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Biomarkers: Implications from Discovery and the development of Microscale Electrochemical Sensing Techniques for Their Detection

A Dissertation Presented to
The Academic Faculty

By

David James Bates

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in the
School of Chemical & Materials Engineering

University of Auckland
10 June 2014
Micron-scale Electrochemical Sensing Techniques for the Discovery and Detection of Biomarkers

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SUMMARY

The majority of scientific advancement is facilitated by curiosity, determination, and tools to measure and analyze the subject and environment in question. Discovery at the bio-relevant level of microns and nanometers requires tools with exceptional sensitivity, selectivity, and repeatability. Among the available methodologies, electrochemical sensing techniques occupy a unique place given their well-understood fundamental principles, relatively low cost, and use of electrons and ions to interact with the target in question. Although the principles of electrochemistry are established and their use as a sensing platform is widespread, there is still a great deal of room for innovation in the packaging, fabrication, and application of electrochemical sensing in next generation devices.

The present study reports on questions from two major scientific fields—biochemistry and molecular biology—and addresses them in part by developing and applying a novel sensing approach using an electrochemical setup. The first is with regard to the biochemical mechanisms involved in microbial metal respiration and was carried out in association with the DiChristina Lab at the Georgia Institute of Technology, School of Biology. Metal respiration is a topic of interest because of its role in biogeochemical cycling and it is the basis of some bioremediation strategies for contamination cleanup by the United States Department of Energy.

In the course of this study an in-gel redox protein detection system was developed using scanning electrochemical microscopy. The identified proteins helped to postulate a mechanism for anaerobic dissimilatory iron respiration by \textit{Shewanella oneidensis} MR-1. An additional finding during this study is the identification of a large outer membrane protein that mediates adhesion of the bacterium to iron oxide particles and may localize itself to the cell surface via Type V protein secretion.
The second question, which relates to molecular biology, is with regard to the epigenetic mechanisms associated with aging. This work was carried out in association with the Wang lab at the University of Louisville, School of Medicine. In this study a review of known epigenetic factors that influence aging or age-related disease was conducted. Following that, a long-lived model was used to identify microRNAs that may influence mid-life decline, which occurs in a large portion of a population in their fifties and sixties and is a phenomenon ascribed to the onset of disruptions to physiological homeostasis that snowball into rapid aging and increased incidence of age-related disease.

The major interest in finding epigenetic factors and particularly microRNAs is that the damage is potentially reversible if detected in time. In other words, microRNA profiles provide an instantaneous snapshot of the cell, tissue, or organism’s response to environmental queues and may provide information in time to reverse the damage by quenching the insult before permanent damage sets in. Current methods to detect and validate microRNA activity require both nucleic acid and biochemical assays that are tedious, expensive, and time-consuming. The desire to develop a sensing device that could carry out all of the necessary work on a single, low-cost and rapid platform led to the design and fabrication of the microelectrode array sensor reported here.
ACKNOWLEDGEMENTS

If I have attained anything, it is the product of God’s mercy, timing, endurance and the people
sovereignly placed in my life. For that reason I would like to thank my love, my springtime, my dear
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a creation to study and marvel at.

Professionally, I thank my mentors through the years: Dr. Thomas DiChristina for teaching
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have helped me along the way.

Raised by an ox & a butterfly; educated by a snake & a badger; refined by a fox & a
bear; healed by a man; loved by an angel; led by a Lamb into glory.
**Key Outputs**

**Patent applications**

Arrays and Methods of Manufacture PCT/NZ2013/000183

**Grants Awarded**


**Publications**


**Meeting abstracts and presentations**


**D. J. Bates**, J. Burns, T. DiChristina. Outer membrane-targeted secretion of the Fe(III) respiration-linked c-type cytochrome MtrC by metal-respiring members of the genus Shewanella. 2007. 107th General Meeting of the American Society for Microbiology, Toronto, ON, Canada.
D. J. Bates and T. J. DiChristina. A decaheme c-type cytochrome isolated from an Fe(III) reductase of Shewanella putrefaciens strain 200 displays high homology to MtrC (OmcB)-like c-type cytochromes in other Shewanella genomes. 2006. 106th General Meeting of the American Society for Microbiology, Orlando, FL.


DiChristina, T., M. Adiga, **D. Bates**, J. Burns, and C. Haller. AQDS electron shuttling pathway rescues the Fe(III) and Mn(IV) respiratory deficiencies of Shewanella oneidensis type II protein secretion (gspD) mutants. 2004. 104th General Meeting of the American Society for Microbiology, New Orleans, LA.
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LIST OF ABBREVIATIONS

ADP     Adenosine diphosphate
ARG1    Arginase 1
ASS1    Argininosuccinate synthetase
AT      Autotransporter
ATP     Adenosine triphosphate
BLAST   Basic Local Alignment Search Tool
CBL     Cystathionine β-lyase
CDD     Conserved Domain Database
DNA     Deoxyribonucleic acid
DQ      Duroquinol
DTT     Dithiothreitol
EDTA    Ethylene diamine tetraacetic acid
ELISA   Enzyme Linked Immunosorbent Assay
ETC     Electron transport chain
FHA     Filamentous hemeglutinin
GH      Growth hormone
GOR     Glutathione oxidoreductase
GSH     Glutathione
GST     Glutathione S-transferase
GSTM    Glutathione S-transferase μ1
GTP     Guanosine triphosphate
HRP     Horseradish peroxidase
IAA     Iodoacteamide
IGF-1   Insulin-like growth factor-1
IM      Inner membrane
LT-HRSEM Low-Temperature High-Resolution SEM
mRNA    Messenger RNAs
miRNAs  MicroRNAs
MALDI-TOF Matrix-Assisted Laser Desorption Time-of-
<table>
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<tr>
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<th>Full Form</th>
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<tr>
<td>MCA</td>
<td>Mercaptocarboxylic acids</td>
</tr>
<tr>
<td>MGMT</td>
<td>O(^6)-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MEA</td>
<td>Microelectrode arrays</td>
</tr>
<tr>
<td>β-MP</td>
<td>β-Mercaptopyruvic acid</td>
</tr>
<tr>
<td>MPA</td>
<td>3-Mercaptoproprionic acid</td>
</tr>
<tr>
<td>MRB</td>
<td>Metal-respiring bacteria</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSA</td>
<td>Mercaptosuccinic acid</td>
</tr>
<tr>
<td>MST</td>
<td>β-Mercaptopyruvate sulfotransferase</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>ODC1</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>PA</td>
<td>Polyamines</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data base</td>
</tr>
<tr>
<td>PMF</td>
<td>Proton motive force</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SAM</td>
<td>Surface Area Monolayer</td>
</tr>
<tr>
<td>SECM</td>
<td>Scanning electrochemical microscopy</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBP</td>
<td>Tri-butylphosphine</td>
</tr>
<tr>
<td>TEA</td>
<td>Terminal electron acceptors</td>
</tr>
<tr>
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<td>Thioglycolic acid</td>
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<td>TR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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CHAPTER ONE

Introduction

“Scientists study the world as it is; engineers create the world that has never been.”

The quote attributed to the late aeronautical engineer and physicist, Theodore von Karman, embodies the commonly assumed distinction between two professions. However, there are many who would contend it’s not simply two separate professions, rather it is two sides of the same coin with each enabling the betterment of the other in an iterative fashion—question, solve, discover; repeat. As was the case with von Karman himself, those who possess the principles and drivers of both have the most profound impact on their respective field and ultimately the world as a whole. Consequently, the earth has seen the rise of the engineering sciences (Fung & Tong, 2001) – bioengineering, biochemical engineering, biomedical engineering, molecular engineering, chemical engineering, etc., which naturally gives rise to generations of engineering scientists churning out of universities to help advance society and tackle the challenges of modern life.

The rise of modern scientific understanding itself is the product of curiosity and analytical tools mixed with a good bit of serendipity and pure stubborn effort. It’s often the frustration associated with the curiosity-satisfying endeavor that leads to innovation and further advance. The adoption of a tool or technique from one field is frequently the catalyst needed to advance another field. This dissertation reports two cases of applying in silico modeling and electrochemical sensing techniques to test scientific hypotheses in two different biological fields: the first in the field of microbial biochemistry to produce a device able to detect and study redox-active proteins, the second in medical molecular biology to produce a microelectrode array able to detect nucleic acid biomarkers implicated in aging and age-
related disease. The microelectrode array has since become a novel sensing platform currently in the process of development and commercialization.

**Redox-Active Proteins of Metal Respiring Bacteria**

Microbiology is the study of microscopic organisms and encompasses the sub-disciplines of bacteriology, virology, mycology and parasitology (M.T. Madigan, Martinko, & Stahl, 2012). Unlike the study focus of the other sub-disciplines, only a relatively small subset of bacteria is responsible for disease while the vast majority are beneficial and even required to sustain life as we know it by keeping vital dynamic processes in flux that surprisingly provide the balance enjoyed by the earth’s ecosystems. An example of one of these critical processes is the chemical cycling between aerobic and photosynthetic organisms; the reactions of which are primarily catalyzed by redox-active proteins. Photosynthetic organisms release vital oxygen in the process of carbon fixation—drawing CO$_2$ from the atmosphere and, with the help of energy absorbed from light, producing sugar molecules, also called simple carbohydrates, like glucose and fructose (e.g., $6\text{CO}_2 + 6\text{H}_2\text{O} + \text{hv} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2$) that make up the larger organic compounds called complex carbohydrates such as starch and cellulose. It is important to note that while a great deal of O$_2$ and carbohydrates are produced by plants, the vast majority are produced by cyanobacteria (e.g., blue-green algae) and algae in the ocean.

The products of the photosynthetic process are consumed by animals and other organisms. In fact, photosynthesis is required to maintain atmospheric O$_2$ levels and supplies all of the carbohydrates and most of the energy necessary for life on Earth (Bryant & Frigaard, 2006). Heterotrophic organisms ingest and breakdown complex carbohydrates back to glucose, which is enzymatically broken down during substrate level phosphorylation and, if allowed to go to catabolic completion (i.e., carbon intermediates not selected for use in any
anabolic processes), is exhaled from the organism as CO$_2$. Obligate aerobes and facultative anaerobes utilize O$_2$ as a terminal electron acceptor (TEA) during oxidative phosphorylation and reduce it back to H$_2$O, which is also exhaled from the organism. Both of these processes are aspects of respiration, or the enzymatic breakdown of chemical bonds to extract and repackage energy originating from the sun, into the high-energy phosphodiester bond of adenosine triphosphate (ATP) or some other nucleotide (e.g., GTP) that is readily stored or used by the cell to power metabolic and physiological processes (or as substrate for anabolic processes).

Substrate level phosphorylation, which includes glycolysis and aspects of the Krebs cycle, utilizes enzymes called kinases to produce ATP. Oxidative phosphorylation, the more efficient of the two, utilizes an electron transport chain (ETC) to build up a charge potential called a proton motive force (PMF) in order to drive molecular motors (e.g., ATPases, GTPases), somewhat analogous to the way that a battery is charged and utilized to power an electric motor. Generation of the PMF requires the safe removal of electrons from the system, which is carried out by redox-active molecules (e.g., NADH, FADH$_2$, quinones, etc.) and enzymes (e.g., dehydrogenases, oxidases, cytochromes, reductases, etc.) in a stepwise fashion that terminates with an electron acceptor (O$_2$) whose reduced product (H$_2$O) carries the electron away. Although aerobic respiration is the reverse of the photosynthetic equation and can therefore be considered a combustion reaction, it is made up of several small redox reactions and thus does not behave like a traditional combustion reaction. Photosynthesis is an anabolic process and respiration is catabolic, each enabling the other to maintain the healthy flux of substrates and products and is a major aspect of carbon cycling (Figure 1.1).
Figure 1.1 Simplified diagram of the dynamic flux sustained between photosynthesis and aerobic respiration. In higher life forms, organelles house the majority of the redox-active proteins and molecules – chloroplasts in photosynthesis and mitochondrion in aerobic respiration.

Other aspects of the carbon cycle occur in the absence of oxygen, one such example is the biogeochemical cycling of minerals. Like aerobic respiration, anaerobic respiration occurs to produce ATP for the organism, which is accomplished most efficiency by generating a PMF across a membrane to drive ATPases. The main difference here is that electrons taken from the catabolic breakdown of the covalent bonds of carbohydrates are dumped onto a transition metal as the TEA instead of oxygen. These reactions are primarily carried out by microorganisms because the electropotentials of these TEAs are insufficient to enable the energy production levels required to sustain higher life forms. Marvelously, microbial respiration of minerals enables the storage, transport, and bioavailability of minerals for anabolic processes in other organisms. Microbial respiration of minerals is a focus of this work and the study of which is the reason behind designing the Respirasomics Reader reported in Chapter 2. The biogeochemical reactions catalyzed by metal-respiring bacteria
(MRB) significantly impact the geochemical and mineralogical reactions of anoxic sediments and redox-stratified aqueous environments. All bio-relevant elements undergo bacterial and chemical cycling (e.g., sulfur and iron in Figure 1.2). As respiration of metals is coupled to the oxidation of organic carbon compounds, MRB also significantly impact the global carbon cycle.

**Figure 1.2** Redox cycles of sulfur (left) and iron (right). Oxidations are shown by yellow outline and reductions by red outline. The oxidation of ferrous iron is carried out aerobically by chemolithotrophs and anaerobically by certain anoxygenic phototrophic bacteria and denitrifying bacteria. (Figure adapted from Madigan, M. et al Brock Biology of Microorganisms 13th Edition p. 705, 10th Edition p. 666).

MRB couple the oxidation of hydrogen or organic carbon to 1) reductive dissolution of solid phase Fe(III) and Mn(IV) and 2) reductive precipitation of relatively soluble (and hence mobile) toxic metals and radionuclides such as Cr(VI), U(VI), and Tc(VII). The second activity is the basis of alternate remediation strategies at radionuclide-contaminated United States Department of Energy (DOE) waste sites. Reductive dissolution by MRB occurs when TEAs exist in a crystalline form or an amorphous (oxy)hydroxide particle presumably unable to be internalized by the cell or contact terminal reductases typically residing in the inner
membrane (IM). To overcome this physiological problem, terminal metal reductases are secreted to the outer membrane (OM) of the cell where they receive electrons from the periplasm through a transmembrane conduit, and ultimately transfer the electrons to the extracellular TEA. Localization of the proteins to the OM is accomplished by at least one of the five known protein secretion systems in Gram-negative bacteria (DiChristina, Moore, & Haller, 2002). The facultative anaerobe, *Shewanella oneidensis* was used as a model in this study. Genome-wide scans of the *Shewanella oneidensis* MR-1 genome reveals genes that encode for three out of the five known secretion systems of Gram-negative bacteria; Type II, Type I, and, as reported in Chapter 2 of this dissertation, Type V protein secretion.

*S. oneidensis* MR-1 is an attractive model system for determining the molecular mechanism of metal oxide respiration. Unlike other metal reducing bacteria (i.e., obligate anaerobes), O₂ is not toxic to the cell and genetic manipulations may be carried out under aerobic conditions. In addition, the availability of the *S. oneidensis* MR-1 genome sequence facilitates the proteomic and genetic approaches outlined in this dissertation (Heidelberg et al., 2002). *S. oneidensis* respires on a suite of TEAs that virtually spans the entire spectrum of reduction/oxidation (redox) potentials found in natural systems. The *S. oneidensis* respiratory system consists of a multifaceted ETC with many branch-points at which electrons are funneled toward TEAs of similar chemical composition with selectivity based on the highest available electro-potentials (Eₜₐ) (DiChristina & DeLong, 1994; C. R. Myers & Nealson, 1988b; Nealson, Belz, & McKee, 2002; Nealson & Saffarini, 1994; Venkateswaran et al., 1999).

Identifying redox-active secreted proteins (secretagogues) is an arduous endeavor. Utilizing secretion system mutants (i.e., mutant strains whose genetic capacity to employ a functional secretion system has been nullified), narrows the field of targets; however, identifying specific secretagogues involved in electron transport and to which redox
substrate/product they act upon remains a challenge. Although traditional chemical assays have been developed to help identify some redox reactions, to our knowledge a universal tool does not exist that is able to scan for and identify redox active proteins. Reported herein is the application of scanning electrochemical microscopy to enable the in-gel detection of redox-active proteins and is coupled with Matrix-Assisted Laser Desorption Time-of-Flight (MALDI-TOF) tandem mass spectrometry (MS/MS) to reveal the identities of the detected proteins.

To date, scientists have made significant strides in identifying redox proteins believed to be involved in iron respiration and proposing electron transport mechanisms; however, a complete mechanistic understanding is lacking, particularly with regard to supramolecular complexation of proteins and their resultant functions including both mechanism of attachment and final electron transfer to iron oxides. By utilizing existing biochemical and *in silico* methods and adapting electrochemical methodology, Chapter 2 of this dissertation: 1) reports on a novel method to detect redox-active protein complexes; 2) reveals the identity of the protein constituents of those complexes; and, 3) postulates a mechanism of attachment and final step of electron transfer to iron oxides. Taken together a better understanding of the potential mechanism of bacterial metal reduction, specifically dissimilatory iron respiration of *Shewanella oneidensis* MR-1, is presented.

**Epigenetic Biomarkers for Age-related Disease Detection**

Advances in medicine also derive from the ongoing process of discovery and innovation. Each iteration opens the door for better understanding of disease pathologies and new ways to treat, prevent and cure them. Oftentimes, the key to disease prevention and/or optimal intervention is early and accurate detection. Such detection is facilitated through diagnostic devices that result from advances in understanding and enabled by tools developed to answer questions following previous discoveries. Accordingly, for the continued advance
of medical knowledge and treatment of disease, a sustained effort must be made on the
development of tools that can faithfully detect and identify threats or aberrancies and offer
insight into the best corrective means. Furthermore, through mindful engineering, these
devices must not only perform well, but must be accessible (i.e., low cost and easily
distributed) and easy to use in order to have the impact and benefit for all of humanity. The
research presented herein displays the process of scientific questions addressed and
engineering principles applied to improve the detection of biomarkers through the design and
development of a diagnostic device that aims to detect the onset of aging and age-related
disease.

**Figure 1.3** Graphic showing types of pre-transcriptional epigenetic modification (left), which
is involved in regulating gene transcription or replication, and post-transcriptional epigenetic
modification, which is involved in regulating gene expression (i.e., protein translation and
copy number).

In 1999, Peter A. Jones and Peter W. Laird suggested that Knudson’s two-hit
hypothesis for cancer should be expanded to include the epigenetic mechanisms of gene
inactivation (P. A. Jones & Laird, 1999). Since that time several studies have reported that epigenetic events do indeed play a major role in the formation of certain cancers (Ando et al., 2008; Shen et al., 2005; Toyota et al., 2008; Ushijima, 2007) and many other age-related diseases (Bates, Liang, Li, & Wang, 2009). Epigenetic events, a term referring mainly to DNA methylation and histone modification events but includes noncoding RNAs, refers to a variety of heritable (i.e., maintained in cellular replication) events that are involved in genetic regulation but that do not alter the DNA sequence. The term field defect, or, field cancerization is an area of abnormal tissue that—although polyclonal and nearly void of monoclonal lesions—is predisposed to or precedes the development of cancer (Shen et al., 2005; Ushijima, 2007). The term field cancerization was first used by Slaughter et al. to describe presence of oral mucosae that are predisposed to cancer development (Slaughter, Southwick, & Smejkal, 1953).

The epigenetic field defect is a term used to describe precancerous tissues that exhibit epigenetic alterations. These cells have also been found adjacent to certain types of cancer (e.g., liver, colon, Barrett’s esophageal, lung, breast, and renal cancers) (Ushijima, 2007). Important to this study, epigenetic events are affected by intrinsic factors (e.g., sex, genetics, aging, endogenous compounds, etc.) and environmental factors (e.g., diet, smoking, alcohol, carcinogens, etc.) and are faithfully replicated during somatic cell replication (Ushijima, 2007). Consequently, with early detection, interventional strategies (e.g., medicinal, dietary and/or lifestyle) may be put in place to try and reverse the effects to potentially prevent or delay the onset of clinical disease.

In cancer cells, epigenetic signatures such as “genome-overall hypomethylation and regional hypermethylation” are almost always present (Ushijima, 2007). Hypomethylation of large regions that are normally methylated can lead to genomic instability and the upregulation of oncogenes as well as the deregulation of noncoding regulatory RNAs (e.g.,
small RNAs). Hypermethylation of certain promoter regions silence necessary genes such as tumor suppressors, microRNAs, and DNA repair genes (Ushijima, 2007). For example in colorectal cancer (CRC), the DNA mismatch repair gene $O^6$-methylguanine-DNA methyltransferase (MGMT) is often found methylated (Shen et al., 2005). A study by Shen et al found that 46% of the CRCs in their sample population displayed hypermethylation of the MGMT promoter region ($hMGMT$), which has been shown to inactivate gene expression that usually results in many unrepaired DNA mutations (Shen et al., 2005). In addition, the majority of the apparently normal tissues adjacent to the $hMGMT$-containing tumors exhibited an hMGMT epigenetic field defect as well (Shen et al., 2005). Epigenetic silencing via promoter hypermethylation has also been reported with the tumor suppressor gene $p16^{INK4A}$, and DNA mismatch repair component, $hMLH1$. The cyclin-dependent kinase inhibitor, $p16^{INK4A}$, is critical in keeping the retinoblastoma (Rb) protein in its active, unphosphorylated state to maintain control of the cell cycle, which is lost in nearly all tumors via disrupted $p16^{INK4A}$ function or mutations of $Rb$ (Baylin & Herman, 2000). Although loss of $p16^{INK4A}$ function can occur through different mechanisms including deletion, point mutation, or promoter hypermethylation, $p16^{INK4A}$ inactivation in CRC is seen only by promoter hypermethylation (Baylin & Herman, 2000). In addition to posttranscriptional epigenetic aberrancies, post-transcriptional epigenetic factors like miRNAs and their dysregulation are known to contribute to age-related disease (Bates et al., 2009). Chapter 3 of this dissertation reviews the roles of microRNA in different aspects of age-related disease and holistically as contributors to aging.

MicroRNAs (miRNAs) are short (19 – 22 nucleotides), noncoding RNAs that play critical roles in post-transcriptional regulation of cellular gene expression (Ambros, 2004a; Bartel, 2004). Regulation via miRNA ranges from development to apoptosis and aging (Bates et al., 2010; E. Wang, 2007); with widespread expression pattern alterations known to
correlate with cancer incidence (Bates et al., 2009). Mature miRNAs function by associating with the RNA-induced silencing complex (RISC) and either i) inhibiting protein translation via incomplete base-pairing of the 3’ untranslated regions (3’-UTR) of target messenger RNA (mRNA) or ii) signaling for degradation of target mRNA via complete homologous binding to the 3’UTR. In either case, post-transcriptional regulation is achieved via silencing the translation of mRNA. The versatility offered by the two mechanisms enables both the targeting of single genes by multiple miRNAs—achieving an additive or fine tuning effect and individual miRNAs can target multiple genes, which contributes to the dynamic redundancies observed in genetic regulation. Any aberrancy in this regulation brings in the potential to set off a cascading effect that increases the propensity and incidence of age-related disease. Reported herein is a review on the known relations of miRNA to age-related disease states and a study evaluating miRNA expression profiles in long-lived Ames Dwarf mouse models versus their wild-type (normal life-expectancy) siblings.

The Ames Dwarf mouse has emerged as an attractive candidate for study in the search for factors that control or affect the process of aging. Although the current knowledge falls short of a complete understanding, many advances have been made as to the role of the endocrine system’s contribution to the process of aging. The Ames dwarf mouse, which is able to live up to 70% longer than its wild-type counterpart, lacks three pituitary hormones (growth hormone, prolactin, and thyrotropin) because of a point mutation affecting a gene (Prop1) whose product is intricately involved in the development of particular cell types in the anterior pituitary gland (Ambros, 2004a; Andersen et al., 1995). Different expression levels of growth hormone (GH) have been shown to directly affect insulin sensitivity (Davidson, 1987; F. P. Dominici, Arostegui Diaz, Bartke, Kopchick, & Turyn, 2000; F. P. Dominici, Hauck, Argentino, Bartke, & Turyn, 2002), polyamine synthesis (A. Gritli-Linde, Bjorkman, Holm, Tornell, & Linde, 1997; D. H. Russell & Snyder, 1969; D. H. Russell,
Snyder, & Medina, 1970; R. K. Sogani, Matsushita, Mueller, & Raben, 1972), and stress resistance (H. M. Brown-Borg, Rakoczy, & Uthus, 2005; S. Murakami, Salmon, & Miller, 2003; A. B. Salmon et al., 2005). In addition, the role of tissue-specific miRNAs in the regulation of endocrine-related functions such as insulin secretion has been demonstrated (Poy et al., 2004). Recent studies establish that the GH/IGF-1/insulin signaling pathway plays a direct role in the rate of aging (V. D. Longo & Finch, 2003; M. Tatar, Bartke, & Antebi, 2003). It is well known in humans that the decline in production of hormones like estrogen and testosterone goes hand in hand with aging. In like manner, GH and insulin-like growth factor-1 (IGF-1) decline with aging (beginning around 30 in humans) and decreased levels of these hormones have been strongly implicated in the physical changes that are associated with aging, such as decreased muscle mass and strength, increased fat mass, and decreased skin thickness (H. M. Brown-Borg, Rakoczy, & Uthus, 2004; Rosen, 2000).

Growth hormone administration has been shown to increase levels of free glutathione (GSH); however, it suppresses levels of the detoxification enzyme, glutathione S-transferase (GST) in the kidneys and liver of Ames dwarf mice, thus reducing the utilization of GSH for toxin removal (H. M. Brown-Borg et al., 2004). Increases in GH have also been linked to increased levels of ornithine decarboxylase (ODC) and polyamines (PA) (A. Gritli-Linde et al., 1997; J. L. Kostyo, 1966; D. H. Russell & Snyder, 1969; D. H. Russell et al., 1970). Imbalances in ornithine metabolism have been linked to a suite of carcinomas, hepatomas, papillomas, and tumorigenesis (Manteuffel-Cymborowska, Chmurzynska, Peska, & Grzelakowska-Sztabert, 1995). Many studies have tried to duplicate the dwarf mouse’s aging phenotype using methods such as targeted gene deletions, caloric restriction, or hormone therapy with promising results (Amirbahman, Sigg, & Gunten, 1997; H. M. Brown-Borg, 2006; H. M. Brown-Borg, Rakoczy, Sharma, & Bartke, 2009; H. M. Brown-Borg et al., 2004; Miller et al., 2002; D. H. Russell & Snyder, 1969; D. H. Russell et al., 1970); however, none
have yet definitively matched or fully explained the dwarf’s uncanny ability to resist normal aging. Furthermore, to our knowledge, biomarkers that are widely accepted as an indicator for inception of mid-life decline as defined in Chapter 3, do not exist. Using the dwarf mouse as a model, Chapter 4 of this dissertation seeks to identify microRNA markers in the mammalian liver that could be used as potential biomarkers for the onset of aging and age-related disease.

**Electrochemical Techniques Used in the Detection of Biomarkers**

Electrochemistry is a branch of chemistry that studies chemical species in solution by measuring and qualifying their electrical interaction with the surface of a conducting electrode. Although the documented study of electricity began as early the 16th Century, it wasn’t until the late 18th Century that Luigi Galvani established the bridge between electricity and chemistry in his essay, "*De Viribus Electricitatis in Motu Musculari Commentarius*" in 1791 (Norris & Ribbons, 1971). Interestingly, Galvani used a biological model to demonstrate this relationship. Many advances were made in the field through the 19th century but it wasn’t until 1898 that Fritz Haber reported the reduction of nitrobenzene in stages by using an electrochemical setup to show that definite reduction products can result from electrolytic processes if the cathode potential is held constant (NobelFoundation, 1966). Four years later, the Electrochemical Society was founded.

In the simplest terms, electrochemistry is the redox (reduction-oxidation) process that occurs when an electron is transferred to or from a molecule or ion. This transfer occurs naturally when two candidate ions (or molecules) are close enough in space and have a large enough spread between their electropotentials (E<sub>th</sub>). Following the transfer of the electron, the E<sub>th</sub> of each species changes with the reduced species (the receiver of the electron) increasing in negative potential and the oxidized (the electron donor) species’ E<sub>th</sub> shifting more positive.
Also changed is the oxidation state each with a -1 change to the reduced species and +1 change to the oxidized species. The change in oxidation state can have a drastic effect on the physical properties of the compound; hence, the premise of the bioremediation strategy for MRBs touched on above and described further in Chapter 2.

This redox event can also be coerced by applying an external potential as is the case with an electrochemical setup. In brief, an electrochemical setup consists of a working electrode at which electrical interactions are measured, a counter electrode to complete the circuit (i.e., hold the potential), and a reference electrode, which serves as a reference to standardize measurements across electrode potentials and therefore useful to identify the different redox species (Figure 1.4). A potentiostat and corresponding software is used to supply potential and current and measure the electrochemical feedback. This setup is powerful for measuring chemical species in bulk solution; however, it wasn’t until around the turn of this century did electrodes become microelectrodes and nanoelectrodes, which greatly enhanced the resolution of measurements and thereby enabled the study of biological interactions at the molecular level. Among other things, this increased granularity gave premise to explore the idea of using a microelectrode array for biomarker detection as reported in Chapter 5.
Current methods used to detect miRNAs or DNA methylation are laborious, time consuming and require dedicated equipment and highly trained technicians to operate. Microelectrode arrays (MEA) may be considered the next generation of microarray and ELISA technology. These technologies primarily utilize target-probe interactions (e.g., DNA-DNA hybridization, antigen-antibody binding) as means for detection; however, microarrays require dedicated equipment with costly time- and labor-intensive PCR amplification and labeling, whereas many MEAs do not (Kukol, Li, Estrela, Ko-Ferrigno, & Migliorato, 2008). ELISA’s are limited in sensitivity and true multiplexing. As reported in Chapter 4 of this study, microRNA arrays are a useful tool to evaluate expression profiles; however, they are subject to the aforementioned constraints which make their utilization as a major diagnostic tool impractical. The main hurdle stems from the construction of a cDNA library and qualitative reverse transcriptase polymerase chain reaction (qRT-PCR), which are both
necessary to assess relative abundance of particular microRNAs in a sample. Furthermore, detection of methylation state has traditionally been done through bisulfite sequencing, which is time consuming and requires expensive, specialized equipment.

Recently, electrochemical sensors have been used to detect and quantify methylation states of DNA (Goto et al., 2010). MEAs taking advantage of microfluidics can provide a field-ready diagnostic that is both robust and highly accurate in performing sample analysis. In addition, the electrochemical platform enables a variety of different techniques to be utilized for measurement (e.g., impedance, resistance, current, potential, linear sweep or cyclic voltammetry, etc.) depending on what is best for the application. To date MEAs have been reported using both Faradaic (Kukol et al., 2008) and non-Faradaic (Yusof, Yanagimoto, Uno, & Nakazato, 2011) electrical impedance spectroscopy (EIS) (Pettit, Goonetilleke, Sulyma, & Roy, 2006), coulostatic pulse technique (Dharuman et al., 2006), as well as a suite of different voltammetries including cyclic (Heinze, 1984), square wave, and linear sweep. The final goal of this study is reported in Chapter 5 and describes the design and fabrication of a next generation microarray that does not require extensive sample preparation and labeling. This low cost device utilizes electrochemical impedance spectroscopy to detect biomarkers in solution using a three-dimensional, micron-scale microelectrode array made of plastic.
REFERENCES


CHAPTER TWO

*Molecular mechanism of metal respiration by Shewanella oneidensis MR-1*

Metal-respiring bacteria play a central role in a variety of globally significant processes, including the biogeochemical cycling of metals, weathering of clays, biomineralization of metal-bearing molecules, and remediation of toxic xenobiotic compounds. The molecular mechanism of bacterial metal reduction, however, is poorly understood. Terminal electron acceptors (TEAs) such as iron (Fe(III)) or manganese (Mn(IV)) oxides are often found in crystalline form or as amorphous (oxy)hydroxide particles presumably unable to contact inner membrane (IM)-localized electron transport systems. To overcome this physiological problem, metal-respiring bacteria are postulated to localize terminal reductases on the outer membrane (OM) of the cell. Protein complexes secreted to the surface of gram-negative bacteria are postulated to function as essential mediators between the cell and its environment, including the import and transport of essential nutrients, the export of waste products, adhesion to host cells or solid surfaces, and the removal of toxic bi-products. DiChristina et al has recently discovered that protein complexes on the bacterial OM may also be involved in anaerobic respiration on metal oxides. One or more polypeptides of these complexes are secreted to the OM where they catalyze the terminal step of electron transfer. The main goal of this portion of my PhD research was to determine the proteins involved in the terminal step of metal oxide respiration by *Shewanella oneidensis* MR-1 via conventional biochemical methods and a novel electrochemical detection system. Identification of the proteins involved in metal-reduction is essential to understanding the pathways for reductive precipitation of heavy metals, an alternate remediation strategy for contaminated United States Department of Energy (DOE) waste sites. Despite the environmental importance of microbial metal respiration, the proteins that
transfer electrons to Fe(III) and Mn(IV) oxides and the mechanisms of electron transfer have not been definitively identified. Research on the molecular mechanism of metal-reduction by *S. oneidensis* was driven by three main hypotheses:

**HYPOTHESIS 1:** Respiratory supercomplexes that span the OM facilitate metal oxide-reduction by utilizing iron-sulfur redox chemistry in parallel with direct electron transfer to chelated-(solubilized) Fe(III).

**HYPOTHESIS 2:** Redox active components on the OM can be detected via SECM in native polyacrylamide gels.

**HYPOTHESIS 3:** Attachment to metal oxide terminal electron acceptors is facilitated by cell secreted proteins.

The experimental procedures, results and discussions will be presented in detail for the three separate hypotheses in the following sections.
HYPOTHESIS 1

Respiratory supercomplexes that span the OM facilitate metal oxide-reduction by utilizing iron-sulfur redox chemistry in parallel with direct electron transfer to chelated-(solubilized) Fe(III).

INTRODUCTION

Molecular mechanism of bacterial metal respiration.

Compared to the wealth of knowledge on the molecular basis of other bacterial respiratory processes (e.g., aerobic respiration, denitrification, sulfate reduction, methanogenesis) (Michael T. Madigan, Martinko, & Parker, 2003), little is known about the molecular details of bacterial metal respiration. With respect to respiration on extra-cellular solid metal oxides, four strategies have been postulated (DiChristina, Fredrickson, & Zachara, 2005; Lovley, Holmes, & Nevin, 2004). The proposed strategies include 1) direct enzymatic reduction of solid Fe(III) and Mn(IV) oxides via OM-localized metal reductases, 2) a two-step, electron shuttling pathway in which exogenous electron shuttling compounds (e.g., humic acids, melanin, phenazines, antibiotics, AQDS) are first microbially reduced and subsequently chemically oxidized by the solid Fe(III) and Mn(IV) oxides in a second (abiotic) electron transfer reaction, 3) an analogous two-step reduction pathway involving endogenous, electron shuttling compounds, and 4) a two-step, Fe(III) solubilization-reduction pathway in which solid Fe(III) oxides are first non-reductively dissolved by bacterially-produced organic ligands, followed by uptake and reduction of the soluble organic Fe(III) forms by periplasmic or OM-localized Fe(III) reductases (DiChristina et al., 2005). Although over 100 metal-reducing bacterial species have been cultivated, the mechanism of electron transfer is best studied in metal-respiring members of the genera Geobacter and Shewanella.
Electron transport coupled to proton translocation begins with oxidation of a variety of electron donors (e.g., H₂, NADH) and transfer of electrons to a quinone pool (C. R. Myers & Myers, 1993b; C. R. Myers & Nealson, 1990; Saffarini, Blumerman, & Mansoorabadi, 2002). Reduced quinone (quinol) diffuses within the inner membrane (IM) to the quinol oxidation site of a 21-kDa tetraheme cytochrome c, designated CymA (C. R. Myers & Myers, 1997; J. M. Myers & Myers, 2000; Schwalb, Chapman, & Reid, 2003). Electrons are then transferred from CymA to a number of periplasmic cytochromes responsible for shuttling electrons across the periplasmic space to a 32-kDa periplasmic decaheme cytochrome, MtrA. 

*mtr*A is part of the *mtr* operon whose gene products are required for respiration on Fe(III) and Mn(IV) oxides (Leang, Coppi, & Lovley, 2003; C. R. Myers & Myers, 2002; C. R. Myers & J. M. Myers, 2003; Pitts et al., 2003). Electrons are then conducted through an as yet unidentified transmembrane conduit to the outer membrane (OM), most likely via other gene products of the *mtrDEF-omcA-mtrCAB* gene cluster (Figure 2.1).
Figure 2.1 Graphical representation of the postulated electron transport chain in Shewanella oneidensis MR-1. The ETC begins with oxidation of NADH (or H2), reduction of menaquinone to menaquinol, oxidation of menaquinol by CymA, a series of electron transfers via soluble periplasmic c-type cytochromes to MtrA, which then translocates electrons to the OM by some unknown mechanism. It is believed that OmcA and MtrC are secreted to the OM via Type II secretion and are directly involved in electron transfer to metal oxides. The exact mechanism of the final electron transfer to the oxide is unknown.

OM proteins involved in the terminal steps of electron transfer to solid Fe(III) or Mn(IV) oxides have not been definitively identified; however, several c-type cytochromes are possible candidates (Lovley et al., 2004). Fe(III) reduction activity is detected in wild-type S. oneidensis OM fractions (DiChristina et al., 2002; C. R. Myers & Myers, 1993a; Shi et al., 2006), an activity that is severely impaired in S. oneidensis mutants lacking OM proteins, including any of the several multi-heme and transmembrane proteins translated from the mtrDEF-omcA-mtrCAB gene cluster. The S. oneidensis genome encodes 42 predicted c-type cytochromes (Heidelberg et al., 2002), 5 of which are encoded in the mtr gene cluster. MtrB is a putative beta-barrel protein postulated to be involved in OM localization of the decaheme
c-type cytochromes OmcA and MtrC (Beliaev & Saffarini, 1998; C. R. Myers & Myers, 2002). mtrB mutants are completely abolished in Mn(IV) reduction activity and are severely impaired in Fe(III) reduction activity, yet retain the ability to reduce all other electron acceptors (Beliaev & Saffarini, 1998). MtrB contains, however, a CXXC motif that may function as a metal or heme-binding site; alternatively, this motif may participate in redox reactions via thiol/disulfide chemistry. MtrB may therefore play a direct role in electron transport. MtrC is an OM decaheme, c-type cytochrome required for both Fe(III) and Mn(IV) reduction activity. The MtrC homolog, MtrF, is translated from the same gene cluster in the S. oneidensis genome. The OM decaheme, c-type cytochrome OmcA is also required for electron transport to Mn(IV) (and not Fe(III)) (J. M. Myers & Myers, 2001). omcA-deficient mutants reduce Mn(IV) at 45% wild-type rates. Interestingly, mtrC over expression in an omcA-deficient mutant restores Mn(IV) reduction activity to greater than wild-type rates, an indication that the functional roles of MtrC and OmcA at least partially overlap in the Mn(IV) reduction pathway (J. M. Myers & C. R. Myers, 2003). MtrC and OmcA form a high-affinity protein complex (K_d < 500 nM). The Fe(III) reductase activity of the MtrC/OmcA Fe(III)-nitrotriacetic acid (NTA) is greater than the sum of the activities of the individual proteins (Shi et al., 2006) suggesting a definite advantage for complexation. Purified OmcA displays reduction activity on solid Fe(III) (hematite) (Xiong et al., 2006), however, such potential has not been established with MtrC.
Although the exact functions of MtrC, OmcA, and MtrB in Fe(III) reduction remain unclear, localization of these proteins to the OM is accomplished via one or more of the five known protein secretion systems in gram-negative bacteria. Genome-wide scans of the *S. oneidensis* MR-1 genome reveals genes that encode for three out of the five secretion systems (Types I, II, and V, Figure 2.2). Type I secretion consists of a three protein complex, in which one of the proteins (TolC) spans the inner and outer membrane to deliver proteins extracellularly directly from the cytoplasm. Type V secretion consists of a single or pair of proteins that transports itself or an exoprotein across the periplasmic space to the OM, and is termed an autotransporter (e.g BrkA in *B. pertussis*) or two partner secretion (Filamenntus hemagglutinin (FHA) from *B. perussis*), respectively. Type V proteins are translocated across the IM via the Sec secretion pathway, as are proteins that are secreted via Type II. Type II secretion consists of a complex multimeric (15 protein) structure that requires coordinated assembly to span the periplasmic space and facilitate exoprotein secretion through an OM.
porin. GspD functions as the OM porin of Type II protein secretion, and results in the laboratory of DiChristina et. al. demonstrate that GspD is required for metal oxide reduction by *S. putrefaciens* (DiChristina et al., 2002). A contiguous cluster of 12 Type II protein secretion genes (*gspC-N* homologs) has also been identified in the *S. oneidensis* MR-1 genome (Heidelberg et al., 2002). A Type II protein secretion mutant of *S. oneidensis* (constructed by targeted deletion of wild-type *gspD*; Justin Burns, Ph.D thesis http://hdl.handle.net /1853/33825), is unable to respire anaerobically on solid Fe(III) or Mn(IV) oxides, yet retains the ability to respire all other electron acceptors, including soluble Fe(III) and AQDS (DiChristina, Adiga, Bates, Burns, & Haller, 2007 submitted). Similar to the Fe(III) respiration-deficient *gspE* insertional mutant of *S. putrefaciens* (DiChristina et al., 2002) a heme-containing Fe(III) reductase (or reductase complex) is present in the proteins peripherally attached to the outside face of the wild-type OM, yet is missing from this location in the Δ*gspD* mutant (Figure 2.3). These results suggest that Type II protein secretion is required for direct enzymatic reduction of solid Fe(III) by all metal-reducing members of the genus *Shewanella* and suggests that other Gram-negative secretion systems could be involved in metal oxide respiration of solid metal oxides. Two-dimensional gel electrophoresis (2DGE comparisons demonstrate that many peripherally attached OM proteins are still localized in the Δ*gspD* mutant (Figure 2.2). Interestingly however, Low-Temperature High-Resolution Scanning Electron Microscopy (LT-HRSEM) images show a lack of OM “nodules” in the secretion mutant (Figure 2.4), which could suggest inability of proper aggregation (i.e., supercomplexation) of proteins. In addition, many pathogenic species of Gram-negative bacteria, functionally couple Type V secreted adhesins to toxins or degradative enzymes to facilitate invasion of eukaryotic cells (Farizo, Huang, & Burns, 2000). *Bordetella pertussis* infection of mammalian trachea for example involves Type I secretion of adenylate cyclase onto the host cell, Type IV secretion of the pertussis toxin into
the extracellular milieu, and Type V secretion of tracheal colonization factor, FHA, and serum resistance protein (BrkA) to the OM. Mutations resulting in loss of function of any of these proteins or secretion systems results in mutants that display activities ranging from a 10-fold loss of infection (R. C. Fernandez & Weiss, 1994) to total loss of virulence (Babu, Bhargavi, Sing Saund, & Saund, 2001; Todar, 2004).

**Figure 2.3.** A. Native PAGE gel after Ferrozine assay showing Fe(III) reductase activity in wild-type but not in mutant OM wash (left) matched with a heme stain of the same gel (right) revealing a c-type cytochrome present in the wild-type reductase but missing in the mutant. B. 2-D GE (12% linear gradient, pl=3-12) of peripheral proteins of wild-type *S. oneidensis*
MR-1 (blue) and type II secretion mutant gspD (red). Proteins common to both are green. (Figure also used in a grant application submitted to the US DOE).

**S. oneidensis MR-1**  
S. oneidensis MR-1::ΔgspD

**Figure 2.4.** LT-HRSEIM images comparing wild-type *S. oneidensis* MR-1 to the type II secretion mutant ∆gspD, which is unable to respire on iron or manganese oxides. The most outstanding feature is the absence of nodules that are present on the wild-type cell.

**MATERIALS & METHODS**

**Chemicals**

All chemicals were purchased from Fischer Scientific (Waltham, MA), unless specified otherwise.

**Protein Harvesting**

Optimal OM complex expression is achieved by quenching growth at peak log phase (*A*<sub>600</sub> ~ 0.85) when O$_2$ grown cells reached [μO$_2$] conditions. Comparisons were made by growing cells anaerobically on a wide range of transition metal TEA’s and harvesting the peripherally attached OM proteins in parallel with μO$_2$ grown cells. Native PAGE in conjunction with an array of staining and activity assays reveals that complexes expressed under μO$_2$ grown were the aggregate of all the tested anaerobic TEAs. In other words, when cells converge from rich oxic conditions to μO$_2$, all the anaerobic machinery for respiration
of transition metal were upregulated. If cells were allowed to continue growth past this A$_{600}$
the vast array of OM complexes will be degraded and only the preferential pathway will
remain.

**KCl Wash and ferrozine assay for Identification of FeR**

*Shewanella* spp. were grown in Westlake media (pH 7.5) supplemented with, 15mM
lactate, and 100mM FeCl$_3$ at 30°C to an A$_{600}$ of 0.8. Cells were centrifuged at 10,000 rpm
for 10 minutes, supernatant was discarded, and then cell pellet was resuspended in 0.5M KCl
buffer. Cell/KCl solution was stirred gently for 1 hour, followed by centrifugation at 10,000
rpm for 10 minutes. Supernatant was filtered with 0.22 um syringe filter, and then
concentrated in a pressure cell with a 2 kDa membrane filter. Concentrated protein sample
was separated and isolated via Native PAGE. Gels were reduced with artificial electron donor
(duroquinol), and then immersed in degassed Tris-Acetate (pH 7.5) containing 30mM Ferric
Citrate. Ferrozine (5mM) was added after 5 minutes. Bands exhibiting Fe(III) reducing
activity in wild-type but not in ΔgspE were excised (adapted from 10). Excised bands
containing protein complexes were separated via SDS-PAGE. Protein bands were excised;
proteins were reduced and alkylated and trypsin degraded (See MALDI MS/MS protocol).
MALDI-TOF analysis was carried out using Applied Biosystems 4700 MALDI-MS
Proteomics Analyzer. MS/MS analysis was performed on intense peaks; algorithms in the AB
software predict amino acid sequence with high confidence.

**Osmotic Shock for Periplasmic Protein Harvesting**

Soluble periplasmic proteins were harvested as previously described (56). Harvested
cells were washed 2X in isoosmotic Buffer (1X WL salts pH 7.5) at 4°C and centrifuged. Cell
pellets were gently resuspended in hyperosmotic solution (Tris-HCl (30mM), EDTA (1mM),
Sucrose 20%, pH 7.5) at 4°C for 20 minutes followed by centrifugation at 4°C. Next, cell
pellet was suspended in Hypoosmotic solution (dH$_2$O, MgCl$_2$ (5mM), pH 7.0) at 4°C and gently swirled for 20 minutes. Solution was centrifuged at 4°C and supernatant (periplasmic fraction) collected and used immediately or diluted with Tris-HCl (60mM) at 1:1 ratio.

**Cystathionine Beta Lyase Activity Assay**

Previously described methods (Zdych, Peist, Reidl, & Boos, 1995) was used to detect the precipitation of PbS resulting from the cleavage of the β-C-S bond and release of sulfide (SH$^-\$). Polyacrylamide gels were submerged in a 100 M Tris-Cl pH 8.2 solution containing 10mM Cysteine and 0.5mM Pb(NO$_2$)$_2$ and placed on shaker for 1 hour to overnight depending on desired level of staining.

**Glutathione Oxidoreductase Assay (GOR)**

The assay reported by Hou et al., was used to detect the disulfide bond reduction of an NAD(P)H-dependent oxidized glutathione reduction (i.e., GSSG → 2GSH). GSH reduces the MTT to a blue color that stains the gel. The GOR or a thioredoxin reductase (TR) will form a glutathione peroxidase (GSH-Px) complex that will appear as a clearing band in the gel. DTNB was employed to silence most other NAD(P)H-dependent oxidoreductases. The gel was washed in 50mM Tris-Cl pH 7 two times. Gel was then immersed in a 50mM Tris-Cl pH 7 solution containing 4mM GSSG, 1.5M β-NADPH, and 2mM DTNB. Next the gel was incubated for 20 minutes followed by a brief washing with 50mM Tris-Cl pH 7 buffer. The gel was then developed for 5-10 minutes in the dark in a 50mM Tris-Cl solution containing 1.2mM MTT and 1.6mM PMS.

**Rhodanese Activity Assay**

The assay reported by Murphy et al., was a negative stain that detects the reduction of dichloroinophenol (DCIP) by sulfite SO$_3^{2-}$. Sulfite evolves from the disproportionation of thiosulfate (S$_2$O$_3^{2-}$) catalyzed by the rhodanese activity of the enzyme in the presence of
cyanide (CN\(^-\)). The DCIP stains the gel blue so only the rhodanese-protein band will remain clear.

The gel was incubated for 5 minutes in a 0.067M phosphate buffer solution containing 0.1M NaS\(_2\)O\(_3\). An equivalent volume of 0.1M KCN in 0.067M phosphate buffer was then added; the gel was incubated for 20 minutes at 30\(^\circ\)C. The reaction was quenched by the addition of 0.5 ml of 37\% formaldehyde (HCOH). The gel was transferred to a solution containing 500\(\mu\)moles of DCIP and 2.5 mg of Phenazinemethylsulfate (PMS) then incubated for 8 minutes. Gel was then placed in buffer and photographed.

**RESULTS**

Peptide mass fingerprinting of the single-stage MALDI-TOF data yielded several exciting, yet unexpected results: 1) the putative respiratory Fe(III) reductase of *S. oneidensis* MR-1 contains a set of at least 14 detectable proteins (Figures. 2.2 A & B), 2) several of the proteins form tightly bound complexes that were not separable via SDS-PAGE, even if the samples were pre-treated by boiling, reduction and alkylation prior to gel electrophoresis, 3) nearly all of the tightly bound complexes contain the same serine protease (Locus No. SO3800) which displays high structural homology to autotransporter-like protein adhesins (Henderson, Navarro-Garcia, & Nataro, 1998), and 4) the 14 detectable proteins include components of the electron transport system and the nearly complete biosynthetic pathway required for assimilatory sulfate reduction in *E. coli* (Lengeler, Drews, & Schlegel, 1999) (Figure 2.3). Taken together, these findings point toward the possibility of *Shewanella* species utilizing a dissimilatory Fe(III) reduction pathway that has adapted aspects of assimilatory sulfate reduction, and that the Fe(III) reduction pathway believed to be purely enzymatic, actually terminates with the abiotic reduction of Fe(III) by organic sulfur compounds produced enzymatically such as mercaptocarboxylic acids (MCA) (e.g., L-
cysteine or β-mercaptopyruvate, a highly reactive intermediate of cysteine metabolism). After electron transfer to Fe(III), the resulting disulfide-containing-compound (e.g., cysteine and RSSR) may be subsequently reduced back to 2 MCA by a putative cysteine reductase, thioredoxin (TrxC), a component identified in the Fe(III) reductase complex (Figure 2.2B), or a glutathione-catalyzed redox reaction. In a separate investigation, x-ray photoelectron spectroscopy of the outer membrane surface of *S. oneidensis* MR-1 and ΔgspD indicated that one of two low binding energy components of the S 2p core region that were present on the wild-type cell surface was absent in ΔgspD.

**Figure 2.5.** A. Ferrozine activity assay of 1D Native PAGE WT vs. Type II secretion mutant. B. 1D SDS-PAGE and MALDI-TOF/peptide fingerprint analysis on Bands. The Fe(III) reductase supercomplex was loaded in a SDS Tris-HCl gel using a BioRad Protean II Cell (Poster: D. Bates, et al., American Society for Microbiology (ASM) 105th Annual National Meeting, Atlanta, GA. June 5-9, 2005).
Figure 2.6. Proteins identified via MALDI-TOF MS/MS peptide fingerprinting constitute aspects of the ETS and the majority of the assimilatory sulfate reduction pathway. Identified proteins are identified by red text.

The binding energy of the missing photopeak was consistent with a mercapto-containing moiety. This phenomenon has also been identified in a type II protein secretion mutant in S. putrefaciens strain 200 (unpublished work with Andy Neal at SREL DOE site).

Glutathione (GSH) was used singly and in combination with duroquinol (DQ) as the electron donor for an in-gel ferrozine activity assay (Fig 2.4A). The GSH-based assay resulted in identification of the reductase identified with DQ and two additional bands, one of which produced a characteristic blue color in the band (Fig 2.4A). The blue colored band was then processed in the same manner as the Fe(III) reductase band. MALDI-TOF analysis in conjunction with peptide mass fingerprinting indicated that the blue band contained additional proteins related to assimilatory sulfate metabolism, including cystathionine β-lyase (or γ-synthase). A variety of in-gel activity assays were used to determine the functional capability of the GSH-dependent Fe(III) reductase complex as well as a comparative analysis of soluble proteins localized on the OM against soluble proteins localized in the periplasm (Fig 2.4).
The activity assays included those that detected activities for 1) rhodanese domain-containing proteins (Murphy, Tilton, Calvert, & Frankenf.J, 1972), 2) glutathione oxidoreductase (GOR) (Hou, Liang, Wang, & Liu, 2004), 3) cystathionine β-lyase (CBL) (Zdych et al., 1995), 4) GSH-dependent Fe(III) reductase, and 5) GSH + DQ-dependent Fe(III) reductase. The rhodanese assay indicated that the blue-colored band a protein that transfers a sulfide (HS⁻) from thiosulfate (S₂O₅²⁻) to cyanide (CN⁻) resulting in sulfite (SO₃²⁻) and thiocyanate (SCN⁻).

The S. oneidensis genome contains five genes whose translated products contain a putative rhodanese domain. These five proteins include 1) β-mercaptopyruvate sulfotransferase (MST), 2), a Sud-like protein (the Sud protein was implicated in polysulfide reduction by catalyzing sulfur transfer from polysulfide to cyanide (Hedrick, Pledger, White, & Baross, 1992), 3) GlpE, 4) CN-hydratase, and 5) a hypothetical rhodanese protein (locus SO0050). The first three proteins have been implicated in organo-sulfur molecule biosynthesis or redox reactions in a variety of previous studies (Nagahara & Katayama, 2005; Ray, Zeng, Potters, Mansuri, & Larson, 2000).

The CBL assay indicated that the blue band contained MetC, a cystathionine β-lyase. The CBL activity assay was based on the interaction of cysteine and lead nitrate (PbNO₃), an interaction that results in C-S bond lysis and PbS formation. The bond between the β-carbon and the thiol group of cysteine (R-Cβ-SH) was cleaved, resulting in sulfide precipitation with lead as lead sulfide (PbS) (brown band in Figure 2.4B). CBL may function to regenerate cysteine via thiocysteine that was formed by cleavage of the β-carbon of one of the two-cysteine components of cysteine. Alternatively, 2 β-mercaptopyruvate may be produced via a pyruvate intermediate formed from MetC cleavage of a disulfide (R-C-S-S-C-R).
Figure 2.7. A. GSH Fe(III) reductase activity assay revealing blue band. B. Native-PAGE in-gel activity assays carried out on OM wash and periplasmic extract first and second lanes for each assay, respectively. From left to right: Rhodanese assay, cystathionine β-lyase assay, glutathione oxidoreductase activity assay, GSH and DQ reduced ferrozine assay, and a coomassie protein stain of the native-PAGE. W = OM Wash, P = Periplasmic extraction.

An in-frame deletion of MetC (ΔmetC) however, did not affect anaerobic growth on Fe(III) (Justin Burns, Ph.D thesis, Georgia Institute of Technology, http://hdl.handle.net/1853/33825). The GOR activity assay detects disulfide bond reduction of an NAD(P)H-dependent oxidized glutathione (i.e., GSSG → 2GSH). GSH reduces MTT to a blue-colored product in native-PAGE gels (Figure 2.3B). GOR or thioredoxin reductase (TR) forms a glutathione peroxidase (GSH-Px) complex that was detected as a clearing band in the gel. Elmans reagent (DTNB) was used to inhibit other NAD(P)H-dependent oxidoreductase activity. The GOR assay indicated that the OM wash protein fraction contained two enzymes capable of reducing disulfide with NADPH as electron-donor.
HYPOTHESIS 2

Detection of Respiratory Supercomplex Activity by Respirasomics Reader - Scanning

Electrochemical Microscopy (RR-SECM)

(Work in conjunction with Douglas Rudolph, Department of Chemistry, Georgia Institute of Technology)

INTRODUCTION

Scanning electrochemical microscopy (SECM) generates signals based on surface induced changes of a Faraday current that results from the hemispherical diffusion of redox-active species to a biased micro-electrode that scans areas in close proximity to the sample surface (Engstrom, Meaney, Tople, & Wightman, 1987; Engstrom, Weber, Wunder, Burgess, & Winquist, 1986; H. Y. Liu, Fan, Lin, & Bard, 1986). The SECM was first introduced in 1986 as an instrument that can examine chemistry near interfaces with high resolution (Engstrom et al., 1987; Engstrom et al., 1986; H. Y. Liu et al., 1986). Today, the micro-electrode probes used were constructed with typical diameters ranging from sub-micron to 25 µm, depending on the application or fabrication capacity. Microelectrodes afford many advantages to macroelectrodes due to their reduced size that enable reduced double layer charging effects, well-defined steady-state currents, enhanced sensitivity; and a reduced ohmic drop, which enables its application as a scanning probe. Furthermore, microelectrode probe positioning relative to the sample surface may be obtained by recording approach curves (i-d curves), as tip-sample distances allow for quantitative characterizations of the Faraday current at the electrode surface. For example, when the electrode tip was several tip diameters away from the sample surface, the steady-state current \(i_{T,\infty}\) can be described by equation 1.

\[ i_{T,\infty} = 4nFDCA \]
Where $n$ is the number of electrons transferred in the tip reaction, $F$ is the Faraday constant, $D$ is the diffusion coefficient of the redox mediator used, $C$ is the mediator concentration, and $a$ is the tip radius. Proper SECM imaging is carried out in close proximity to the sample surface ($\sim 1 – 2$ microelectrode radii away) and is positioned by recording current-distant readings called approach curves. As the microelectrode tip approaches the insulating surface, $i_T$ decreases due to the increasing inability of the redox mediator to diffuse from the bulk solution to the microelectrode tip, i.e., there is negative feedback as the microelectrode tip approaches the sample surface. Alternatively, if the sample substrate is conductive, $i_T$ increases as it draws closer to the surface in a positive feedback manner.

SECM has proven its utility as an electrochemical imaging tool in a suite of applications ranging from corrosion studies to food and beverage and biomedical research (Barker, 1999; Liljeroth, 2002; Mirkin, 1992; Wittstock, 1997). The superb redox species characterization rendered by SECM makes it an attractive candidate for a plethora of bioanalytical applications, including quantitative measurements of concentration (Figure 2.6A) and reaction rates of substrate depletion and/or product formation.
Figure 2.8. A. Square-wave voltammograms obtained at 25 µm Pt/Hg microelectrode in bulk solution calibrated for Fe$^{2+}$ and Mn$^{2+}$ in 10 mL degassed Tris-Acetate buffer (pH 7.5) with standard addition of 25 µL of 40 mM MnCl$_2$ and 20 mM FeCl$_2$ solutions. B. Fe$^{2+}$ and C) Mn$^{2+}$ calibration recorded at the Pt/Hg amalgam microelectrode. (Collaboration with Douglas Rudolph)

![Figure 2.8](image)

Figure 2.9. Square-wave voltammograms obtained in bulk solution via 25 µm Pt/Hg microelectrode in 10 mL of 20 mM Ferrozine to which 25 µL of 20 mM S$^2-$ stock solution (standard addition) was added (0.5 M KCl supporting electrolyte).

Identification of enzymatic complexes peripherally attached to the outer membrane of metal-reducing Gram negative bacteria is crucial to understanding the mechanism of microbial metal reduction. These complexes are often composed of several different polypeptides which have been secreted to the outer membrane where they may undergo a multitude of post-translational modifications. Previous methods used to identify the protein constituents of these unknown complexes are time-consuming, tedious, or require prior knowledge of the identity of at least one component. This study outlines a novel method for identification of individual proteins of Fe(III) reductase complexes peripherally attached to metal-reducing *Shewanella*—an SECM-based polyacrylamide gel reader represented in Figure 2.8.
Figure 2.10. The “Respirasomics Reader.” SECM setup to scan 1-D and 2-D PAGE gels for redox active protein complexes by measuring substrate depletion/product formation over time.

MATERIALS & METHODS

Cell Growth and Protein Harvesting

Cells were grown and proteins harvested as described in previous section.

SECM Instrumentation and Setup

An electrochemical workstation (CH 660, CH Instruments, Austin, TX) equipped with an electrochemical cell in a three-electrode configuration (a silver/silver chloride reference electrode, a Platinum (Pt) counter electrode, and an amalgam of gold/mercury or platinum/mercury working ultramicroelectrode (UME) is used for SECM imaging and performed in a Faraday cage. A PC outfitted with an A/D-D/A board (DAS1602-16, Plug-In-Electronic, Eichenau, FRG) was used to control the micropositioning system (SPI Scientific Precision Instruments, Oppenheim, FRG) and bi-potentiostat (PG100, IPS-Jaissle,
Waiblingen, FRG) of the SECM. Mira software from G. Wittstock (University Oldenburg, FRG) was used to control the positioning system and analysis/visualization software (IDL, Creaso, Munich, FRG) was used data acquisition and evaluation.

**Microelectrode Fabrication and Calibration**

Microelectrode fabrication and calibration was provided by Douglas Rudolph and described in his Master’s thesis (Rudolph, D.A., Georgia Institute of Technology [http://hdl.handle.net/1853/7133](http://hdl.handle.net/1853/7133)).

**SECM Approach Curves**

Working electrodes were positioned above the gel surface in an optimal distance to eliminate bulk effects by recording z-approach curves. These curves were graphed by measuring the reduction of oxygen in 0.1M Tris-acetate buffer, pH 7.5. This is a negative feedback curve as current is reduced as the working electrode approaches the insulating gel surface. The optimal distance ends up being between 15-30 µm.

**Redox Active Enzyme Detection**

Native polyacrylamide gels containing size-separated, duraquinol- or glutathione-reduced proteins were submerged in argon-degassed ferric citrate (10mM)/ Tris-acetate (0.1M) buffer, pH 7.5. An approach curve was taken to assure optimal distance between working electrode and gel surface. Square wave voltammogram scans were made across gel lanes at set points moving the working electrode in the y-direction.

**RESULTS**

Peripheral protein complexes were detached from *Shewanella* cell surfaces, separated via non-denaturing PAGE and exposed to an artificial electron donor and Fe(III). Fe(III)
reductase complexes were identified by scanning electrochemical microscopy (SECM), in
which the gel surface was scanned vertically for Fe(II) production with a Pt-Hg thin film
amalgam micro-electrode (Pt/Hg-ME). Pt/Hg-ME’s are ideally suited for detection of
dissolved metal ions and other redox-active species (e.g. Fe$^{2+/3+}$, Mn$^{2+/4+}$, SO$_3^{2-}$, H$_2$S, etc.)
using square wave (SWV) or linear sweep voltammetry (LSV). By employing SWV in a
potential range of -0.2 to -1.6 V vs. Ag/AgCl reference electrode, redox-active species
generated by or involved in respiratory reductase supercomplexes from metal-reducing
bacteria can be simultaneously detected following separation in native gels without the need
of staining for detection.

Positioned directly above an iron reductase band (validated via ferrozine assay) SWV
at time zero showed a Fe$^{3+}$ peak at -0.4 V vs. reference electrode (RE) (Figure 2.9Aa).
Emerging shoulder peaks indicative of a FeS complex (-0.55V vs. RE) and Fe$^{2+}$ (-1.4 vs. RE)
can be seen at 7 minutes (Figure 2.9Ab). The Fe$^{3+}$ peak diminishes as the FeS and Fe$^{2+}$ peaks
continue to evolve in magnitude and are truly evident after 10 minutes (Figure 2.9Ac-d). As a
comparison, Figure 2.10 shows SWV over a redox inactive protein band and over an active
band.

Fe(III) reductase complexes identified in this manner were excised from the non-
denaturing gel, reduced, alkylated, and then separated by 2-D SDS-PAGE (Figure 2.11). The
individual proteins were identified by MALDI-TOF mass spectrometry and peptide mass
fingerprinting. Results indicate that the Fe(III) reductase complex contains omc an outer
membrane c-type cytochrome (Figure 2.12) as well as the previously reported proteins. The
newly developed technique is currently being used to identify other redox-active enzymatic
complexes peripherally attached to the Shewanella cell surface.
Figure 2.11. A. The square wave voltamograms shown in (a-d) cover a period of 20 min and were recorded at a 25 μm Pt/Hg amalgam UME positioned in close proximity above the PAG in inert Ar atmosphere containing approx. 2.5 ppm oxygen. (a) start of measurements at 0 min, (b) after 7 min, (c) after 10 min, and (d) after 12 min. All potentials are reported vs. Ag/AgCl reference electrode. (Bates, D. ASM 2005). B. Single scan over an active reductase band from wild-type OM wash displaying Fe(III) (-0.38V), R-SH (-0.45V), R-S-S-R (-1.2V), and Fe(II) (-1.45) peaks. (American Society for Microbiology

Figure 2.12. A. SECM over redox active protein complex displaying Fe(III) reduction, FeS complexation, and Fe(II) evolution. B. SECM over inactive protein complex showing only the presence of Fe(III) and trace amount of sulfur species in the buffer. (Bates et al., 2005).
**DISCUSSION**

Organic Fe(III) complexes were observed in both whole cell and Fe(III) reductase band SECM experiments. Fe(III)-citrate was not detectable by the Ag/Hg or Pt/Hg electrode in an polymeric form, however, a few minutes after Fe(III)-citrate introduction, and only in
the presence of active metal-respiring *Shewanella* or directly over the reductase band, an organic Fe(III) complex (Fe(III)-L) peak begins to form. The organic Fe(III) complex may be the result of a ligand exchange event. Mercaptocarboxylic acids (MCA) [e.g., cysteine (Cys), β-mercaptopyruvic acid (βMP), thioglycolic acid (TGA), mercaptosuccinic acid (MSA), and 3-mercaptoproprionic acid (MPA) (Figure 2.13)] dissolve Fe(III) oxides by generating reactive surface complexes in which the ligand was bound to surface Fe(III) centers via both –COO− and –S− groups (Borghi, Morando, & Blesa, 1991). TGA (mercaptoacetate), for example, forms tight complexes with Fe(III), especially in alkali media where both Fe(II) and Fe(III) are complexed, the latter reduced via an intramolecular electron transfer and formation of disulfide, O2CCH2SSCH2CO (Baumgartner, Blesa, & Maroto, 1982). Upon formation of disulfide, Fe(II) was released into solution. In addition, Fe(III)-L in solution is rapidly reduced directly by c-type cytochromes (Shi et al., 2006). The chemical properties of various MCA affect adsorption to, and reductive dissolution of the Fe(III)(hydr)oxides. If the affinity for the oxide is high (i.e., an aggressive complexant), the MCA will adsorb strongly and may passivate the surface. On the other hand, reagents that adsorb poorly (e.g., cysteine) are considered non-aggressive, and reductive dissolution rates at pH 4 are dependent solely on diffusion (Borghi et al., 1991). At circumneutral pH the amino group (pKₐ 10.29) is likely deprotonated upon adsorption to the Fe(III) oxide-surface and forms a negatively charged, surface-adsorbed cysteine species. The pKₐ of the amino group of cysteine decreases by 2.8 pH units upon adsorption to the hydrous ferric oxide (HFO)-surface. Cysteine is therefore a moderately aggressive species (Amirbahman, Sigg, & vonGunten, 1997), ideal for efficient reductive dissolution of Fe(III) oxides. The trend in adsorption affinity is most likely driven by the number of carbon atoms; 2-3 carbon atoms yield the most efficient relationship between affinity and reductive dissolution rates, although MCA affinity for aqueous Fe(III) follow a different trend (Borghi et al., 1991). Models of MCA surface complexes via both
Fe—O(O)R and Fe—SR correlate with the results of SECM analyses; peaks associated with soluble Fe(III) and Fe(II) species are detected simultaneously followed by R—SH and R—S-S—R peaks. Also, the blue colored complex associated with the GSH-reduced protein complex is similar to the previous finding that in an aqueous solution, Fe(III) and MCA form a blue-colored Fe(III)-L⁺ species in which the metal ion is probably bound to S-R and C(O)O⁻ (Borghi et al., 1991).

**Figure 2.15.** Structures of possible mercaptocarboxylic acids involved in Fe-chelation and Fe(III) reduction. From left to right: Cysteine, β-mercaptopyruvate, thioglycolic acid, mercaptsussinic acid, and mercaptopropionic acid.

Respiratory supercomplexes are well-suited for carrying out the complex set of redox reactions postulated to occur in the *Shewanella* Fe(III) reductase complex: 1) catalytic activity may be enhanced by reducing diffusion distances between the large number of substrates, 2) substrate channels may render redox reactions less dependent on bulk properties of the substrate pools (e.g., mid-point redox potentials), 3) biochemical reactions resulting in the highly reactive, deleterious intermediates invariably produced in metal-based redox systems may be controlled, 4) branchpoints of the complex, overlapping electron transport pathways of *Shewanella* may be regulated, and 5) the autotransporter-like serine protease SO3800 may act as a molecular scaffold (in the same manner as scaffolding in cellulosomes) to stabilize the overall architecture of the multi-protein Fe(III) reductase respiratory complex, or as an adhesin to anchor the complex to the metal-oxide; further limiting diffusion distances.
Figure 2.16. Postulated mechanism of Fe(III)-oxide reduction- combining electron transport (red arrows) with MCA biosynthesis (blue arrows). MST – mercaptopyruvate sulfotransferase; GOR – glutathione oxidoreductase (Supercomplex simplified for clarity).

These results suggest that the respiratory Fe(III) reductase complex of *Shewanella* is part of a larger respiratory supercomplex that includes the systems required for anaerobic growth on sulfite, thiosulfate, tetrathionate, elemental sulfur and potentially other partially-oxidized sulfur compounds (*Shewanella* does not respire sulfate). In addition, all Fe(III)-respiring bacteria also display Mn(IV) respiration capability, and all Fe(III) respiration-deficient mutants lose the ability to respire Mn(IV) (Arnold, DiChristina, & Hoffmann, 1986; Burnes, Mulberry, & DiChristina, 1998; DiChristina & DeLong, 1994), suggesting that *Shewanella* respires Mn(IV) as terminal electron acceptor by employing either 1) a (purely chemical) double electron-shuttle system that begins with the MCA-catalyzed reduction of Fe(III) to Fe(II), and terminates with the Fe(II)-catalyzed chemical reduction of Mn(IV), or 2) an electron shuttle where a solubilized Fe(II)-L reduces Mn(IV) following biotic reduction of the soluble Fe(III)-L; or perhaps both. In either scheme, electrons entering the anaerobic respiratory system via CymA are sent through a series of periplasmic and OM cytochromes to
MtrC, OmcB, and MtrB. Electrons from the electron transport chain may also be funneled, via NADPH and GSH, into the assimilatory sulfate reduction pathway to produce an MCA that solubilizes and/or chemically reduces Fe(III) to Fe(II), which in turn, transfers electrons to Mn(IV) in the terminal reduction step. The resulting oxidized intermediate components of the double electron shuttle to Mn(IV) (i.e., cystine or RSSR, and Fe(III)-L) may then be re-reduced in the respiratory supercomplex, with disulfide reduced via MtrB (CXXC motif), TrxC, or GOR, and Fe(III)-L complexes directly reduced via MtrC/OmcA. Taken together, these identified proteins complete an enzymatic pathway that suggests the mechanism of Fe(III)- and Mn(IV)-oxide reduction (Figure 2.16).

**Figure 2.17.** Assimilatory sulfate reduction pathway as postulated with respect to metal-oxide reduction. Enzymes in red text were identified in the DQ-reduced Fe(III) reductase via MALDI-TOF and peptide fingerprinting. Enzymes in blue text were identified in the GSH-reduced blue band displaying Fe(III) reduction activity via MALDI-TOF and or in-gel activity assay.
The DiChristina lab and collaborators have continued to pursue this hypothesis, but in bulk solution using a geochemical voltammetry setup (Taillefert et al., 2007). This work showed that an organic Fe(III) solubilizing complex that is strong enough to destabilize either Fe(III) oxides or Fe(III) citrate is produced by *Shewanella*. Jones et al. used an electrochemical platform to show that *S. onedensis* MR-1 mutants selected for the inability to produce soluble organic-Fe(III) complexes are unable to respire Fe(III) as an aerobic electron acceptor (M. E. Jones, Fennessey, DiChristina, & Taillefert, 2010). Furthermore, Fennessey et al., using voltammetric and siderophore detection assays, showed that siderophores are not involved in Fe(III) solubilization during anaerobic Fe(III) respiration (Fennessey, Jones, Taillefert, & DiChristina, 2010). Taken together, there is strong evidence in support of the two hypotheses set forth thus far in this chapter.
HYPOTHESIS 3

Adhesion to Iron(III) oxide by Shewanella oneidensis MR-1 is facilitated by an autotransporter-like adhesin

[Premise and some aspects are reported in Burns, J. et al., Outer Membrane-Associated Serine Protease Involved in Adhesion of Shewanella oneidensis to Fe(III) Oxides. 2010. Environmental Science & Technology. 44, 68-73 © 2010 and are adapted or reprinted here with permission from the American Chemical Society]

INTRODUCTION

Protein complexes secreted to the surface of Gram-negative bacteria are postulated to function as essential mediators between the cell and its environment, including the import and transport of essential nutrients, the export of waste products, the removal of toxic bi-products, as well as facilitating attachment to solid surfaces and adjacent cells and providing protection from predation, host defense systems, and microbicidies. Bacteria often adhere to specific molecular targets through the presentation of protein structures on the cell surface (e.g., fimbriae) with distinct surface-binding capacities (Veiga, de Lorenzo, & Fernandez, 2003). Many adhesins have been identified in a variety of Gram-negative pathogens anchoring directly to the OM of the host cell resulting in an intimate target cell contact (Altschul, Gish, Miller, Myers, & Lipman, 1990; Benz & Schmidt, 1992a; DiChristina et al., 2005; DiChristina & J., 1994; Gaspard, Vazquez, & Holliger, 1998; Henderson et al., 1998; Hu, Cheng, Li, Zou, & Xu, 1999; Lovley et al., 2004; Marchler-Bauer et al., 2007). This close contact adhesion facilitates host infection by closing the gap for invasins to interact with recognition proteins of the host cell and by reducing the diffusion distance of secreted toxins and serum resistance proteins. Most of these adhesins belong to the Type V secretory pathway and appear to work in concert with other
known secretion systems in Gram-negative bacteria with respect to extracellular interactions. *Bordetella pertussis* infection of mammalian trachea for example involves Type I secretion of adenylate cyclase onto the host cell, Type IV secretion of the pertussis toxin into the extracellular milieu, and Type V secretion of tracheal colonization factor, filamentous hemagglutinin (FHA), and serum resistance protein (BrkA) to the OM. Mutations resulting in loss of function of any of these proteins or secretion systems results in mutants that display activities ranging from a 10-fold loss of infection (R. C. Fernandez & Weiss, 1994) to total loss of virulence (Babu et al., 2001; Todar, 2004). Adhesins make attractive candidates for vaccines because of the critical role exhibited in host attachment and subsequent infection.

The autotransporter (AT) family is an important class of secreted proteins in Gram-negative bacteria. The AT secretion system is named such because it involves the self-exportation of two-module polypeptides; one module being the C-terminal domain (called transporter or β-module) that is inserted into the OM, the other module is termed the passenger domain, which denotes the protein moiety externally presented on and often anchored to the cell surface (F. M. Fernandez et al., 2003). AT secretion involves a sequence of events commencing with secretion across the IM into the periplasm, transport across the periplasm is generally facilitated by periplasmic chaperones (e.g. DsbA), followed by formation of a C-terminus β-barrel and insertion into the OM as either a monomer or in an oligomeric form with other β-modules; in either case an outer membrane pore is formed. The N-terminal domain then passes through this pore as the functional domain of the AT and is localized on the outer surface of the OM. Based on standard autotransporter-defining criteria—(i) N-terminal signal peptide, (ii) a functional passenger domain, and (iii) a C-terminus β-barrel-forming β-module—it is believed that serine protease SO3800 of *Shewanella*
*Pseudomonas* *oneidensis* MR-1 is a member of the Type V secretion family with a particular role in adhesion. Testing this hypothesis demonstrates that this protein not only has a role in adhesion, but also displays a specificity for ferric iron oxide concurrent with previous studies on the Fe(III)-specific affinity displayed at the cell surface (F. Caccavo, Schamberger, Keiding, & Nielsen, 1997; Lower, Hochella, & Beveridge, 2001).

**MATERIALS AND METHODS**

**Bioinformatic analysis**

Bioinformatic analysis was used to determine sequence and structural information of SO3800 by computer-assisted comparisons with experimentally characterized proteins. National Center for Biotechnology Information’s (NCBI) Basic Local Alignment Search Tool (BLAST) algorithms (Altschul et al., 1990; Altschul et al., 1997; Madden, Tatusov, & Zhang, 1996) were used to compare the SO3800 peptide sequence (obtained from The Institute of Genomic Research (TIGR)) against the 1) non-redundant database, 2) the entire microbe genome, 3) sequenced *Shewanella* species, and 4) known AT-containing pathogenic bacteria. NCBI conserved domain algorithms were used to identify conserved domains and motifs (Marchler-Bauer et al., 2005; Marchler-Bauer et al., 2007; Marchler-Bauer et al., 2003; Marchler-Bauer & Bryant, 2004; Marchler-Bauer et al., 2002). The bioinformatic tool, Prosite (Gattiker, Gasteiger, & Bairoch, 2002), was used to search the Prosite database (Hulo et al., 2006), which was useful in describing protein domains, families and functional sites as well as associated patterns and profiles to identify them. The T-COFFEE program was used to compare SO3800 peptide sequence with selected sequences from Table 1 (Notredame, Higgins, & Heringa, 2000).
The NCBI BLAST algorithm was used to search the protein databases for the most appropriate PDB structures to serve as templates for modeling. PDB files were cleaned-up using PDB Vacuum in the Modeler Toolbox, located at http://genomics.biology.gatech.edu/motool. ClustalW was used to generate a multiple sequence alignment of the serine protease with templates that corresponded to conserved domains. The final model of the autotransporter was generated with the FORTRAN program Modeller (http://salib.org/modeller/modeller/html). The autotransporter homology model was validated with PROCHECK (http://deposit.pdb.org/cgi-bin/validate/adit-session-driver).

**Protein Harvesting**

To confirm the presence of SO3800 on the OM, proteins were harvested from the cell surface following growth on a variety of TEAs. Optimal OM complex expression was achieved by quenching growth at peak log phase (A_{600} \approx 0.85) when O_{2} grown cells reached micro-aerobic (\mu_{O_{2}}) conditions. Comparisons were made by growing cells anaerobically on a wide range of transition metal (e.g., hydrous ferric oxide, ferric citrate, manganese pyrophosphate) TEAs and harvesting the peripherally attached OM proteins in parallel with \mu_{O_{2}} grown cells.

**KCl Wash for harvesting of peripherally attached OM proteins**

*Shewanella oneidensis* MR-1 was grown in SM media (pH 7.5) supplemented with, 15 mM lactate, and 100 mM FeCl₃ at 30 °C to an A_{600} of 0.8 (DiChristina & DeLong, 1994; DiChristina et al., 2002; C. R. Myers & Nealson, 1988a). Cells were centrifuged at 10,000 rpm for 10 minutes, the supernatant was discarded, and then cell pellet was re-suspended in 0.5 M KCl buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 7.2) (Gaspard et al., 1998; Ohlendieck, 1996). Cell/KCl solution
was stirred gently for 1 hour, followed by centrifugation at 10,000 rpm for 10 minutes. Supernatant was filtered with 0.22 um syringe filter, and then concentrated in a pressure cell with a 1 kDa membrane filter (Milipore Corp. Billerica, MA). Cells and solutions were maintained at or below 4°C whenever possible. Concentrated protein sample was separated and isolated via native PAGE on both Protean II and criterion cells (BioRad Hercules, CA). A ferrozine activity assay was used to determine Fe(III) reducing bands as previously described (DiChristina et al., 2002). Native gels were photographed using BioRad image station or digital camera (Cannon, Melville, NY) and desired protein bands were excised. Excised bands containing protein complexes were separated into individual protein components via SDS-PAGE. Resulting protein bands were excised and proteins were reduced and alkylated [20mM tributylphosphine (TBP) and 40mM iodoacetamide (IAA), respectively (Sigma Aldrich, St. Louis, MO)] followed by trypsin digestion at 37°C for 6 hours.

**MALDI-TOF MS/MS Analysis**

Trypsin degraded peptide fragments were concentrated using C_{18} micro-ZipTips (Millipore, Billerica, MA 01821) and eluted into a 50:50 Millipore dH2O: acetonitrile solution containing 0.1% formic acid and 10 mg/ml of α-cyano-4-hydroxy cinnamic acid (Sigma Aldrich, St. Louis, MO). Peptide fragment-containing solution was spotted onto a 192 well plate (Applied Biosystems (AB), Foster City, CA) for single-stage MALDI-TOF analysis (Georgia Tech Bioanalytical Mass Spectrometry Facility) in 1.0 μl volumes. MALDI-TOF analysis was carried out using an AB 4700 MALDI-TOF-TOF Proteomics Analyzer. This instrument combines a MALDI source with a TOF-TOF tandem mass analyzer to acquire both peptide mass fingerprint and MS-MS spectra of peptide ions via high-energy collisionally-induced dissociation,
which was fed into GPS Explorer algorithms that render sequence predictions with high fidelity. This instrument was equipped with Explorer instrument control software and GPS Explorer 3.5 software suite for proteomics data analyses. Protein identifications were confirmed by uploading mass spectrometry peak values (peptide mass fingerprint) into the PROFOUND website (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe) and comparing the resulting protein identity to that yielded by the GPS Explorer 3.5 inquiry.

**Gene replacement mutagenesis**

Performed by Justin Burns and reported in Burns et al. 2010.

**Confirmation of in-frame deletion**

Performed by Justin Burns and reported in Burns et al. 2010.

**RESULTS**

**Bioinformatic analysis**

NCBI BLAST analyses of SO3800 against known autotransporter-containing species (Pseudomonas spp., Bordetella spp., H. influenza spp., Yersinia spp., and Escherichia spp.) indicates that MR-1 contains homologous AT C-terminal domains with identities greater than 30% as well as sequence similarities greater than 40% with known AT adhesins (e.g AIDA-1 E. coli, AT adhesin from Yersinia spp. and Haemophilus spp.) (Table 2.1). The structural similarity algorithm, T-COFFEE, was used to compare SO3800 and the known ATs, BrkA, from Bordetella pertussis, AIDA-I from Escherichia coli O157:H7 strain Sakai, AT adhesin from Actinobacillus pleuropneumoniae L20, and AT adhesin (YadA-like) from Yersinia pestis Angola. Aligning the sequences of both BrkA and AIDA-I with SO3800 generated T-
COFFEE scores of 100%. T-COFFEE alignments of SO3800 with the AT adhesins from Actinobacillus pleuropneumoniae and Yersinia pestis yielded much lower scores—51 and 54, respectively (Table 2.1). Alignments with the highest sequence homology among Shewanella species (peptidase S8 and S53, subtilisin, kexin, sedolisin, Shewanella baltica) yielded a maximum score of 100 (Figure 2.19), however, alignments of SO3800 with the highest homology hits outside of Shewanella yielded lower scores ranging from 70 to 91% from peptidase S8 and S53, subtilisin, kexin, sedol of Geobacter lovleyi and a serine protease from Pseudoalteromonas tunicata, respectively. Insertion of the SO3800 sequence into NCBI’s Conserved Domain Database (CDD) revealed a serine protease domain, a subtilisin-like motif, a cadherin domain, a flageller hook-cap domain (cellular motility and secretion), an AT-β-domain, and glycosyl hydrolase family domains 28, 47, 49, and 76 (Table 2.2). Interestingly, the Prosite scan of the SO3800 sequence revealed the presence of two serine protease active sites (one Asp and one Ser), a P-loop motif, and a TonB-dependent receptor protein signature (Table 2.3). Also, the amino acid sequence displayed an unusually large overall residual negative charge with an excess of more than 50 acidic amino acids (e.g., Asp and Glu). Analysis of predicted secondary structure demonstrated the even-number (twelve) stranded β-sheet C-terminus typical of monomeric ATs, with an α-helix linker region adjacent to the N-terminus of the β-barrel domain (Henderson, Navarro-Garcia, Desvaux, Fernandez, & Ala’Aldeen, 2004) (Figure 2.20) (www.tigr.org). Three dimensional structural modelling of the SO3800 sequence yielded a tertiary structure similar to the solved crystal structures of known autotransporters (Figure 2.21).
Table 2.1 Known AT-containing species

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Identity (Positives%)</th>
<th>T-COFFEE</th>
<th>Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bordetella pertussis</em> Tohama I</td>
<td>serum resistance protein (BrkA)</td>
<td>27 (41)</td>
<td>100</td>
<td>968-1174</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>Large exoproteins involved in heme utilization or adhesion</td>
<td>23 (40)</td>
<td>N/A</td>
<td>926-1188</td>
</tr>
<tr>
<td><em>Actinobacillus pleuropneumoniae</em> L20</td>
<td>autotransporter adhesin</td>
<td>24 (37), 26 (36), 35 (37)</td>
<td>51</td>
<td>925-1179</td>
</tr>
<tr>
<td><em>Actinobacillus pleuropneumoniae</em> serovar</td>
<td>autotransporter adhesin</td>
<td>26 (37), 24 (36)</td>
<td>51</td>
<td>991-1186, 193-401</td>
</tr>
<tr>
<td><em>Yersinia pestis</em> Angola</td>
<td>Autotransporter adhesin (YadA-like)</td>
<td>24 (38), 23 (37)</td>
<td>54</td>
<td>922-1089, 207-592</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7 str. Sakai</td>
<td>putative invasin</td>
<td>23 (40), 22(37)</td>
<td>N/A</td>
<td>926-1176, 1072-1180</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>adhesin</td>
<td>21 (38)</td>
<td>N/A</td>
<td>916-1187</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>HMW2A, high molecular weight adhesin 2</td>
<td>22 (38)</td>
<td>N/A</td>
<td>873-1123</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7 str. Sakai</td>
<td>AidA-I adhesin-like protein</td>
<td>26 (41)</td>
<td>100</td>
<td>905-1188</td>
</tr>
<tr>
<td><em>Bordetella parapertussis</em></td>
<td>putative hemolysin</td>
<td>25 (36)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.2 Conserved Domains via CDD search.

<table>
<thead>
<tr>
<th>Conserved Domain</th>
<th>Coordinates</th>
<th>Pfam or COG ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidase S8</td>
<td>149-371</td>
<td>00082.12</td>
</tr>
<tr>
<td>PA domain</td>
<td>379-471</td>
<td>02225.12</td>
</tr>
<tr>
<td>Peptidase S8</td>
<td>472-568</td>
<td>00082.12</td>
</tr>
<tr>
<td>FlgD (flagellar hook-cap)</td>
<td>400-490</td>
<td>03963.8 COG1843.2</td>
</tr>
<tr>
<td>Cadherin</td>
<td>1000-1100</td>
<td></td>
</tr>
<tr>
<td>Glycosyl hydrolase Family domains</td>
<td>50-200, 140-290, 160-450, and 500-630, respectively</td>
<td>01532.12 03718.10 03663.10 00295.12</td>
</tr>
<tr>
<td>AT β-domain</td>
<td>900-1200</td>
<td>03797.9 COG5571</td>
</tr>
</tbody>
</table>

### Table 2.3 Conserved motifs via Prosite search.

<table>
<thead>
<tr>
<th>Conserved motif</th>
<th>Residues</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TonB-dependent receptor proteins</td>
<td>1-118</td>
<td>MKTKLSLAISAALLTSAAVAGTTAQYNTT NQTTDKYAGLSVTKNSNEQKQAVAAWM VKLKAPSLAQSQLKGFKQSVMSQIEESS QTKVKNAITSMDADL</td>
</tr>
<tr>
<td>Ser Protease Asp active site</td>
<td>169-180</td>
<td>VAVLDTGVDYTH</td>
</tr>
<tr>
<td>Ser Protease Ser active site</td>
<td>532-542</td>
<td>GTSFSSPITAG</td>
</tr>
<tr>
<td>ATP/GTP-binding motif A(P-loop)</td>
<td>902-909</td>
<td>AEADLGTK</td>
</tr>
</tbody>
</table>
Figure 2.18. Secondary structure comparison: Autotransporters of Bordatella pertussis (BrkA) and S. oneidensis MR-1 are nearly identical (Notredame et al., 2000).

```plaintext
Figure 2.18. Secondary structure comparison: Autotransporters of Bordatella pertussis (BrkA) and S. oneidensis MR-1 are nearly identical (Notredame et al., 2000).
```
Figure 2.19. Secondary structure prediction via TIGR website. Cylinders represent predicted alpha helices and arrows represent predicted beta sheets.
Figure 2.20. Tertiary structure prediction of S. oneidensis MR-1 autotransporter via homology modeling (Bates, D., et al., ASM poster 2005).

**Electron Acceptor utilization of MR-1::ΔSO3800**

MR-1::ΔSO3800 respired all electron acceptors at wild-type rates (Burns et al., 2010). Although ΔSO3800 grew at wild-type rates on dimethylsulfoxide (DMSO, \((\text{CH}_3)_2\text{SO}\)), thiosulfate \((S_2\text{O}_3^{2-})\), or elemental sulfur \((S^0)\) as electron acceptor and formate \((\text{HCOOH})\) as electron donor, a 24-hour lag prior to onset of growth was discovered.
Proteomic Analysis of MR-1::ΔSO3800

RC/DC protein assays carried out on protein samples extracted from the extracellular-side of the OM revealed a significant reduction in the overall amount of protein localized to the surface of the OM with 4.65 µg/ml from ΔSO3800 compared to 5.49µg/ml of WT. Protein complex separation of ΔSO3800 samples was met with much less resistance and increased protein-band resolution was achieved (Figure 2.22).

Figure 2.21. SDS-PAGE of excised Fe(III) reductase band showing better separation in the absence of SO3800

Cassien and Gelatin zymogram polyacrylamide gels (BioRad) were run under native and denaturing conditions. Native gels revealed proteolytic activity in the majority of peripherally attached protein complexes (Figure 2.23) while denatured gels varied depending on the zymogram protein used (i.e., either casein or gelatin)
with casein-containing zymogram gels (12% T) showing 3-5 protease-active bands in succession around 90 kDa, all of which were missing in Δ3800, but present in the Type II secretion mutant ΔgspD. Gelatin-containing zymogram gels (10% T) showed 4-5 well separated bands with ΔSO3800 missing only two bands around 100 and 75 kDa (Figure 2.24). Separation of native protease-active OM protein complexes into individual protein components by SDS-PAGE, in conjunction MALDI-TOF MS analysis of the excised bands revealed the presence of SO3800 in several of the OM complexes (Figure 2.25). Protein complexes were never fully separated due to the strong adherence of SO3800 to other OM proteins despite several attempts including 9.2 M Urea treatment, boiling, reducing agents (dithiothreitol (DTT), β-mercaptoethanol, or tri-butylphosphine (TBP)) followed by alkylation via iodoacteamide (IAA), and the use of a variety of different solubilization and gel stringency (%T) concentrations.

![Image](image.png)

**Figure 2.22.** Native PAGE of MR-1 OM wash (left) set beside Zymogram PAGE run under native conditions, demonstrating that prominent bands display protease activity.
Figure 2.23. Zymogram SDS-PAGE analyses of OM wash of MR-1, ΔSO3800, and ΔgspD. 4A 10%, gelatin-containing gel; 4B 12%, casein-containing gel.

Figure 2.24. 2-Stage PAGE of OM complexes followed by MALDI-TOF analysis of boxed bands, all of which were identified as SO3800. The native PA gel was laid on top of subsequent SDS-PA gel indicating complex separation in the SDS-PAGE lane directly below the native band.
Adhesion Assay

The ability of both strains to attach to hematite (α-Fe₂O₃) and corundum (α-Al₂O₃) was tested. The assay was carried out with three separate batches of cells, in each case, attachment to three replicate iron- and aluminum-oxide surfaces was estimated by counting cells at ten randomly chosen sites on each surface. The resulting estimates of cell density were analyzed using a nested analysis of variance.

The results of attachment assays were shown in Figure 2.26. For both surfaces, cell density estimates were significantly lower for the ΔSO3800 mutant (F₁,₁₁⁴ = 212.8; p < 0.001 and F₁,₁₁⁴ = 21.4; p < 0.001 for hematite and corundum respectively) compared to wild type cells. On hematite, the mean cell density observed across the three batch trials was 1.16x10⁶ cells cm⁻² for wild type cells compared to 0.55x10⁶ cells cm⁻² for the ΔSO3800 mutant. For corundum the difference between means was smaller, 0.88x10⁶ compared to 0.72x10⁶ cells cm⁻² for the wild type and mutant cells respectively. The difference in cell density between the wild type and mutant strain was therefore considerably greater on the hematite surface than corundum.
Figure 2.25. (Top) Results of adhesion assay demonstrating a reduction of > 50% in hematite attachment (Bottom) Control showing adhesion to corundum was considerably less than that of its structural ferric analog, hematite (Burns et al. ©2010, American Chemical Society, used with permission.)

SEM analysis

LT-HRSEM requires neither fixation nor dehydration of cells and thus permits visualization of cells in a fully hydrated state. Surfaces of fumarate-grown MR-1 wild type cells are free of exopolymers (Figure 2.26 A & B), an observation confirmed at high magnification (Figure 2.26 C). The only surface-associated features observed on wild type cells are polar flagella (Figure 2.26 B); otherwise the cell surface remains
largely featureless. The surfaces of MR-1::ΔSO3800 cells are distinctly different from the wild type cells (Figure 2.26 D - F). Two major differences are observed: firstly, pilus-like structures are associated with cells not observed with wild-type cells; secondly, both the cell surface and the surface of the pilus-like structures of ΔSO3800 display a textured surface compared to the smooth appearance of wild-type cells.

![Figure 2.26](image)

**Figure 2.26** LT-HRSEM images of MR-1 cells (Top row) compared to ΔSO3800 (bottom row) (Burns et al. ©2010, American Chemical Society, used with permission)

**DISCUSSION**

Most research into the mechanism of anaerobic metal oxide respiration has concentrated on electron transport machinery and mechanism of (DiChristina et al., 2005; Lovley et al., 2004), with four possible mechanisms proposed 1) direct contact,
2) exogenous electron shuttling, 3) an endogenous electron shuttle, and 4) a solubilizing (or chelating) mechanism to bring the TEA into the cell where it was reduced via a periplasmic reductase. Although only one of the proposed mechanisms would require direct adhesion to the Fe(III)-oxide, all would benefit by adhesion. Direct cellular adhesion to the oxide particle greatly reduces diffusion distances, preserves desirable shuttle concentrations resulting in sufficient rates of electron transfer, and limits loss of shuttling compound; especially beneficial if the shuttle is being manufactured by the cell at an energetic cost. Despite the advantages adhesion poses to metal-oxide reduction, relatively few studies have examined the mechanism of adhesion or the physiological interaction between MRB and metal-oxide particles.

The majority of the studies focused on adhesion of *Shewanella* spp. point toward or suggest, but do not identify, an OM protein (or proteins) with adhesive specificity for Fe(III)-oxides (F. Caccavo, Jr., 1999; F. Caccavo et al., 1997; Lower et al., 2001). In these studies, as well as in this study, adhesion assays were employed with iso-structural analogs (ISA) (e.g., hematite (α-Fe₂O₃) and corundum (α-Al₂O₃), goethite (α-FeOOH) and diaspore (αAlOOH)) as well as other non-iron metal compounds (e.g., ZnSe, SiO₂) to demonstrate specificity for adhesion to Fe(III)-oxides. All experiments demonstrated an increased affinity for Fe(III)-minerals over the corresponding ISAs. All these studies show that iron is not essential for surface adhesion, but Fe(III)-associated surfaces display much higher adhesion density that is consistently higher than that of the corresponding non-iron ISA (Lower et al., 2001). The slight loss in adhesion of ΔSO3800 when compared to wild-type on corundum can be attributed to the overall negative surface charge decrease of the SO3800 mutant. The presence of the TonB-dependent receptor protein signature on the N-terminus of SO3800 and the homology to other Fe-compound binding proteins
suggests that SO3800 has an inherent affinity for ferric compounds. Proteins that are currently known or presumed to interact with TonB are involved one way or another with binding and acquisition of iron or iron-bound chelates (Schalk, Yue, & Buchanan, 2004). The TonB protein interacts with OM receptor proteins involved in high-affinity binding and energy-dependent uptake of specific substrates (i.e., Fe-containing compounds) into the periplasmic space. Without a TonB interaction, however, these receptors simply bind their substrate and do not carry out active transport into the cell (i.e., in the absence of a TonB interaction, the TonB dependent signature-containing protein is essentially an iron binding protein). This implied specificity in conjunction with the structural homology displayed with other AT adhesins along with the phenotypic characterizations present a strong case implicating SO3800 as a Fe(III)-binding AT adhesin.

Results from electrophoretic mobility estimates demonstrate that deletion of the SO3800 gene results in the conversion from the relatively hard, ion-impenetrable cell surface of wild-type cells to that of the a soft, ion-permeable cell surface of the SO3800 mutant, which suggests a considerable increase in OM capsule (J. L. Burns et al., 2010). The presence of an increased capsule is confirmed visually with the LHRSEM imaging and fluorescent micrographs of TRITC-ConA stained cells of ΔSO3800 compared to wild-type (J. L. Burns et al., 2010). These results correlate with another study carried out with Shewanella algae BrY (F. Caccavo et al., 1997), in which an adhesion deficient mutant, S. algae RAD20, displayed a 50% reduction in adherence and a significant increase in EPS. Although S.algae RAD20 and S. oneidensis MR-1::ΔSO3800 display some analogous phenotypes, cell surface charges were similar for both the wild-type and mutant strain in S. algae and considerably different in S. oneidensis, thereby casting doubt on the notion of a homologous
protein nullification. A different study involving Ag43 and AIDA-I, two AT adhesins of *E. coli*, showed that an increased capsule structure shields the function of these adhesins (Schembri, Dalsgaard, & Klemm, 2004). This could be applied to the aforementioned adhesion mutants of BrY and MR-1, suggesting that the increased capsular layer could shield the function of protein adhesins on the cell surface of these mutants. Conversely, a study carried out with *Pseudomonas* sp. strain 200 (now *Shewanella putrefaciens* strain 200) used SEM to show that what appeared to be extracellular polymer was being used to anchor the cell to Fe(III) minerals such as hematite and goethite (Arnold, DiChristina, & Hoffmann, 1988). It was later reported that EPS does not appear to have the same role in *Shewanella algae* BrY, but rather a proteinacious mechanism of adhesion is employed (F. Caccavo, Jr., 1999). These different results demonstrate the possibility of species-specific mechanisms of adhesion to Fe(III)-oxides as well as the potential for multiple mechanisms of adhesion to these particles.

The increased EPS observed in this study may result from the loss of the four glycosyl hydrolase domains of SO3800 and their possible role in limiting capsule thickness by degrading polysaccharides that come in contact with these proteins. Concanavalin A, used to visualize the capsule layer of ΔSO3800, binds specifically to α-mannose, α-glucose, and N-acetylated α-glucose, which are the targets of three of the four hydrolase families (47 and 76 are α-mannosidase and an endo-α-mannosidase, and 49 is a dextranase that cleaves α-1, 6-glucosidic bonds of dextran). Capsule shielding is less likely considering the stark contrast between losses of ΔSO3800 adhesion on hematite verses that of corundum. In either case, questions as to the role(s) of increased capsule in these adhesion mutants remain. Also, the question of differential expression of capsule and adhesins of pathogens posed in the
Schembri et al., study should be expanded to include potential analogous roles of adhesins and capsules in metal-reducing Gram-negative bacteria.

The ability for ΔSO3800 to respire Fe(III)-oxides at wild-type rates correlates as well with the results of the previous studies with *S. algae* RAD20 (F. Caccavo, Jr., 1999). Considering the retained ability for efficient electron transfer despite an increased capsule size, it is doubtful that the first of the four postulated mechanisms of metal reduction, that of direct enzymatic reduction, is the sole mechanism employed by *Shewanella oneidensis* MR-1. A direct enzymatic reduction mechanism implies that the metal oxide particle comes within 1.4 nm of the redox center of the enzyme for feasible electron transfer. The increased capsule would prevent this type of electron transfer by inhibiting metal-oxides from obtaining the distance needed for transfer. The increase of electrophoretic softness infers an increase in capsule by 2 nm—a minimum estimate resulting from Oshima’s theory only probing to the depths of the Debye-Huckle layer, ultimately suggesting a larger capsule increase (J. L. Burns et al., 2010).

Proteomic analyses of SO3800 demonstrate a strong resistance to separation from complexed proteins. A study conducted with *P. gingivitis* demonstrated that some of its ATs, called gingipains, mediate adhesion to tissues in the oral cavity (Potempa, Sroka, Imamura, & Travis, 2003). This AT possess both adhesin and proteinase activities as well as an incredible ability to remain stable under the extremely varied extracellular conditions experienced in the oral cavity (i.e. hot and cold foods and beverages, dental hygiene, etc.). This trait is therefore, not unique to SO3800 and would an expected characteristic of oral cavity ATs, as well as any others residing in environments of dynamic or harsh conditions.
Zymogram analysis of the cell surface proteins of ΔSO3800 compared to wild-type reveals a number of protease-active bands present in the wild-type, but missing in the mutant. The missing bands were matched with coomassie stained gels; the wild-type bands were identified via MALDI-TOF MS analyses as serine protease SO3800. SO3800-containing bands were also extracted from the OM of ΔgspD, the Type II secretion mutants, demonstrating that the presentation of SO3800 on the OM surface is a Type II secretion system-independent process. Presumably, if SO3800 were an allocrit of the Type II protein secretion system, SO3800 would not be expected to be found on the OM of ΔgspD, but in the periplasm where other Type II-secreted OM proteins (e.g., cytochromes) have been found (DiChristina et al., 2002). The presence of an N-terminal Sec-secretion signal suggest that SO3800 is translocated across the IM by the Sec translocase system, and is thereby not a likely candidate for Type I protein secretion. Therefore, based on bioinformatic and phenotypic characterization as well as deductive reasoning, SO3800 is secreted via Type V protein secretion.

The CDD blast revealed the presences of an AT β-domain (or β-module), which typically consists of amphipathic β-sheet structures that spontaneously form an even number (typically 12 or 14) stranded β-barrel. The even number of beta strands, with the first and last forming antiparallel hydrogen bonds to close the ring, results in the N-terminus facing towards the periplasm. Computer assisted prediction of the secondary structure of SO3800 reveals all the AT-defining structural criteria is encompassed in its gene (Figure 2.14). Several ATs, including SO3800, exhibit a short α-helix preceding the β-barrel, which may facilitate movement of the passenger domain through the β-barrel (Henderson et al., 1998), as well as partially block the pore (Oomen et al., 2004). Other studies have broadened the spectrum of AT criteria by demonstrating that the β-barrel can also be an oligomer of β-modules, as is the
case with the AT, YadA from \textit{Yersinia pestis}, who’s solved crystal structure is in the Protein Data Base (PDB). YadA contains a \( \beta \)-module consisting of four \( \beta \)-strands that forms a 12-stranded \( \beta \)-barrel upon trimerization (Roggenkamp et al., 2003). The low T-COFFEE score between SO3800, despite its relatively high sequence homology, could be attributed to the need of the \( \beta \)-module to form a trimer for proper secretion of YadA, whereas SO3800 is monomeric with respect to secretion like AIDA-I and BrkA. Insertion of the AT \( \beta \)-barrel into the OM is believed to be assisted by periplasmic chaperones (e.g. Skp) or proteins known to have chaperone-like activity (e.g. DsbA and DsbC) (Hu et al., 1999; Jacob-Dubuisson, Striker, & Hultgren, 1994; Zheng, Quan, Song, Yang, & Wang, 1997). DsbA is a probable candidate serving as a chaperone of SO3800 because of the ability of DsbA to keep the proprotein in a reduced form, preventing disulfide bond formation. SO3800 has a relatively high number of cysteine residues when compared to other ATs (6 of 1215 in SO3800), some of these residues may offer assistance with adhesion to Fe(III)-minerals. Although one study with \textit{S. flexneri} demonstrated that the chaperone activity of DegP (a periplasmic protease that functions as a chaperone at lower temperatures) is required for proper expression and localization of IcsA (Purdy, Hong, & Payne, 2002), periplasmic chaperones have not been identified for most ATs (Henderson et al., 2004; Henderson et al., 1998).

The N-terminal, or passenger domain, of ATs may contain one, two, or three functional domains, as well as, several different functional motifs. The passenger domain is typically hydrophilic and in its unfolded state is suspected to form a temporary hairpin structure until self-translocation through the C-terminus pore. The hairpin-like structure is postulated to act in conjunction with a short \( \alpha \)-helical linker region to pull the passenger domain through the \( \beta \)-barrel pore (Maurer, Jose, &
Meyer, 1999). The short α-helical linker region has been shown in the *B. pertussis* AT, BrkA, to be essential for proper folding and activity (Oliver, Huang, & Fernandez, 2003; Oliver, Huang, Nodel, Pleasance, & Fernandez, 2003). This α-helical linker, as aforementioned, is present in the predicted secondary structure of ΔSO3800 and is postulated to have the same mechanistic function as in BrkA. Analysis of predicted amino acid sequences reveals the presence of a P-loop nucleotide-binding motif in several members of the AT family, an indication that energy may be required for OM translocation (Henderson et al., 1998). Prosite analysis of the SO3800 sequence reveals the presence of an ATP/GTP binding site motif A (P-loop) at residues 902 – 909 (AEADLGKT), which is adjacent to the N-terminus of the β-module—an ideal location for energy-coupled secretion.

To avoid proteolytic degradation by OM proteases (e.g. OmpT) the AT passenger domain must fold into native conformation in conjunction with translocation or immediately following translocation to the cell surface. Recent studies have shown that a conserved region known as the “junction region” is necessary for initiation of proper folding and activity following translocation across the OM (Oliver, Huang, Nodel, et al., 2003; Surana, Cutter, Barenkamp, & St Geme, 2004). There exists a divergence in further processing of the passenger domain following arrival of native conformation. Some ATs, such as Hsr of *Helicobacter mustelae* and Hia of *Haemophilus influenzae*, are postulated to remain covalently attached to the β-module as one large protein with the C-terminal domain spanning the OM and the N-terminal domain extending into the extracellular environment (Henderson et al., 2004; Henderson et al., 1998; Newman & Stathopoulos, 2004; O'Toole, Austin, & Trust, 1994; St Geme & Cutter, 2000). Another mechanism, seen with Ag43, AIDA-I, and the pertactins P.68, P69, & P70, involves passenger domain
cleavage from the β-module but remaining attached via noncovalent interaction with the β-module (Benz & Schmidt, 1992a, 1992b; Henderson & Nataro, 2001; Henderson et al., 1998; Leininger et al., 1991; Newman & Stathopoulos, 2004; Owen, Meehan, de Loughry-Doherty, & Henderson, 1996). The third mechanism involves the cleavage, by means of autoproteolysis or an OM protease, and release of the passenger domain into the extra-cellular environment (Henderson & Nataro, 2001; Henderson et al., 1998; Newman & Stathopoulos, 2004). SO3800 is believed to fall in line with the second mechanism due to the readiness of SO3800-containing complexes to detach from the OM when exposed to high ionic strength buffers (e.g. incubations with 0.5 M KCl buffer). Also, the size of SO3800 is approximately 126 kDa, however, when the OM wash is denatured and separated via SDS gel SO3800 is displayed in multiple bands ranging from 80-90kDa, which may be the passenger domain that has been cleaved from the transporter region. This size reduction may, however, be an illusion due to the hearty overall negative charge of SO3800 as is reported the case with Aae, an AT involved in adhesion of *Actinobacillus actinomycetemcomitans* (Rose, Meyer, & Fives-Taylor, 2003).

The study of functional domains and motifs of ATs is a recent and continually developing contribution to the overall mechanistic understanding of bacterial surface-attachment as well as host invasion in the cases of pathogenic bacteria. ATs have been shown to function as proteases, adhesins, toxins, elastases, mucinases, lipases, serum resistance proteins, and mediators of intracellular motility (Henderson & Nataro, 2001). Many ATs are known to contain more than one of these functional properties (Timpe, Holm, Vanlerberg, Basrur, & Lafontaine, 2003). The SO3800 passenger domain is no exception, displaying many conserved domains and motifs, as well as an unusually large overall residual negative charge with an excess of more...
than 50 acidic amino acids (e.g., Asp and Glu) contributing to a high net negative charge, which may contribute to electrostatic interaction to positively charged metal-oxide surfaces (e.g., Ferric- and manganese-oxide particles).

SO3800 is an OM localized protein that exhibits two important activities related to iron reduction. Principally, we have shown that SO3800-deficient cells do not adhere to Fe(III)-oxide surfaces (J. L. Burns et al., 2010). Secondly, SO3800 has glycosyl hydrolase activity, which maintains the cell surface free of capsule material. Although we have not unequivocally demonstrated that SO3800 is the only mechanism by which *S. oneidensis* adheres to Fe(III)-oxides, all the evidence presented implicates SO3800 strongly in the recognition and adherence to Fe(III)-oxide particles, as well as maintaining the cell membrane free of capsule, thus promoting intimate association with the iron mineral; ultimately facilitating maximum efficiency in a direct electron transfer mechanism.
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CHAPTER THREE

*MicroRNA biology and its implications on aging and age-related disease*

**SUMMARY**

As the molecular mechanisms associated with aging become more understood, it is apparent that the normal processes involved in the development and metabolism of an organism are subject to changes that upset its crucial homeostatic balance, which in turn sets in motion the weakening and disease-prone process of senescence. This imbalance is the result of a variety of effectors, such as environmental insults, endogenous toxins, and genetic mishaps. In addition, it is highly probable that posttranscriptional regulatory events play a large role in the changes associated with aging. The emerging knowledge of posttranscriptional regulation is redefining our understanding of the complexities of cellular systems biology and genetics. The implications of the impact that small regulatory RNAs have on the many facets of developmental and molecular biology should be included as part of our current understanding of the biochemistry involved in these processes. These molecular regulators—along with other epigenetic events—restrict the flow of genetic expression, thus affording the cell an adjustable and tempered homeostatic balance control. Recent findings in the fields of organismal development, cancer, and aging indicate that small noncoding RNA play a greater role than previously believed in orchestrating the changes associated with these processes. Furthermore, any misappropriation of these regulatory resources could lead to age-related diseases, and are therefore promising targets for prophylactics and therapeutics to combat maladies associated with aging. Here we report a brief overview of noncoding RNA as well as the potential roles of microRNAs in biochemical equilibriums where imbalance contributes to the many phenotypes of aging.
INTRODUCTION

In recent years, a paradigm shift has been taking place regarding the study of the molecular mechanisms of aging, i.e., the epigenetic control of the regulation of physiological processes dictating changes observed late in the animal lifespan. Until recently, the focus of most studies has been on risk factors associated with genetic insults, predisposition, and inheritability. Success in these areas has laid the foundation for a new focus on epigenetic and posttranscriptional factors that contribute to aging and age-related disease. Four major mechanisms are recognized as determining epigenetic forces that modulate the expression of genes and the signaling pathways in which they are embedded: DNA methylation, acetylation, genomic stability, and microRNA-dependent regulation. Each of these can affect cellular signaling; their aberrancy is becoming recognized as a causative factor for age-dependent changes. Here we focus on some of the factors contributing to the aging phenotypes, specifically the role of microRNAs in their manifestation, since this small molecular species is a member of the noncoding RNA (ncRNA)—a class of nucleic acids, which regulates gene expression yet does not encode any kind of translated product.

The discovery of small non-coding RNAs has opened the door to a previously unexplored world of posttranscriptional regulation that affects virtually every facet of developmental and cellular biology. From differentiation to senescence (Schickel, Boyerinas, Park, & Peter, 2008; Schommer et al., 2008), oncogenesis to tumor suppression (Schickel et al., 2008), and age extension to lifespan shortening (Boehm & Slack, 2005), these small RNAs are believed to regulate more than 60 percent of all human genes (Wu, Sun, Zou, & Chen, 2007). While most studies on noncoding RNA regulation have focused on roles in cancer and various other specific diseases, a few demonstrate a direct correlation between noncoding RNA regulation and aging in animals such as worms (Boehm & Slack, 2005; Boehm & Slack, 2006) and mice (O. C. Maes, An, J., Sarojini, H., Wang, E., 2008), as well
as in humans (Drummond, McCarthy, Fry, Esser, & Rasmussen, 2008). A thorough understanding of the posttranscriptional and epigenetic factors involved in both normal aging and age-related disease (e.g., cancers, diabetes, neurodegenerative and cardiovascular diseases, etc.) will illuminate new strategies and approaches to diagnose, treat, or suppress many aspects of age-dependent frailty.

Our understanding of the biology of aging has advanced remarkably over the past century, largely due to breakthroughs in genomic technologies combined with the establishment of key model systems. These model systems support not only descriptive work on changes in signaling dysfunction over the course of an animal’s lifespan but also genetic manipulation to correct such malfunctioning signaling, which may result in longevity extension. Several well established models, such as yeast, flies, and different rodent species—including long-lived dwarf mice, transgenic mice, and calorie-restricted animals—have revealed the crucial roles of the endocrine system, oxidative defense, and toxin response as key biochemical factors for lifespan determination (Kenyon, 2005). In particular, specific dietary alterations, such as caloric restriction or supplementation (e.g., amino acids or hormones), have helped target particular pathways that significantly affect lifespan (Kenyon, 2005). From all these studies, an understanding has emerged of the now well-known central signaling axes, including pathways controlled by growth hormone, insulin-like growth factor 1 (IGF-1), and insulin (Bartke et al., 2001; Bartke, Masternak, Al-Regaiey, & Bonkowski, 2007; H. M. Brown-Borg, 2005; H.M. Brown-Borg, 2006; H. M. Brown-Borg, 2008; H. M. Brown-Borg, Rakoczy, S.G., Sharma, S., Bartke, A.,, 2008; Kenyon, 2005). These all converge to trigger downstream signaling targets (e.g., serine/threonine kinases like Akt/PKB), which in turn alter stress resistance in the organism (V. D. Longo, Finch, C.E., 2003). The connection of these signaling phenomena to the function of noncoding RNAs, specifically microRNAs, are based on findings demonstrating that this molecular species can be regulated by hormones.
(Poy et al., 2004) and also influence hormonal regulation (Fiedler, Carletti, Hong, & Christenson, 2008; Poy et al., 2004), and toxic defense gene expression (O. C. Maes, An, J., Sarojini, H., Wang, E., 2008). In this report we focus on three recognized phenomena—oxidative stress and inflammation, intermediate metabolism, and cancer—to review some of the molecular mechanisms of aging including the functional impact of ncRNAs, particularly microRNAs.

**DISCUSSION**

**Small RNA biology and epigenetics**

**Noncoding RNA**

Of the 3 billion base pairs comprising the human genome, only 1.5 to 2 percent is known to encode proteins; the remaining regions were formerly referred to as “junk DNA” (Bartke et al., 2001). A drastic shift in thinking regarding the extent of usefulness of these uncoded regions occurred after the ENCODE project consortium revealed that more than 90 percent of the human genome is transcribed—mostly into non-protein coding RNA (ncRNA) (Bartke et al., 2007). Although it is very possible that some of the transcriptional output is merely random nonfunctional noise, it is also very likely that the majority of this output is regulatory RNAs that form networks, functioning to direct genetic information at all levels of cellular processes. These regulatory networks appear to have coevolved with a sophisticated protein infrastructure that supports RNAs and acts on their instructions (Mattick, 2007). It is even proposed that feed-forward RNA regulatory networks primarily program the direction of epigenetic events, such as differentiation and development, and that most of the information required for multicellular development is embedded in these networks (Poy et al., 2004). Therefore, it is possible that molecular degeneration of the organization and/or communication within or between such regulatory networks is responsible for many of the physiological manifestations associated with aging.
The term “noncoding RNA” (ncRNA) refers to both structural (e.g., transfer, ribosomal, and small nuclear RNAs) and the recently discovered regulatory RNAs (e.g., siRNA and miRNA), which are comprised of both long (>200 nt) and small (<200 nt) ncRNAs. We focus here on regulatory ncRNAs, their processing, mechanism, function, and possible impact on aging. Regulatory RNAs function at both transcriptional and posttranscriptional levels. However, our understanding of these regulatory ncRNAs has only begun; much of the transcriptional regulation, biogenesis, and functional mechanisms still remain to be explored.

**Long ncRNA**

Noncoding regulatory RNAs greater than 200 nucleotide bases in size are called long ncRNA. While it is believed that there is a large number of these ncRNAs, only a small subset has clearly defined functions. Also, it is believed that an appreciable portion of long ncRNAs serves as precursors for small ncRNAs (Beltran et al., 2008). Long ncRNAs target different aspects of RNA transcription, some functioning as activators and others as repressors, and quite possibly, some serving as both. Some long ncRNAs target components of transcription such as RNA polymerase (RNAP) II (Wilkinson, Davies, & Isles, 2007). Together with transcription factors, these ncRNAs may constitute a regulatory network that helps maintaining the delicate balance of gene expression—either through activation or repression—in complex eukaryotes. Two examples of such cooperative relationships at the transcriptional level can be seen with long ncRNA, Evf-2, which functions as a co-activator for the homeobox transcription factor, Dlx2 (Nagano et al., 2008); and with Alu RNA, which prevents the formation of active pre-initiation complexes via high-affinity binding to RNAP II (Schoeftner & Blasco, 2008).

Noncoding RNAs have been shown to facilitate various aspects of posttranscriptional messenger RNA (mRNA) processing (Beltran et al., 2008). Examples of this processing have
been shown with some natural antisense transcripts (J. Chen et al., 2004). Natural antisense transcripts (NAT), the majority of which are noncoding RNAs, are either cis or trans, depending on the origin of transcription, and encompass another emerging field of posttranscriptional regulation. The cis-Natural antisense transcripts (cis-NAT) are transcribed from the opposite strand of the same locus as the sense DNA and exhibit a long complementary overlap with the sense transcript (J. Chen et al., 2004). Messenger RNA processing was demonstrated by a study showing that the cis-NAT that complements the splice site of the 5’-UTR intron of Zinc finger E-box protein 2 (Zeb2) is required for Zeb2 splicing (Beltran et al., 2008). The trans-NATs, such as miRNAs, are transcribed from all over the genome, target multiple transcripts, have short complementary regions, and allow for a few mismatches (Carmichael, 2003). This category of ncRNAs will be touched on later in this review.

Possibly the most recognized function of long ncRNA is to regulate the epigenome by modifying chromatin structure. Long ncRNAs regulate chromatin by activating or silencing cis or trans genes through a variety of ways, e.g., a single promoter, a gene cluster, or an entire chromosome (Whitehead, Pandey, & Kanduri, 2008). A brilliant study by Rinn et al demonstrated long-distance gene regulation of Homeotic (Hox) genes—a class of mammalian genes that establish the body plan during early development—by ncRNA. This study shows that a Hox locus (HOXD) is silenced by a ncRNA functioning in trans from a different chromosome containing the HOXC locus in which the ncRNA, called HOTAIR, was encoded (Rinn et al., 2007). HOTAIR is a 2.2 kb ncRNA that interacts with Polycomb Repressive Complex 2 (PRC2), recruiting it for HOXD loci targeting and H3 histone lysine-27 trimethylation (H3K27) (Rinn et al., 2007). The Rinn et al study identified 231 such transcripts, which represent putative ncRNAs called Hox ncRNAs. The expression of Hox ncRNAs, which are dispersed along developmental axes and contain unique sequence motifs,
demarcates chromosomal domains of differential histone methylation and RNA polymerase accessibility (Rinn et al., 2007). *Hox* ncRNAs are an example of potentially other ncRNAs functioning in long-range gene silencing in *trans*; the implications of which are vast and warrant further exploration.

Long ncRNAs also function in *cis*-acting epigenetic processes such as genomic imprinting. Imprinting is an epigenetic process in which one of a set of autosomal genes is expressed in a parent-of-origin-specific manner (Wilkinson et al., 2007). Nagano et al reported an inspiring study with *cis*-acting long range ncRNA regulation. These researchers showed that the long ncRNA called *Air* interacts with the *cis*-linked *Slc22a3* gene promoter region and helps recruit the H3K9 histone methyltransferase, G9a, to the promoter region to epigenetically silence transcription, thus orchestrating the allele-specific silencing of *Slc22a3* (Nagano et al., 2008). Nagano et al suggested that a similar mechanism of epigenetic silencing might be employed by the well-known long ncRNA, *Xist*, on the X chromosome. Taken together, these studies on both *Hox* and *Air* (and perhaps many other long ncRNAs) demonstrate long ncRNA interacting with chromatin to mediate targeted recruitment of histone-modifying proteins for long-range epigenetic gene regulation both in *cis* and *trans* (Nagano et al., 2008; Wilkinson et al., 2007).

The recent discovery of telomeric ncRNAs (TelRNAs) suggests that long ncRNAs may play a direct role in the molecular mechanisms of aging (Azzalin, Reichenbach, Khoriauli, Giulotto, & Lingner, 2007; Schoeftner & Blasco, 2008). Stefan Schoeftner and Maria Blasco reported that TelRNAs are structural components of telomeric chromatin that block the activity of telomerase *in vitro*. Their report goes on to suggest that TelRNAs may regulate telomerase activity at chromosome ends, which may directly or indirectly play a role in cancer and aging (Schoeftner & Blasco, 2008). While these studies with long ncRNA represent only a very small portion of the available knowledge, they do offer some insight
into the innumerable possibilities of the intertwining roles between long ncRNA and the biological processes of development and aging. Furthermore, any mishap in such regulation may initiate the homeostatic imbalance that leads to commonly encountered disease states.

**Small ncRNA**

In general, small ncRNAs stem from the molecular processing of long ncRNA precursors. Classifications of small regulatory ncRNAs include short interfering RNAs (siRNA), microRNAs (miRNA), Piwi-interacting RNAs (piRNA), repeat-associated small interfering RNAs (rasiRNAs), heterochromatin small RNAs (hcRNA), NAT-derived siRNAs (natsiRNA), trans-acting siRNAs (tasiRNA), 21U-RNA, tiny noncoding RNAs (tncRNA) (Farazi, Juranek, & Tuschl, 2008), and small modulatory double strand RNAs (dsRNA) (Kuwabara, Hsieh, Nakashima, Taira, & Gage, 2004). Although each class stems from distinct precursors and binds to distinct members of the Argonaute/Piwi protein family, they all form ribonucleoprotein complexes that recognize either near perfect or partially complementary homology with nucleic acid targets to mediate a variety of regulatory processes, such as transcriptional and posttranscriptional gene silencing.

Of the small ncRNAs, microRNAs are currently the most recognized class in animals. MicroRNAs range from 18 to 25 nucleotides long, and usually begin with a 5'-terminal U. According to the latest release of Sanger miRBase (release 12.0), 866 different mature miRNAs are identified in humans, and around 627 in mice (S. Griffiths-Jones, Saini, van Dongen, & Enright, 2008). MicroRNAs are encoded all over the genome, in intergenic regions, introns, exons, exon overlaps, and UTR regions, and are found many times in clusters (Bonawitz, Rodeheffer, & Shadel, 2006). Although some miRNA clusters are found interspersed among Alu repeats (e.g., human chromosome 19 miRNA cluster) and are therefore transcribed by RNA polymerase III (RNAP III) (Borchert, Lanier, & Davidson, 2006), most miRNAs are transcribed into 5'-cAPPED poly(A)-tailed primary miRNAs (pri-
miRNA) by RNAP II (Kim, 2005). The biogenesis of mature miRNAs and the mechanisms of their function are summarized in Figure 3.1. After transcription inside the nucleus, pri-miRNAs fold into clusters of imperfect 3 helical-turn (33bp) stem-and-loop structures that are recognized by DGCR8 (DiGeorge syndrome critical region 8, also known as Pasha in some species) and Drosha, which functions to cut pri-miRNAs 22 bp from the loop to release a precursor miRNA (preRNA) of about 70 nucleotides (Han et al., 2006). Exportin 5 and Ran (ras-related nuclear protein) GTPase then transport pre-miRNAs into the cytoplasm where they are further processed into double-strand processing intermediates by the enzyme Dicer. The guide strand, because of its less stable 5’ end, is thermodynamically favored for incorporation into the Ago effector complexes, while the remaining passenger strand, is degraded (Takeda, Iwasaki, Watanabe, Utsumi, & Watanabe, 2008). Mature miRNAs function in the RISC complex to suppress target gene expression by either inhibiting mRNA translation or signaling for mRNA degradation via binding to either the 3’ UTR or coding region of the target mRNA. Alternative miRNA biogenesis mechanisms have been reported. Such is the case of intronic miRNAs, called mirtrons, whose 3’2 end of the stem-loop precursor structure is cleaved by nuclear pre-mRNA splicing rather than by Drosha (Berezikov, Chung, Willis, Cuppen, & Lai, 2007).
Most miRNA gene regulation occurs at the posttranscriptional level, by either translation inhibition or mRNA degradation, depending on which Ago protein binds the miRNA and the degree of complementarity between the miRNA and target mRNA. MicroRNAs with near-perfect complementarity usually signal for mRNA cleavage, whereas miRNAs that exhibit more mismatches usually inhibit translation and/or trigger the transport of mRNA to mRNA-processing bodies (P-bodies, or cytoplasmic GW-bodies) where the message is stored or degraded (Du & Zamore, 2007). MicroRNA recognition of target sites normally depends on Watson-Crick pairing to the 5’ region of the miRNA, primarily nucleotides 2-7, which is known as the miRNA “seed”. There are five types of seed matches that have been described to date (Baek et al., 2008; Friedman, Farh, Burge, & Bartel, 2009; Grimson et al., 2007).
Although miRNAs typically bind to the 3'-UTR, Tay et al. recently reported that mouse transcription factors (TF), Nanog, Oct4 (also known as Pou5f1), and Sox2, have naturally occurring miRNA target sites within their peptide-coding sequences (PCS). Tay’s report showed that miRNAs up-regulated by retinoic acid-induced differentiation (miRs-124, 296, and -470) target these TFs, and that the upregulation of these miRNAs led to the transcriptional and morphological changes characteristic of differentiating mouse embryonic stem cells (Tay, Zhang, Thomson, Lim, & Rigoutsos, 2008). The role of miRNA has not only been shown to affect development but also to be a major factor in the control of aging, as has been demonstrated in Caenorhabditis elegans.

The first miRNA and gene target pair to be described were lin-4 and the transcription factor, lin-14. This pair was found to control the timing of larval development in Caenorhabditis elegans (Lee, Feinbaum, & Ambros, 1993). Over a decade later it was reported that the pair are also involved in life span regulation in the adult (Boehm & Slack, 2005). Slack and Boehm demonstrated that reducing lin-4 expression accelerates tissue aging and shortens life span, whereas increasing lin-4 expression or reducing lin-14 activity led to increased longevity (Boehm & Slack, 2005). This study suggests that the extension of life span conferred by a reduction in lin-14 activity is dependent on two other transcription factors, DAF-16 and HSF-1, which means that the lin-4/lin-14 pair affects life span via the insulin/insulin-like growth factor-1 pathway (Boehm & Slack, 2005).

**Oxidative Stress and Inflammation**

Major intrinsic contributors to homeostatic imbalance and its resulting complications include mitochondrial dysfunction, oxidative stress, inflammation and their interrelatedness. Some of these may result from epigenetic and posttranscriptional regulatory events. Several reviews cover the functional impact of miRNAs on the developmental path and their essential
role in the determination of cell lineage (Aberdam, Candi, Knight, & Melino, 2008; Erson & Petty, 2008; Schickel et al., 2008). The following describes examples of these contributors to aging in skin, heart, brain, and vasculature while connecting miRNA functions with oxidative stress and inflammation in and subsequent consequences on the age-dependent phenotypes seen in these tissues.

Mitochondrial dysfunction can lead to a destructive spiral of continued cellular insults that perpetuates and intensifies the age-dependent physiological deterioration. This dysfunction can arise from reduced oxidative phosphorylation gene expression or efficiency, which has been shown to increase levels of reactive oxygen species (ROS)—molecules responsible for oxidative stress and known to contribute significantly to aging (Bonawitz et al., 2006). Oxidative stress is caused by ROS imbalance: the cell is overwhelmed by or loses the ability to deal with these reactive molecules that damage nucleic acids and proteins, as well as many other essential macromolecules in cells (Lesnefsky & Hoppel, 2006). An example of oxidative stress can be seen in human skin, which possesses exceptionally efficient antioxidant activities; however, during aging, anti-oxidant capacity decreases and ROS levels increase (Callaghan & Wilhelm, 2008). The loss of antioxidant capacity stems from a reduction in oxidative defense proteins, which could be the result of posttranscriptional suppression by miRNAs.

The effects of oxidative stress on aging skin are under continued study and many reports have been published relating ROS to skin aging. The effects of ROS are evident as skin becomes increasingly thin, dry and blemished; with an obvious loss of elasticity and architectural integrity due in part to the degradation of collagen and other ROS-induced skin matrix changes (Callaghan & Wilhelm, 2008). Oxidative stress and induction of transcription factor AP-1 is thought to impair and degrade collagen types I and III in the dermis, which promotes skin aging (Callaghan & Wilhelm, 2008). Although specific studies of miRNAs and
aging skin have yet to be reported, miRs-146a, 203, 21 and 125b have reported roles in phenomena such as hair follicle morphogenesis, psoriasis, eczema, and carcinogenesis, with miR-203 demonstrating skin-specific expression (Bostjancic & Glavac, 2008).

Mitochondrial dysfunction is attributed to the aging cardiac phenotype, by the increase of ROS production and the release of cytochrome c (Suarez, Fernandez-Hernando, Pober, & Sessa, 2007; Suh, Heath, & Hagen, 2003). A recent study by Brink et al showed that four of the five electron transport chain complexes—the main constituents of the oxidative phosphorylation pathway—exhibit decreased expression in aged mouse heart (Brink, Demetrius, Lehrach, & Adjaye, 2008). Interestingly, recent studies show direct involvement of miRNAs, such as miR-195, in cardiac hypertrophy and heart failure, and demonstrate that a cardiac-specific miRNA, miR-208, is required for cardiomyocyte hypertrophy, fibrosis, and expression of betaMHC in response to stress and hypothyroidism (van Rooij et al., 2006; van Rooij et al., 2007). It is tempting to speculate that miRNAs target crucial components of oxidative phosphorylation with increasing suppression, which gives rise to the observed aging phenotype.

The effects of oxidative stress are observed at all macromolecular levels in aging brains (Petrosillo, Matera, Casanova, Ruggiero, & Paradies, 2008) and are reported to cause brain dysfunction (B. Martin, Mattson, & Maudsley, 2006). Recent reports have shown correlations between mitochondrial complexes and aging brains. For example, aging rat brains exhibited mitochondrial complex I dysfunction (Petrosillo et al., 2008), and mitochondrial complexes I and IV displayed a marked decrease in expression in the aging hippocampus (Navarro et al., 2008). Reductions of F0F1-ATP synthase subunits in rat aging brains were also reported (Dencher, Frenzel, Reifschneider, Sugawa, & Krause, 2007). Despite the significance of these recent findings and the impact they have on the overall understanding of natural aging processes, current knowledge fails to provide an adequate
understanding of the causative factors and regulatory events leading to the decrease in oxidative phosphorylation gene expression in aging brains. However, recent findings with neurodegenerative disease have shed some light on possible causative regulatory factors in age-associated neural decline. For example, in Alzheimer’s disease a key miRNA responsible for suppression of the detrimental beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1) is found to be decreased in the early stages of the disease (W. Wang et al., 2008). The decreased expression of this miRNA, miR-107, allows for increased expression of its target protein, BACE1, the enzyme responsible for the generation of β-amyloid, which plays an early role in Alzheimer’s disease (W. Wang et al., 2008), an age-related disease that involves similar mechanisms found with inflammation.

Increased inflammation is another major characteristic of aging experienced by an aging individual, which can result in a suite of different ailments depending on the type, location, and origin of inflammation. Oxidative stress—including both ROS and reactive nitrogen species (RNS)—has been tied to inflammation in skin (Okayama, 2005), prostate (Khandrika, Kumar, Koul, Maroni, & Koul, 2009), pancreas (Leung & Chan, 2009), and lung (Azad, Rojanasakul, & Vallyathan, 2008), as well as in vasculature (Lubos, Handy, & Loscalzo, 2008) and the brain (Lukiw, Zhao, & Cui, 2008; Munhoz et al., 2008). These different types of tissue inflammation lead to, or result in a number of disease states, most of which are associated with aging. Moreover, oxidative stress is linked to autoimmune related inflammation such as rheumatoid arthritis (RA) (Hitchon & El-Gabalawy, 2004).

Interestingly, miRNAs -146a/b and -155 are found to be upregulated in RA compared with patients with osteoarthritis (OA) (Sonkoly & Pivarcsi, 2009; Stanczyk et al., 2008). It has been shown that miR-155 is under the regulation of tumor necrosis factor alpha (TNF-α)—a pro-inflammatory cytokine—in synovial fibroblasts of RA patients (RAFS) (Stanczyk et al., 2008). MicroRNA-155 is believed to be involved in the suppression of the extracellular
matrix-degrading proteins MMP-3 and -1 (Stanczyk et al., 2008), two suspected contributors to complications associated with RA. Necrosis factor kappa B (NF-κB) has been shown to regulate miR-146a expression, which, although upregulated in RA, is believed to be malfunctioning, seeing as how it does not effectively suppress its target genes, tumor necrosis factor receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK-1) (Pauley et al., 2008). Repression of TRAF6 and IRAK-1 expression has been shown to reduce TNF-α expression by as much as 86 percent; hence, it is believed that miR-146a is a malfunctioning feedback loop in RA cases (Pauley et al., 2008). Conversely, in Alzheimer’s disease, miR-146a exhibits increased expression with inverse expression of its target gene, complement factor H (CFH)—an important inflammatory response repressor (Lukiw et al., 2008). As shown in Figure 2 (adapted from (Hitchon & El-Gabalawy, 2004)), the intertwining of miRNA, TFs, and other signaling molecules is unavoidable, and the imbalance of these cascades, the probability of which undoubtedly increases with age, results in molecular signaling and regulatory complications that is manifested as different disease states.
Numerous studies of model systems of delayed aging show an enhanced ability to deal with ROS and toxic stress that stem from variations in the GH/IGF-1/insulin signaling pathway (H. M. Brown-Borg & Rakoczy, 2003; H. M. Brown-Borg, Rakoczy, Romanick, & Kennedy, 2002; H. M. Brown-Borg, Rakoczy, S.G., Uthus, E.O., 2004; Kenyon, 2005). However, this enhanced ability may stem not only from endocrine variations and environmental stresses but also from posttranscriptional dys-regulation of upstream factors resulting from the improper balance of miRNA expressions. Disequilibrium of key miRNAs may cause inappropriate expression of genes critical to various signaling mechanisms required for the operation or protection of individual tissue functions. The above examples
are by no means comprehensive of the present knowledge regarding the factors contributing to aging but rather serve as a brief overview of some of the major factors to some of the disequilibriums that lead to aging, and to open the door to discover the many possible ways that variations in miRNA expression affects aging.

**Intermediate metabolism**

The posttranscriptional regulation of intermediate metabolism by miRNA function, although currently underexplored, may introduce a new avenue of research for the study of the biology of aging. Key model systems, such as the Ames dwarf mouse and calorie restricted mice, have laid the foundation for the role of intermediate metabolism as a major contributor to the process of aging (H. M. Brown-Borg & Rakoczy, 2005; H. M. Brown-Borg et al., 2004, 2005; H. M. Brown-Borg, Rakoczy, S.G., Sharma, S., Bartke, A., 2008; Kenyon, 2005). The Ames dwarf mouse is an attractive model system to study the molecular mechanisms of aging because of its remarkable ability to live 70 percent longer than its wild-type siblings (Anderson, 1995; Schaible, 1961). This ability stems from a point mutation affecting the anterior pituitary that causes deficiencies in growth hormone, prolactin, and thyrotropin, which in turn affect downstream IGF-1 and insulin expression, ultimately leading to the long-lived phenotype (V. D. Longo, Finch, C.E., 2003). This pathway is the central axis that contributes to extended longevity in calorie restricted animals as well (Parr, 1997). Several studies report that the dwarf mouse exhibits heightened oxidative and toxic defense proteins such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione S-transferase (H. M. Brown-Borg & Rakoczy, 2003; H. M. Brown-Borg et al., 2002). Interestingly, our previous study of changes of miRNA expression in mouse liver during aging showed that the levels of miR-669c and miR-709 gradually increase with age beginning around 18 months, followed by upregulation of miR-93 and miR-214 in extremely aged liver (33 months). These upregulated miRNAs target genes associated with
detoxification and regenerative capacity, functions known to decline in old liver. In particular, these upregulated miRNAs may contribute to the age-related decline in oxidative defense, by targeting various classes of glutathione S-transferases (O. C. Maes, An, Sarojini, & Wang, 2008).

Intermediate metabolism is intricately related to known contributors to aging and age-related disease and involves a homeostatic balance that degrades with aging. As an example, several studies have shown that arginase—a rate limiting enzyme of the urea cycle—competes with nitric oxide synthase (NOS) for L-arginine (Durante, Johnson, & Johnson, 2007; Santhanam, Christianson, Nyhan, & Berkowitz, 2008; Santhanam et al., 2007; Steppan et al., 2006) despite a much lower binding affinity (K_m 1-5 mM) than NOS (K_m ~ 2 – 20 M) (Santhanam et al., 2007). Arginase regulates NOS via substrate depletion because of a much higher catalytic turnover that increases with S-nitrosylation (Durante et al., 2007; Romero et al., 2008) despite a much lower binding affinity (K_m 1-5 mM) than NOS (K_m ~ 2 – 20 M) (Santhanam et al., 2007). Arginase regulates NOS via substrate depletion because of a much higher catalytic turnover that increases with S-nitrosylation (Durante et al., 2007; Romero et al., 2008). NOS in turn regulates arginase via the principle intermediate, N-hydroxyarginine (NOHA), in the conversion of arginine to nitric oxide (NO) and citrulline, which acts as a modest arginase inhibitor (Ignarro et al., 2001). Yet, NO enhances arginase activity via S-nitrosylation of cysteine residues 168 and 303, which yields a six-fold decrease in arginase K_m affording arginase an enhanced ability to bind substrate (Santhanam et al., 2007). These studies point out that arginase expression and S-nitrosylation both increase with aging; therefore, arginase increasingly outcompetes NO synthase—resulting in a decrease of bioavailable NO (Santhanam et al., 2008; Santhanam et al., 2007). This decline in bioavailable NO is suggested to contribute to endothelial dysfunction in the aging cardiovascular system (Santhanam et al., 2007) and numerous other conditions such as wound healing in diabetes and possibly many more of the clinical complications associated with diabetes (Romero et al., 2008), as insulin has been shown to reduce arginase activity (Kashyap, Lara, Zhang, Park, & DeFronzo, 2008) and is known to represses urea cycle gene
expression (Romero et al., 2008). Increased arginase expression also has a speculated role in heart failure (Durante et al., 2007) and increased smooth muscle cell and tumor cell proliferation (Ignarro et al., 2001; Wei, Wu, Morris, & Ignarro, 2001). The arginase review by Durante et al points out that arginase-mediated impairment of NO synthesis is implicated in several other pathological conditions, including cystic fibrosis, psoriasis, asthma, glumerulonephritis, arthritis, and sickle cell disease.

The reciprocated regulation between arginase and NOS is crucial for homeostatic balance. Nitric oxide is an important signaling molecule playing a crucial role in circulation, platelet and leukocyte activation, regulation of smooth muscle cell proliferation, inhibition of tumor cell proliferation, extracellular matrix deposition and endothelial cell death (Durante et al., 2007; Ignarro et al., 2001; Loscalzo & Welch, 1995). Despite its importance, elevated levels of NO lead to apoptosis (Brune, 2003; Brune, Zhou, & von Knethen, 2003), and hence, arginase plays a vital role in the regulation of bioavailable NO and vascular aging. Recent studies have reported that diabetes-induced impairment of vasorelaxation to acetylcholine is correlated with increases in reactive oxygen species (ROS) and arginase activity in both the aorta and liver (Romero et al., 2008; Santhanam et al., 2008). The tight coupling between oxygenase and reductase domains of NOS maximizes NO formation rather than side products; however, ROS such as superoxide, peroxynitrite, and hydrogen peroxide are produced when substrate L-arginine (or tetrahydrobiopterin (BH4) with endothelial NOS) is limited, as is the case with increased arginase expression (Berka, Wu, Yeh, Palmer, & Tsai, 2004). Romero et al demonstrated the effectiveness of posttranscriptional regulation in limiting arginase expression in a recent study. This group was able to prevent the rise in arginase activity and normalize NO signaling in bovine coronary endothelial cells by transfecting them with arginase small interfering RNA (siRNA) (Romero et al., 2008).
In fact, vascular aging is attributed in part to endothelial cells that become dysfunctional because of the reduction in bioavailable nitric oxide (NO), which in turn alters the production of prostanoids, causes the impairment of endothelium-dependent hyperpolarization, and increases the release of endothelin-1 (Feletou & Vanhoutte, 2006). Studies have shown that miR-221 and miR-222 may reduce the expression of endothelial nitric oxide synthase (eNOS) (Suarez et al., 2007), which may lead to a reduction in bioavailable NO, the results of which are the same as increased arginase activity listed above. With regard to vascular inflammation—a suggested contributor to vascular diseases such as atherosclerosis—miR-126 expression is shown to inversely correlate with TNF-α-stimulated vascular cell adhesion molecule 1 (VCAM1) expression (Urbich, Kuehbacher, & Dimmeler, 2008). TNF-α-activated TFs, NF-κB and IRF-1, induce VCAM1 upregulation. VCAM-1 is an intracellular adhesion molecule that mediates leukocyte adhesion to endothelial cells (Sonkoly & Pivarcsi, 2009). It is the adherence of these leukocytes to the endothelial cells constituting the vascular walls that, built up over time with certain types of lipoproteins, forms plaques that cause the narrowing and hardening of arteries. Functional experiments demonstrated that overexpression of miR-126 decreased TNF-α-induced leukocyte adherence to umbilical vein endothelial cells (HUVEC), and vice versa, inhibition of miR-126 increased VCAM1-mediated leukocyte adherence to HUVEC cells (Harris, Yamakuchi, Ferlito, Mendell, & Lowenstein, 2008). MicroRNA-155 is also believed to have a role in cardiovascular disease; however, unlike what is observed in RA, it is the decrease in expression of miR-155 that is suggested to cause the problem, as it has been shown to translationally repress angiotensin II type 1 receptor (AT1R) (M. M. Martin, Lee, Buckenberger, Schmittgen, & Elton, 2006). When AT1R is activated by angiotensin II, a cascade of events is set in motion that includes vascular inflammation, altered vascular tone,
structural remodeling, and endothelial dysfunction, all of which are complications associated with cardiovasculature and kidney disease.

This posttranscriptional regulation maintains the critical balance of intermediate metabolites and may contribute to the delayed aging phenotype. Experimentally determined miRNA expression represents a snapshot of how a cell or tissue is attempting to maintain or reestablish homeostatic balance. Whether or not the miRNA expression levels observed in aged tissues or cells are causative or reactionary to functional decline varies case by case; however, a thorough understanding of these mechanisms could help us develop therapeutics and prophylactics to stabilize existing or potential imbalances that lead to disease. As the study of miRNA and aging advances, it is possible that we will see miRNA regulation intertwined with every known biochemical pathway system, and that these miRNA either contribute to or fight against the functional decline suffered by every organism during aging.

**Cancer**

With the exception of a few childhood cancers, cancers known to afflict humans are generally manifested after mid-life; thus, cancer has always been considered an age-dependent disease, and the risk of cancer increases with age. Many groundbreaking studies demonstrate the relationships between epigenetic events, posttranscriptional regulation and cancer. While miRNA studies in cancer constitute a most promising field for new diagnostic and therapeutic discovery, it remains to be seen how preventive medicine may succeed in reducing cancer risk factors by manipulating patterns of miRNA expression before the early pathological steps of this devastating disease ever occur. In other words, a delayed upregulation of unwanted miRNA expression might reduce or slow cancer development or even prevent cancer from ever occurring. The dwarf mouse is an excellent example; its long-
lived phenotype is attributed in part to its ability to stave off cancer (Ikeno, Bronson, Hubbard, Lee, & Bartke, 2003).

Some of the most promising studies on miRNAs and cancer have focused on the “guardian of the genome”, the p53 tumor suppressor; p53 is central to many cellular protection mechanisms, including cellular senescence. Ablation of Dicer and loss of mature miRNAs in embryonic fibroblasts causes upregulation of p19Arf and p53, inhibition of cell proliferation, and induction of a premature senescence phenotype; this phenotype is also observed in vivo after Dicer ablation in developing limbs and skin of adult mice. Deletion of the Ink4a/Arf or p53 locus rescues fibroblasts from premature senescence induced by Dicer ablation (Mudhasani et al., 2008). As a direct (mutual) transcriptional target of p53, the miR-34 family can induce morphological alterations characteristic of cellular senescence (He et al., 2007). Nutlin-3, an MDM2 inhibitor, activates p53, resulting in upregulation of the miR-34 family, and consequently cell senescence (Kumamoto et al., 2008). Cellular senescence may be induced by miR-34 through modulation of the E2F pathway (Tazawa, Tsuchiya, Izumiya, & Nakagama, 2007).

MicroRNA regulation functions in both tumorigenesis and tumor suppression, as seen in the case of two tumor suppressors, p16INK4a and p19Arf, whose expression increases during aging and replicative senescence. Expression of miR-let-7b progressively increases in the cerebrum with age, down-regulating its target gene, HMGA2, and in turn up-regulating p16INK4a and/or p19Arf (Nishino, Kim, Chada, & Morrison, 2008). MiR-24 is predicted to associate with the p16INK4a mRNA coding and 3'-untranslated regions; it suppresses p16INK4a expression in human diploid fibroblasts and cervical carcinoma cells. Increased p16INK4a expression, seen in replicative senescence, is also associated with decreased levels of miR-24 (Lal et al., 2008). MiR-20a posttranscriptionally suppresses Leukemia/lymphoma Related Factor (LRF), which in turn allows for increased p19ARF transcription, and
consequently induces senescence in mouse embryonic fibroblasts (MEF) (Poliseno et al., 2008). MicroRNA expression profiling reveals upregulation of hsa-mir-371, hsa-mir-369-5P, hsa-mir-29c, hsa-mir-499 and hsa-let-7f in replicative senescence of mesenchymal stem cells (Wagner et al., 2008). Inhibition of miR-17-5p leads anaplastic thyroid cancer (ATC) cells to cellular senescence (Takakura et al., 2008).

Recent emerging evidence has rekindled the interest in the relationship between inflammation and cancer—two age related factors—with the newly discovered variable of miRNA suppression joining these usual suspects (Hussain & Harris, 2007). A study reported by Chen et al demonstrated the role of has-miR-199a as a regulator of IκB kinase-β (IKKβ) expression (R. Chen et al., 2008). This study not only identifies IKKβ as a major factor in promoting the TLR4-MyD88-NF-κB pathway in epithelial ovarian cancer (EOC) cells, which enables them to secrete proinflammatory and protumor cytokines and thus promote tumor progression, but also shows that increased IKKβ expression may yield chemoresistance as well (R. Chen et al., 2008). Two EOC cell types, Types I & II, were shown to exhibit differential expression of has-miR-199a. Type I EOC cells exhibited significantly higher IKKβ expression compared to Type II EOC cells, as well as a suite of inflammatory cytokines that were elevated in Type I and undetectable in Type II cells. Type I cells were also much more proliferative and chemoresistant than Type II cells. Fascinatingly, Type II cells exhibited elevated has-miR-199a levels, whereas Type I exhibited a deficiency in has-miR-199a expression, which was shown to allow for increased IKKβ expression and the cascade of proinflammatory and growth-promoting events and possible chemoresistance that follows (R. Chen et al., 2008). Chen and colleagues were able to prove that it was indeed has-miR-199a responsible for the deficient IKKβ expression in Type II cells and thus opened the door for the development of effective therapies to circumvent chemoresistant ovarian cancers using miRNA-based means.
The insightful studies above, and many others like them, shed more light on the molecular mechanisms of cancer and offer clues as to how we can employ these natural regulators to suppress aging and age-related disease. MicroRNA and other noncoding RNA regulation are essential tools employed by cells to both maintain stability and to counter insults that cause imbalance; however, their employment may also propagate or instigate instability. This understanding has broadened our view of the many overlapping pathways and redundancies built into the cells dynamic and tempered array of biochemical constellations and of why diseases such as cancer remain so elusive to cure.

**Predominant Upregulation of MicroRNA Expression**

Our results seem to show a tendency toward predominant upregulation of miRNA expression during aging (O. C. Maes, An, J., Sarojini, H., Wang, E., 2008; O. C. Maes et al., 2007) and unpublished data). Tissue examination has been limited to aging liver thus far; however, although human tissues are diverse in function and cellular composition, all tissues and the cells populating them share a common thread—the necessity to maintain oxidative defense to ward off insults from reactive oxygen species and toxins. Our observation of the trend towards predominant upregulation of miRNA expression during aging may yet prove valid beyond the liver; after all, most lead miRNAs identified as upregulated in liver target genes that are functionally classified in the six families of oxidative defense, DNA repair, intermediate metabolism, cytoskeletal organization, cell cycle control, and apoptosis. By no means is degenerative signaling during aging limited to these six families of genes; nor is universal deterioration shared among all tissues. Nevertheless, it is likely that these six families represent an Achilles heel whereby system networks are damaged during aging, and this damage induces a cascade effect upon other networks controlled by families of genes beyond these six families.
Downregulation of essential gene expression to maintain the signaling contributed by the function of the above six families of genes is observed repeatedly in tissues during aging by numerous reports and documented in many excellent reviews. The predominant upregulation of miRNA expression that we report in the systems studied so far, liver, introduces yet another layer of molecular control beneath gene expression—the posttranscriptional level of control for these genes’ expression. Our observation of predominant miRNA upregulation introduces a new paradigm for the study of the biology of aging and asserts that during aging: (1) degenerative signaling may be determined by a few key miRNAs functionally coordinating together for changes in an entire programmatic shift; (2) individual tissues may have their own sets of miRNAs for this programmatic shift, some of which are shared with other tissues, while some are tissue-specific; and (3) tissue-specific changes of miRNAs may then be used as essential biomarkers to define specific pathway(s) leading to that particular organ’s molecular degeneration during aging.

The above paradigm (shown in Figure 3.3 and 3.4) is: how do long-lived animals counter the trend of predominant upregulation of miRNA expression? Two scenarios occur to us: (1) quantitative delay of upregulation of specific unwanted miRNA expression; and (2) qualitative counteraction of unwanted miRNA expression by the activation of another group of miRNAs, whose downstream action on target genes may counteract deleterious effects. Taken together, the longevity-assurance phenotype may include a reduction in rate of onset or intensity of miRNA expression disequilibrium. Irregular or unrestrained miRNA expression may cause subsequent dysregulated posttranscriptional control. This not only may include the up-regulation of specific miRNAs involved in the suppression of oxidative defense, DNA repair, etc., but also may enlist additional miRNA upregulation, further propagating the unwanted signaling, and spiraling decline observed in some aspects of aging. This molecular engineering caters to the need for a long-term survival strategy in various vital organs.
In conclusion, we suggest that during aging, miRNA posttranscriptional regulation of expression of genes involved in dynamic signaling may affect many intertwining networks, tampering with and deforming various regulatory networks. During development, miRNAs play important roles in cell lineage determination. For example, for neuronal precursors to reach the terminally differentiated state, a specific set of miRNAs is activated to silence the unwanted non-neuronal gene expression, and another set of miRNAs is downregulated to allow the proper neuronal-specific genes to be expressed. Thus, with individual cell types, there is a specific balance of two sets of miRNAs, whose own activation and deactivation
induce the silencing or expression of their target genes. Failure to maintain this dynamic equilibrium of appropriate miRNA expressions may lead to a molecular degeneration, seen first at a modest level with one or two changes in miRNA expression, which eventually cascades into a larger number of miRNAs shifting their expression to deleterious levels, as shown in Figure 4. As with the various subsets of miRNA determining differential cell lineage determination, a “one size fits all” mechanism is unlikely to be responsible for the failure of controlling the equilibrium of miRNA expression states; rather, a tissue-specific deterioration may be manifested by the unique combination of special subsets of miRNAs, whose expression pattern gradually drifts from homeostasis. Some of these miRNAs are shared among different tissues and others are tissue-specific.

Figure 3.4. Model of miRNA disequilibrium starting at mid-life, with a few upregulated miRNAs escaping from tempered homeostatic expression; later in life greater preponderance of deleterious miRNA upregulation is observed. (Bates et al. 2009 © Elsevier, used with permission).
We suggest that during aging, the balance of key mRNA expression is tilted towards predominant upregulation, with a downstream effect of posttranscriptional suppression of target genes, bringing about the signaling abnormalities manifested in the aging phenotype. A key question remains: what causes the trend towards predominant upregulation of miRNA expression? Future studies should answer this question, which may open a Pandora’s Box of unsuspected detail as to how posttranscriptional control, specifically in the form of noncoding RNA such as miRNAs, is involved in life span determination as well as age-dependent physiological changes. Finally, the use of miRNA as diagnostics and therapeutics promises to become a fertile field for clinical intervention. This newly appreciated molecular species may provide epigenetic molecular secrets to delay aging, and reduce or slow down the perils of age-dependent diseases.
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CHAPTER FOUR

*MicroRNA regulation in Ames dwarf mouse liver may contribute to delayed aging*

**SUMMARY**

The Ames dwarf mouse is well known for its remarkable propensity to delay the onset of aging. Although significant advances have been made demonstrating that this aging phenotype results primarily from an endocrine imbalance, the posttranscriptional regulation of gene expression and its impact on longevity remains to be explored. Towards this end, we present the first comprehensive study by microRNA microarray screening to identify dwarf-specific lead microRNAs, and investigate their roles as pivotal molecular regulators directing the long-lived phenotype. Mapping the signature microRNAs to the inversely expressed putative target genes, followed by *in situ* immunohistochemical staining and *in vitro* correlation assays, reveal that dwarf mice posttranscriptionally regulate key proteins of intermediate metabolism, most importantly the biosynthetic pathway involving ornithine decarboxylase and spermidine synthase. Functional assays using 3'UTR reporter constructs in co-transfection experiments confirm that microRNA-27a indeed suppresses the expression of both of these proteins, marking them as probable targets of this microRNA *in vivo*. Moreover, the putative repressed action of this microRNA on ornithine decarboxylase is identified in dwarf mouse liver as early as two months of age. Taken together, our results show that among the altered aspects of intermediate metabolism detected in the dwarf mouse liver — glutathione metabolism, the urea cycle, and polyamine biosynthesis — microRNA-27a is a key posttranscriptional control. Furthermore, compared to its normal siblings, the dwarf
mouse exhibits a head start in regulating these pathways to control their normality, which may ultimately contribute to its extended healthspan and longevity.

**INTRODUCTION**

Organismal senescence, the inevitable process of aging faced by every organism, may be characterized by a decreasing ability to handle stress (both exogenous and endogenous), and the increasing homeostatic imbalance, genetic malfunction, and risk of disease. Recent studies demonstrate that genetically-altered organisms with significantly longer life-spans also possess an enhanced ability to respond to environmental and cellular stresses, and minimize genetic alterations or error (Kenyon, 2005). Understanding the mechanistic and molecular processes that enable these organisms to live longer may yield insight into ways to decrease the rate of aging and minimize its detrimental effects.

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs, usually between 18 to 25 nucleotides in length, involved in the regulation of cellular and developmental processes through posttranscriptional gene repression (Alvarez-Garcia, 2005; Ambros, 2004b). MicroRNAs typically bind to partially complementary regions of the 3' untranslated region (UTR) of messenger RNAs (mRNAs), to induce either translation inhibition or signal for mRNA degradation. A more complete understanding of the role miRNAs play in the process of aging could lead to gene-based strategies to diagnose, suppress, or cure aging-related symptoms and disease.

The Ames dwarf mouse has been an attractive animal model in the search for genomic factors that control or affect the process of aging. Mice of this strain live up to 70% longer than wild-type counterparts, because of a deficiency in three pituitary hormones (growth hormone, prolactin, and thyrotropin), resulting from a point mutation affecting the *Prop1* gene (Anderson, 1995; Schaible, 1961). Recent findings demonstrate that some miRNAs either regulate (Poy et al., 2004) or are regulated by endocrine-related functions (Fiedler et

Although many facets of the dwarf mouse warrant study (e.g., tumor suppression, altered metabolism, increased memory, etc.), little is known about the posttranscriptional regulation of gene expression directing these phenotypic features. In this study, variations in protein and miRNA expression were investigated in dwarf mice of different ages, and contrasted against their wild-type counterparts via global proteomic profiling in conjunction with miRNA microarray analyses, followed by functional analysis of specific lead miRNA/target relationships. To study the effects of aging, we selected the liver because of its role in detoxification, hormone degradation, and its direct correlation to aging in mammals (Cao, 2001; O. C. Maes, An, J., Sarojini, H., Wang, E., 2008; Schmucker, 2005).
Here we report the up-regulation of ten miRNAs in dwarf mouse liver, with microRNA-27a emerging as the leading significant miRNA species in this context. Parallel proteomic profiling reveals that genes targeted by these miRNAs exhibit reciprocal down-regulation, and are in protein families associated with intermediate metabolism—specifically glutathione metabolism, the urea cycle, and polyamine biosynthesis. *In situ* immunohistochemical staining and *in vitro* reporter and endogenous expression assays demonstrated an inversely correlated expression between miR-27a and two of its putative targets involved in polyamine biosynthesis—ODC1 and spermidine synthase (SRM). In particular, the reciprocal expression of miR-27a and ODC1 can be observed as early as 2-month old livers of dwarf mice, giving these mice a head start over their control wild-type brothers. Thus, the up-regulated miRNAs, led here by miR-27a, suggest that noncoding RNAs serve as posttranscriptional regulatory factors, functionally vital to longevity determination.

**MATERIALS & METHODS**

**Chemicals**

Chemicals were purchased from Sigma Aldrich unless mentioned otherwise.

**Experimental Procedures**

Comparative proteomic and miRNA expression analysis were carried out in triplicate for each of 15 mice divided evenly into five categories: 2-month-old control, 2-month-old dwarf, 24-month-old control, 24-month-old dwarf, and 33-month-old dwarf. These ages represent mature, old, and extremely old age groups.

**Mouse strain and samples**

Male Ames dwarf mice and their wild-type siblings were produced in the Southern Illinois University animal facility. Tissue samples were collected from Ames dwarf and
control mouse livers at 2, 24, and 33 (dwarf mice only) months of age. Comparative proteomic and miRNA expression analysis was carried out in triplicate for each of 15 mice divided evenly into five categories: 2-month-old control, 2-month-old dwarf, 24-month-old control, 24-month-old dwarf, and 33-month-old dwarf. Samples were rapidly frozen at -80°C until analysis. Tissue samples were processed as described below.

**Total and small RNA extractions**

Total RNA was extracted from frozen tissue blocks by grinding them in Trizol according to the manufacturer’s instructions. Small RNA enrichment was carried out according to Park et al. (Park, 2002). Briefly, total RNA was adjusted to 400 µL with RNase-free water. Next, 50 µL of NaCl (5M) and 50µL PEG 8000 (v/v 50%) were added. Samples were incubated on ice for 2 hours followed by centrifugation for 10 min. at 13,000 rpm at 4°C. The supernatant was transferred to a microcentrifuge tube, and 50 µL sodium acetate (3M, pH 4.6) and 1 mL of 100% ethanol were added. Samples were vortexed and incubated at -20 °C for 2 h, then centrifuged at 12,000 g for 10 min. at 4 °C. The supernatant was discarded, and 1 mL of cold 75% ethanol was used to wash the pellet. Samples were centrifuged again at 12,000 g (10 min, 4 °C), the supernatant was discarded, and the RNA pellet was dried and dissolved in 12 µL of RNase-free water at 60 °C for 10 min. Samples were quantified using spectrophotometry (260 nm), and stored at -80 °C.

**MicroRNA profiling**

Samples of small RNA were labeled on their 3’ end with digoxigenin (DIG) using the DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche Diagnostics, Indianapolis, IN). 1.0 µg of small RNA was labeled in a total volume of 20 µL, as described by Wang et al. (E. Wang, Lacelle, Xu, Zhao, & Hou, 2002). Mouse miRNA microarrays (MMChips) bore 367 anti-sense DNA sequences of mouse miRNAs obtained from miRBase
Hybridization and detection of miRNAs were carried out as previously described (Schipper, 2007).

Hybridization intensities were measured using an Expression 1680 scanner (Epson, Long Beach, CA); Array-Pro Analyzer 4.5 software (Media Cybernetics, Bethesda MD) was used for data acquisition. Whole cell area measurements were used to derive net intensity levels; the mean intensity of ring background around the spots was used for correction. Array-Pro Analyzer software was also used for normalization of MMChips, using mean signal intensity of all cells. SAM software, Version 3.02 (Significance Analysis of Microarrays, Stanford University, Stanford, CA) was used for microarray data analyses, including pairwise comparison ($T$-statistics) between strains and age groups from young to old. Kolmogorov-Smirnov statistics were generated using the Gene Set Enrichment Analysis (GSEA) software (Subramanian, 2005). Unsupervised hierarchical clustering using Pearson’s correlation was carried out with GenePattern software (www.broad.mit.edu/cancer/software/genepattern/; Broad Institute, Cambridge, MA).

**qRT-PCR validation**

QRT-PCR was carried out as described by Maes et al. (O. C. Maes, An, J., Sarojini, H., Wang, E., 2008). Briefly, 0.1 µg of small RNA was quantified using the NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) via real-time PCR. Mature DNA sense sequences (obtained from miRBase http://microrna.sanger.ac.uk/) were used as forward primers. Validation miRNAs were chosen, one from each postulated target gene. MicroRNA primer sequences used were mmu-miR-27a (ttcacagtggctaatggcgc), mmu-miR-669b (agttttgtgtgcatgtgcatgt), mmu-miR-22 (aagctgccagttgaagaactgt), mmu-miR-96 (ttgctgactacacttttgct), and mmu-miR-501-3p (aatgcacccggcagaggatttg). As a reference sequence, 5S rRNA was used, probed using an internal forward primer (cagggcggcctggtagttacttg). MicroRNA expression fold changes between ages were
calculated using the delta Ct method, relative to controls following normalization with levels of 5S rRNA.

**Protein extraction**

To obtain total protein, tissues were diced, then homogenized in 2 volumes (g/mL) of RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, pH 8.0) containing 1 x Protease Inhibitor (Calbiochem, San Diego, CA) and centrifuged at 10,000 x g for 10 minutes; the supernatant was harvested. One volume of RIPA buffer was added to the pellet and sonicated 3 x 10 s, centrifuged, and supernatants pooled. The Bradford method was used to quantify total protein concentration with BioRad reagents (BioRad, Hercules, CA).

**Western Blot Analysis**

Total protein was resolved via SDS-PAGE, and blotted on nitrocellulose membrane Protran BA 85 (Whatman Schlecher & Schuell, Springfield Mill, Maidstone, Kent, UK) as previously described (Sarojini H, 2007). Rabbit anti-Ornithine Decarboxylase (ODC1), anti-Arginase 1 (ARG1), and goat anti-Argininosuccinate synthetase (ASS1) primary antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA), rabbit anti-Vimentin (normalization control) was produced in-house, and anti-Glutathione S-transferase µ1 (GSTM) was purchased from Aviva Systems Biology (San Diego, CA). Secondary antibodies used were either sheep anti-rabbit horseradish peroxidase (HRP) or donkey anti-goat HRP, obtained from Santa Cruz Biotechnologies. Films were developed in a dark room, and proteins were quantified using densitometry software (ImageQuant version 5.2, Molecular Dynamics, [www.mdyn.com](http://www.mdyn.com)), using vimentin as a normalization control. Three samples for each group were detected and analyzed.
Total protein (150 μg) was precipitated with 6 volumes of cold acetone (-20 °C), then allowed to decant at -20°C. Proteins were denatured, reduced, and alkylated according to manufacturer’s instructions (iTRAQ™ labeling kit, Applied Biosystems). Samples were Trypsin-digested (Trypsin from Promega, Madison, WI) for 16h at 37°C. Mouse peptides of control 2 months, dwarf 2 months, control 24 months, and dwarf 24 months were labeled with iTRAQ reagents 114, 115, 116, & 117, respectively. Labeled peptides were then identified and quantified via tandem mass spectrometry. Because the limitation of the quadruplex labeling, the 33-month proteomic was not performed.

Fractionation of peptides was performed on a cation PolySulfoethyl A column (4.6 nm x 20 cm, PolyLC Inc., Columbia, MD), using a BioCAD workstation (Applied Biosystems) as described by Cong et al. (Cong, Fan, & Wang, 2006). C18 ZipTips (Millipore Corporation, Billerica, MA) were used to desalinate fractions prior to LC-MS/MS. A QSTARTM XL hybrid liquid tandem mass spectrometry (LC-MS/MS) system (Applied Biosystems), interfaced with an 1100 Series Capillary LC system (Agilent, Sta. Clara, CA) with an analytical Vydac HPLC Column (75 μm x 150 mm; Vydac MS C18 300A, Alltech Associates Inc., Nicholasville, KY) was used for peptide fractionation and identification. MS TOF scans were acquired from m/z 350 to 1600, with up to two precursors selected for MS/MS from m/z 60 – 2000, using information-dependent acquisition and rolling collision energy applied to promote fragmentation.

Nanospray MS and MS/MS data were analyzed using ProteinPilot™ Software 2.0.1 (Applied Biosystems) for iTRAQ identification and quantification between samples. N—termini, Lys, Tyr, and Cys modifications were selected as fixed, Met oxidation as variable, one missed cleavage allowed, precursor error tolerance was set at <0.15 Da, and product ion
error tolerance at <0.1 Da. For quantification of iTRAQ labeled peptides between age groups, the cutoff was set at >95%, with a ProtScore threshold of 1.30.

**Data mining and statistical analysis for candidate targets of lead miRNAs**

MicroRNA target predictions were acquired and downloaded from the miRBase website [http://microrna.sanger.ac.uk/](http://microrna.sanger.ac.uk/) (S. Griffiths-Jones, Saini, H.K., Dongen, S.V., Enright, A.J., 2008). Each target prediction was inversely correlated with proteomic and gene expression data. Three other databases, TargetScan ([http://www.targetscan.org/mmu_50/](http://www.targetscan.org/mmu_50/)), RNA22 ([http://cbcsrv.watson.ibm.com/rna22 Targets.html](http://cbcsrv.watson.ibm.com/rna22_targets.html)) and PicTar ([http://pictar.mdc-berlin.de/](http://pictar.mdc-berlin.de/)) were used for selected miRNAs and their targets. For RNA22, a stringency cutoff was set up with the following parameters: the maximum number of allowed UN-paired bases was 0 in a seed/nucleus of 6 nucleotides, the minimum number of paired-up bases in heteroduplex was 14; and the maximum folding energy for a heteroduplex was -25 Kcal/mol.

Predictions of microRNA/gene target correlations were assessed on the following three levels: 1) significance of experimentally determined expression changes (p-value and q-value %); 2) probability of microRNA and target gene relationship; and 3) correlation between miRNA expression and gene expression changes (Partial correlations).

On the first level, the microarray data were processed using SAM software, Version 3.02, which yields fold change comparisons and a q-value percentage to demonstrate the confidence of a real expression difference. To supplement and confirm the microarray analysis, qPCR was carried out in triplicate, with the single best candidate miRNAs — determined by level two analyses, described below — that exhibited significant variation in microarray analysis, and experimentally determined significant inverse expression with its predicted target protein. Target protein expression was determined using global proteomic profiling, as specified above, with greater than 95% confidence (p-value reported in Table 4.1), and confirmed via Western blot analysis on a few select proteins.
On the second level of statistical analysis, the miRBase Targets Version 5 and miRanda algorithms were employed to demonstrate a high theoretical correlation between the selected miRNAs and their target proteins. These correlations are reported in Table 4.2. According to John et al, the percentage of false positives is 30%, 24%, 19% and 9% for transcripts with 3, 4, 5 and 10 target sites (John et al., 2004). These calculations are based on running real miRNA sequences that were randomly shuffled through the same predictive algorithms to measure their predicted targeting potential. The results of this exercise were compared to the results of the real miRNA targeting calculations. All of the miRNA/targets listed in our tables have greater than 10 target sites. P values given are the best P value of the selected miRNA for a transcript.

As described above, besides miRanda algorithms, we also used the TargetScam, RNA22 and PicTar to do the relation prediction between miR-27a and ODC1/SRM (Table 4.5). In the TargetScan database, for each input protein, the list of miRNA families will be demonstrated with the target sites. For specific sequences that are matched between miR-27a and ODC1 or SRM, we used RNA22. In other words, we loaded the sequence of mmu-miR-27a and 3’-UTR sequences of ODC1 and SRM (sequences obtained from the NCBI genebank) to cover all the possible target sites as revealed by RNA22. PicTar database is provided publicly for human and Drosophila miRNA target predictions. Like miRanda, by inputting the miRNA name, it will list all the targets. Since the current study is for mouse microRNAs, we only used it for a supporting searching in addition to the first 3 databases.

The third level of statistical correlation is a direct linear regression using ANOVA statistical analysis (via SPSS software version 13.0), to calculate partial correlation coefficients between the five miRNAs and their corresponding predicted target gene expressions, obtained experimentally via qPCR, MMChip and Western Blot, or proteomics, respectively (ASS1 and CPS1 did not undergo Western Blot analysis).
variables were miRNA expression change, target gene expression change, and age of mouse sample.

For all above tasks, attribution for genes and proteins to a particular gene family was made according to the SOURCE (http://source.stanford.edu) and BRENDA (http://bRENDA-enzyme.info) databases.
Table 4.1. Fold changes of microRNA and protein expression

<table>
<thead>
<tr>
<th>microRNA &amp; Target protein</th>
<th>vs. 2-month-old control</th>
<th>vs. 24-month-old control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (q-value%)</td>
<td>D (q-value%)</td>
</tr>
<tr>
<td>mmu-miR-501-3p</td>
<td>1.02 ( &gt;10 )</td>
<td>1.22 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.07)</td>
<td>(σ=±.08)</td>
</tr>
<tr>
<td>CPS1 (p-value = 0.04)</td>
<td>1.0 ( &gt;10 )</td>
<td>1.07 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.005)</td>
<td>(σ=±.006)</td>
</tr>
<tr>
<td>mmu-miR-22</td>
<td>1.08 ( &gt;10 )</td>
<td>1.15 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>mmu-miR-127*</td>
<td>0.92 ( &gt;10 )</td>
<td>1.00 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>mmu-miR-411*</td>
<td>1.08 ( &gt;10 )</td>
<td>1.31 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>mmu-miR-470</td>
<td>1.15 ( &gt;10 )</td>
<td>1.13 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>ASS1 (p-value = 0.01)</td>
<td>0.89 (σ=±.11)</td>
<td>1.41 (σ=±.15)</td>
</tr>
<tr>
<td></td>
<td>(σ=±.05)</td>
<td>(σ=±.09)</td>
</tr>
<tr>
<td>mmu-miR-669b</td>
<td>1.20 ( &gt;10 )</td>
<td>1.46 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>mmu-miR-29b*</td>
<td>1.34 ( &gt;10 )</td>
<td>1.40 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>mmu-miR-382*</td>
<td>0.88 ( &gt;10 )</td>
<td>1.05 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>mmu-miR-676</td>
<td>1.53 ( &gt;10 )</td>
<td>1.75 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>ARG1 (p-value = 0.015)</td>
<td>1.37 (σ=±.09)</td>
<td>1.52 (σ=±.21)</td>
</tr>
<tr>
<td></td>
<td>(σ=±.08)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>mmu-miR-27a</td>
<td>0.99 ( &gt;10 )</td>
<td>1.50 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>ODC1 (from Western Blot)</td>
<td>0.75 (σ=±.19)</td>
<td>0.68 (σ=±.01)</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>mmu-miR-96</td>
<td>0.95 ( &gt;10 )</td>
<td>0.90 ( &gt;10 )</td>
</tr>
<tr>
<td></td>
<td>(σ=±.00)</td>
<td>(σ=±.00)</td>
</tr>
<tr>
<td>GST μ1 (p-value = 0.01)</td>
<td>1.32 (σ=±.10)</td>
<td>1.28 (σ=±.22)</td>
</tr>
<tr>
<td></td>
<td>(σ=±.34)</td>
<td>(σ=±.12)</td>
</tr>
</tbody>
</table>

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Table 4.2. MicroRNA target predictions via miRBase

<table>
<thead>
<tr>
<th>miRs</th>
<th>Target Gene Name</th>
<th>P-value</th>
<th>UTR Length (bps)</th>
<th>Total Sites</th>
<th>No. miRNAs predicted to target</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-27a</td>
<td>Odc1</td>
<td>0.0428941</td>
<td>333</td>
<td>17</td>
<td>49 [+]</td>
</tr>
<tr>
<td></td>
<td>Srm</td>
<td>0.0150736</td>
<td>312</td>
<td>15</td>
<td>56 [+]</td>
</tr>
<tr>
<td>mmu-miR-669b</td>
<td>Arg1</td>
<td>0.00010411</td>
<td>389</td>
<td>19</td>
<td>41 [+]</td>
</tr>
<tr>
<td>mmu-miR-22</td>
<td>Ass1</td>
<td>0.0225366</td>
<td>255</td>
<td>11</td>
<td>29 [+]</td>
</tr>
<tr>
<td>mmu-miR-96</td>
<td>Gstm1</td>
<td>0.0298408</td>
<td>431</td>
<td>13</td>
<td>36 [+]</td>
</tr>
<tr>
<td>mmu-miR-501</td>
<td>Cps1</td>
<td>0.018076</td>
<td>1000</td>
<td>12</td>
<td>16 [+]</td>
</tr>
<tr>
<td>mmu-miR-127*</td>
<td>Ass1</td>
<td>0.0266144</td>
<td>255</td>
<td>11</td>
<td>29 [+]</td>
</tr>
<tr>
<td>mmu-miR-411*</td>
<td>Ass1</td>
<td>0.0315475</td>
<td>255</td>
<td>11</td>
<td>29 [+]</td>
</tr>
<tr>
<td>mmu-miR-470</td>
<td>Ass1</td>
<td>0.0372748</td>
<td>255</td>
<td>11</td>
<td>29 [+]</td>
</tr>
<tr>
<td>mmu-miR-29b*</td>
<td>Arg1</td>
<td>0.00169758</td>
<td>389</td>
<td>19</td>
<td>41 [+]</td>
</tr>
<tr>
<td>mmu-miR-382*</td>
<td>Arg1</td>
<td>0.0316054</td>
<td>389</td>
<td>19</td>
<td>41 [+]</td>
</tr>
<tr>
<td>mmu-miR-676</td>
<td>Arg1</td>
<td>0.0453475</td>
<td>389</td>
<td>19</td>
<td>41 [+]</td>
</tr>
</tbody>
</table>

**In situ hybridization with mmu-miR-27a locked-nucleic acid (LNA) probe**

MicroRNA 27a in situ hybridization followed the protocol described by Obernosterer et al. (Obernosterer, Martinez, & Alenius, 2007). Wild type mouse liver and dwarf mouse liver tissue (2-month-old) were collected and fixed in 4% paraformaldehyde for 2 hrs at 4 °C. Tissues were kept in PBS with 30% sucrose overnight at 4 °C, frozen in Tek O.C.T. (Sakura Finetek, CA) on dry ice, and sectioned at 10 µm on the cryostat (Leica, Germany). In brief, tissue sections were washed in PBS for 10 minutes, then placed in Acetylation solution (98% DEPC, 1.3% triethanolamine (Fluka, St. Louis, MO), 0.175% HCl, 12% acetic acid (Sigma, St. Louis, MO)) for 20 min. Next, the sections were digested by Proteinase K (25 µg/mL,
Sigma P2308) for 5 min at room temperature, washed in PBS for 5 minutes, and prehybridized at 50 °C for 4 hrs. The mmu-miR-27a LNA probe bearing an embedded Digoxigenin (DIG) sequence was purchased from Exiqon (Woburn, MA). Probes (1 nM) were denatured with denaturing hybridization solution at 95 °C for 5 min, then added to the slides and hybridized at 50 °C overnight. The next day, the slides were washed in 5 x SSC at 60 °C for 5 min, and 0.2 x SSC 60 °C for 60 min. After blocking for 1 hr (2% FCS), sections were incubated with anti-DIG antibody (Roche, Indianapolis, IN, 1: 2000) overnight at 4 °C. Localization of the DIG antibody-labeling was performed by further reaction via NBT (Nitro blue tetrazolium chloride)/BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) for color development 1 or 2 days at room temperature. The images were examined on a Zeiss fluorescence microscope (Carl Zeiss, Brighton, MI) and AxioVision Rel. 4.6 imaging system.

**ODC1 Immunohistochemical staining**

Sections of the same frozen tissue specimens used above for the in situ hybridization studies were blocked and incubated with ODC1 antibodies (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Alexa 594 goat anti-rabbit IgG (1:400; Invitrogen) was incubated with sections for 40 min at 37 ºC. The images were examined on a Zeiss fluorescence microscope (Carl Zeiss, Brighton, MI) and AxioVision Rel. 4.6 imaging system.

**Mouse miR-27a preparation and the 3’-UTRs of ODC1 and SRM cloning**

Mouse miR-27a expression clone and scrambled control were purchased from GeneCopoeia (Germantown, MD). The 3’-UTRs of mouse ODC1 and SRM were amplified from mouse genomic DNA (727 bp and 337 bp, respectively). The amplified sequence for the 3’-UTR of ODC1 is from 2 to 728 bp downstream of the stop codon, lacking the 25 bp upstream of the 3’-terminal of UCSC gene uc007ncv.1. The amplified sequence for the 3’-
UTR of SRM is from 23 bp upstream to 311 bp downstream of the stop codon, which is 1 bp upstream of the 3’-terminal of UCSC gene uc008vuw. According to miRanda database (Table 4.5), miR-27a target sites are located at bp positions 287-307 and bp positions 26-44 downstream of the stop codon in the final reporter clones for 3’-UTRs of ODC1 and SRM respectively. The following primers were used for cloning:

ODC1-EcoRI-5.1, 5’-GAATTCTTAAATGCCATTCTTTTAGCCTTTGC-3’,

ODC1-BamHI-3.1, 5’-GGATCCGGAAAGTTGACTGCCGATGTT-3’,

SRM-EcoRI-5.1, 5’-GAATTCTTAAAGGAAGGCGCTCAATGACATA-3’

SRM-BamHI-3.1, 5’-GGATCCAGAGGTCATGACTGAGCTTTGT-3’

(Underlined: restriction site, bold: in-frame stop codon)

The amplified DNA fragments were cloned into the pDrive cloning vector (Qiagen, Valencia, CA), then subcloned into pHcRed1-C1 vector (Clontech, Mountain View, CA) using EcoRI and BamHI sites. All constructs were confirmed by DNA sequencing.

**Cell transfection/co-transfection**

HEK 293 and NIH/3T3 cells at 70-80% confluence (ATCC, Manassas, VA) were used for transfection experiments with the cell line nucleofector kit V (Lonza Walkersville Inc., Walkersville, MD). Briefly, the cells were kept in culture for 48 hrs, trypsinized and collected. 1 × 10^6 cells were resuspended in 100 μl nucleofector solution. 5 μg of plasmid was loaded into a cuvette for each transfection/cotransfection. The experimental groups included: control, miR-27a, miR-27a+ ODC1 3’-UTR, miR-27a+ SRM 3’-UTR, control+ ODC1 3’-UTR, and control+ SRM 3’-UTR. Program Q-001 or A-033 was applied for HEK 293 cells or mouse NIH/3T3 cells, respectively. The cells were then immediately plated out in pre-warmed medium, supplemented with 10% FBS, into 35 mm glass bottom Petri dishes (MatTek, Ashland, MA). 72 hrs (for 293 cells) or 48 hrs (for NIH/3T3 cells) later, the cells were fixed with 4% paraformaldehyde in PBS, and imaged on a Zeiss fluorescence
microscope (Carl Zeiss, Brighton, MI) and AxioVision Rel. 4.6 imaging system. For the endogenous ODC1 detection, scrambled control or miR-27a-transfected 293 and 3T3 cells were fixed and incubated with ODC1 antibody and Alexa 594 goat anti-rabbit, as described above.

In general, the transfection efficiency is ~50% in all our experiments; therefore, we were not able to perform grind-and-find experiments to examine the transfected gene’s impact on its targets in terms of the expression levels of either endogenous target, ODC1-protein, or co-transfected 3’-UTR constructs, e. g. ODC or SRM 3’-UTRs. (Endogenous SRM was not examined, because no commercial antibody to this protein is available for immunocytochemistry). To resolve this problem, the putative reciprocal expression between the expression of transduced miR-27a and that of endogenous ODC1 or co-transfected 3’-UTR constructs was determined via quantitation of respective intensities between the former and latter by measurements of individual cells—green versus red fluorescence intensities, or green versus ODC antibody staining intensities. Individual cell intensity was generated in digitized output by tracing the cell circumference; the final measurement was obtained using densitometry software (ImageQuant version 5.2, Molecular Dynamics, www.mdyn.com). Fluorescence intensity of individual cells was obtained by this method; for each experimental condition described above, ten separate fields of ~30 cells were measured, and their digitized numerical values were recorded and used for statistical analysis for significance. The results are presented in the histograms shown in right panels of Figures 4.7, 4.8 and 4.9, with photographic images showing the represented fields, as in the left panels of the above figures, used for the quantitation of the fluorescence images.
RESULTS

MicroRNA microarray analysis

Comparative expression analysis of screening by miRNA microarray (MMChips) for each of 15 mice, divided evenly into five categories (control 2-month-old [m.o.], dwarf 2-m.o., control 24-m.o., dwarf 24-m.o., dwarf 33-m.o.), reveals that the vast majority of miRNAs exhibiting variation in dwarf mice compared with controls are up-regulated (Figure 4.1A-F and Figure 4.13). Only two miRNAs, microRNA-29c (miR-29c) and miR-707, are downregulated in the aged dwarf compared with controls (Figure 4.1E & F). Most miRNAs that exhibit variations in expression during aging typically increase in expression with age, and much more markedly in the dwarf. MicroRNA-27a exhibits the most drastic disparity between dwarf and control mice (Figure 4.2A); this miRNA increases with age in the dwarf, whereas there is a slight decrease with age in controls. MicroRNAs postulated to target genes of the urea cycle (part of arginine metabolism) display increased expression with age (Figure 4.2B and Figure 4.13).

qRT-PCR Confirmation

MicroRNA microarray expression analysis was qualitatively confirmed by qRT-PCR of miRNAs mmu-miR-27a, -669b, -22, -96, and -501-3p. These five miRNAs were selected because they exhibit the strongest theoretical correlation with target genes that also exhibit inverse expression experimentally (Table 4.2 shaded in gray). MicroRNA-27a displays significantly increased expression in the dwarf mouse at 24 months of age over controls. A disparity exists between MMChip results and qPCR in miR-27a expression in the 24-month-old control compared to the 2-month-old control; the chip shows decreased expression in the older control, whereas qPCR displays increased expression (Figure 4.3A). qPCR also shows miR-27a expression in 33-month-old dwarfs on par with 24-month-old controls, whereas the
chip displays increased expression in the dwarf at that time point. In general, microarray screening is less sensitive, and often presents data according to analysis of data from the entire array; even with all the positive and negative controls, the results are the outcome of hundreds of loci on the arrays. The microarray is therefore less sensitive than qPCR, because with qPCR the data output quantifies a single miRNA. Mmu-miR-669b displays a similar pattern of expression, with levels peaking at 24 months in the dwarf and dropping sharply at 33 months of age, but still remaining much higher than the 24-month-old control (greater than fourfold) (Figure 4.3B). Mmu-miR-22 displays expression similar to miR-27a, with maximum expression at 24 months of age, six times higher in the dwarf than in the same-aged control, followed by plummeting expression at 33 months, to levels nearly equal to 24-month-old controls (Figure 4.3C). Mmu-miR-96 qualitatively corroborates the microarray data except for expression at 24 months of age in the dwarf mouse, which is on average 3.76-fold less than same age controls (Figure 4.3D). Interestingly, 33-month-old dwarfs exhibit miR-96 expression nearly equal to that of controls at 24 months of age. Mmu-miR-501-3p displays increased expression in the dwarf at 2 and 33 months of age (1.75 and 3.74-fold compared to 24-month-old controls, respectively), whereas 24-month-old dwarfs exhibit miR-501-3p expression on par with same-aged controls (Figure 4.3E).
Figure 4.1. Heat map demonstration of expression levels with respect to age and mouse category (control, C or dwarf, D). Intensity of color equals relative expression, with blue representing decreasing expression, and red representing increasing expression. A.) Expression comparison of dwarf versus control at 2 months of age, B.) 24 months, C.) 24 month-old control vs. 24- and 33-month-old dwarf. Values are averages of 3 mice per age group and category. (Bates *et al.* 2009 © John Wiley and Sons used with permission)
Figure 4.2. A.) Top: Graphical representation of miRNA, mmu-miR-27a, which exhibits the greatest fold change in dwarf mice versus wild-type controls. B.) Bottom: Graphical representation of key miRNA expression of dwarf mouse miRNA versus control. Samples are represented by age in months (2, 24, or 33) and C or D (control or dwarf mouse, respectively). (Bates et al. 2009 © John Wiley and Sons used with permission)
Figure 4.3. Quantitative Real-Time PCR (qRT-PCR) confirmation of MMChip results. A) Predicted target of ODC, microRNA mmu-miR-27a, which displays maximum increased fold change; B) predicted target of ASS1, mmu-miR-22; C) a postulated target of ARG1, mmu-miR-669b; D) postulated target of GST μ1, mmu-miR-96; and E) postulated target of CPS1, mmu-miR-501-3p. The C point on the x-axis represents C24 for the red line, or C2 for the blue line. (Error bars = Standard Deviation, [σ]). (Bates et al. 2009 © John Wiley and Sons used with permission)

Proteomic Results

Comparative proteomics of the Ames dwarf mouse and its wild-type counterpart reveals striking differences in expression of proteins involved in intermediate metabolism and detoxification. Enzymes involved in arginine and ornithine metabolism—specifically the urea cycle—show significant variation. Arginase 1 (ARG1), argininosuccinate synthase (ASS1), and carbamoyl-phosphate synthetase (CPS1) exhibit particularly varied expression patterns (Figure 4.4A – C). In the young dwarf (2 months of age) these enzymes are considerably
upregulated compared to the young control. Expression of these proteins in dwarf mice decreases significantly with age, whereas expression in controls holds constant or decreases slightly. Carbamoyl-phosphate synthetase, ASS1, and ARG1 exhibit elevated expression in the young dwarf mouse (22%, 41%, and 52%, respectively), but ultimately end up with expression levels at 33 months of age equal to (ASS1 and ARG1) or slightly higher than (CPS1 at 10% above) control mouse levels at 24 months.

The detoxification enzyme, glutathione S-transferase (GST) isoform μ1, displays increased expression in the dwarf mouse at 24 months compared to controls by 46% (Figure 4.4E). In both dwarf and control, GST μ1 expression increases with age; however, in the dwarf mouse the expression at 33 months drops towards the levels measured in wild-type mice at 24 months. The oxidative defense enzyme, Cu,Zn-superoxide dismutase (SOD), is upregulated 21 and 35% (σ = ± 3% and ± 16%, respectively) in the dwarf at 24 and 33 months, respectively (Figure 4.4F). Another oxidative defense enzyme, catalase, is slightly downregulated in the dwarf mouse from 2 to 24 months compared to same-age controls (9% ± 6% and 13% ± 1%), as well as decreasing in expression with age until 24 months (12% decrease, data not shown). Surprisingly, catalase expression in 33-month-old dwarfs increases to slightly above that in 24-month-old controls.
Figure 4.4. Graphical representation of protein expression in dwarf mice versus controls. Samples are represented by age in months (2, 24, or 33) and C or D (control or dwarf mouse, respectively). The C point on the x-axis represents C24 for the red columns, or C2 for the blue columns. (Bates et al. 2009 © John Wiley and Sons used with permission)

Figure 4.5 A) Western Blot analysis of ornithine decarboxylase (ODC1), Arginase 1 (ARG1), glutathione S-transferase (GST) μ1, superoxide dismutase (SOD), and Vimentin (VIM) as normalization control. B) Histogram of the average densitometry measurements of
Western Blot Analysis

Western Blot analysis of four key proteins, ornithine decarboxylase (ODC), ARG1, GST μ1, and SOD was carried out. The latter three qualitatively confirm results of proteomic analysis, with the exception of ARG1 expression not increasing in the 33-month-old dwarf. Arginase 1 expression is significantly increased in the young dwarf compared to controls by 30%, followed by a drastic decrease in expression in the dwarf mouse at 24 months of age, with expression levels at 59% of those measured in the same-aged control. 33-month-old dwarfs have slightly lower ARG1 expression (~8%) than 24-month-old dwarfs. Ornithine decarboxylase, a protein expected to be down-regulated in the dwarf by biochemical pathway analysis and miRNA targeting, exhibits significantly decreased expression in the dwarf at both 24 and 33 months of age, compared to 2 and 24-month-old controls (Figure 4.5 A, B). Dwarf ODC1 expression at 2, 24 and 33 months is 75, 56 and 64%, respectively, of the values measured in 2-month-old controls. Similar, Dwarf ODC1 expression at 24 months is 82% that of the values measured in 24-month-old controls, and at 33 months is 94% that of 24-month-old controls (p<0.01). GST μ1 is greatly over-expressed in 2- and 24-month-old dwarf mice (412% and 136% compared to same age controls, respectively); however, at 33 months of age the expression level drops drastically, to 76% of that in 24-month-old controls. Superoxide dismutase displays increased expression (~2X) in the dwarf mouse that decreases with age, whereas in the controls SOD expression increases with age—though never quite reaching dwarf levels (Densitometry in Figure 4.12).
**Statistical Correlation**

Theoretical calculations reported by database algorithms of miRBase Targets Version 5 ([http://microrna.sanger.ac.uk/targets/v5/](http://microrna.sanger.ac.uk/targets/v5/)) reveal strong correlations between the five qPCR-tested miRNAs and the respective target proteins (Table 4.2 and Table 4.4). Partial correlations calculated demonstrate significant correlation between experimental expression of miRNAs and target proteins (Table 4.3). All partial correlations have a calculated $|r|$ value greater than $|0.6|$.

**Table 4.3.** Statistical correlation between miRNA and predicted target gene expression.

<table>
<thead>
<tr>
<th>MicroRNA and target gene</th>
<th>r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-27a qPCR/Odc1 Western blot</td>
<td>-0.683</td>
<td>0.074</td>
</tr>
<tr>
<td>miR-669b qPCR/Arg1 Western blot</td>
<td>-0.752</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-96 qPCR/Gstm1 Western blot</td>
<td>-0.776</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-22 MMchip/Ass1 proteomic</td>
<td>-0.82</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-501-3p MMchip/CPS1 proteomic</td>
<td>-0.662</td>
<td>0.009</td>
</tr>
</tbody>
</table>

**Table 4.4.** miRanda scores for predicted miRNA and target gene correlation

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Target miRs</th>
<th>Score</th>
<th>Base P</th>
<th>Poisson P</th>
<th>Org P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ass1</td>
<td>mmu-miR-22</td>
<td>16.828</td>
<td>0.023</td>
<td>0.023</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>mmu-miR-127*</td>
<td>16.828</td>
<td>0.027</td>
<td>0.027</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>mmu-miR-411*</td>
<td>15.919</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>mmu-miR-470</td>
<td>16.630</td>
<td>0.038</td>
<td>0.037</td>
<td>0.037</td>
</tr>
<tr>
<td>Odc1</td>
<td>mmu-miR-27a</td>
<td>16.605</td>
<td>0.043</td>
<td>0.043</td>
<td>0.043</td>
</tr>
<tr>
<td>Arg1</td>
<td>mmu-miR-669b</td>
<td>16.956</td>
<td>0.040</td>
<td>0.040</td>
<td>0.000</td>
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<tr>
<td></td>
<td>mmu-miR-29b*</td>
<td>16.845</td>
<td>0.042</td>
<td>0.002</td>
<td>0.002</td>
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<td>-------------------</td>
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<tr>
<td></td>
<td>mmu-miR-382*</td>
<td>16.291</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>mmu-miR-676</td>
<td>15.930</td>
<td>0.046</td>
<td>0.045</td>
<td>0.045</td>
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<tr>
<td>Gstm</td>
<td>mmu-miR-96</td>
<td>15.867</td>
<td>0.056</td>
<td>0.055</td>
<td>0.030</td>
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<tr>
<td></td>
<td>mmu-miR-501-3p</td>
<td>15.750</td>
<td>0.046</td>
<td>0.045</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>mmu-miR-27a</td>
<td>17.718</td>
<td>0.015</td>
<td>0.015</td>
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</tr>
</tbody>
</table>

**Table 4.5.** The predicted relation between mmu-miR-27a and ODC1/SRM by different databases

<table>
<thead>
<tr>
<th></th>
<th>ODC1 3'-UTR</th>
<th>SRM 3'-UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TargetScan</strong></td>
<td>5'-target-3' ACUGUGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UGACACU</td>
<td></td>
</tr>
<tr>
<td><strong>RNA22</strong></td>
<td>5'-target-3' AGTTTTGGGAT---GTCTTTTGTGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CG---CCUUGAAUCGGUGACACUU</td>
<td></td>
</tr>
<tr>
<td><strong>Pictar</strong></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td><strong>miRanda</strong></td>
<td>5'-target-3' AAUCGGUGACACUU</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGACCCATTGTGAA</td>
<td></td>
</tr>
</tbody>
</table>

**Bioinformatic Analysis of Key microRNAs and their Candidate Target Proteins.**

Microarray analysis of miRNAs extracted from the livers of Ames dwarf and wild-type mice reveal strong correlations with the proteomic data from the same sample set (Table 4.1). The highest fold change detected by miRNA profiling between dwarf and control is seen with miR-27a. According to the miRBase Targets Version 5 database
(http://microrna.sanger.ac.uk/), both ODC1 and SRM key enzymes of polyamine biosynthesis, are targeted by miR-27a. Three other databases were employed with the hope to further confirm the predicted relationship between miR-27a and ODC1/SRM. Our searching results show that using the TargetScan, (http://www.targetscan.org/mmu_50/) database, miR-27a is reported to target the 3’-UTR of SRM but not of ODC1. The RNA22 database (http://cbcsrv.watson.ibm.com/rna22_targets.html) predicts the 3’-UTRs of both ODC1 and SRM to be targeted by miR-27a, with a stringent cutoff setup (maximum number of allowed UN-paired bases is 0 (ODC1)/ 2 (SRM) in seed/nucleus of 6 nucleotides, minimum number of paired-up bases in heteroduplex is 14; and the maximum folding energy for heteroduplex (Kcal/mol) is -25). No relation between miR-27a and ODC1/SRM is found in PicTar (http://pictar.mdc-berlin.de/) thus far (Table 4.5). This may be due to the fact that PicTar largely concentrates on human miRNA and its target prediction. Based on this exercise of algorithmic prediction, ODC1 and SRM were chosen for further functional studies as shown later in Figures 4.7, 4.8 & 4.9.

The relationship between miR-27a and ODC prompted us to examine further other candidate miR/target pairs. Towards this end, we performed comparative analysis of lead microRNAs selected by our array screening results with data obtained from Tandem Mass proteomic profiles. We found that, in addition to miR-27a, many other lead microRNAs are postulated to target key proteins of the urea cycle by gene family analysis, and furthermore their targets are not only identified by our proteomic profile results as lead proteins but also show inverse expression patterns (Table 4.1). For example, arginase 1 and ASS1 are each targeted by four different miRNAs (mmu-miRs-29b*, -676, -382*, & -669b target ARG1, and mmu-miRs-22, -127*, -470, & -411* target ASS1), all of which display relatively similar expression patterns and inverse correlations between miRNA and target gene expression. Expression levels of miR-669b increase with age in both mouse types; however, the dwarf
increases at a much higher rate (2X that of the control) before leveling off between 24 and 33 months of age (Figure 4.2Bvi, 4.3B, & 4.4C). MicroRNA-501-3p is postulated to target CPS1, and displays significantly increased expression over that of the controls (Figure 4.2Bi, 4.3E, & 4.4A). Expression of miR-501-3p increases in the dwarf significantly at 33 months, while at the same time, CPS1 decreases to its lowest level. MicroRNA 96 targets GST μ1, and shows decreased expression in dwarf mice, thus allowing for increased gene expression; however, expression in the dwarf at 33 months of age returns to levels similar to 24-month-old controls (Figure 4.3D & 4.4E).

![Figure 4.6](image)

**Figure 4.6.** *In situ* hybridization (ISH) of mmu-miR-27a (upper panels) and immunohistochemical staining of ODC1 (lower panels). Increased hybridization of mmu-miR-27a is observed in dwarf liver tissue (middle upper panel) *versus* control mouse tissue (left upper control), whereas decreased ODC1 staining is observed in the dwarf tissue (middle lower panel) *versus* control mouse tissue (left lower control). (Bates et al. 2009 © John Wiley and Sons used with permission)
Figure 4.7. miR-27a/ODC1 cotransfected 293 cells. Panel I showing phase contrast (a, d, g, & j) and green (b, e, h, & k) and red fluorescence (c, f, i, & l) images of the same field. Panel II: quantification of fluorescence in co-transfected cells (ODC1 3’UTR: upper, SRM 3’UTR: lower). (Bates et al. 2009 © John Wiley and Sons used with permission)
Figure 4.8. miR-27a/ODC1 cotransfected NIH-3T3 cells. Panel I showing phase contrast (a, d, g, & j) and green (b, e, h, & k) and red fluorescence (c, f, i, & l) images of the same field. Panel II: quantification of the fluorescence in co-transfected cells (ODC1 3’UTR: upper, SRM 3’UTR: lower). (Bates et al. 2009 © John Wiley and Sons used with permission)

In situ detection of mmu-miR-27a and Immunostaining of ODC1 in wild type and dwarf mouse liver

MicroRNA-27a expression patterns were further determined by in situ hybridization (ISH), using LNA (locked nucleic acid) probes (Figure 4.6). Hybridizations were carried out with liver sections from 2-month-old mice (both wild type control and dwarf mice). Consistent with our MMchip and qPCR validation results, we observed stronger in situ signals for miR-27a in dwarf than in wild type mouse livers (Figure 4.6, upper panels). ODC1, the predicted target of miR-27a, was detected by immunohistochemistry, as described in Materials and Methods; more ODC1 staining was observed in wild type than in dwarf mouse livers (Figure 4.6, lower panels). An antibody to SRM was not commercially
available, and therefore the inverse relationship of miR-27a and its targets was evaluated solely with ODC1 for this assay, as well as for the transduced expression of this miRNA in the endogenous target study described in the following section. Taken together, the correlated inverse expression of miR-27a and its predicted target protein (ODC1) was confirmed in situ. Furthermore, these results provide additional verification that ODC1 is a candidate target of miR-27a repression at the post-transcriptional level.

**Figure 4.9. Panel I:** Immunocytochemistry analysis of miR-27a suppression of endogenous ODC1 in both 293 (a to f) and NIH-3T3 cell strains (g to l). The cells shown with phase contrast (a, d, g, j), plasmid transfected (green fluorescence; b, e, h, k) and ODC1 immunostaining (red fluorescence; c, f, i, l) are from the respective identical fields. **Panel II:** quantification of the fluorescence for plasmid transfection and ODC1 staining. (293 cells: upper, NIH-3T3 cells: lower). (Bates *et al.* 2009 © John Wiley and Sons used with permission)
**Reporter functional assay of miR-27a with ODC1 and SRM**

The 3’-UTRs of ODC1 and SRM were cloned and inserted into pHcRed1-C1 vectors, with the HcRed1 fluorescent protein (red fluorescence) as the reporter. Mouse miR-27a expression may be assayed by the transfected expression of green fluorescence (GFP) by a plasmid containing both mmu-miR-27a and GFP. To study the functional impact of miR-27a on the expression of either ODC1 or SRM, co-transfection experiments were carried out using miR-27a expression plasmids and either of the two cloned 3’-UTR reporter constructs.

In co-transfected 293 and NIH/3T3 cells, we observed strong green and red fluorescence intensity at 24 hours after co-transfection, reflecting the input construct expression. Repression of the transfected miR-27a was observed beginning at 48 hours in 293 cells (Figure 4.7), and at 72 hours in NIH/3T3 cells (Figure 4.8); this is evidenced by the reduced intensity of red fluorescence from miR-27a +ODC1 3’-UTR or +SRM 3’-UTR (Figure 4.7 & 4.8, Panel I, a-c and g-i). This reduction is not observed in the plasmid construct bearing scrambled sequences, instead of miR-27a, in either ODC1 3’-UTR or SRM-3’-UTR cells (Figure 4.7 and 4.8, Panel I, d-f and j-l), indicating that the expression of the red fluorescence protein is repressed by miR-27a *via* its binding to the 3’-UTRs of ODC1 or SRM. Due to the co-transfection regimen, transfection efficiency was not homogenous; only ~50% of the cell population showed positive inclusion. This prevented us from doing Western blotting; instead, we used quantitative image analysis to show the repression level by ratio comparison between transfected miR-27a and 3’-UTR constructs. As shown in Panel II.a, cells transfected with control plasmids containing the scrambled sequence exhibit no red fluorescence suppression via complementary binding to the ODC1-3’UTR; thus no difference was found between the intensities of the two fluorophores representing the two transfected plasmids. However, this was not the case when plasmids bearing the miR-27a insert were used. Here, the level of transfected ODC1-3’UTR intensity is significantly repressed to
almost undetectable levels. Similar observations were seen with miR-27a/SRM and that of the scrambled control vector/SRM 3’-UTR (Panel II.b).

Next, endogenous ODC1 expression was measured in 293 and NIH/3T3 cells before and after miR-27a transfection. The results show that ODC1 expression indeed decreases in miR-27a-transfected 293 and NIH/3T3 cells, compared to scrambled control plasmid-transfected cells. Our transfection efficiency of ~50% provided the opportunity to study experimental versus control conditions by comparing fluorescence intensities among neighboring cells. For example, seen in the same field, the specific cell indicated by a double arrow with transfected miR-27a expression (Figure 4.9, Panel I. b) shows almost no detectable fluorescence intensity (Figure 4.9, Panel 1. c). In contrast, the neighboring cell (indicated by a single arrow), which shows no transfected miR-27a expression, retains its endogenous ODC1 expression (Figure 4.9, Panel 1. c). Similar differential fluorescence intensities between transfected cells and their endogenous ODC1 levels were not observed with plasmid constructs containing the scrambled sequence; scrambled sequence-transfected cells and their un-transfected neighbors show the same levels of fluorescence intensity. Again, we observed marked differences of fluorescence intensity between the transfected expression of miR-27a and endogenous ODC1 detected by antibody staining. In both cases, ODC1 is significantly lower than its normal level in miR-27a transfected cells, and not so in those carrying the scrambled sequence. This is particularly true in 293 cells, where endogenous ODC1 is almost undetectable in miR-27a-transfected cells (Figure 4.9 Panel 1.c.). Thus, the specific repression by transfected miR-27a on endogenous ODC1 levels is indeed due to miRNA action, and not nonspecific binding per se. Similar observations were recorded when the same experiment was performed in NIH-3T3 cells (Figure 4.9, Panel I g-l). Quantitation of repression by transduced miR-27a was measured as described for Figure 4.7 and 4.8, Panel II, with 10 representative fields totaling more than 300 cells. Taken
together, our results show that miR-27a represses ODC1 and SRM 3’-UTR reporters, and moreover, endogenous ODC1 expression is similarly affected by transfection with this miRNA. Therefore, the relationship between miR-27a and ODC1/SRM is not only estimated by software analysis, but also validated in our experimental functional assays.

**Figure 4.10.** Urea cycle/ornithine metabolism pathway diagram, showing proteins and the miRNAs that are postulated to posttranscriptionally regulate target protein expression. In order of the cycling pathway: 1) Carbamoyl-phosphate synthase (CPS1), 2) Ornithine carbamoyltransferase (OCT), 3) Argininosuccinate synthase 1 (ASS1), 4) Argininosuccinate lyase (ASL), 5) Arginase (ARG), 6) Nitric oxide synthase (NOS), 7) Ornithine decarboxylase (ODC), and 8) Ornithine aminotransferase (OAT). (Bates et al. 2009 © John Wiley and Sons used with permission)

**DISCUSSION**

Most studies conducted to date on Ames dwarf mice and aging focus on growth hormone (GH), and more specifically, on the downstream reduction of the insulin-like growth
factor (IGF-1), increased insulin sensitivity, and increased stress resistance and tumor suppression capabilities (H. M. Brown-Borg, 2008; H. M. Brown-Borg et al., 2005; H. M. Brown-Borg, Rakoczy, S.G., Sharma, S., Bartke, A., 2008; F. P. Dominici, Hauck, S., Argentino, D.P., Bartke, A., Turyn, D., 2002; E. Wang, 2007; Z. Wang, Masternak, Al-Regaiey, & Bartke, 2007). Most of these features are also noted in studies demonstrating that the Ames dwarf mouse’s long-lived phenotype can be observed in altered GH, insulin/glucose metabolism, or IGF-1 by either gene deletion (e.g., GH receptor knock out) or caloric restriction. Furthermore, increased longevity due to decreased GH or IGF-1 signaling led us to investigate the post-transcriptional regulation of genes involved in this desired phenotype—the delayed onset of aging—while bypassing undesirable effects associated with these hormonal deficiencies (e.g., reduced size, impaired reproductive capacity, etc.). Our results show that control of expression of genes specifically involved in intermediate metabolism and toxin defense is altered early on in the dwarf mouse. Our identification of up-regulated key miRNAs, led by miR-27a, suggests that these miRNAs suppress protein expression to levels similar to those of the aged control, but with a nine-month delay.

At present, several databases with their particular algorithms are commonly used to predict miR/target pair relationships. However, target identification from these different algorithms may yield discordant results. For example, in our study, ODC1 is identified as a target for miR-27a by miRanda and RNA22, but not by the popular TargetScan program. Therefore, attempts to identify specific targets for a particular miRNA need to employ at least four programs (miRBase target version 5 (miRanda), TargetScan, RNA22, and PicTar) in the public domain, each with its own unique features. The first two databases are popular for miRNA target prediction. Targetscan considers the miRNAs sharing the same seed sequence as a family and presents the predicted target proteins for each of the miRNA families. However, this database does not incorporate rapidly updated miRNAs, as does miRanda.
RNA22 is a powerful miRNA analysis algorithm with applicability similar to Targetscan as described above but with the additional utility for one to one prediction, *i.e.* one special interest miRNA for target proteins as we have presented in this report for miR-27a. Finally, Pictar is mainly providing the human and Drosophila miRNA target predictions. All four algorithms were used in this report and helped strengthen our hypothesis that protein members of the polyamine biosynthetic pathway were targeted by miR-27a (Table 4.5). Besides these programs available in the public domain, many personalized programs are also available in different bioinformatic laboratories; too many to be discussed here. Notwithstanding these programs and utilities, final determination of a particular miRNA/target relationship must be validated by functional studies, as shown in our results with miR-27a and its two targets, ODC1 and SRM, using reporter (Fig 4.7A and B) and repression of endogenous gene expression assays (Figure 4.7C).

The drastically reduced ornithine decarboxylase expression in dwarf mouse liver at 24 months of age, yet afterwards surprisingly increases towards the levels measured in aged controls (Figure 4.5 A and B) is very intriguing. Ornithine decarboxylase is the key rate-determining enzyme in polyamine biosynthesis, and catalyzes the reaction of L-ornithine to putrescine—a precursor of spermidine and spermine. MicroRNA-27a is implicated in a suite of studies pointing towards a role in cancer proliferation, transcription factor regulation, promotion of MDR1 expression, protective protein suppression and gastric adenocarcinoma, and gastric mucosal atrophy (Arisawa et al., 2007; T. Liu, Tang, Lang, Liu, & Li, 2008; Mertens-Talcott, Chintharlapalli, Li, & Safe, 2007; X. Wang et al., 2008; Zhu et al., 2008). In dwarf mouse liver, miR-27a expression increases with age, and is predicted to target ODC1 with significant complementary binding to the 3’-UTR of ODC1 mRNA and the 3’-UTR of SRM mRNA (the successive enzyme in the pathway to spermidine synthesis) (Table 4.5). Our *in situ* and *in vitro* assays support this prediction, with ISH combined with
immunohistochemical staining demonstrating an inverse expression disparity between miR-27a and ODC1 from wild-type controls (Figure 4.6). \textit{In vitro} assays show that the 3’-UTRs of both ODC1 and SRM are apparently bound by miR-27a, and translation of the red fluorescent gene product is suppressed (Figure 4.7). \textit{In vitro} endogenous expression assays show in two different cell types that ODC1 expression is reduced after transfection with the miR-27a vector, which is likely due to miR-27a binding to the 3’-UTR of endogenous ODC1 mRNA; thus inhibiting translation (Figure 4.9). Taken together, it is reasonable to suggest that polyamine synthesis in the dwarf mouse liver is posttranscriptionally suppressed \textit{via} miR-27a.

Besides the essential function of polyamine biosynthesis in normal cell processes, increased polyamine biosynthesis resulting from an imbalance of arginine/ornithine metabolism or abnormal hormonal fluctuation has been linked to tumor cell proliferation, a variety of cancers, lesions, polyps and various other abnormal growths (Byun, Choi, Moon, Kong, & Chul Chung, 2008; A. Gritli-Linde, Bjorkman, U., Holm, I., Tornell, J., Linde, A., 1997; Ignarro et al., 2001; Manteuffel-Cymborowska et al., 1995; R. K. Sogani, Matsushita, S., Mueller, J.F., Raben, M.S., 1972). Part of the ability of dwarf mice to suppress or avoid tumor or cancer growth may be attributed to the decreased polyamine biosynthesis resulting from reduced ARG1, ODC1, and possibly also SRM expression. Reduced expression of ODC1 could also be a result of the hormonal deficiencies in the dwarf mouse; ODC1 expression has been directly correlated with hormonal expressions of GH, PRL, testosterone, and estrogen (Byun et al., 2008; Gonzalez et al., 1991; A. Gritli-Linde, Bjorkman, U., Holm, I., Tornell, J., Linde, A., 1997; Kondo, Toyama, Sugiura, Fujii, & Yamashita, 2008; Manteuffel-Cymborowska et al., 1995). It is tempting to speculate that a relationship exists between hormone levels and miR-27a expression. What remains to be seen is why ODC1 expression increases in the aged dwarf (33-m.o.) towards the values measured in aged wild-
type (24-m.o.) mice—correlating with sharply decreasing miR-27a expression detected by qPCR (Figure 4.12A)—but with a presumed lack of hormonal stimulation. This may suggest that another factor is at play, working in coordination with miR-27a, ultimately allowing levels of ODC1 in the aged dwarf (33 m.o.) to reach levels observed in the aged control (24 m.o.). Further study using functional means (e.g., knock-in and knock-out of miR-27a) may demonstrate that the observed expression contributes significantly to the dwarf’s delayed aging.

Interestingly, CPS1, ASS1, and ARG1 exhibit increased protein expression in the young dwarf, followed by increasing miRNA suppression with age. This ultimately suppresses proteins to levels equal to or below those of older controls. It is well known that insulin represses transcriptional expression of proteins involved in urea synthesis, and many diabetes-associated complications arise from increased expression of ARG1 (Romero et al., 2008). The dwarf mouse, which exhibits reduced insulin levels compared to normal mice, would be expected to have over-expressed urea cycle proteins such as CPS1, ASS1 and ARG1; but because of miRNA regulation, CPS1 and ASS1 are either held slightly higher than, or suppressed to levels below, those of controls, depending upon the age examined. Unlike CPS1 and ASS1, ARG1 exhibits sharp miRNA suppression in Ames dwarf mice, to well below control levels, but then protein levels increase to reach—at 33 months of age—values equal to levels expressed in the 24-month-old control, thus displaying a 9 month lag before reaching parity with aged expression levels between dwarf and control, as also observed with ODC1. This pattern, occurring either fortuitously or because of dwarf physiology, should allow for adequate L-arginine production—a crucial substrate for nitric oxide production—while moderating its downstream use as a critical substrate of polyamine biosynthesis. Alternatively, the posttranscriptional suppression of these urea cycle proteins
may result from the reduced downstream expression—and thus reduced demand for the L-ornithine substrate—of critical polyamine biosynthesis proteins such as ODC1 and SRM.

**Figure 4.11.** Model of dwarf mouse endocrine deficiencies, and proposed effect on intermediate metabolism and microRNA regulation. (Bates *et al.* 2009 © John Wiley and Sons used with permission)
Figure 4.12. Histogram of average densitometry measurements of proteins (error bars ± σ). A.) arginase 1 (ARG1); B.) glutathione S-transferase μ1 (GST μ1); C.) superoxide dismutase (SOD). (Bates et al. 2009 © John Wiley and Sons used with permission)
A recent study shows that ODC1 activity and IGF-I and IGF-binding protein reductions result from dietary deficiencies of L-arginine (Cremades, Ruzafa, Monserrat, Lopez-Contreras, & Penafiel, 2004). Arginine can act as a secretagogue—a substance that promotes secretion of another substance—of GH and insulin in humans and other mammals (Morimoto, Fernandez-Mejia, Romero-Navarro, Morales-Peza, & Diaz-Sanchez, 2001; Rosenfeld, Rosenbloom, & Guevara-Aguirre, 1994). Taken together, these findings further emphasize a possible relationship between dwarf GH/IGF-1/insulin effects, miRNA regulation, and arginine/polyamine metabolism. Enhanced regulation of polyamine biosynthesis would be expected to contribute to the dwarf mouse’s decreased incidence of cancer compared with wild-type mice (Ikeno et al., 2003).

The dwarf mouse is well known for its heightened oxidative and toxic defense capabilities. The marked over-expression of GST μ1 in the dwarf mouse demonstrates its
ability to conjugate toxic compounds with free glutathione (GSH) for removal. After embryonic development, GST μ1 is continuously expressed throughout its life at levels higher than wild-type controls. A previous study on wild-type aging in mouse liver showed an increase with age in GST-targeting miRNA (O. C. Maes, An, J., Sarojini, H., Wang, E., 2008). However, in this study one GST-targeting miRNA, miR-96, displays significantly decreased expression in the dwarf mouse at 24 months of age, whereas the controls’ expression remains relatively constant. At 33 months of age, however, miR-96 expression in the dwarf mouse returns to levels close to that of the wild-type, at the same time point that dwarf GST expression is similar to that of aged controls. Moreover, a previous study showed that dwarf mice display increased Cu,Zn-SOD and catalase activity in their youth compared to controls, but this activity declines with age (Hauck & Bartke, 2000). In like manner, catalase expression decreased with age in our study; however, unlike in the previous study, it is expressed slightly less—instead of slightly more—than controls, and increases in expression at 33 months, although this extreme old age was not tested in the previous study.

The increased expression of SOD and GST μ1 in the young dwarf may suggest that enhanced oxidative and toxic defense in early life contributes to the delayed onset of aging observed with dwarf mice.

In conclusion, our study shows that miRNAs upregulated in dwarf mice correspond to alterations in target genes shown by our proteomic profiling, and reveal the level of posttranscriptional control that is presumably related to longevity determination. Specific miRNA upregulation associated with aging differs between Ames dwarf mice and their wild-type counterparts. This difference may well determine the slower rate of aging, since the dwarf seems to eventually arrive at proteomic expression levels similar to aged controls, but with a nine-month lag. Our work presented here implicates the function of miRNAs in delayed aging in the Ames dwarf mouse long-lived phenotype. Additionally, the prominent
miR-27a and its reciprocal relationship with ODC1 expression, seen as early as 2 months of age, suggests a head-start in the dwarf mouse’s ability to control the rate of intermediate metabolism signaling, and thereby potentially extend its life span. Future work with miRNAs, such as knock-in or knock-out transgenic mice with identified miRNAs such as miR-27a and its sisters, will reveal their functional impact in extending life span without hormonal deficiencies.
REFERENCES


CHAPTER FIVE

Next generation electro-analytical diagnostic device

INTRODUCTION

In the case of many age-related disease states, early detection is the paramount factor to decisive intervention and favorable prognosis. Early detection of such disease states typically requires analysis of multiple metrics whose combined confidence intervals provide a high enough confidence level to warrant intervention or further cost-intensive diagnostic testing. As such, diagnostic devices that aim to realistically address this need for the general population must provide high sensitivity, repeatability, and robust multiplexing capabilities all at a price point that invites the standardization of its equipment and protocol.

Microarray technology is generally more powerful than traditional diagnostic techniques due to its ability to produce qualitative and relative quantitative (when comparing two or more samples) information for a suite of biomarkers, which significantly increases specificity and confidence in the result. For instance, microarrays can detect pathogens down to the sub-type level, which is beyond the capabilities of many standard methods (Fang et al., 2010). However, next generation sequencing enables higher sensitivity and is forecasted to offer nearly similar price points, constituting it a credible threat to replace microarray technologies in the foreseeable future (Mardis, 2008). Both of these technologies still require RT-PCR amplification (the former also requires fluorescent labeling), which is only possible with dedicated equipment available in a diagnostic lab and thus precluding their deployment in doctor’s offices, small clinics, and in the field.

Functionalized electro-analytical chips may enable rapid, on-site detection of a variety of disease states by offering the combination of high sensitivity, heterogeneous multiplexing,
and rapid detection capabilities. Electrical impedance spectroscopy (EIS), for example, negates the need for expensive and time-consuming sample preparation procedures, and has been shown to detect DNA and RNA hybridization with picomolar sensitivity (Ihalainen et al., 2014). In addition, microsensor arrays allow increased current flow—compared to a standard ultramicroelectrode—and thus may significantly enhance detection sensitivity (Morf & de Rooij, 1997). Furthermore, microelectrode array chips afford heterogeneous multiplexing abilities, which enables detection of a variety of different markers including nucleic acids, proteins, metabolites, and ligands as well as redox-active species that can be detected and quantified to increase the confidence of the diagnosis.

Sensitivity and reproducibility are fundamental considerations when assessing the viability of a biosensor. In an electrochemical setup, both of these attributes are dependent upon spatial configuration. As such, a basic understanding of the different possible spatial configurations and their effects on these two parameters is needed prior to fabricating microelectrode array prototypes.

This chapter details:

Part 1. The use of COMSOL to model the different configurations of the microelectrode setup.

Part 2. The fabrication of a novel microarray sensor
PART 1. Microelectrode Array Sensors – Mathematical Modeling

Hypothesis: Increasing the ratio of surface area of electrode to volume of solution using droplet geometry instead of bulk solution will greatly enhance the electrical impedance sensitivity.

(Work in association with: Karthik Kannappan, Cather Simpson, Ashton Partridge)

DESIGN

The concept behind the sensor design involves two movable electrodes comprising of arrays of gold-coated tips precisely aligned over each other (Figure 5.1). The buffer solution which electrically connects the electrodes can either be micro droplets as shown in the figure, or fill the entire voids between the electrodes. All the tips on the lower arrays are electrically connected to form a common cathode / reference electrode while the tips on the upper arrays are electrically separated from each other enabling them to be individually addressed working electrodes (WEs). The WE tips are functionalized with biological capture agents before insertion into the chamber. The electrical resistance between the electrodes is firstly measured with the control buffer solution as the baseline, and subsequently the buffer solution containing target analytes for sampling. The measured difference in the resistance between the two cases is used to characterize the analytes that are bound to the electrode surface.
Figure 5.1 Design of the microarray sensor: The dome shaped microarrays are patterned on to a polymer which is coated with copper/gold conductive layers and then covered with epoxy to expose just the tips.

**Electrochemical Impedance Spectroscopy (EIS)**

The straight forward way of measuring the electrical resistance between electrodes separated by solution, is by applying a voltage and measuring the current. This process involves transport of charge across the electrode-solution interface. In the electrodes the charge is transported by the movement of electrons, while in the solution it is transported through the movement of ions. The charge transfer from the electrode to the ions in solution occurs mainly due to the oxidation and reduction reactions of the ionic species. The redox reactions that occur at the interface are faradaic in nature and the current passed is proportional to the applied voltage. In order to measure this current, a standard redox couple such as ferri/ferrocyanide is added to the solution. When an analyte is bound to the electrode surface, it impedes the charge transfer and therefore reduces the current passed through electrodes. The reduction in current is proportional to the amount of the analyte bound on the surface, which can be measured. The ease with which the charge transfer occurs can be defined by the charge transfer resistance across the interface, $R_{ct}$. When the system is at electrochemical equilibrium, $R_{ct}$ can be defined as in Equation 1.
\[ R_{ct} = \frac{RT}{F I_0} \]  

Where \( R \) is gas constant, \( T \) is temperature, \( F \) is Faraday’s constant and \( I_0 \) is exchange current across the interface.

Under certain conditions, such as in the absence of a redox couple, there will be no charge transfer across the electrode-solution interface. However, when a potential is applied, the external circuit will detect a flow of transient current due to the charging of the capacitive element that is present at the interface. This transient current changes when there is adsorption or desorption of analytes and these processes are non-faradaic in nature. This capacitance at the interface is due to the formation of ion layers separated by the solvent molecules and is known as double layer capacitance. This capacitance is represented as \( 1/jwC_{dl} \). Here, \( w \) is the frequency and \( C_{dl} \) can be defined by Equation 2.

\[ C_{dl} = \frac{A}{D_{dl}} \left( \varepsilon_0 \varepsilon_r \right) \]  

Where \( A \) is the area of the electrode, \( D_{dl} \) is the height of the double layer and \( \varepsilon_0 \) and \( \varepsilon_r \) are vacuum and relative permittivity, respectively. These two faradaic and non-faradaic components along with the solution resistance \( (R_s) \) contribute to a complex resistance of the system called the impedance. The simplified Randles circuit shown in Figure 5.2 best represents the equivalent circuit model of impedance imparted by these elements.
The total impedance of the equivalent circuit model with \(1/j\omega C_{dl}\) and \(R_{ct}\) in parallel which is in series with solution resistance \((R_s)\) can be given by Equation 3.

\[
Z(\omega) = R_s + \frac{R_{ct}}{1 + j\omega R_{ct} C_{dl}}
\]

This impedance data is a complex number which is usually measured for wide range of frequencies. The change in the impedance value when an analyte adsorbs to the electrode surface varies depending on the frequency of the applied signal. There are two different ways to represent the impedance data: one is Nyquist plot where the real part of the impedance \(Z'(\omega)\) is plotted against the imaginary part \(Z''(\omega)\); the other is the bode plot where the magnitude or the phase is plotted against the frequency.
Mathematical model

In order to approximate the effect of geometry on the sensitivity of the tips to detect target analytes, two different geometries were set up in COMSOL multiphysics, one with droplet formed between the two tips and the other with buffer filling the entire chamber. The total impedance between the two electrodes is the sum of the impedance at the electrode-electrolyte interface and the impedance of the electrolyte solution. In order to measure the impedance changes due to change in the geometry of the system, the total impedance of the system was simulated by solving the modified Laplace’s equation for two different geometries.

\[
-\nabla V (\sigma + j\omega \varepsilon_0 \varepsilon_r) = J - J_e
\]

\[
\nabla \cdot J = Q_j
\]

Where \( V \) is the voltage, \( J \) is the current generated within the system, \( J_e \) is the external current source, \( Q_j \) is the total charge within the system, \( \sigma \) is the conductivity of the solution, \( \varepsilon_0 \) is permittivity of free space and \( \varepsilon_r \) is the relative permittivity of the solution.

The total current across the interface is the product of potential difference and the total impedance across the interface.

\[
J = -\frac{A}{D_{dl}} \left( \frac{1}{R_{ct}} + j\omega \varepsilon_0 \varepsilon_r \right) (V_e - V_s)
\]

The modelled geometry along with its domain equations and boundary conditions is shown in Figure 5.3.
Figure 5.3 Modelled geometry for the case of droplet between the electrodes showing the domain equations and the boundary conditions that were solved

RESULTS

An AC potential of 1 V was applied between the upper and lower electrodes and total impedance was calculated from the current distribution for a frequency range of 1 KHz to 1x10^6 KHz. The buffer is considered as a solution with conductivity of 0.02S/m and a relative permittivity of 80. Impedance was calculated for various radius of droplet formed along with various distances between the two tips. The results are illustrated in the figures below. The top of Figure 5.4 demonstrates basic model setup in comparing droplet versus fill charge potential distribution analysis. The 2D figure below displays both an electropotential
heatmap and current density (indicated by arrows) distribution. Surprisingly, the two geometries were quite similar as is further shown in the 3D rendering.

**Figure 5.4** Two different geometries (a) droplet formation b) chamber filled with electrolyte showing potential distribution (Red -1V Blue 0-V) and current density vectors (arrows) in 2D and 3D domain
The modelling results allow a number of variables associated with the system to be estimated including:

- Effect in the impedance of varying the electrolyte size and shape
- Variations in the double layer capacitance
- Variation in charge transfer resistance

**Variations in electrolyte geometry**

Figure 5.5 reveals significant variation in impedance sensitivity with electrode separation distance for smaller droplet size. The magnitude of the variation decreases with increasing volume of solution. Figure 5.6 demonstrates relatively minor variations in impedance sensitivity with increasing distance between electrodes when the volume is assumed as infinite.

Figures 5.7 and 5.8 show the greatest difference in impedance sensitivity between the droplet and filled geometries. Figure 5.7 shows a 12-fold increased sensitivity when the droplet radius is 5μm versus the filled geometry; however, a 3 fold increase of droplet radius reduces sensitivity to only twice that of the filled geometry. The Bode plot in Figure 5.8 shows that this great disparity in sensitivity between droplet geometry with a 5μm radius versus fill geometry was only seen at the lower frequency ranges.
Figure 5.5 Impedance for various parameters of geometry (a) (Droplet formation)

Figure 5.6 Impedance for various parameters of geometry (b) (Fill)
Figure 5.7 Impedance comparison between two geometries. Red showing fully immersed geometry (b), other two showing the droplet formation with different radius (Geometry (a))

Figure 5.8 Frequency vs. Impedance (Bode plot) for two different geometries. Red showing geometry b) and green showing droplet with radius 5 um
Variations in double layer capacitance

For sensor applications, the changes in the interface can be detected by either measuring the faradaic changes or the non-faradaic signals at the interface. Non faradaic electrochemical impedance spectroscopy (EIS) involves neglecting any changes due to redox reactions and measures the capacitance changes due to changes in the double layer thickness (Ma, Zhou, Zoval, & Madou, 2006; Yusmeeraz Yusof 2000). In order to determine the total impedance change of the system due to changes in double layer thickness, model simulations were carried out for various double layer thicknesses \( D_{dl} \). For all the cases \( R_{ct} = 1M \text{ ohm} \).

Figures 5.10 – 5.11 show the results for various cases of droplet and filled scenarios.

The Nyquist plot in Figure 5.9 reveals that although there was greater resistance observed in the droplet geometry, variations in double layer thickness does not have a significant impact. Importantly, Figure 5.11 shows that differences between the impedance due to changes in double layer thickness were the same for both fill and droplet geometries except in the low frequency range.
Figure 5.9 Impedance (Nyquist plot) for various double layer lengths of the interface for two different geometries (Red - Fill / Blue – Droplet)

Figure 5.10 Bode plot showing difference in the Real Z (ohms) for two different double layer thickness.
Variations in charge transfer resistance

Another method to detect the changes at the interface is by measuring the redox reaction at the interface. When there is a change in the interface due to biological capture agents, the rate at which the redox reaction takes place changes. This changes the current at the interface which consequently changes the charge transfer resistance ($R_{ct}$) of the system. The ($R_{ct}$) values vary for different interfaces (Franks, Schenker, Schmutz, & Hierlemann, 2005). The impedance changes of the system simulated for various $R_{ct}$ are illustrated in Figures 5.13 – 5.15.

Figure 5.13 shows the difference in impedance changes between the two geometries at several different $R_{ct}$ values ranging from 1K to 1M ohms. As expected, the droplet geometry has slightly enhanced impedance sensitivity across the $R_{ct}$ values tested. Figure 5.14 displays the differences in impedance of the droplet geometry at different $R_{ct}$ values and
reveals that the most drastic impedance difference across the selected frequencies occurs with a 10K ohms $R_{ct}$. Figure 5.15 overlays the impedance differences at 1M and 100K ohms $R_{ct}$ of the two geometries across the given frequencies. Interestingly, the two geometries have very similar impedance changes at the specified charge transfer resistances.

**Figure 5.12** Change in the impedance due to changes in charge transfer resistance ($R_{ct}$) for the two different geometries (Red- Fill/ Blue- Droplet).
Figure 5.13 Change in the impedance due to change in Rct for the droplet geometry

Figure 5.14 Difference between the impedance due to change in Rct from 1M ohm to 100K for the two geometries
DISCUSSION

Modelling variations of two parameters: the distance between the electrodes and the volume of the electrolyte (as either a droplet between the tips or as a solution that completely covers the tips), showed that sensitivity was inversely related to both the distance between the electrodes and the electrode area, but not significantly affected by the volume of the electrolyte at the micron scale, which is presumably due to diffusion limitations in bulk solution at the microelectrode interface. The top of Figure 5.4 maintains droplet radii while separating the distance between electrodes which expands the volume and electrode separation distance. COMSOL modelling used to test the hypothesis suggests that sensitivity is increased, particularly at lower frequencies; however, not sufficiently enough to warrant undertaking the engineering challenge of fabricating a device which has uniform micro-droplet formation and preservation across tens of thousands of electrode tips. Consequently, the decision was made to design a microelectrode array on a single chip, as opposed to the initial design using upper and lower electrode arrays diagrammed in Figure 5.1, using an interdigitated arrangement of working and counter electrodes. This design is preferred as it greatly reduces complexity, cost, and the necessity for extreme precision, and is more in line with the requirements of a next generation device listed below.
PART 2. Microelectrode Array Fabrication

**Hypothesis:** A three dimensional microelectrode array with multiplexing capabilities can be fabricated out of plastic and capable of detecting nucleic acid biomarkers.

**INTRODUCTION**

The fabrication of microelectrodes and arrays of electrodes is not new and there are many techniques that have been employed to carry out their production. These techniques include: different forms of printing (e.g., screen printing or ink jet printing), lithographic techniques (e.g., soft-lithography, photolithography, ion and electron beam lithography), direct electrodeposition (i.e., deposition of wires), patterning of carbon nanotube/fiber arrays and assembly techniques (e.g., wires set in an epoxy resin) (McDonald et al., 2000; McDonald & Whitesides, 2002; Ng, Gitlin, Stroock, & Whitesides, 2002). However, these known methods have a number of limitations. In particular, they are cumbersome to carry out and it is difficult to accurately define the arrays over a large surface area. Moreover, on the millimeter to nanometer scale the lack of definition leads to poor resolution, which ultimately affects sensitivity and reproducibility. Furthermore, issues of scale up and cost arise particularly with nanoscale arrays as the time and care required limit fabrication to small batch processes.

Notwithstanding, the prospect of arrays on the micrometer to nanometer scale over large surface areas with improved accuracy of definition is particularly attractive in the areas of sensing, electrochemistry, and catalysis. In a typical electrochemical detection process it is generally preferable to employ an array of smaller electrodes as opposed to a single large electrode. Reasons for this include:

- the ability to use smaller sample volumes;
• application in both in vivo and in vitro measurement;
• low depletion rate of target molecules;
• low background charging due to their reduced surface area;
• reduced iR drop;
• High current density arising from enhanced mass transport to the electrode surface as a result of convergent diffusion; and
• Enhanced control over probe-analyte interaction probability.

Furthermore, the ability to add a multiplexing function to the array, wherein simultaneous testing or measurement of multiple analytes or biomarkers can be conducted, significantly enhances value of the sensing platform. Such a system could be used to detect known multiple different biomarkers relevant to a specific disease, organ or system and thereby increase confidence in the diagnosis. Toward this end, we set out to design a low cost microelectrode array constructed of plastic and a thin layer of gold that hopes to offer all the aforementioned benefits.

Initial Work in Microelectrode Array Fabrication

Overview

The basic design of the Microelectrode Array (MEA) chip includes micro-patterned substrate of polymethyl methacrylate (PMMA) that was fabricated using hot embossing. The micro-patterned sheets were then coated with a thin conductive layer of gold. The microelectrode arrays were produced by laser scribing through the conductive layer to electrically isolate defined regions of conductive tips in such a way to enable electrical connection from one edge of the chip (Figure 5.15).
Figure 5.15 Cross section of gold coated PMMA chip showing laser scribes for electrical isolation of electrode arrays. Scribes shown here are made with a CO2 laser and were not ideal because of the depth. Decision was made to switch to femtosecond laser for controlled depth and width (~50um x 50um).

Once the pattern of the electrodes has been scribed into the conductive layer electrical isolation of the tips was achieved by laying down an insulating layer to fill in the valleys between the tips (Figure 5.16). Tips were cleaned by plasma etching followed by electrochemical cleaning to remove any insulating material or adsorbed organics. The basic concept of the chip design was outlined in Figure 5.17.
**Figure 5.16** Patterned PMMA with a thin layer of gold deposited. Insulating material used to isolate tips seen filling in the valleys from the top right.

**Figure 5.17** Proof of concept device: MEA chip. Working area comprises interdigitated track 4 mm (w) x 10 mm (l) scribed by laser. Interdigitation alternates Counter and Working electrode(s) arrays of tips. Electrical connections to the electrodes were made on flanking regions of working area. Insulating material fills in the valleys. Future embodiments will reduce tip size for expected heightened sensitivity.
**Chip Production**

The patterned PMMA sheets were laser cut into circular wafers with 9.0 cm diameters (Figure 5.18A). Wafers were washed thoroughly with DI water and dried in vacuum for 2 hours. 14 ml of EDC solution (800 mg EDC in 14 ml H₂O) and 14 ml of NHS solution (800 mg of NHS in 14 ml H₂O) were prepared separately. 7-ml of the prepared EDC and NHS solutions were mixed together in two different vials and to each vial a 0.5ml of SAM-COOH solution was added. Each wafer was then placed into a large petri dish into which 14 ml of EDC/NHS/SAM-COOH solution was added, then shaken at room temperature overnight. Wafers were washed thoroughly and dried in vacuum for 2 hours. Dried wafers were sputter coated with a 45nm layer of Au (2 mins x 3 times) using an Edwards Sputter Coater (Model S150B). Electrical separation to form working electrode(s) and counter electrode is achieved by scribing a 50 um thick track in an interdigitated pattern through the gold into the plastic using the femtosecond laser (Coherent Legend Elite, Coherent Inc. Santa Clara, CA. USA) at the Photon Factory (University of Auckland). The 9cm wafers were cut into forty-seven 1-cm² chips using a 40W CO₂ laser (Full Spectrum, Las Vegas, NV) (Figure 5.18B). Chips were then annealed to relieve heat stress around the edge brought on by the laser by immersing in 80°C water and then cooling to room temperature. Annealed chips were treated with an O₂ plasma (Diener Electronic –Femto Plasma Cleaner, Ebhausen, Germany) for 5 minutes on both the sides, then placed in a glass petri dish and a 15 ml of 10% Allyamine solution (13.5 ml H₂O +1.5 ml Allyamine) is added and shaken at 65°C for 1.5 hours.
Initially, several different off-the-shelf tip-isolating/insulating reagents were explored ranging from White Out to Rustcoat and various inks and epoxies. Additionally, different viscosities of the same compounds were tested in attempts to develop a process of inexpensive and reproducible tip isolation. A particular type of Rustcoat (Dulux) was close (Figure 5.19) to meeting the criteria but falls short in the end because of flaking off when submerged into organic solvent-based reagents that were used in the process of the Self Assembled Monolayer (SAM) deposition. Realizing a stable, inert compound that can be applied evenly and consistently was fundamental to the success of this sensor, a decision was made to utilize spin coating of SU-8, which is common practice in the semi-conductor industry and proves a very effective solution (Figure 5.20).
A 75 um layer SU-8 2025 (Microchem, Newton, MA) was applied over the gold via spin coating at 2000 RPM for 50 seconds. The epoxy was cured (UV exposure 2 mins) then etched away using reactive ion etching for 180 seconds to expose and clean 25 um gold protrusions, thus forming the prefunctionalized MEA wafer (Figure 5.15). MEA chips were immediately submerged in SAM solution (10mM mercaptohexanol) or dual SAM solution (10uM mercaptohexanol, 10uM carboxy-6-mercaptohexane), depending on desired probe attachment chemistry. Immediate submersion prevents unwanted organic adsorption to contaminate the gold surface.

**Figure 5.19** Image showing substrates with a) pure Au coating and b) Au with Rustcoat layer just exposing the tips, in different magnifications
Gold Stability

Gold surface stability was an issue as it can flake off the PMMA tips, which disrupts repeatability of measurements. Several deposition methods of gold onto PMMA was carried out and the most promising methods were tested using the tape test according to ASTM D1000 (Annual Book of ASTM Standards, Vol. 10.01) and reported in Table 5.2. Table 5.1 shows the ranking criteria according to the ASTM standard.
Table 5.1 ASTM D1000 Classification of Results of Crosscut Adhesion test.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Percentage of area removed</th>
<th>Surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>5B</td>
<td>0% None</td>
<td></td>
</tr>
<tr>
<td>4B</td>
<td>Less than 5%</td>
<td></td>
</tr>
<tr>
<td>3B</td>
<td>5% to 15%</td>
<td></td>
</tr>
<tr>
<td>2B</td>
<td>15% to 35%</td>
<td></td>
</tr>
<tr>
<td>1B</td>
<td>35% to 65%</td>
<td></td>
</tr>
<tr>
<td>0B</td>
<td>Greater than 65%</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 Comparison of different pre-treatment and deposition methods. (Booth, Kannapan, Pandeya, Bates, & Partridge, 2014)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Before Tape Test</th>
<th>After Tape Test</th>
<th>Ranking</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><img src="image1" alt="" /></td>
<td><img src="image2" alt="" /></td>
<td>0B</td>
</tr>
<tr>
<td></td>
<td>Heated to 70 °C for 10 min between first and remaining three coatings (1 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td><img src="image3" alt="" /></td>
<td><img src="image4" alt="" /></td>
<td>0B-1B</td>
</tr>
<tr>
<td></td>
<td>Heated to 70 °C for 30 min between first and remaining</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step</td>
<td>Description</td>
<td>Image</td>
<td>Notes</td>
</tr>
<tr>
<td>------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>III</td>
<td>Ethanol cleaned then 2 x 1 min coatings (~15 nm)</td>
<td><img src="1B-2B.jpg" alt="Image" /></td>
<td>1B-2B</td>
</tr>
<tr>
<td>IV</td>
<td>Coating for 1 min (~7.5 nm)</td>
<td><img src="0B.jpg" alt="Image" /></td>
<td>0B</td>
</tr>
<tr>
<td>II b</td>
<td>1 s microwave</td>
<td><img src="0B.jpg" alt="Image" /></td>
<td>0B</td>
</tr>
<tr>
<td>III b</td>
<td>2 s microwave</td>
<td><img src="0B.jpg" alt="Image" /></td>
<td>0B</td>
</tr>
<tr>
<td>V</td>
<td>Gold then 80°C (30 min), gold then 80°C (30 min) then 2 x 1 min gold coating.</td>
<td><img src="0B.jpg" alt="Image" /></td>
<td>0B</td>
</tr>
<tr>
<td>I b</td>
<td>3 s microwave after 1 gold layer – cracked gold</td>
<td><img src="3B-4B.jpg" alt="Image" /></td>
<td>3B-4B</td>
</tr>
<tr>
<td>VI b 90°C heated after 1 gold layer – warped plastic shape</td>
<td>4B-5B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 a-c Clean, plasma etch, 1 layer of gold (1 min), heat at 80°C for 30 min</td>
<td>3B-4B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 a-c Clean, plasma etch, 1 layer of gold (1 min), heat at 80°C for 30 min, second layer of gold (1 min)</td>
<td>3B-4B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 5.3** Further comparisons of gold adhesion techniques using the tape test, post-deposition sonication, and CVs (Booth et al., 2014)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Au thickness (nm)</th>
<th>Resistance through metal mask (Ω)</th>
<th>CVS-EC</th>
<th>Tape test</th>
<th>Notations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Smooth layer/Smooth layer (Ω)</td>
<td></td>
<td>Before</td>
<td>After 1 min</td>
</tr>
<tr>
<td>Clean, Plasma, gold sputter, gold sputter</td>
<td>7.5</td>
<td>6.7 ± 1.2/8.9 ± 2.4 (Ω)</td>
<td><img src="image1.png" alt="Image" /></td>
<td>Before</td>
<td>After 1 min</td>
</tr>
<tr>
<td>Sample 1 and 2</td>
<td>15</td>
<td>3.3 ± 0.7/3.8 ± 1.2 (Ω)</td>
<td><img src="image2.png" alt="Image" /></td>
<td>Before</td>
<td>After 1 min</td>
</tr>
<tr>
<td>Cleaned PSMA, UV, 1st, then gold sputter</td>
<td>7</td>
<td>1.0 ± 0.3/ / (Ω)</td>
<td><img src="image3.png" alt="Image" /></td>
<td>Before</td>
<td>After 1 min</td>
</tr>
<tr>
<td>Sample 4</td>
<td>15</td>
<td>2.7 ± 0.1/4.0 ± 0.9 (Ω)</td>
<td><img src="image4.png" alt="Image" /></td>
<td>Before</td>
<td>After 1 min</td>
</tr>
</tbody>
</table>

**Chip cleaning**

The prepared chip was placed in a silicone electrochemical cell (Figure 5.21) with stainless steel counter and AgCl reference electrode. H$_2$SO$_4$ (0.5N) was circulated into the chamber using a peristaltic pump and a 1.65V potential was applied to the chip for 15s. Next, the chip was subjected to cyclic voltammograms from 0V to 1.65V with the same 0.5 N H$_2$SO$_4$ solution in circulation. The CV should show Au oxidation and reduction peaks at 1.5V and 0.55V, respectively (Figure 5.22). After the electrochemical cleaning the chip was washed by circulating DI water through the electrochemical chamber for 2 minutes with the pump setting at 150 rpm.
Comparing reference electrodes

Three reference electrodes were examined in bulk solution in attempts to optimize cost and performance dynamics (Figure 5.23). The working electrodes and counter electrode were both on the chip (laser scribed, using one side).

As expected, a slight shift in the potentials was observed; however, each reference electrode showed clear CVs. The Ag wire in this case was not Ag/AgCl, just Ag acting as a pseudo reference electrode. Ferrocene solution was used as a standard to quantify changes in potential between the three electrodes. Going forward, Ag/AgCl was used for convenience, although any of the three could be used in a commercial device.
**Figure 5.22** CV showing Au oxidation and reduction peaks for 1cm square substrates, coated with epoxy with just tips exposed. The CV is run in a silicone electrochemical cell. (AgCl reference electrode)

**Figure 5.23** CVs of gold chip using three different reference electrodes: Platinum wire, plain silver wire, and commercial Ag/AgCl reference electrode

**Substrate treatment**

Following cleaning, 0.1 M SAM-COOH solution was introduced into the electrochemical flow cell and circulated for 1 hour. The chip was washed thoroughly by
circulating water through the cell for 3 minutes. The chip was then characterized by introducing 5mM K₃FeCN₆ solution containing 0.1M KCl into the chamber and running a CV from -0.1V to 0.3 V against Ag/AgCl electrode. The substrate was washed thoroughly by circulating water.

Both 0.75 ml of EDC solution (50 mg EDC in 0.75 ml H₂O) and 0.75 ml of NHS solution (50 mg of NHS in 0.75 ml H₂O) were prepared separately; both solutions were mixed together to get 1.5 ml of EDC\NHS solution. The mixed solution was then run through the electrochemical cell for 1.5hr to activate the SAM which was adsorbed onto Au tips. The substrate was then quickly washed with water.

6-Amino acid solution was prepared by mixing 1g in 7 ml of water. This solution was run though the electrochemical chamber for 1 hour. The cell was washed with water and then characterized using cyclic voltammetry and Faradaic impedance spectroscopy by introducing 5mM K₃FeCN₆ / 0.1M KCl solution in the chamber and then thoroughly washed with water. Results are shown in Figure 5.24 and Figure 5.25.

![Graph](image)

**Figure 5.24** CV showing the K₃FeCN₆ oxidation and reduction for the substrate with SAM-COOH and SAM-COOH with additional biolinker attached (amino acid) (AgCl reference electrode)
The aminoacid linker was then activated by again circulating the EDC\NHS solution prepared previously for 1.5 hr. The substrate was washed with water. 500 ul of 1 um blue bead solution was mixed with 4 ml of PBS buffer solution. The bead suspension was circulated through the cell for 15 min and then washed with water. The substrate is then examined using microscope (Figure 5.26).

Figure 5.25 Impedance data for the substrate with SAM-COOH and SAM-COOH with additional biolinker attached (amino acid). Frequency range 10MHz to 0.1Hz, Amplitude 0.1V.
It was determined that further characterization was needed to enhance SAM deposition, biolinker attachment and ultimately probe attachment.

**Incorporation of chips into improved PDMS molds**

A more suitable PDMS mold was fabricated to encase the MEA chip and support the electrical connections in a standardized way, which enables improved repeatability and ease of use in performing electrochemical measurements. Initially, problems with connectivity were found between the chip and the PCB. Several attempts were made to improve connectivity including: 1) the addition of plastic pillars to mechanically push the PCB down on to the chip; 2) using a standard clamp to increase pressure; 3) conductive tape on the PCB connection; and ultimately, 4) mold re-design to try to improve this connection. The final configuration includes the new mold design and conductive tape (Figure 5.27).
Figure 5.27 (Left) Assembly configuration of PDMS mold with PCB and solution channel intact. Arrows show where conductive tape was used to assure contact between conductive panels on the PCB and the electrodes of the MEA chip (shown in top right). (Right) Diagrammatic representation of the MEA chip with three working electrodes (WE 1-3), counter electrode (CE), and an outline to show where sample solution crosses the electrodes guided by the preformed channel of the mold.

Initially, an electrochemical signal was not visible when running CVs; however, all the CVs demonstrate very high currents (high μA to mA range), which suggests solution was leaking out of the mold channel and electrically shorting the working electrodes together. An O-ring was cut from PDMS and set in the mold to better seal the channel (Figure 5.28).

Figure 5.28 Picture showing mold assembly with PDMS O-ring in place.

CVs were performed with the O-ring in place and a good signal was seen (Figure 5.29A). Interestingly, the gold reduction peak was visible at 770mV as opposed to the 900mV observed previously in bulk solution. This shift was easily attributable to the change
in reference electrode between the two setups. Cycling over time causes the gold reduction peak to increase as expected (Figure 5.29B). Slight differences were also seen in the oxidation curves, with some unexpected jumps in current, which may result from shifts in the electrode potential arising from minor solution leakage. Careful attention was paid to this matter and epoxy was sometimes used to assure the absence of leaks.

![Figure 5.29 A. Gold reduction peak was seen at 770 mV, slightly lower than the 900 mV previously observed in the bulk solution. B. Increases in gold reduction peak over time as expected.](image)

**Characterization of thiol monolayers on gold electrodes**

**Behavior of Au electrode in ethanol-based K₃FeCN₆ solution**

A water and ethanol solution was tested against pure water to determine if the better solvent for the SAM solution (i.e., water:ethanol) yields better electrochemical sensitivity and functionality than water alone. If successful, it may enable real-time monitoring of SAM deposition during functionalization. To compare baselines, the Au electrode was subjected to CV in K₃FeCN₆ in a 1:1 water:ethanol solution and compared to K₃FeCN₆ in pure water. The results show a reduced current and a less pronounced ferricyanide peak with 1:1 water:ethanol than the CV performed with K₃FeCN₆ in pure water. Next, SAM was adsorbed on the Au surface by immersing the electrode in a 1:1 water ethanol solution containing
SAM-OH for 5 minutes. The electrode containing SAM was studied again with CV in water solution without ethanol compared to the 1:1 water:ethanol solution, both in the presence of potassium ferricyanide. The ferricyanide peaks of the Au electrode with SAM disappeared in the water solution without ethanol, whereas the peaks were reduced but still present in the ethanol and water solution (Figure 5.30).

![Figure 5.30 CVs of Au electrode with and without SAM in the water-based and the water:ethanol-based K₃FeCN₆ solution.](image)

**Real-time monitoring of SAM adsorption**

Experiments were carried out to determine the viability of consistent, high-throughput deposition of SAM layers for potential commercial production scenarios. The gold electrodes were cleaned electrochemically using H₂SO₄ solution as before and cycled from 0V to 0.5V in a solution containing 5mM K₃FeCN₆ in 1:1 water:ethanol with KCl support electrolyte. A highly concentrated SAM solution (5 mM) was added at the 14th and 28th cycles. The CV results show a decrease in the current immediately after the addition of SAM solution indicating the adsorption of SAM onto Au layer. Subsequent cycles after the addition of SAM indicate the layer remains and was stable (Figure 5.31).
Figure 5.31 Real time CV measurement during SAM addition. 1ml of Concentrated SAM (5 mM) in ethanol solution is added at 14th and 28th cycle.

**SAM Adsorption Behavior**

The gold electrodes were subjected to cyclic voltammetry in Potassium ferricyanide (K₃FeCN₆) solution. The CV shows a standard ferricyanide oxidation and reduction peak at 0.35 V and 0.15 V respectively. As reported by Collman et al, cyclic voltammetry was utilized to put on and take off the SAM layer (Collman et al., 2009). Basically, the freshly cleaned electrodes were immersed in solution containing 5mM SAM-OH in 1:1 ethanol:water solution. The electrodes were then taken out periodically and washed with water and subjected to cyclic voltammograms in 5mM Pottasium ferricyanide (K₃FeCN₆) solution with KCl as supporting electrolyte. The CV shows gradual disappearance of the ferricyanide peak over time with the electrodes immersed in the SAM solution indicating the gradual adsorption of SAM onto gold electrodes, i.e., the decreased peaks were indicative of reduced current flow resulting from increased SAM build up (Figure 5.32).
After a period of 20 minutes the current reaches a steady state value showing that the electrodes were saturated with SAM. In other words, the CV of the electrode after 20 minutes of SAM adsorption has similar characteristics as the CV of the electrode after 2 hours of SAM adsorption.

**SAM desorption behavior**

The SAM was removed from the electrode electrochemically using a method described previously by Collman et al which entails holding the electrode at 1.8 V in 0.5M sulfuric acid for 30s. The electrodes were then subjected to CV in 0.5mM K$_3$FeCN$_6$ solution and the CV shows the return of the ferricyanide redox peaks indicating the removal of SAM. However, the redox peaks after the removal of SAM show a slight shift from the redox peaks from the clean Au electrodes (Figure 5.33).
The impedance was also measured in Potassium ferricyanide (K$_3$FeCN$_6$) solution containing KCl supporting electrolytes. The frequency was varied from 0.1 Hz to 10 KHz for both with and without SAM. The data without SAM was performed with a lower resolution scan and hence has fewer data points than the data with SAM but both the data were in the same frequency range (Figure 5.34). The data shows impedance components $Z'$ and $Z''$ varying over the frequency, where the total impedance was $Z'+j Z''$. Resistance and capacitance information can be derived from the impedance data, which was reported in Figure 5.35.
Figure 5.34 Figure showing the difference in impedance between the Au electrode with and without SAM. The frequency range is from 10KHz to 0.1Hz.

Figure 5.35 Figure showing the resistance and capacitance derived from the impedance data of the Au electrodes with and without SAM.
Multiplexing and Functionalization

**Multiplexing**

Multiplexing of the MEA requires the controlled deposition of probes to individual working electrodes and will enable one MEA chip to support several capture agents to detect multiple targets simultaneously, thereby increasing confidence in the diagnosis. Initial experiments use a conducting polymer - polypyrrole (PPy), to confirm selective deposition, as the deposited polymer appears black due to conjugated backbone. Growing PPy on individual electrodes was achieved by running cyclic voltammetry (CV) from -0.5 to 9 V, for 3 cycles in 0.1 M PPy, 0.1 M of the dopant Dodecylbenzenesulfonate (DBS) in water solution (adapted from (Tabard-Cossa, Godin, & Beaulieu, 2005)). Optical microscopy confirms that PPy only grows on the working electrode as was expected (Figure 5.36). Figure 5.37 shows a close up of a tip from one electrode compared to an adjacent electrode to which no potential was applied.
Figure 5.36 Pictures of 2 MEA chips under two magnifications with PPy growth on specific electrodes while adjacent electrodes to which potential is not applied remain bare. (Top) PPy was grown by cyclic voltammetry. (Bottom) PPy grown by applying constant potential (as reported in Tabard-Cossa et al. 2005). Electrode separation (i.e., laser scribing lines) was depicted by the dashed line.

Figure 5.37 Picture of MEA chip the red line indicating the area of the channel and hence exposed tips A. PPy growth in the fluidic channel – below the red line; and B. showing electrode-specific growth with no growth on adjacent electrodes – between dashed black lines.
Figure 5.38 Picture of two adjacent tips demonstrating electrical deposition of functionalizing agent on one electrode array while a neighboring tip 200 µm away remains bare.

**Functionalization**

Prior to specific probe functionalization, several background experiments were carried out in order to characterize the surface and chemistries associated with functionalization. Blue polystyrene beads (1 µm diameter) possessing amine groups were chemically attached to carboxylated SAM via NHS-catalyzed coupling to the SAM’s carboxyl group in order to visually assess surface coverage of the SAM. This time coverage was significantly improved (Figure 5.39 compared to Figure 5.26).
Finally, DNA-functionalization of tips was visually observed using a region in the promoter of the brain-derived neurotropic factor (BDNF) gene with a fluorescent tag (FITC) to validate coverage. This capture agent was deposited on the tip by mixing the probe at 1:9 (probe:SAM) ratio as reported in literature (Kukol et al., 2008) to allow appropriate spacing and assure proper orientation by preventing unwanted adsorption of the phosphate backbone. Configurations tested are: 1) probe without label to be bound with labeled target; 2) probe without label to be tested with scramble probe control (test for non-specific binding); and 3) labeled probe to show coverage (Figure 5.40).
**EIS of nucleic acid biomarker on MEA sensor platform**

To prove the concept of using the MEA as a diagnostic sensor, a DNA sequence specific to a region in the BDNF promoter was deposited as a capture agent on the working electrode as previously described. To electrochemically validate the MEA chip, experiments were run in parallel with a commercially acquired macro gold electrode (designated MacroAu) (Pine Instruments, Durham, NC, USA). The tested sequences are:

- **Probe:** 5` - [Thiol] GCC ACA TGC TGT CCC CGA GAA [Fluo] - 3` with thiol SS-C6 and fluorescein (FITC) functionalization

- **Target:** 5` - [Fluo] TTC TCG GGG ACA GCA TGT GGC - 3` with FITC functionalization

- **Scramble DNA:** 5` – [Fluo] CAG TAG CTT CCG AAT ATG TGA - 3` with FITC functionalization.
MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Aldrich, unless otherwise mentioned.

Electrochemical Setup

Electrochemical setup consists of an Ag/AgCl external reference electrode (3 M NaCl), a Pt wire counter electrode (both purchased from BASi Ltd.), and either the MEA or MacroAu as working electrode. For consistency, functionalization of the sensors was performed in bulk solutions by pipetting aliquots of solution on top of the electrode surface.

Surface preparation

MEA sensors were initially cleaned by placing them in HNO₃ (10% v/v in H₂O) overnight. Afterward, the sensors were cleaned by CV, running 15 cycles in 0.5 M H₂SO₄ solution between -0.2 and 1.65 V (vs. Ag/AgCl). The CV results in Figure 5.41 give an indication of the cleanliness of the surfaces.

Figure 5.41 Example CVs of electrodes following cleaning in 0.5 M H₂SO₄ solution of A) MEA and B) MacroAu.
**Functionalization**

The clean electrodes were incubated at ambient temperature with 10 µM thiol-functionalized, single-stranded DNA (ssDNA) (Gene Link, Hawthorne, NY), 90 µM mercaptohexanol (MCH) in phosphate buffered saline (PBS) solution overnight. Next, the electrodes were washed with PBS buffer and placed in 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in PBS buffer for measurements. Impedance measurements were performed on a pocket STAT from Ivium Technologies, at a bias potential of 0.23 V, between the frequencies 100 kHz to 0.02 Hz for MacroAu, and 100 kHz to 0.1 Hz for the MEA, with an amplitude of 0.01 V. Data was analyzed with the inbuilt fitting program in the Iviumsoft software (Ivium Technologies, Eindhoven, the Netherlands), fitted using two modified Randels’ equivalent circuits to extract the charge transfer resistance value, $R_{ct}$. The impedance reading at this point gives the “probe” reading.

After collecting a “probe” measurement, the electrode surface was washed, and target DNA of differing concentrations (from 0.1 nM to 1 µM in saline-sodium citrate buffer) was pipetted on to the electrode surface. The electrode and solution were heated in a sealed chamber in a water bath set at 70 °C for 5 min to denature any hybridization or tertiary structure. After that, the electrode was left in the chamber at room temperature for a further 55 min. Subsequently the electrodes were washed and an impedance measurement was taken. Although absolute sensor responses were not identical, the observed trends were reproducible. Therefore, changes in the signal were described as opposed to absolute signal values. Signal change can be normalized against the $R_{ct}$ value for the “probe” surface prior to target incubation ($R_{ct}^0$), giving $\Delta R_{ct}/R_{ct}^0$.

When necessary the electrodes were stored in nuclease free water overnight at 4 °C. To regenerate the surface, and to remove any remaining hybridized target DNA, wash buffer
solution (SSC buffer) was heated to 90 °C then placed on the electrode surface for 6 min in total with five changes in solution. Another measurement was taken at with different probe concentration and compared to the original “probe” signal.

RESULTS

Probe attachment

Attaching ssDNA and MCH on the gold electrode surface via Au-S bond gives rise to an increase in charge transfer resistance for the electrodes, as can be seen in Error! Reference source not found.2. This indicates that a monolayer of ssDNA/MCH was formed on the electrode surface. While absolute probe densities were not measured, the ratio of ssDNA to MCH was assumed to be proportional to the relative concentrations of the incubating solution. The modelling of the impedance results was performed (Error! Reference source not found.2), and although different equivalent circuits were required, both models appear to fit the data well. The MEA sensors showed higher $R_{ct}$ values as well as greater noise in their signals.
Figure 5.42 Impedance results displayed as Nyquist plots for A) and B) the MacroAu electrode, and C) and D) the MEA sensor. Figures A) and C) show impedance data for i) the bare gold electrodes (black circles), and ii) the ssDNA:MCH functionalized surfaces (green circles). Meanwhile, B) and D) show the impedance data after fitting. The two different equivalent circuits used for fitting can be seen in the insets. In these graphs the symbols represent the experimental data while the solid lines indicate the fitted data.

Target incubation

Incubations with target DNA gives rise to decreases in charge transfer resistance, $R_{ct}$, with higher DNA concentrations giving lower $R_{ct}$ values. The sensors appear to saturate at about 500 nM of target DNA. Both sensors show similar behavior, though the low concentrations (<200nM) are not tested on the MEA sensor (Figure 5.44). The developed MacroAu sensor shows sensitivity in the low nanomolar range.
**Figure 5.43** Impedance results displayed as Nyquist plots i) after immobilization of ssDNA:MCH, giving the “probe” surface, ii) after incubation with target concentrations of ii) 200 nM, iii) 500 nM and iv) 1000 nM, for A) the MacroAu electrode, and B) a DSL VZ electrode. The symbols represent the experimental data while the solid lines indicate the fitted data.

**Figure 5.44** Calibration curves corresponding to the changes in charge transfer resistance for A) MacroAu electrode, and B) MEA sensor (data points were averaged from the three working electrodes on a sensor), after incubation with target DNA of different concentrations (0.5 – 1000 nM).

**Non-specific binding**

Control experiments were performed to investigate the role of non-specific binding on the DNA sensor. The “probe” surface was exposed to scramble DNA of a random sequence. A small change in Rct was observed; albeit, a much smaller decrease than that observed for
the same concentration of target DNA. These results suggest the probe, and therefore sensor system, has significant specificity for the target DNA.

**Figure 5.45** A. Impedance results displayed as a Nyquist plot i) the “probe” surface, ii) after incubation with scramble DNA (200 nM), iii) after incubation with target DNA (200 nM), B. a bar chart showing the relative signal changes for target DNA and scramble DNA.

**Regeneration**

Regeneration was performed on sensor surfaces as required. Regeneration shows reasonable reproducibility, with the signal slightly lowering after successive regenerations as shown in Error! Reference source not found.6.

**Figure 5.46** A. Impedance results for i) the initial “probe” surface, and for ii) the regenerated “probe” surface, B. the percentage of original signal remaining following successive regenerations.
DISCUSSION

Electrochemical impedance spectroscopy (EIS) was a noninvasive technique that effectively measures bioaffinity events by quantifying the change in charge transfer resistance at the electrode surface (Dubuisson, Yang, & Loh, 2011). EIS has proven a powerful technique in the rapid and label-free detection of nucleic acid biomarkers with high sensitivity (Kukol et al., 2008). In fact, EIS has proven itself to be an efficient analytical method for many chemical, physical, and biological processes (Sezginturk & Uygun, 2012). Such processes include identification of membranes; antigen-antibody, receptor-ligand, DNA-DNA and RNA-RNA interactions; and catalytic reactions such as enzyme-substrate interactions (Sezginturk & Uygun, 2012). This study reports the fabrication of a new electrochemical sensor that advances the field by incorporating multiple micron-scale electrode arrays of three-dimensional structures built out of low cost plastic substrate.

The design of the MEA reported herein attempts to build on previously developed EIS techniques and innovate in both manufacturing and sensing to reduce the cost of the former while enhancing the capability of the latter. By utilizing inexpensive plastic substrates and leveraging a proprietary high throughput, low cost processing platform exclusively licensed to Digital Sensing Limited (Auckland, New Zealand), the manufacturing costs are reduced to a fraction of that of silicon processing. By using micron-scale, and eventually nanoscale, 3D electrical sensing structures arranged in electrode arrays, the sensing capability was enhance because of the focused resolution of measuring changes in $R_{ct}$ in the microenvironment of one microscopic tip (i.e. detecting the bioevent at the ultramicroelectrode) but goes further to average the detection of these discrete bioevents across thousands of tips, which renders increased confidence in detection. Additionally, the multitude of tips enables enough aggregate current flow to offer favorable signal strength (i.e., better signal to noise ratio) (Morf & de Rooij, 1997). Furthermore, enabling multiple electrodes on the same sensor chip
offers the ability for multiplexing, which by using EIS, unlike many other sensing techniques, enables several different classes of probe-target interaction (e.g., DNA-DNA, antibody-antigen, receptor-ligand, enzyme-substrate, redox species, etc.) to be performed simultaneously on the same MEA chip; therefore, greatly increasing the confidence in the overall diagnosis. However, the process to develop such a platform does not come without its challenges.

One of the major frustrations associated with the materials selected for this MEA was the ability to maintain a durable and consistent gold surface. Unlike with silicon-based sensors, the thin (5 – 25nm) gold deposition atop the PMMA substrate flakes after repeated use. As one of the priorities is to assure easy, low cost fabrication, a polymer substrate was required—both for cost of material and cost of processing. Therefore, extensive examination of gold stability on top of a PMMA substrate is undertaken (Booth et al., 2014). It is determined that there seems to be the normal tradeoff between extensive preparations and therefore cost, and durability. The most durable process was to assure an ultraclean PMMA surface, lay down a thick layer of gold (50nm – 100nm), and heat the gold-coated polymer to insure optimal adherence. Thicker layers of gold help mask defects in the polymer incurred during processing and they also yield better resistance values (e.g., 50nm was better than 5nm). Three ohms (3 Ω) was the set ideal target value (Song et al., 2012). Unfortunately, increased gold thickness increases the cost of materials and time of processing—both increased gold deposition time and increased laser time to scribe the electrodes. Currently, other polymer chemistries are being explored to try and find the optimal combination between gold adhesion, the ability to process for nanoscale patterning, and use under EIS conditions (i.e., buffers, solvents, electricity, thermal fluctuations, etc.). Ideally, the MEA can be subjected to extensive cycling, not merely for repeatability and reusability but for functionalizing a multiplexed array.
Among the many possible advantages of the MEA reported here, the ability to multiplex with different categories of capture agents and targets may prove the most powerful. To our knowledge a low cost platform that enables the label-free detection of proteins, substrates/products, nucleic acids, antibodies, drugs, hormones, microbes, etc. concurrently on the same platform does not exist. If successful, and in combination with upstream sample processing and downstream data analysis, this MEA has the potential to cause a major shift in point of care diagnostic abilities. The vision is to build on the work reported here with PPy deposition, where the capture agent (e.g., antibody, DNA, etc.) is tethered to a conductively-deposited polymer like PPy. MEA chips are “dipped” in homogenous solutions of Probe1-PPy, potential is applied to the desired electrode(s) and consequently functionalizing the specific electrode(s) with Probe1. The MEA chip is then washed, dipped in Probe2-PPy solution, and another electrode or set of electrodes is functionalized with Probe2, and so on. Such a process could be automated and therefore maintain the prospect of also meeting the low cost objective.

Finally, to realize the potential of this MEA at a commercial scale will require significant resources; however, the work reported here in detecting DNA hybridization and electrode-specific PPy deposition demonstrates the potential for multiplexing and warrants a dedicated focus toward this goal. At present there are several projects in the lab of Dr. A.C. Partridge running concurrently to build upon this MEA platform in its ability to detect: 1) pathogens; 2) hormones; 3) enzymes; and 4) epigenetic signatures (e.g. methylation state, miRNA interactions, etc.). Furthermore, work is underway to enhance the ability to regenerate the MEA platform for multiple uses, which may help further reduce its cost. Taken together, the novel electrochemical device described here has the potential to become a low cost diagnostic device for both human and animal healthcare as well as food and agricultural
monitoring. Of particular interest is to use this device for detecting the onset of mid-life decline as well as biomarkers correlating to age-related disease.
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evoke cardiac hypertrophy and heart failure. *Proc Natl Acad Sci U S A*, 103(48), 18255-18260.


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CHAPTER SIX

Conclusion

Nanotechnology has come a long way since Richard Feynman formally introduced the concept in his famous, “There’s Plenty of Room at the Bottom,” lecture in 1959. Today this field is evolving more rapidly than ever and it’s truly at the cusp of redefining the world as we know it. Of the many areas that will be revolutionized, none could be more predisposed than the biological sciences as fundamental life has been utilizing “nanotechnology” for the past few billion years; science and technology are merely beginning to catch up. Consequently, the degree to which we understand and influence basic biological processes that are occurring at the nanoscale is naturally contingent upon our ability to interact, measure, and manipulate at that same scale.

The body of research presented in this dissertation begins with the study of proteins involved in electron transfer from organic carbon to metal oxides, with a specific focus on the final step of electron transfer to the terminal electron acceptor. Dissimilatory metal reduction, i.e., anaerobic respiration that terminates with the reduction of a metal compound outside of the organism, is a little known phenomenon but plays a major role in both the biogeochemical cycling of minerals and the global carbon cycle in anoxic environments. To better understand this mechanism research and experiments were carried out to answer to basic questions: 1) how does the metal-reducing bacteria make contact with the metal oxide? And 2) how are electrons transferred from the bacterium to metal oxides located outside of the cell? A possible answer to the first question was revealed by identifying an outer membrane protein called SO3800 and postulating its function as an autotransporter involved in iron oxide adhesion and in supercomplexation of the iron reductase complex. This finding was enabled through the use of standard
biochemical techniques, computer modelling, genetic manipulation and electrochemical assays and was reported in *Applied Environmental Microbiology* in 2010 (Justin L Burns & DiChristina, 2009). The answer to the second question is much more complex.

The study of proteins involved in electron transfer lends itself to an electrochemical setup. However, to our knowledge, this study reported here is the first to use a microelectrode for the *in gel* detection of redox active proteins. Scanning electrochemical microscopy was used to scan native PAGE gels of outer membrane protein complexes for redox activity with iron, manganese and sulfur species. This technique identified complexes that are able to directly reduce sulfur and iron, which was corroborated by a suite of biochemical assays. Ultimately, this relationship was confirmed by using mass spectrometry to determine the identity of the proteins constituting these complexes, which revealed a set of conserved proteins whose function is known. The protein complexes shown to reduce iron and sulfur were made up of proteins that are known to be involved in the electron transport chain and others involved in sulfur reduction, biosynthesis, and assimilation. From the protein identities and the electrochemical and biochemical data, a mechanism for iron oxide and manganese oxide reduction was proposed, which suggests that the production and reduction of organosulfur species (e.g., mercaptocarboxylic acids) is used to mediate the final step of electron transfer to these metal oxides. This proposal has been strengthened by a number of subsequent studies (Fennessey et al., 2010; Taillefert et al., 2007).

Shifting focus to a more medically relevant topic, the second major focus of this study was to better understand the molecular mechanisms associated with aging and age-related disease, with particular effort spent searching for epigenetic biomarkers indicative of mid-life decline. Understanding the molecular mechanisms that effect aging is paramount to assembling a diagnostic strategy; while focusing specifically on the
epigenetic mechanisms contributing to aging presents the possibility, with accurate and timely diagnosis, for the contributing insult(s) to be reversed. Noncoding RNAs are well suited for use as biomarkers as the identification of nucleic acids is a well-known craft. A review of known non-coding RNAs associated with aging and age-related disease was reported in *Biochemica Biophysica Acta* in 2009 (Bates et al., 2009).

Studying the Ames dwarf mouse as a model for long life, i.e., reduced aging and age-related disease, by comparing its microRNA profiles to its age- and gender-matched siblings enabled the identification of key microRNAs believed to be associated with long life. Among the identified microRNAs, mmu-miR-27a and mmu-miR-96 were identified as candidate biomarkers. On a sensing platform, the decline of miR-27a expression could suggest systemic pressures heading toward accelerated aging and increased prevalence of age-related disease; whereas the increase of miR-96 could suggest a declining ability to deal with stress and toxins. The identification of these and other microRNA biomarkers potentially associated with aging was initially achieved using microRNA microarrays. After candidates were selected, qRT-PCR was required to quantitatively confirm what was qualitatively suggested by the microarray. Once this was confirmed, proteomic analysis was performed to narrow down the targets of the individual microRNAs as suggested by bioinformatics and *in silico* modeling. Target protein expressions suggesting inverse correlation with microRNA expression was then validated via Western blot. Following this validation, *in vitro* functional assays were performed to confirm the predicted inverse relationship between microRNA expression and putative target protein expression. Lastly, *in situ* hybridization was used to confirm correlations *in situ*. This procedure and the results were reported in *Aging Cell* in 2009 (Bates et al., 2010).

The frustrations associated with the extensive molecular and biochemical experimentation required to identify and validate a biomarker served as motivation to help
design and fabricate the microelectrode array reported in Chapter 5, which has the potential to simultaneously detect and quantify microRNA biomarkers and target protein levels in the same sample. A traditional microarray is insufficient as a single source diagnostic when it comes to biomarkers like microRNA because of the redundancies built into genetic regulation, which doesn’t necessarily correlate directly to a specific phenotype. In other words, multiplexing is critical when using epigenetic biomarkers because resultant phenotype is not necessarily a binary response; it is more like a dimmer switch (Bates et al., 2009). As such multiple data points (biomarkers) should be used; preferably a mixture of nucleic acids and target proteins.

Current methodologies for determining the state of epigenetic status are time consuming and expensive. In order to make epigenetic sensing available as a routine diagnostic there is a need to develop inexpensive and rapid diagnostic tools. In association with Digital Sensing Ltd (DSL) a plastic-based sensing methodology was developed that allows low levels of target analytes to be measured by exploiting physical properties at the micron and eventually nanoscale. The last major aim of this research was to develop the DSL-based electrochemical sensors, and push the boundaries of cost and sensitivity. Toward this end, a three-dimensional microelectrode array was designed, fabricated and tested with promising results that merit continued development in the hopes of commercializing a low cost, high-sensitivity platform able to rapidly detect and differentiate epigenetic signatures, particularly related to aging.
REFERENCES


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  - Nature of contribution by PhD candidate: cell growth, protein harvesting, protein separation, protein detection, mass spec, modelling
  - Extent of contribution by PhD candidate (%): 50%

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<td>co-PI</td>
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<td>Dr. Andrew Neal</td>
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Outer Membrane-Associated Serine Protease Involved in Adhesion of Shewanella oneidensis to Fe(III) Oxides

Author: Justin L. Burns, Brian R. Ginn, David J. Bates, Steven N. Dublin, Jeannette V. Taylor, Robert P. Apkarian, Samuel Amaro-Garcia, Andrew L. Neal, and Thomas J. D'Christian

Publication: Environmental Science & Technology
Publisher: American Chemical Society
Date: Jan 1, 2010

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MicroRNA regulation in Ames dwarf mouse liver may contribute to delayed aging. Chapter 4 discovery of targets and background on use of marine microRNAs 27a, and 124 as targets for electrode probe.

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<td>Dr. A. Barrie</td>
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<td>Dr. H. Sarneki</td>
<td>transferion of miRNA for functional assay in BSL facility</td>
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Nature of contribution by PhD candidate: Design of electrochemical sensor, experimental planning, execution, oversight and writing

Extent of contribution by PhD candidate (%): 60%

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<td>Dr. Karthik Kanapjan</td>
<td>Sensor fabrication, mathematical modeling, flow modeling, channel design, functionalization, electrochemistry</td>
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<td>Dr. Marshika Booth</td>
<td>Sensor fab, functionalization, electrochemistry, stellar experimentation and write up</td>
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<td>Shweta Pandya</td>
<td>Sensor fab, experimentation, exceptional lab management</td>
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