and looked for the presence of *ama-1*. Reanalyzing the intensity spots of *ama-1* in the 1 and 4 hour microarray data sets, it was revealed that *ama-1* was up-regulated in both sets of data. So, the intensity of the cDNA bands were measured to calculate a ratio of treated intensity over control for each of the expression levels of *cdr-4* and *vem-1*. Although this

experiment is not completely accurate without a true control, it was shown that *cdr-4* was increasing expression 2-fold and *vem-1* was increasing expression 4-fold in 1 hour treated cDNA versus control. These experiments will be performed again once I identify a proper internal control by analyzing the microarray data and identifying a gene not changing expression during treatment.

Another goal for the summer was to create a GFP (green fluorescent protein) transgenic worm for future experiments to discover the role of each gene. GFP attached to a gene's promoter region would serve as a visual non-quantifiable aid of specific gene up-regulation. More importantly, it would allow for the location and identification of specific cells or subsets of cells that may express the gene. This identification would further narrow the location for ethanol response and could be applied to other experiments to identify its role. This summer, I was successful in making two GFP constructs ready to inject into *C. elegans* worms. *cdr-4* and *vem-1* (K07E3.8a) were the two of four gene promoters that I successfully fused with the GFP template, but due to limited time the promoter::GFP was not injected into the worms. This will be attempted next summer.

Three mutant strains correlating with my genes of interest were ordered from the *C. elegans* Genetics Center (CGC). These strains were used to look at behavioral assays that may identify mutants with observable behavioral change due to 500mM ethanol in comparison to wild type (N2). We placed ten young adult worms from each strain within copper rings on an unseeded agar plate for each treatment: 0mM and 500mM ethanol. Two minute movies were recorded at 10, 30, and 50 minutes for each treatment. I tracked the worms in each movie to determine a mean speed of each strain at each time point within each treatment using Image-Pro Plus software. We performed two replicates, but the results of each were inconsistent and there was an observable difference in expected and observed wild type (N2) behavior. This suggested that the behavioral assay was not working. These behavioral assays will be attempted again next summer.

During this summer I did not perform a complete analysis with every gene, that is my goal for next summer. I will brainstorm over the academic year and return verify the microarray data and begin asking more questions about the role of each gene in response to ethanol.

Academic Year:

During this upcoming academic year (2006-2007), the goal is to perform microarray experiments and identify genes that are significantly changing expression in response to anoxia. The fall semester will be used to practice RNA isolation and cDNA labeling to prepare for hybridization to microarrays. Throughout this time, protocols will be modified accordingly. Since the microarray experiments can only be performed once, practicing and being certain of high-purity RNA from treated and untreated worms will be necessary. Once all protocols have been mastered, in the spring semester, the microarray experiment will be performed. The experiment will consist of two time points of anoxic treatment as well as controls at both 1 hour and 24 hours. The chips will be sent to Washington University Microarray Core Facility to be analyzed. The data received from the microarrays will be further analyzed to identify any genes with significant change in expression that may play a role in enhancing organismal survival using SAM and/or MAGICTool Software. A few genes may be selected for verification of the microarray analysis results by RT-PCR using ThermoScript™ RT-PCR Systems (Invitrogen).

Plan for Academic Year:

1. Perfect RNA isolation and identify the best method for producing the highest/purest yield
2. Analyzing public microarray data involving hypoxia in *C. elegans*
3. Perform microarray experiment exposing *C. elegans* to anoxic periods at 0 and 24 hours
4. Microarray analysis using SAM and other programs such as MAGIC Tool
5. Confirming microarray results with RT-PCR using ThermoScript RT-PCR Kit (Invitrogen)

Works Cited:

Ameri, K., C. Lewis, M. Raida, H. Sowter, T. Hai, A. Harris. 2004. Anoxic induction ofATF-4 through HIF-1–independent pathways of protein stabilization in human cancer cells. *Blood* 103: 1876-1882.

Berg, J., J. Tymoczko, S. Lubert. 2002. Biochemistry. New York: W. H. Freeman. <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Search&db=books&doptcmdl=GenBookHL&term=oxygen+cancer+tumors+AND+stryer%5Bbook%5D+AND+216179%5Buid%5D&rid=stryer.section.2245#2262/>.

Consortium TCeGS. 1998. Genome sequence of the nematode *Caenorhabditis elegans:* A platform for investation biology. *Science* 282: 2012-2018.

Genome Sequencing Center. GSC: *Caenorhabditis elegans*. <http://genome.wustl.edu/genome/celegans/microarray/ma_gen_info.cgi>.

Hajeria, V., J. Trego, P. Padilla. 2005. Characterization of sub-nuclear changes in Caenorhabditis elegans embryos exposed to brief, intermediate and long-term anoxia to analyze anoxia-induced cell cycle arrest. BMC Cell Biology 6: 47.

Nystul, T., M. Roth. 2004. Carbon monoxide-induced suspended animation protects against hypoxic damage in Caenorhabditis elegans. PNAS 101: 9133-9136.

Richard Glickman-Simon, MD. 2005. Learning about health & illness. Massachusetts General Hospital. <http://healthgate.partners.org/browsing/LearningCenter.asp?fileName=96472.xml&title=>.

Runko, E., Z. Kaprielian. 2004. Caenorhabditis elegans VEM-1, a Novel Membrane Protein, Regulates the Guidance of Ventral Nerve Cord-Associated Axons. The Journal of Neuroscience 24: 9015-9026.

Van Voorhies, W., S. Ward. 2000. Broad oxygen tolerance in the nematode *Caenorhabditis elegans*. *The Journal of Experimental Biology* 203: 2467-2478.

WormBase. <www.wormbase.org>.

Budget:

Item	Company	Catalog Number	Price
ThermoScript™ RT-PCR Systems	Invitrogen	111146-024	$149.99
UltraPure™ Agarose 1000	Invitrogen	10975-035	$217.00
AnaeroJar	Fisher	OXAG0025A	$237.62
Trizol	Invitrogen		
RNeasy Kit			