



# From Neural Stem Cells to Children: Secreted Phosphoprotein 1 in Lead Neurotoxicity

## Citation

Wagner, Peter John. 2015. From Neural Stem Cells to Children: Secreted Phosphoprotein 1 in Lead Neurotoxicity. Doctoral dissertation, Harvard University, Graduate School of Arts & Sciences.

## Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:17464950>

## Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA>

## Share Your Story

The Harvard community has made this article openly available.  
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

# **From Neural Stem Cells to Children: Secreted Phosphoprotein 1 in Lead Neurotoxicity**

A dissertation presented

by

**Peter John Wagner**

to

**The Committee on Higher Degrees in Biological Sciences in Public Health**

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

**Biological Sciences in Public Health**

Harvard University

Cambridge, Massachusetts

May 2015

© 2015 Peter Wagner  
All rights reserved.

**From Neural Stem Cells to Children: Secreted Phosphoprotein 1 in Lead Neurotoxicity****Abstract**

Lead (Pb) exposure in the earliest stages of neurodevelopment leads to lasting deficits in cognitive function and behavior. Neural stem cells (NSCs) are multipotent stem cells and the first cells of the central nervous system, but little is known of their molecular response to Pb exposure. We exposed human NSCs to 1 $\mu$ M Pb for 24 hours and performed RNA sequencing. 16 of 19 differentially expressed transcripts are upregulated, and of these 10 are known targets of NRF2, the master transcriptional regulator of the cellular antioxidant response. One of the top Pb-induced genes, Secreted Phosphoprotein 1 (*SPP1*), was not a previously known NRF2 target. We show *SPP1* is induced following activation of NRF2 by other known activators and by knockdown of its negative regulator KEAP1. In addition, the induction of *SPP1* by Pb was attenuated after siRNA-mediated knockdown of NRF2. We identified a putative Antioxidant Response Element in the *SPP1* promoter and confirmed its function by Chromatin immunoprecipitation (ChIP) of NRF2. To further investigate the role of *SPP1* in neurodevelopment, we examined *SPP1* single nucleotide polymorphisms (SNPs) in a longitudinal birth cohort of infants profiled at 24 months for the mental and psychomotor development indices (MDI and PDI, respectively) of the Bayley Scales of Infant Development. Sixteen single nucleotide polymorphisms (SNPs) in and near *SPP1* were tested for main effect and effect modification. For main effect on MDI, eight SNPs are associated at  $p < 0.05$ , but none pass multiple testing correction. In PDI analyses, SNP rs4693923 shows statistically significant effect modification on the PDI response to second trimester Pb exposure. A-allele carriers of this SNP show a positive correlation between Pb exposure and PDI, which is not seen among GG homozygotes. Together our studies show *SPP1* is a novel target of NRF2 and suggest a critical role for *SPP1* in modulating prenatal Pb neurotoxicity.

# Table of Contents

List of Figures .....	v
List of Tables .....	v
Acknowledgements .....	vi
CHAPTER 1: Introduction.....	1
1.1. Lead (Pb) is Ubiquitous in the Environment .....	2
1.2. Pb is an Environmental Toxicant .....	2
1.3. Prenatal Pb Exposure Leads to Lasting Neurological Deficits .....	4
1.4. Neural Stem Cells (NSCs) May Play a Role in the Prenatal Critical Exposure Window.....	5
1.5. Pb Induces Cellular Oxidative Stress .....	6
1.6. NRF2 Activation Helps the Cell Recover from Oxidative Stress.....	7
1.7. Pb is a Non-Competitive Antagonist of the NMDA Receptor.....	9
1.8. Transcriptome Profiling Allows for Discovery of New Biology in Pb toxicity .....	10
1.9. Genetic Epidemiology Allows for Discovery of Mediators of Pb Neurotoxicity .....	12
1.10. References.....	14
CHAPTER 2: Secreted Phosphoprotein 1 (SPP1) is a Novel NRF2 Target Upregulated by Pb in Human Neural Stem Cells.....	20
2.1. Abstract.....	21
2.2. Introduction.....	22
2.3. Materials and Methods.....	23
2.4. Results .....	28
2.5. Discussion.....	36
2.6. Acknowledgements.....	38
2.7. References.....	39
CHAPTER 3: Association of Secreted Phosphoprotein 1 ( <i>SPP1</i> ) SNPs with Mental and Psychomotor Development in a Lead-Exposed Population .....	44
3.1. Abstract.....	45
3.2. Introduction.....	46
3.3. Materials and Methods.....	47
3.4. Results .....	50
3.5. Discussion.....	55
3.6. Acknowledgements.....	57
3.7. References.....	57
CHAPTER 4: Conclusions and Perspectives.....	60
<i>SPP1</i> is a Mediator of Lead (Pb) Neurotoxicity in Early Neurodevelopment.....	61
<i>SPP1</i> is a Novel Target of NRF2.....	63
More Opportunities in the Transcriptomic Profile of NSCs .....	65
An Integrative Approach to Study Environmental Exposures .....	66
References .....	67

## List of Figures

2.1 Differential expression in human NSCs exposed to 1 $\mu$ M Pb and vehicle by RNA sequencing .....	29
2.2 All SPP1 splice variants are upregulated following Pb exposure and upregulation is confirmed at the protein level .....	32
2.3 SPP1 is upregulated in an NRF2-dependent mechanism .....	33
2.4 NRF2 binds directly to an ARE in the SPP1 promoter .....	35
3.1 LD patterns around SPP1 in the study population and analysis of the rs46923* $\log_e$ Pb interaction .....	53

## List of Tables

2.1 Summary of primer sequences used in this study.....	26
2.2 Differential expression of human NSCs exposed to 1 vs 0 $\mu$ M Pb by RNA sequencing.....	30
3.1 Summary of SPP1 SNP association effect sizes and p-values in determining MDI and PDI .....	51
3.2 Summary of variables in the ELEMENT birth cohort.....	54

# Acknowledgements

Since I interviewed Jane Goodall for my middle-school newspaper in 2001, environmental protection has been a core value of mine. In college I dedicated myself to the environment academically by majoring in environmental biology major. In the course of my studies, I started to feel that the greatest reason for preserving the environment was for the good of human health. After college I built a strong foundation in laboratory and population biology at the Institute for Molecular Medicine Finland. Quan Lu and David Christiani then offered me a truly unique opportunity to study the effects of environmental metal exposures by integrating cell and molecular biology with human population data at Harvard. I could not have asked for a PhD project better suited to my interests.

I would like to thank Quan Lu for having me in his lab and advising me through the PhD. My favorite thing about Quan is his excitement and willingness to try novel and emerging cell-based screening technologies, and as a result our lab has been at the cusp of the innovation. For instance, at Quan's suggestion I became one of the first persons at the School of Public Health to perform RNA sequencing. Quan taught me not to shy away from endeavors where the learning curve is steep. In those challenges, Quan was always accessible and took a great deal of time to learn, strategize, and troubleshoot with me. I also thank Quan for a countless number of meals and celebrations he hosted over the years. Szechuan cuisine has become a new favorite of mine and is now as synonymous to me with special occasions as sparkling wine.

I also thank David Christiani, my secondary advisor. David's work originally piqued my interest in coming to Harvard, and he was a terrific sounding board for my thinking already when I was choosing among graduate programs. David gave me the opportunity to steer my project exactly as I have wanted to while providing intellectual help, logistical support, and access to some of the most incredible gene-environmental interaction datasets on the planet. David never left unread any draft I sent him, and I recently joked to a friend that he is the kind of person who can edit an entire thesis on his iphone while taking an overnight flight from Bangladesh.

My parents, Hanna and David, have never compromised on education and have made sure that I have had every opportunity to achieve my full potential. It goes without saying that I would not have made it this far without them. My parents have also made life outside school these last few years a pleasure. They helped me buy my first home and took me on many memorable trips. Together we have been to West Africa and South America in addition to several trips home to Finland. My parents have also been very supportive for me throughout my PhD. Research is an emotional process fraught with seemingly more failure than success, and my parents have always been there to let me talk through my frustrations. My brother Karl has always been so proud and supportive of my academic achievements, and I am so happy he came all the way from Togo to see my thesis defense. It would not have been the same without him. Thanks to my grandmother, Lena Aarnio, and her late sister, Airi Salmela, for all their love and support, especially while I was living in Finland. Thanks also to my American grandmother, Ethel Wagner, for being a consistent source of encouragement to pursue a career in science.

A lab is social environment where one learns a great deal from fellow lab members. Ruoxi Hu helped me in innumerable ways through my PhD. Outside my advisors, she did the most to shape my PhD project and helped me troubleshoot my way through many experimental roadblocks. In addition, she was a great inspiration for me in making career choices and understanding opportunities after the PhD. I am thrilled to be working with her next year, and look forward to tackling many new challenges with her in the future. Joe Nabhan was my first mentor in the Lu Lab and was an active mentor of mine even after his departure from the Lab. Xiaofeng Jiang is the only member of the lab to have been in the lab for the entire time I was a member, and he was an incredible resource for planning a wide variety of experiments. Xiaofeng is one of the most careful and experienced bench scientists I know, and I learned so much from him. Ray Oh, another early mentor of mine in the Lu Lab, helped shape much of my thinking on the molecular biology of metals. Qiyu Wang was a more recent addition to the lab, but his incredible work ethic has been motivating, and I have really enjoyed getting to know him. I owe thanks to my summer intern, Lucy Suarez, whose success and friendship since her internship made my time mentoring her so rewarding. I would also like to thank other members of the lab for their own



contributions to my time in the Lu Lab: Wen Chi Pan, James Lao, Shiwen Huang, Ron Panganiban, and Maoyun Sun.

I would like to thank Les Kobzik for being the chair of my preliminary qualifying exam (PQE) committee, degree advisory committee (DAC) and thesis defense committee. His ability quickly to synthesize various experiments and theories into an actionable plan is incredible, and it is a quality I strive to emulate. Bob Wright deserves thanks for starting the Harvard Superfund Program, within which I did all my graduate work, and for serving on my PQE committee. I would also like to thank Tom Guilarte, our collaborator at Columbia University and one of my DAC members, who always generously provided tissue samples and animal data to inform my work. He is a leader in lead neurotoxicity, and his vetting of my work was incredibly enriching. I have a great deal of gratitude for all the time taken by Bernardo Lemos on my PQE and defense committees; Takao Hensch and Mark Weisskopf on my DAC; and Maitreyi Mazumdar and Vishal Vaidya on my defense committee.

In the Environmental Health department, I would like to thank Sally Bedugnis for all her help with various lab issues and for being a good friend. Patrice Ayers was always a breath of fresh air on the third floor, and I am going to miss her positive energy. Juliana Rosario, Alissa Wilcox, and Marshall Katler were enormously helpful administrators in the department and were often there for me when I have needed help. Fellow graduate students Rose Filoramo, Christine Ordija, and Helen Cho have been wonderful classmates in the department. I would also like to thank the late Jere Mead, his widow Dorothea Mead, and Joe Brain whose funds supported me during my time as a graduate student.

Some of my best friends in Boston have been from my BPH class: Alex Sakatos, Allison Demas, Caeul Lim, Eylul Harputlugil, Jordan Becker, Lear Brace, Michelle Rooks, and Sri Kalyanaraman. In addition, significant others, Burak Tekin, Noah Becker, Brian Hopkins, and Peter Watson, have been great extensions to the friend group. I would especially like to thank Michelle Rooks, who was my writing buddy while preparing our PQE and DAC proposals. She gave me thorough edits and substantial emotional support through those hard times. I would like to thank Allison and Caeul for taking unforgettable trips with me to DC and Finland. BPH assistant directors Holly Southern and Deirdre Duckett were enormously helpful in navigating both the School of Public Health and the Graduate School

of Arts and Sciences. I would like to thank Marianne Wessling-Resnick and Brendan Manning for putting so much time into the BPH program. BPH is very generous to its students, and I had the pleasure of organizing and attending many events that have made this program truly special.

I would like to thank Iny Jhun, Amy Labar, Kelsey Gleason, and Jami King, with whom I coordinated the Roxbury Prep Tutoring Program. The tutoring program sends volunteer tutors to the Roxbury Prep charter school in Mission Hill. Tutoring through the program was one of the most refreshing and rewarding escapes from the lab. I would like to thank the school and the teachers who volunteer their time to host tutoring. I would also like to thank Venus Israni and Dale Trevino in the Office of Diversity and Inclusion, where the coordination of the tutoring program is housed. Dale and Venus are inspirational leaders for the causes of diversity and inclusion within the school, and it was a pleasure to work with them in pursuit of those ideals.

I owe my previous advisors, Aarno Palotie, Leena Palotie, Kaisa Silander, and Brian Mailloux, a great deal of gratitude for all their help throughout the years. Aarno was the Principal Investigator in my first research group. He has continued to be an informal mentor of mine during both my two years in the lab of his late wife, Leena, and my years in graduate school. I really value all his honesty, insight, and support. Working in Leena's lab was one of the best experiences I have had, and I honor her for building a world-class human genetics research unit in Finland. I learned so much in those two years from working with huge data sets, new technologies, and the world's leading geneticists. I owe a great deal of what I learned to my advisor in the lab, Kaisa Silander. Kaisa put a lot of faith in me and gave me all the tools I needed rapidly to become familiar with a number of complex questions, methods, and theories in the field. The people who worked there made the work environment in Finland very special, and I would especially like to thank Anne Vikman, Annu Näkki, Ansku Sulonen, Karola Rehnström, Olli Pietiläinen, Markus Perola, Samuli Ripatti, Emmi Tikkanen, Ida Surakka, Antti Sarin-Pekka, Marine Largeau, Mikko Muona, and Virpi Leppä.

While writing our theses, Chris Aakre and I explored a number of Harvard and MIT libraries. Thesis writing with Chris made an otherwise lonely and drawn out process a very pleasant one. It has been a great pleasure getting to know Chris, and his fiancé Jonathan, through our job search and final

year in graduate school. Of my Boston friends, I would especially like to thank Ben Weinryb-Grohsgal, who was my roommate in junior year of college and then one of my closest friends in Boston. Ben always has the best ideas of what to do, and I have immensely enjoyed trekking around the city with him. I would also like to thank many other friends in Boston including Tom McHale, Oliver Sellars-Garcia, Jyri Eskola, Taru Tukiainen, John D'Amico, Harry Reyes, and Tze Tan. You have all made Boston a great place to live!

Finally, I would like to thank some of my best friends. Thank you to Jennifer Wilson, whose example I followed when coming to graduate school. David Coates has been one of my closest friends, and he is becoming a great travel partner even though he lives so far away in Hong Kong. David Spector, with whom, along with Danny Grossman and Danny Rubens, I have had so many adventures, deserves special thanks. I would like to thank Atossa Abrahamian for being a great friend and to her family for hosting me in Brooklyn my last summer in college while I was working on my senior thesis. I would also like to thank Anna Law and look forward to our travels together. Thanks also to: Amrita Casie-Chetty, who is my closest friend from high school and has graciously hosted me several times in New York; Simon Lax, with whom I have had some of the most relaxing weekends in Maine; Kate Ruskin, who has taken me through salt marshes in Maine in boots and up mountains in New Hampshire in snowshoes; and Laura Palotie, who straddles the US and Finland like me, and has become a great friend.

While I put so much work into this thesis, it did not happen without the help, support and friendship of so many individuals. To those I forgot to thank, I sincerely apologize. So many people have made my time at Harvard and in Boston so terrific. I hope you all enjoy this thesis, and that the result is worthy of all the help and support you all have given me.

# **CHAPTER 1: Introduction**

### **1.1. Lead (Pb) is Ubiquitous in the Environment**

Pb is easily extracted from ore and has been a part of human civilization since the earliest days of metallurgy. The earliest record of Pb use is in the form of metallic beads, tubes, rings and tools from 6400 BCE found in excavations of the ancient city of Çatal Höyük in modern day Turkey (Heskel, 1983). Pb, as a byproduct of silver smelting, began to be used widely in the Roman Empire where yearly production reached eighty thousand tons according to data derived from Greenland ice cores (Hong et al., 1994). Materially Pb was used in various applications in construction, mainly in piping and cooking utensils. As a result of rampant exposure in the Roman Empire, Pb is suggested to underlie the known mental illness and medical problems of many Roman emperors (Nriagu, 1983).

It was not until the use of tetraethyl Pb in gasoline beginning in the 1920's, however, that the level of Pb in the environment started becoming truly ubiquitous. While a certain degree was already known of Pb's toxicity, the benefits provided by tetraethyl Pb in gasoline were thought to outweigh the risks. As an extremely effective antiknock agent, tetraethyl Pb ushered in a new generation of combustion engines. In addition to gasoline, Pb has been added to paint for centuries. The "white lead" compound ( $2\text{PbCO}_3 \cdot \text{Pb}(\text{OH})_2$ ) is naturally occurring and makes a good pigment due to its opacity and texture. Flaking and peeling paint is a common component of household dust, which can be breathed in, and is often ingested by infants who spend more time on the floor and practice hand-in-mouth behavior. Pb leaching from piping solder, canning and pottery glaze into water and food comprises another important source of Pb exposure.

### **1.2. Pb is an Environmental Toxicant**

It was not until the 1970's that epidemiological understanding of Pb's affects, particularly in children, was able to force legislative change. The Clean Air Act, Safe Drinking Water Act and the Toxic Substances Control Act have banned or limited Pb additives in gasoline, piping solder and paint, respectively, and have been enormously successful in abating Pb exposure. According to National Health and Nutrition Examination Survey (NHANES) data, in the 15-year period from 1976 and 1991 mean blood

Pb levels declined 77% (13.7 to 3.2 micrograms/dL) and 72% (20.2 to 5.6 micrograms/dL) for non-Hispanic white and non-Hispanic black children aged 1 to 5, respectively (Pirkle et al., 1994).

However, Pb is persistent in the environment and remains an important public health concern in the United States (US) and elsewhere. Modern-day sources of Pb, especially in the US, are predominantly a legacy of the metal's historic uses. Urban areas have higher Pb levels resulting from older housing and infrastructure, in which it is more common to have leaded paint, Pb piping and more tetraethyl Pb deposition due to a historically high density of car traffic (Datko-Williams et al., 2014). Currently Pb is second of 275 compounds on the Substance Priority List of the Agency for Toxic Substances and Disease Registry (ATSDR). The substance priority list ranking is based on frequency, toxicity and potential for human exposure at highly contaminated sites listed on the National Priorities List of the US Environmental Protection Agency.

The phase-out of Pb from gasoline has been slower in some parts of the world and as of 2013 leaded gasoline was still available in six countries. The United Nations is assisting countries in their phase-outs, and this decade is expected to see the last sales of leaded gasoline worldwide. The slower transition to unleaded gasoline and products has led to enduring elevations in blood Pb in many countries. For example, mean blood Pb levels have been recently reported to be 13 µg/dL in China (Ye et al., 2007), 15 µg/dL in Bangladeshi urban populations (Kaiser et al., 2001, Wasserman et al., 2007) and 11 µg/dL in urban Indian children (Roy et al., 2009). However, as economically lesser-developed countries tackle Pb phase-outs from gasoline and products like paint, new challenges are arising. Perhaps most notably, Pb is common in electronic waste, which is a rapidly growing disposal challenge worldwide. For instance in Guiyu, a poorly regulated electronic waste destination in China, 70.8% of children have blood Pb levels above the 10µg/dL (Zheng et al., 2008). 10µg/dL was the threshold recommended by the Centers for Disease Control and Prevention (CDC) up to the year 2012 when the reference level was halved to 5 µg/dL.

### **1.3. Prenatal Pb Exposure Leads to Lasting Neurological Deficits**

Appreciation for Pb's more pronounced acute toxicity in children began in the late 19<sup>th</sup> century with physician reports and gained a great deal of traction through the 20<sup>th</sup> century. As the acute toxicity of Pb became widely established, however, the significance of chronic, low-dose Pb exposure remained a matter of debate. A seminal paper in 1979 by Herbert Needleman in the *New England Journal of Medicine* showed that children's Pb levels in recently shed teeth were significantly negatively associated with measures of attention, verbal and auditory processing, and classroom behavior (Needleman et al., 1979). Needleman's findings have been substantially reinforced by several follow-up studies (Wasserman et al., 1997, Needleman et al., 1996, Fulton et al., 1987). Worryingly, as longitudinal studies began to address these questions it was found that the changes in behavior and cognitive functioning brought about by Pb exposure were persistent into later life with a critical window of toxicity seen prenatally and within the first few years of life. Two biologic factors that make prenatal Pb exposure particularly important are that Pb is freely able to cross the placenta (Goyer, 1996) and that release of Pb in pelvic bone remodeling during pregnancy leads to a spike in blood Pb that can affect the fetus (Gulson et al., 2003). A longitudinal study showed prenatal Pb exposure was associated with lower Mental Development Index (MDI) scores of the Bayley Scales of Infant Development through two years of age (Bellinger et al., 1987). In another study, Pb exposure in children under 7 years old was associated with lower IQ scores at 11-13 years of age (Tong et al., 1996). Dramatically, a recent study also revealed that adults in Boston aged 28-30 had IQ's statistically significantly inversely associated with blood Pb at 6 months, 4 year and 10 years of age; and at each time point blood Pb averaged below 10 µg/dL (Mazumdar et al., 2011).

Prenatal Pb exposure also increases risk of schizophrenia, a disease with onset in late adolescence and early adulthood (Opler et al., 2004, Opler et al., 2008). Arguably consistent with these findings of cognitive and behavioral difficulties, a causative relationship in the US between the peak in environmental Pb levels in the early 1970's and the peak in violence crime twenty years later in the early 1990's has been theorized (Nevin, 2000, Mielke and Zahran, 2012, Nevin, 2007). A longitudinal analysis of a birth cohort in Cleveland has shown that prenatal Pb exposure assessed in maternal blood during

pregnancy was better correlated with the rate of arrest by 19 to 24 years of age than 6-year old or average childhood blood Pb (Wright et al., 2008). Currently, the only treatment for Pb exposure is chelation therapy, which reduces blood Pb levels but does not prevent the progressive cognitive or behavioral deficits of Pb exposure at a young age (Rogan et al., 2001, Dietrich et al., 2004).

Pb does not have a safe threshold, and in fact the incremental effect sizes of the exposure may be higher at lower doses than higher doses (Canfield et al., 2003, Gilbert and Weiss, 2006, Lanphear et al., 2005). According to a 2003 New England Journal of Medicine study, the overall effect of every 1 µg/dL increase in blood Pb results in a decrease of 0.87 IQ points, but below 10 µg/dL blood Pb every 1 µg/dL increase results in a 1.37 IQ decrease (Canfield et al., 2003). While these statistics may not pose a great deal of significance clinically, the economic loss to society is considerable. Due to the gains in IQ's alone from environmental Pb abatement in the US, it was estimated that the economic benefit for each year's cohort of 3.8 million US 2-year olds is \$110-\$319 billion in year 2000 dollars (Grosse et al., 2002). Economic gains resulting from reduced anti-social and delinquent behaviors and other Pb-related medical complications would drive this economic benefit estimate even higher.

#### **1.4. Neural Stem Cells (NSCs) May Play a Role in the Prenatal Critical Exposure Window**

In early embryogenesis the ectoderm thickens and folds to create the neural tube, which is the precursor of the central nervous system. The cells of the neural tube are multipotent stem cells that can differentiate into any of the cell types of the central nervous system, and are referred to in this thesis as NSCs. NSCs begin to proliferate rapidly in weeks 5-6 of gestation and continue to do so until the end of the first trimester. In the second trimester, these cells start to migrate and differentiate primarily into neurons, astrocytes, and oligodendrocytes. The early stages of development, especially in the nervous system, are believed to be particularly vulnerable to toxicant insult (Mendola et al., 2002).

In predictive models of 24-month MDI outcomes, first trimester Pb exposure assessed in maternal blood was the most statistically significant predictor when compared to maternal blood from the second and third trimesters, cord blood and child's blood at 12 and 24 month's of age (Hu et al., 2006). The timeline presented by Hu et al. (2006) suggests a potential role for NSCs in the etiology of Pb-related



cognitive deficits in early life. Laboratory experiments show Pb slows proliferation of NSCs *in vivo* (Gilbert et al., 2005, Schneider et al., 2005, Verina et al., 2007, Breier et al., 2008) and *in vitro* (Huang and Schneider, 2004, Breier et al., 2008). In rat ventral mesencephalon-derived NSCs, proliferation decreased by almost 50% after six days of exposure to 1  $\mu$ M Pb (Huang and Schneider, 2004). In addition, Pb exposure in rats altered dendrite morphology in NSCs differentiating into neurons, but did not influence their cell-type fate (Verina et al., 2007). While these studies reveal dramatic effects on NSCs under Pb exposure, the mechanisms underlying the effects remain unknown.

While the existence of an *in utero* critical time point is supported by many studies cited here, it is worth noting that in a cohort of Yugoslavian children, blood Pb at 4, 5 and 7 years of age is more predictive of IQ than *in utero* exposure assessed in maternal blood (Wasserman et al., 2000). Differences in assessing the Pb exposure, population-specific confounders and differences in outcome measurements may describe the discrepancy. The toxicokinetics of the passage of Pb from mother to fetus are not well described, and the exact timing of fetal exposure and assessment may complicate association studies at this exposure time point.

### **1.5. Pb Induces Cellular Oxidative Stress**

Oxidative stress occurs in cells experiencing elevated levels of reactive oxygen species (ROS). ROS are reactive oxygenated compounds that damage cells by reacting with proteins, lipids and DNA. Common ROS include superoxide anions ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical (HO). HO are by far the most reactive of these, directly causing lipid peroxidation, DNA oxidation and amino acid oxidation. The toxicity of  $O_2^-$  and  $H_2O_2$  largely results from their capacity to become HO. The main source of  $O_2^-$  is oxidative phosphorylation in mitochondria. In oxidative phosphorylation, oxygen acts as the terminal electron receptor and generally forms water molecules by binding protons simultaneously. Under physiologic conditions, however, 1-3% of the oxygen molecules in the mitochondria interact with electrons that escape the electron transport chain resulting in  $O_2^-$ . Alone  $O_2^-$  are not particularly reactive with cellular components. However, superoxide dismutase converts  $O_2^-$  to  $H_2O_2$  in a reaction that can also be spontaneous or catalyzed by flavins and quinones.  $H_2O_2$  is capable of oxidizing Cys and Met residues

in its own chemical form, but is otherwise not especially destructive to the cell. The HO generated from H<sub>2</sub>O<sub>2</sub> via the Fenton reaction is responsible for H<sub>2</sub>O<sub>2</sub> toxicity. The Fenton reaction involves the oxidation of unbound divalent iron and the reduction of H<sub>2</sub>O<sub>2</sub> into a HO and a hydroxide molecule (HO<sup>-</sup>).

Redox-cycling metals, such as iron, copper, chromium and cobalt, undergo reduction-oxidation reactions with cellular components that result in O<sub>2</sub><sup>-</sup> and nitric oxide (Jomova and Valko, 2011). Pb, however, like cadmium and arsenic, are redox-inactive metals and do not directly generate ROS. Instead redox-inactive metals tend to deplete cellular glutathione through a variety of mechanisms. Glutathione is a key component of the cell's ROS detoxification system. As a result, endogenous ROS accumulate leading to oxidative stress. Pb-associated oxidative stress is seen in the form of elevated malondialdehyde (a oxidative stress biomarker) levels in brains of rats exposed pre- and perinatally to Pb acetate in drinking water (Baranowska-Bosiacka et al., 2012) and in the blood of people with occupational Pb exposure (Gurer-Orhan et al., 2004).

#### **1.6. NRF2 Activation Helps the Cell Recover from Oxidative Stress**

Basal levels of ROS are a natural consequence of oxidative phosphorylation, and cells have inducible defense mechanisms to deal with elevated cellular ROS. The primary cellular response to an increase in ROS is activation of the cap 'n' collar-bZIP transcription factor NRF2 (Nuclear factor (erythroid-derived 2)-like 2, aka. NFE2L2). Under normal circumstances NRF2 is held in the cytoplasm and ubiquitinated through its redox-sensitive interaction with KEAP1 (Kelch-like ECH-associated protein 1.) KEAP1 serves as an adaptor for a cullin-3/ring-box-1/E3 ubiquitin ligase complex that ubiquitinates NRF2, thereby targeting it for proteasome degradation. Modification of KEAP1 Cys151 by ROS leads to conformational changes in the KEAP1 protein causing the dissociation of NRF2 from KEAP1. As a result, NRF2 stabilizes and translocates to the nucleus where it acts as a transcription factor. NRF2 binds to Antioxidant Response Elements (AREs) in the promoter regions of target genes. The core ARE has a consensus sequence of 5'-RTKAYnnnGCR-3' (Erickson et al., 2002). A more comprehensive probability weight matrix describing a 21-basepair long ARE has been calculated using experimentally validated AREs

(Wang et al., 2007). A chromatin immunoprecipitation sequencing study has functionally identified 242 AREs in the human genome (Chorley et al., 2012).

NRF2 target genes generally fall into 5 categories: glutathione-mediated detoxification, thioredoxin-mediated detoxification, NADPH production, iron sequestration and quinone detoxification. Glutathione (GSH) is the main ROS detoxifying agent, and NRF2 targets genes encoding proteins involved in glutathione production, use and regeneration. GSH is used by several glutathione peroxidases and glutathione-S-transferases in the reduction and detoxification of ROS. Oxidized GSH dimerizes to form Glutathione disulfide (GSSG), which is regenerated back to GSH by Glutathione reductase (*GSR*, a NRF2 target) and NADPH. Thioredoxin 1 (*TXN1*, a NRF2 target) reduces disulfide linkages created by ROS to restore cysteine residues. *TXN1* is oxidized in the process and restored by thioredoxin reductase 1 (*TXNRD1*, a NRF2 target). *TXNRD1* also catalyzes the reduction of cystine to cysteine, which is used for GSH synthesis. Regeneration of GSSG to GSH and of oxidized *TXNRD1* to active *TXNRD1* is achieved via NADPH oxidation. NRF2 increases NADPH production by targeting all enzymes involved in NADPH generation (Mitsuishi et al., 2012). Conversely, NAD(P)H dehydrogenase (quinone) 1 (*NQO1*, a NRF2 target gene) reduces NADPH in the detoxification of quinones, which catalyze reactions creating  $O_2^-$  and  $H_2O_2$ , to less reactive hydroquinones. NRF2 encourages free iron sequestration to reduce HO generation from  $H_2O_2$  via the Fenton reaction. Iron is sequestered in the ferritin complex comprising the ferritin light chain (FTL) and ferritin heavy chain (FHL) proteins, and heme-stored iron is released for sequestration in the ferritin complex by heme oxygenase 1 (HMOX1); *FTL*, *FHL* and *HMOX1* are all NRF2 target genes.

Several antioxidants are NRF2 inducers, and therefore are protective by enhancing the cells' own defenses against oxidative stress. These include DL-Sulforaphane found in cruciferous vegetables and curcumin, which is found in the South Asian spice turmeric. DL-Sulforaphane is shown to alleviate toxic affects of metals in several in vitro and in vivo settings. For instance, in primary mouse hepatocytes treatment with DL-Sulforaphane prior to mono-methyl mercury exposure suppressed mercury accumulation in the cell and decreased cytotoxicity in wildtype but not *NRF2* knockout cells (Toyama et al., 2011). Similarly, *in vivo* injection of DL-Sulforaphane before mono-methyl mercury exposure resulted

in a decrease in brain and liver mercury levels in wildtype but not *NRF2* knockout mice (Toyama et al., 2011).

Some antioxidants work independently of the NRF2 pathway. For instance, antioxidants can work directly on ROS like Vitamin E as a  $O_2^-$  scavenger. The drug N-Acetylcysteine is a precursor to GSH and ameliorates oxidative stress conditions by replenishing cellular glutathione levels. Pretreatment of the SH-SY5Y neuroblastoma cell line with N-Acetylcysteine attenuates Cadmium-induced apoptosis (Kim et al., 2013) and Manganese induced DNA lesions (Stephenson et al., 2013). Clinically N-Acetylcysteine is used regularly to treat acetaminophen toxicity and as a mucolytic agent, because of its ability to break disulfide bonds to liquidate mucus.

### **1.7. Pb is a Non-Competitive Antagonist of the NMDA Receptor**

Many promising putative toxicological mechanisms of Pb have been described, and most notable among them relate to Pb's non-competitive antagonism of the N-methyl-D-aspartate (NMDA) receptor (Alkondon et al., 1990, Guilarte and Miceli, 1992). The NMDA receptor is a ligand-gated ion channel that plays an essential role in brain development, synaptic plasticity and Long Term Potentiation (LTP). LTP, the enhanced postsynaptic response following high frequency stimulation, is thought to be a central mechanism of learning and memory. The NMDA receptor permits the passage of calcium ions, unlike other ion channels, and is only activated upon depolarization of the postsynaptic membrane and the binding of glutamate and glycine. Calcium serves as a second messenger in a variety of signaling cascades that promote LTP. One such signaling cascade activates the cAMP element binding transcription factor (CREB), which drives the expression of brain-derived neurotrophic factor (BDNF) (Hardingham et al., 2001). This pathway has been linked to the Pb-induced reduction of BDNF expression in and consequent secretion from postsynaptic neurons, which causes decreased trk-B activation and vesicular release by BDNF in the presynaptic neuron (Stansfield et al., 2012, Neal et al., 2011, Neal et al., 2010). The resulting dysregulation of the BDNF – trk-B signaling axis results in a decrease in synapses formed in synaptogenesis.

The relationship between Pb and the NMDA receptor has effects on synaptogenesis and synaptic pruning, explaining some of the Pb-associated cognitive deficits. However, the relevancy of the Pb-NMDA relationship in NSCs is less clear. While some evidence shows the NMDA receptor regulates rat hippocampal neural progenitor cell proliferation (Joo, Kim et al. 2007 & Fan, Gao et al. 2012), the Pb effect on neural proliferation has not been shown to rely on the NMDA receptor.

### **1.8. Transcriptome Profiling Allows for the Discovery of New Biology in Pb toxicity**

The transcriptome is the sum total of all RNAs transcribed from the genome. Changes in gene expression are examined on a global level through transcriptome profiling in order to discover new biology underlying phenotypes or exposure responses. Traditionally, transcriptomics employed microarrays to observe global gene expression changes. RNA Microarrays are surfaces imprinted with DNA oligos complimentary to known RNAs. Upon loading of the sample, RNAs bind to the corresponding DNA oligos in a concentration-dependent manner. The degree of binding is quantified usually by detection of a fluorophore-labeled target and compared between samples in order to calculate fold change of expression.

Microarrays have potentiated a revolution of gene expression studies and propelled our understanding of gene regulation forward significantly. Of the global gene expression studies performed on Pb, only two were performed in humans. The first study analyzed blood mRNA and Pb profiles in an autism case-control study. The population was minimally exposed to Pb (average 1.1  $\mu\text{g}/\text{dL}$ ) and the number of participants was very small ( $n=37$  cases with autism and  $n=15$  controls) (Tian et al., 2011). These factors along with the differences observed in autism cases and controls complicate the relevancy of this data to Pb-exposed populations. The second human study looked at adults whose early life Pb exposure had been characterized approximately 30 years earlier (Mazumdar et al., 2012). Among this sample the expression of genes related to  $\beta$ -Amyloid production and deposition, including ADAM9, RTN4 and LRPAP1, were inversely correlated with Pb exposure. Gene network analysis of differential expression based on prenatal Pb exposure revealed enrichment of nerve growth and cell development gene sets.

A microarray study of whole 21 day-old mouse brain revealed a total of 350 genes affected by Pb-exposure, which were enriched for pathways pertaining to focal adhesion, extracellular matrix receptor interaction, Fc epsilon R1 signaling, glycan structures-biosynthesis 1, purine metabolism, N-glycan biosynthesis, and VEGF signaling (Kasten-Jolly et al., 2012). Another microarray study by the same research group reported significant differential expression patterns in rats with peri- and post-natal Pb exposure, which differed by timing of exposure and gender (Schneider et al., 2012). Pathway analysis in this study identified dysregulation of pathways pertaining to ion binding, regulation of RNA metabolic processes and positive regulation of macromolecule biosynthetic processes. Two recent papers have addressed differential expression in Pb-exposed zebrafish embryos (Peterson et al., 2011, Yang et al., 2007). Pb elicits both a unique set of expression changes as well as changes shared among zebrafish embryos exposed to other environmental toxicants (Yang et al., 2007). Expression changes included induction of chaperone proteins, like heat shock protein 70 (*Hsp70*) and oxidative stress-related proteins including glutathione-s-transferase omega 1 (*GSTO1*). Expression changes among zebrafish embryos vary by embryonic stage and include many genes involved in neuronal development, like the down regulation of cell-adhesion genes *NRXN2* and *CELSR3* (Peterson et al., 2011). None of these genome-wide studies has followed up with further mechanistic investigation of the differentially expressed genes, which is a major goal of this thesis following our own discovery approach.

In this thesis, we employ next-generation RNA sequencing, a game-changing technology in the field of transcriptomics. RNA sequencing harnesses advances in DNA sequencing technologies for the study of transcriptome profiling. In preparing cDNA libraries from RNA samples, a number of processing steps are required, which include ribosomal RNA depletion, RNA fragmentation, cDNA synthesis, adaptor ligation and PCR amplification. The adaptors used in RNA sequencing are bar-coded such that several samples can be multiplexed on a single lane of a sequencing machine for cost efficiency. The resulting reads are aligned to the genome. Gene count tables are compiled based on gene annotations. Various packages exist for analyzing data, including DESeq (Anders and Huber, 2010) and edgeR (Robinson et al., 2010). Generally, the packages normalize read depth among samples and call differential expression. Read normalization corrects for sequencing depth differences that could result from library quantification

error and small differences in sequencer loading amounts. Various methods have been proposed for read normalization, and one that is widely used is the trimmed mean of M values (TMM) normalization method (Robinson and Oshlack, 2010). After normalization count data is fit to the negative binomial distribution. The negative binomial distribution predicts greater dispersion of count data than the Poisson distribution, and in practice is shown to fit RNA sequencing data better (Anders and Huber, 2010). A generalized linear model and a Benjamini-Hochberg multiple testing correction is used to determine differential expression and evaluate statistical significance.

The advantages of next-generation sequencing over traditional microarray approaches are manifold. Most importantly, gene expression studies using microarrays are limited to the probes included and are thus unable to provide full transcriptome coverage. Secondly, the relative abundance of splice variants cannot be detected by microarray. In the context of metal toxicity, BDNF has as many as 11 different splice variants (Aid et al., 2007) and these splice variants are differentially regulated by Pb exposure (Stansfield et al., 2012). Therefore, RNA sequencing provides a platform to study the complexities of intra-gene variation that may have previously been overlooked. Thirdly, RNA sequencing has a broader dynamic range than microarrays making it a better method to detect fold changes in genes very lowly and very highly expressed. Finally, microarrays intrinsically have lower sensitivity than next-generation sequencing due to variable hybridization efficiencies (Irizarry et al., 2005). The lack of sensitivity may often preclude the use of low and physiologic doses of chemicals in determining transcriptomic responses. The dose of any toxicant at high concentrations likely involves different biology than a lower dose exposure.

### **1.9. Genetic Epidemiology Allows for Discovery of Mediators of Pb Neurotoxicity**

Reproducible evidence showing that genetic variants in conjunction with Pb exposure are associated with measurable cognitive discrepancies in human populations gives strong supporting evidence for the role of gene-Pb interactions in human neurocognitive development. Furthermore, inter-individual susceptibility to Pb exposure has been observed (Bellinger, 2004), and genetic differences likely play a large role in innate differences in susceptibility.

In genetic epidemiology, known polymorphic loci in the genome are used to map unknown causative genomic variants underlying phenotypes of interest. The first polymorphic markers used widely were microsatellites, which are 2-5 base-pair long repeat sequences of variable lengths in populations. Single Nucleotide Polymorphisms (SNPs) are now more commonly used because they occur at far higher density in the genome and are easier to genotype in large multiplexed assays. SNPs are almost exclusively bi-allelic, usually coding for a substitution mutation, but they sometimes encode a deletion or duplication mutation. SNP genotypes are usually used in regression analysis in the form of minor allele copy number (0, 1 or 2). Mapping is done in either families using linkage analysis or populations of unrelated individuals by association analysis. While statistical power favors family-based linkage mapping, difficulties in biomarker and phenotype collection across generations make linkage mapping an unviable option for most studies. Therefore, SNP association studies have become the standard technology for approaching questions in gene-environment interaction studies.

In evaluating the role of a SNP in the etiology of a trait, one starts with the main effect of the SNP. The SNP main effect measures the effect of the minor allele copy number on the outcome of a trait or outcome in regression analysis. This approach assumes that the effect of the genetic polymorphism is independent of environmental conditions. The second test involves an interaction term between the SNP and the environmental variable. A multiplicative model is most common in such analyses, where the product of each input variable is used in regression analysis alongside the minor allele copy number and the environmental variable included individually. Interaction studies in genetic epidemiology are a mechanism to approach and better understand the heterogeneity among genotype and exposure classes in trait and disease outcomes. A significant interaction is evidence of genetic effect modification, in which the expected effect of an exposure on an outcome depends on the individual's genotype.

Several epidemiological studies have identified genetic variants as possible effect modifiers of neurological outcomes related to Pb exposure, including variation in the genes: Delta-Aminolevulinic Acid Dehydratase (*ALAD*) (Bellinger et al., 1994, Pawlas et al., 2012), Vitamin D Receptor (*VDR*) (Krieg et al., 2010, Pawlas et al., 2012), Dopamine receptor D2 (*DRD2*) (Roy et al., 2011), Dopamine receptor D4 (*DRD4*) (Froehlich et al., 2007) and Apolipoprotein E (*APOE*) (Wright et al., 2003). *ALAD* is a main Pb



binding protein, and the polymorphism associated in interaction with Pb exposure is a variant known to influence Pb binding efficiency and blood Pb level (Scinicariello et al., 2007). DRD2 and DRD4 are regulators of the dopamine system and APOE is a fatty acid metabolism regulator, which indicate a wide variety of pathways implicated in Pb neurotoxicity. However, it is important to note that these findings, while promising, need further replication and molecular characterization. Replication is difficult and it is worth noting, for instance, that while the Pb-ALAD interaction in cognitive outcomes is observed in two reports, it could not be replicated in one large study (n=842) (Krieg et al., 2009). Failure to replicate should not be considered a total annulment of a previous association as differences in study design and population linkage structure may be responsible. Because comprehensive and detailed replication of many epidemiological findings is costly and impractical, integration of data across disciplines may be a more fruitful approach. By tying together lines of evidence from more than one field, one is better able to detect true associations and toxicant susceptibility variants.

#### **1.10. References**

- AID, T., KAZANTSEVA, A., PIIRSOO, M., PALM, K. & TIMMUSK, T. 2007. Mouse and rat BDNF gene structure and expression revisited. *Journal of Neuroscience Research*, 85, 525-35.
- ALKONDON, M., COSTA, A. C., RADHAKRISHNAN, V., ARONSTAM, R. S. & ALBUQUERQUE, E. X. 1990. Selective blockade of NMDA-activated channel currents may be implicated in learning deficits caused by lead. *FEBS Letters*, 261, 124-30.
- ANDERS, S. & HUBER, W. 2010. Differential expression analysis for sequence count data. *Genome Biol*, 11, R106.
- BARANOWSKA-BOSIACKA, I., GUTOWSKA, I., MARCHLEWICZ, M., MARCHETTI, C., KURZAWSKI, M., DZIEDZIEJKO, V., KOLASA, A., OLSZEWSKA, M., RYBICKA, M., SAFRANOW, K., NOWACKI, P., WISZNIEWSKA, B. & CHLUBEK, D. 2012. Disrupted pro- and antioxidative balance as a mechanism of neurotoxicity induced by perinatal exposure to lead. *Brain Research*, 1435, 56-71.
- BELLINGER, D., HU, H., TITLEBAUM, L. & NEEDLEMAN, H. L. 1994. Attentional correlates of dentin and bone lead levels in adolescents. *Archives of Environmental Health*, 49, 98-105.
- BELLINGER, D., LEVITON, A., WATERNAUX, C., NEEDLEMAN, H. & RABINOWITZ, M. 1987. Longitudinal analyses of prenatal and postnatal lead exposure and early cognitive development. *New England Journal of Medicine*, 316, 1037-43.
- BELLINGER, D. C. 2004. Lead. *Pediatrics*, 113, 1016-22.

- BREIER, J. M., RADIO, N. M., MUNDY, W. R. & SHAFER, T. J. 2008. Development of a high-throughput screening assay for chemical effects on proliferation and viability of immortalized human neural progenitor cells. *Toxicological Sciences*, 105, 119-33.
- CANFIELD, R. L., HENDERSON, C. R., JR., CORY-SLECHTA, D. A., COX, C., JUSKO, T. A. & LANPHEAR, B. P. 2003. Intellectual impairment in children with blood lead concentrations below 10 microg per deciliter. *N Engl J Med*, 348, 1517-26.
- CHORLEY, B. N., CAMPBELL, M. R., WANG, X., KARACA, M., SAMBANDAN, D., BANGURA, F., XUE, P., PI, J., KLEEBERGER, S. R. & BELL, D. A. 2012. Identification of novel NRF2-regulated genes by ChIP-Seq: influence on retinoid X receptor alpha. *Nucleic Acids Res*, 40, 7416-29.
- DATKO-WILLIAMS, L., WILKIE, A. & RICHMOND-BRYANT, J. 2014. Analysis of U.S. soil lead (Pb) studies from 1970 to 2012. *Sci Total Environ*, 468-469, 854-63.
- DIETRICH, K. N., WARE, J. H., SALGANIK, M., RADCLIFFE, J., ROGAN, W. J., RHOADS, G. G., FAY, M. E., DAVOLI, C. T., DENCKLA, M. B., BORNSCHEIN, R. L., SCHWARZ, D., DOCKERY, D. W., ADUBATO, S. & JONES, R. L. 2004. Effect of chelation therapy on the neuropsychological and behavioral development of lead-exposed children after school entry. *Pediatrics*, 114, 19-26.
- ERICKSON, A. M., NEVAREA, Z., GIPP, J. J. & MULCAHY, R. T. 2002. Identification of a variant antioxidant response element in the promoter of the human glutamate-cysteine ligase modifier subunit gene. Revision of the ARE consensus sequence. *J Biol Chem*, 277, 30730-7.
- FROELICH, T. E., LANPHEAR, B. P., DIETRICH, K. N., CORY-SLECHTA, D. A., WANG, N. & KAHN, R. S. 2007. Interactive effects of a DRD4 polymorphism, lead, and sex on executive functions in children. *Biological Psychiatry*, 62, 243-9.
- FULTON, M., RAAB, G., THOMSON, G., LAXEN, D., HUNTER, R. & HEPBURN, W. 1987. Influence of blood lead on the ability and attainment of children in Edinburgh. *Lancet*, 1, 1221-6.
- GILBERT, M. E., KELLY, M. E., SAMSAM, T. E. & GOODMAN, J. H. 2005. Chronic developmental lead exposure reduces neurogenesis in adult rat hippocampus but does not impair spatial learning. *Toxicol Sci*, 86, 365-74.
- GILBERT, S. G. & WEISS, B. 2006. A rationale for lowering the blood lead action level from 10 to 2 microg/dL. *Neurotoxicology*, 27, 693-701.
- GOYER, R. A. 1996. Results of lead research: prenatal exposure and neurological consequences. *Environ Health Perspect*, 104, 1050-4.
- GROSSE, S. D., MATTE, T. D., SCHWARTZ, J. & JACKSON, R. J. 2002. Economic gains resulting from the reduction in children's exposure to lead in the United States. *Environ Health Perspect*, 110, 563-9.
- GUILARTE, T. R. & MICELI, R. C. 1992. Age-dependent effects of lead on [3H]MK-801 binding to the NMDA receptor-gated ionophore: in vitro and in vivo studies. *Neuroscience Letters*, 148, 27-30.
- GULSON, B. L., MIZON, K. J., KORSCH, M. J., PALMER, J. M. & DONNELLY, J. B. 2003. Mobilization of lead from human bone tissue during pregnancy and lactation--a summary of long-term research. *Sci Total Environ*, 303, 79-104.

- GURER-ORHAN, H., SABIR, H. U. & OZGUNES, H. 2004. Correlation between clinical indicators of lead poisoning and oxidative stress parameters in controls and lead-exposed workers. *Toxicology*, 195, 147-54.
- HARDINGHAM, G. E., ARNOLD, F. J. & BADING, H. 2001. Nuclear calcium signaling controls CREB-mediated gene expression triggered by synaptic activity. *Nature Neuroscience*, 4, 261-7.
- HESKEL, D. L. 1983. A Model for the Adoption of Metallurgy in the Ancient Middle East. *Current Anthropology*, 24, 362-366.
- HONG, S., CANDELONE, J. P., PATTERSON, C. C. & BOUTRON, C. F. 1994. Greenland ice evidence of hemispheric lead pollution two millennia ago by greek and roman civilizations. *Science*, 265, 1841-3.
- HU, H., TELLEZ-ROJO, M. M., BELLINGER, D., SMITH, D., ETTINGER, A. S., LAMADRID-FIGUEROA, H., SCHWARTZ, J., SCHNAAS, L., MERCADO-GARCIA, A. & HERNANDEZ-AVILA, M. 2006. Fetal lead exposure at each stage of pregnancy as a predictor of infant mental development. *Environmental Health Perspectives*, 114, 1730-5.
- HUANG, F. & SCHNEIDER, J. S. 2004. Effects of lead exposure on proliferation and differentiation of NSCs derived from different regions of embryonic rat brain. *Neurotoxicology*, 25, 1001-12.
- IRIZARRY, R. A., WARREN, D., SPENCER, F., KIM, I. F., BISWAL, S., FRANK, B. C., GABRIELSON, E., GARCIA, J. G., GEOGHEGAN, J., GERMINO, G., GRIFFIN, C., HILMER, S. C., HOFFMAN, E., JEDLICKA, A. E., KAWASAKI, E., MARTINEZ-MURILLO, F., MORSBERGER, L., LEE, H., PETERSEN, D., QUACKENBUSH, J., SCOTT, A., WILSON, M., YANG, Y., YE, S. Q. & YU, W. 2005. Multiple-laboratory comparison of microarray platforms. *Nat Methods*, 2, 345-50.
- JOMOVA, K. & VALKO, M. 2011. Advances in metal-induced oxidative stress and human disease. *Toxicology*, 283, 65-87.
- KAISER, R., HENDERSON, A. K., DALEY, W. R., NAUGHTON, M., KHAN, M. H., RAHMAN, M., KIESZAK, S. & RUBIN, C. H. 2001. Blood lead levels of primary school children in Dhaka, Bangladesh. *Environ Health Perspect*, 109, 563-6.
- KASTEN-JOLLY, J., PABELLO, N., BOLIVAR, V. J. & LAWRENCE, D. A. 2012. Developmental lead effects on behavior and brain gene expression in male and female BALB/cAnNTac mice. *Neurotoxicology*, 33, 1005-20.
- KIM, S., CHEON, H. S., KIM, S. Y., JUHNN, Y. S. & KIM, Y. Y. 2013. Cadmium induces neuronal cell death through reactive oxygen species activated by GADD153. *BMC Cell Biol*, 14, 4.
- KRIEG, E. F., JR., BUTLER, M. A., CHANG, M. H., LIU, T., YESUPRIYA, A., DOWLING, N. & LINDEGREN, M. L. 2010. Lead and cognitive function in VDR genotypes in the third National Health and Nutrition Examination Survey. *Neurotoxicology and Teratology*, 32, 262-72.
- KRIEG, E. F., JR., BUTLER, M. A., CHANG, M. H., LIU, T., YESUPRIYA, A., LINDEGREN, M. L. & DOWLING, N. 2009. Lead and cognitive function in ALAD genotypes in the third National Health and Nutrition Examination Survey. *Neurotoxicology and Teratology*, 31, 364-71.
- LANPHEAR, B. P., HORNING, R., KHOURY, J., YOLTON, K., BAGHURST, P., BELLINGER, D. C., CANFIELD, R. L., DIETRICH, K. N., BORNSCHEIN, R., GREENE, T., ROTHENBERG, S. J., NEEDLEMAN, H. L., SCHNAAS, L., WASSERMAN, G., GRAZIANO, J. & ROBERTS, R. 2005. Low-level environmental lead

exposure and children's intellectual function: an international pooled analysis. *Environ Health Perspect*, 113, 894-9.

MAZUMDAR, M., BELLINGER, D. C., GREGAS, M., ABANILLA, K., BACIC, J. & NEEDLEMAN, H. L. 2011. Low-level environmental lead exposure in childhood and adult intellectual function: a follow-up study. *Environ Health*, 10, 24.

MAZUMDAR, M., XIA, W., HOFMANN, O., GREGAS, M., HO SUI, S., HIDE, W., YANG, T., NEEDLEMAN, H. L. & BELLINGER, D. C. 2012. Prenatal lead levels, plasma amyloid beta levels, and gene expression in young adulthood. *Environ Health Perspect*, 120, 702-7.

MENDOLA, P., SELEVAN, S. G., GUTTER, S. & RICE, D. 2002. Environmental factors associated with a spectrum of neurodevelopmental deficits. *Ment Retard Dev Disabil Res Rev*, 8, 188-97.

MIELKE, H. W. & ZAHRAN, S. 2012. The urban rise and fall of air lead (Pb) and the latent surge and retreat of societal violence. *Environ Int*, 43, 48-55.

MITSUISHI, Y., TAGUCHI, K., KAWATANI, Y., SHIBATA, T., NUKIWA, T., ABURATANI, H., YAMAMOTO, M. & MOTOHASHI, H. 2012. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell*, 22, 66-79.

NEAL, A. P., STANSFIELD, K. H., WORLEY, P. F., THOMPSON, R. E. & GUILARTE, T. R. 2010. Lead exposure during synaptogenesis alters vesicular proteins and impairs vesicular release: potential role of NMDA receptor-dependent BDNF signaling. *Toxicological Sciences*, 116, 249-63.

NEAL, A. P., WORLEY, P. F. & GUILARTE, T. R. 2011. Lead exposure during synaptogenesis alters NMDA receptor targeting via NMDA receptor inhibition. *Neurotoxicology*, 32, 281-9.

NEEDLEMAN, H. L., GUNNOE, C., LEVITON, A., REED, R., PERESIE, H., MAHER, C. & BARRETT, P. 1979. Deficits in psychologic and classroom performance of children with elevated dentine lead levels. *N Engl J Med*, 300, 689-95.

NEEDLEMAN, H. L., RIESS, J. A., TOBIN, M. J., BIESECKER, G. E. & GREENHOUSE, J. B. 1996. Bone lead levels and delinquent behavior. *JAMA*, 275, 363-9.

NEVIN, R. 2000. How lead exposure relates to temporal changes in IQ, violent crime, and unwed pregnancy. *Environmental Research*, 83, 1-22.

NEVIN, R. 2007. Understanding international crime trends: the legacy of preschool lead exposure. *Environmental Research*, 104, 315-36.

NRIAGU, J. O. 1983. Saturnine gout among Roman aristocrats. Did lead poisoning contribute to the fall of the Empire? *N Engl J Med*, 308, 660-3.

OPLER, M. G., BROWN, A. S., GRAZIANO, J., DESAI, M., ZHENG, W., SCHAEFER, C., FACTOR-LITVAK, P. & SUSSER, E. S. 2004. Prenatal lead exposure, delta-aminolevulinic acid, and schizophrenia. *Environmental Health Perspectives*, 112, 548-52.

OPLER, M. G., BUKA, S. L., GROEGER, J., MCKEAGUE, I., WEI, C., FACTOR-LITVAK, P., BRESNAHAN, M., GRAZIANO, J., GOLDSTEIN, J. M., SEIDMAN, L. J., BROWN, A. S. & SUSSER, E. S. 2008. Prenatal exposure to lead, delta-aminolevulinic acid, and schizophrenia: further evidence. *Environmental Health Perspectives*, 116, 1586-90.

- PAWLAS, N., BROBERG, K., OLEWINSKA, E., PROKOPOWICZ, A., SKERFVING, S. & PAWLAS, K. 2012. Modification by the genes ALAD and VDR of lead-induced cognitive effects in children. *Neurotoxicology*, 33, 37-43.
- PETERSON, S. M., ZHANG, J., WEBER, G. & FREEMAN, J. L. 2011. Global gene expression analysis reveals dynamic and developmental stage-dependent enrichment of lead-induced neurological gene alterations. *Environmental Health Perspectives*, 119, 615-21.
- PIRKLE, J. L., BRODY, D. J., GUNTER, E. W., KRAMER, R. A., PASCHAL, D. C., FLEGAL, K. M. & MATTE, T. D. 1994. The decline in blood lead levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). *JAMA*, 272, 284-91.
- ROBINSON, M. D., MCCARTHY, D. J. & SMYTH, G. K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26, 139-40.
- ROBINSON, M. D. & OSHLACK, A. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol*, 11, R25.
- ROGAN, W. J., DIETRICH, K. N., WARE, J. H., DOCKERY, D. W., SALGANIK, M., RADCLIFFE, J., JONES, R. L., RAGAN, N. B., CHISOLM, J. J., JR. & RHOADS, G. G. 2001. The effect of chelation therapy with succimer on neuropsychological development in children exposed to lead. *New England Journal of Medicine*, 344, 1421-6.
- ROY, A., BELLINGER, D., HU, H., SCHWARTZ, J., ETTINGER, A. S., WRIGHT, R. O., BOUCHARD, M., PALANIAPPAN, K. & BALAKRISHNAN, K. 2009. Lead exposure and behavior among young children in Chennai, India. *Environ Health Perspect*, 117, 1607-11.
- ROY, A., HU, H., BELLINGER, D. C., MUKHERJEE, B., MODALI, R., NASARUDDIN, K., SCHWARTZ, J., WRIGHT, R. O., ETTINGER, A. S., PALANIAPAN, K. & BALAKRISHNAN, K. 2011. Hemoglobin, lead exposure, and intelligence quotient: effect modification by the DRD2 Taq IA polymorphism. *Environmental Health Perspectives*, 119, 144-9.
- SCHNEIDER, J. S., ANDERSON, D. W., TALSANIA, K., METTIL, W. & VADIGEPALLI, R. 2012. Effects of developmental lead exposure on the hippocampal transcriptome: influences of sex, developmental period, and lead exposure level. *Toxicological Sciences*, 129, 108-25.
- SCHNEIDER, J. S., ANDERSON, D. W., WADE, T. V., SMITH, M. G., LEIBRANDT, P., ZUCK, L. & LIDSKY, T. I. 2005. Inhibition of progenitor cell proliferation in the dentate gyrus of rats following post-weaning lead exposure. *Neurotoxicology*, 26, 141-5.
- SCINICARIELLO, F., MURRAY, H. E., MOFFETT, D. B., ABADIN, H. G., SEXTON, M. J. & FOWLER, B. A. 2007. Lead and delta-aminolevulinic acid dehydratase polymorphism: where does it lead? A meta-analysis. *Environ Health Perspect*, 115, 35-41.
- STANSFIELD, K. H., PILSNER, J. R., LU, Q., WRIGHT, R. O. & GUILARTE, T. R. 2012. Dysregulation of BDNF-TrkB signaling in developing hippocampal neurons by Pb<sup>2+</sup>: Implications for an environmental basis of neurodevelopmental disorders. *Toxicological Sciences*.
- STEPHENSON, A. P., SCHNEIDER, J. A., NELSON, B. C., ATHA, D. H., JAIN, A., SOLIMAN, K. F., ASCHNER, M., MAZZIO, E. & RENEE REAMS, R. 2013. Manganese-induced oxidative DNA damage in neuronal SH-SY5Y cells: attenuation of thymine base lesions by glutathione and N-acetylcysteine. *Toxicol Lett*, 218, 299-307.

- TIAN, Y., GREEN, P. G., STAMOVA, B., HERTZ-PICCIOTTO, I., PESSAH, I. N., HANSEN, R., YANG, X., GREGG, J. P., ASHWOOD, P., JICKLING, G., VAN DE WATER, J. & SHARP, F. R. 2011. Correlations of gene expression with blood lead levels in children with autism compared to typically developing controls. *Neurotox Res*, 19, 1-13.
- TONG, S., BAGHURST, P., MCMICHAEL, A., SAWYER, M. & MUDGE, J. 1996. Lifetime exposure to environmental lead and children's intelligence at 11-13 years: the Port Pirie cohort study. *BMJ*, 312, 1569-75.
- TOYAMA, T., SHINKAI, Y., YASUTAKE, A., UCHIDA, K., YAMAMOTO, M. & KUMAGAI, Y. 2011. Isothiocyanates reduce mercury accumulation via an Nrf2-dependent mechanism during exposure of mice to methylmercury. *Environ Health Perspect*, 119, 1117-22.
- VERINA, T., ROHDE, C. A. & GUILARTE, T. R. 2007. Environmental lead exposure during early life alters granule cell neurogenesis and morphology in the hippocampus of young adult rats. *Neuroscience*, 145, 1037-47.
- WANG, X., TOMSO, D. J., CHORLEY, B. N., CHO, H. Y., CHEUNG, V. G., KLEEBERGER, S. R. & BELL, D. A. 2007. Identification of polymorphic antioxidant response elements in the human genome. *Human Molecular Genetics*, 16, 1188-200.
- WASSERMAN, G. A., LIU, X., LOLACONO, N. J., FACTOR-LITVAK, P., KLINE, J. K., POPOVAC, D., MORINA, N., MUSABEGOVIĆ, A., VRENEZI, N., CAPUNI-PARACKA, S., LEKIC, V., PRETENI-REDJEPI, E., HADZIALJEVIC, S., SLAVKOVICH, V. & GRAZIANO, J. H. 1997. Lead exposure and intelligence in 7-year-old children: the Yugoslavia Prospective Study. *Environ Health Perspect*, 105, 956-62.
- WASSERMAN, G. A., LIU, X., PARVEZ, F., AHSAN, H., FACTOR-LITVAK, P., KLINE, J., VAN GEEN, A., SLAVKOVICH, V., LOIACONO, N. J., LEVY, D., CHENG, Z. & GRAZIANO, J. H. 2007. Water arsenic exposure and intellectual function in 6-year-old children in Arai hazar, Bangladesh. *Environ Health Perspect*, 115, 285-9.
- WASSERMAN, G. A., LIU, X., POPOVAC, D., FACTOR-LITVAK, P., KLINE, J., WATERNAUX, C., LOIACONO, N. & GRAZIANO, J. H. 2000. The Yugoslavia Prospective Lead Study: contributions of prenatal and postnatal lead exposure to early intelligence. *Neurotoxicol Teratol*, 22, 811-8.
- WRIGHT, J. P., DIETRICH, K. N., RIS, M. D., HORNUNG, R. W., WESSEL, S. D., LANPHEAR, B. P., HO, M. & RAE, M. N. 2008. Association of prenatal and childhood blood lead concentrations with criminal arrests in early adulthood. *PLoS Med*, 5, e101.
- WRIGHT, R. O., HU, H., SILVERMAN, E. K., TSAIH, S. W., SCHWARTZ, J., BELLINGER, D., PALAZUELOS, E., WEISS, S. T. & HERNANDEZ-AVILA, M. 2003. Apolipoprotein E genotype predicts 24-month bayley scales infant development score. *Pediatric Research*, 54, 819-25.
- YANG, L., KEMADJOU, J. R., ZINSMEISTER, C., BAUER, M., LEGRADI, J., MULLER, F., PANKRATZ, M., JAKEL, J. & STRAHLE, U. 2007. Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo. *Genome Biol*, 8, R227.
- YE, X., FU, H. & GUIDOTTI, T. 2007. Environmental exposure and children's health in China. *Arch Environ Occup Health*, 62, 61-73.
- ZHENG, L., WU, K., LI, Y., QI, Z., HAN, D., ZHANG, B., GU, C., CHEN, G., LIU, J., CHEN, S., XU, X. & HUO, X. 2008. Blood lead and cadmium levels and relevant factors among children from an e-waste recycling town in China. *Environmental Research*, 108, 15-20.

## **CHAPTER 2: Secreted Phosphoprotein 1 (SPP1) is a Novel NRF2 Target Upregulated by Pb in Human Neural Stem Cells**

This work is currently unpublished.

Peter Wagner performed all laboratory work.

Rory Kirchner performed RNA sequencing quality control, genome alignments and generation of gene-count tables.

Peter Wagner performed differential expression analysis of RNA sequencing data and analyses of all other datasets.

## 2.1. Abstract

Neural stem cells (NSCs) are multipotent stem cells that actively proliferate in the first trimester and differentiate in the second trimester of pregnancy. The presence and activity of NSCs in the first and second trimesters corresponds to an emerging critical window of lead (Pb) neurotoxicity. However, the molecular mechanisms explaining how these cells are affected by Pb exposure are not well understood. To better understand how NSCs react to Pb exposure, we exposed human NSCs to a physiologically relevant dose of Pb (1 $\mu$ M) and performed next-generation RNA sequencing to uncover the primary transcriptional response of NSCs to Pb. We identified 16 significantly upregulated genes, among which 10 are known targets of NRF2. One of the top Pb-induced genes, *SPP1*, was not a known NRF2 target. Arsenic and a canonical NRF2 inducer, DL-Sulforaphane, also induced *SPP1* expression in a manner consistent with *NQO1* differential expression, a bona fide NRF2 target gene. In addition, activation of NRF2 by knockdown of its negative regulator KEAP1 led to an increase in *SPP1* expression. The induction of *SPP1* by Pb was attenuated by siRNA-mediated knockdown of NRF2. Using a position weight matrix we identified a high-scoring putative Antioxidant Response Element (ARE) in the *SPP1* promoter, 623 bases upstream of the *SPP1* transcription start site. Chromatin immunoprecipitation (ChIP) of NRF2 demonstrated that the ARE was functional in human NSCs. Our study thus identified *SPP1* as a novel direct target of NRF2 that may play a role in modulating Pb neurotoxicity in early life.



## 2.2. Introduction

The confluence of genetic and environmental risk factors in early life is increasingly recognized as critical in determining a number of neurodevelopmental outcomes and traits, such as autism spectrum disorders and indices of mental and psychomotor development. Pb is one such environmental risk factor, which is ubiquitous following centuries of Pb mining and use. While legislation prohibiting the use of Pb has been successful in causing precipitous declines in Pb levels in US children, no safe level of Pb is known and US experts have recently halved the high blood Pb reference level from 10 to 5  $\mu\text{g}/\text{dl}$ . In addition, Pb levels in children and the environment remain high in many countries where Pb has not – or has only recently – been phased out of gasoline, paint and other applications. Pb predominantly targets the nervous system leading to many neuropathies, and at high levels affects adults and children alike. However, low-dose exposure in early life has more pronounced effects on neurological outcomes among young children. Some studies have begun to show that in utero Pb exposure is even more predictive than childhood blood Pb of Mental Development Index (MDI) scores at age 2 (Hu et al., 2006), schizophrenia (Opler et al., 2004, Opler et al., 2008) and likelihood of arrest in later life (Wright et al., 2008). Currently, the only treatment for Pb exposure is chelation therapy, which reduces blood Pb levels but does not prevent the progressive cognitive or behavioral deficits of Pb exposure at a young age (Rogan et al., 2001, Dietrich et al., 2004).

NSCs are the progenitor cells of all cell types of the central nervous system, including astrocytes, neurons and oligodendrocytes. The majority of the differentiation of NSCs to terminally differentiated cells occurs through the second trimester of pregnancy. The growth and differentiation of NSCs are important components of synaptogenesis. Disruptions to synaptogenesis leads to lasting deficits in neurocognitive functioning. Pb slows proliferation of NSCs *in vivo* (Gilbert et al., 2005, Schneider et al., 2005, Verina et al., 2007, Breier et al., 2008) and *in vitro* (Huang and Schneider, 2004, Breier et al., 2008). In rat ventral mesencephalon-derived NSCs, proliferation decreased by almost 50% after six days of exposure to 1  $\mu\text{M}$  Pb (Huang and Schneider, 2004). Pb exposure in rats alters dendrite morphology in NSCs differentiating into neurons, but does not influence their cell-type fate (Verina et al., 2007). Pb's

effects on the developing brain may be explained, in part, by the decrease in NSC proliferation resulting in reduced synaptogenesis. Despite these studies establishing the detrimental effects of Pb on NSCs, the underlying molecular mechanisms remain poorly understood.

We have performed next-generation RNA sequencing on human NSCs in order to determine their primary transcriptional response to Pb. To our knowledge, this is the first study to use RNA sequencing to study Pb neurotoxicity and the first to use human neural tissue in a genome-wide study. The advantages of deep sequencing over traditional microarray approaches are manifold. Most importantly, gene expression studies using microarrays are limited to the probes included and are thus unable to provide full transcriptome coverage. For instance, long non-coding RNAs (lncRNAs) are traditionally not included in microarray platforms, but are highly expressed in the brain and are dynamically regulated throughout development. RNA sequencing is a platform to explore lncRNAs changes alongside protein-coding genes in hNSCs. Furthermore, the sensitivity of RNA sequencing allows for the use of a physiologic concentration of Pb exposure. The concentration used in this study, 1  $\mu$ M, is roughly quadruple the CDC level of concern of Pb in blood and a reasonable blood Pb concentration in exposed populations (Pirkle et al., 1994, Zheng et al., 2008).

### **2.3. Materials and Methods**

#### *Human NSCs (NSCs) in cell culture*

NSCs derived from NIH-approved H9 (WA09) human embryonic stem cells were purchased from Life Technologies and cultured according to supplier's protocol. Briefly, cells were maintained in an undifferentiated state on CELLStart-coated polystyrene plates in KnockOut DMEM/F12 media supplemented with 2 mM GlutaMAX-I, 20 ng/mL bFGF, 20 ng/mL of EGF and 2% StemPro Neural Supplement (all reagents from Life Technologies). 500k/well NSCs were plated in six-well plates 24 hours before exposure for sequencing and replication studies. For siRNA transfection, cells were plated at 350k/well the day before transfection. All experiments were performed in passage 3 cells. An aqueous solution of 1 mM Pb acetate trihydrate (cat #316512, Sigma Aldrich) stock is used for all experiments.

### *MTT Cell Number Assay*

MTT, or (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), is a chemical with a tetrazolium ring that is cleaved by mitochondrial dehydrogenases in viable cells to form aqueous-insoluble formazan crystals that are purple in color. Once dissolved, their density can be measured spectrophotometrically for a readout of viable cell number. For the MTT assay, cells were seeded 24 hours prior to exposure at 10k cells per well of a 96 well plate. Exposure to 0, 0.5, 1, 2, 5 and 10  $\mu\text{M}$  Pb was performed in 8 replicate wells. The assay was performed according to the MTT manufacturer's protocol (Sigma Aldrich). Briefly, 0.05 mg of MTT was added to each well for 3 hours. Formazan crystals are solubilized in 10% Triton X-100 plus and 0.1 N HCl in anhydrous isopropanol after repeated pipetting. Absorbance was read at 570 nm, and background at 690 nm removed. Mean absorbance, which is correlated with cell number, is reported along with the standard error of the mean of 8 replicate samples.

### *Illumina RNA TruSeq Library Preparation*

Poly-adenylated RNA species were isolated from 1  $\mu\text{g}$  of total RNA from each sample and converted to a cDNA library for RNA sequencing using the Illumina TruSeq RNA v2 kit. Sample preparation involves isolating poly-adenylated RNA molecules using poly-T oligo-attached magnetic beads, RNA fragmentation, cDNA synthesis, ligation of adapters, PCR amplification using one primer with a unique DNA barcode and finally library validation by Agilent Technologies 2100 Bioanalyzer. Quantification of the cDNA library samples was performed using the KAPA SYBR Fast Universal Quantification Kit (KAPA Biosystems). Four samples were multiplexed into a single lane of the Illumina HiSeq 2000 for paired-end reads of 100 bp. Sequencing was performed at the Bauer Core Illumina Sequencing Facility (FAS Center for Systems Biology, Cambridge MA).

### *Data Processing and Quality Control of RNA Sequencing Results*

Low quality reads (<25 phred), adaptors and poly-A tails were trimmed with cutadapt (Martin, 2011). Read pairs with one or more reads shorter than 20 base pairs were removed using an in-house script (Rory Kirchner). Quality of reads was assessed using FASTQC (Babraham Bioinformatics). Tophat2 (Trapnell et al., 2009) aligned read pairs to human genome build 19. RSeQC (Wang et al., 2012) was run following alignment to confirm alignment quality. Reads were compiled into count tables using

HTseq-count (Simon Anders, EMBL Heidelberg, Germany). Counts are normalized in edgeR (Robinson et al., 2010). Differential expression is determined by a generalized linear model, which takes into account unexposed-exposed paired information such that baseline differences between the replicates are subtracted out. edgeR output includes information on fold change, abundance and statistical significance. We report only statistically significantly differentially regulated transcripts following a Benjamini-Hochberg multiple testing correction ( $q < 0.05$ ) that have a greater than  $\pm 0.2$  fold change and a minimum counts per million mapped of one.

#### *Differential Expression by qPCR*

RNA is reverse transcribed using SuperScript III reverse transcriptase and oligo-dT (Life Technologies). The resulting cDNA is amplified using 2x SYBR mix (Qiagen) and 3 mM of each primer in a StepOne Plus Thermocycler (Applied Biosystems) in Quantitative Reverse Transcriptase Polymerase Chain Reaction (qPCR). Melt curves are checked for single-length amplification products. Fold changes are calculated using the  $2^{-\Delta\Delta Ct}$  method. *GAPDH* is the housekeeping gene used for normalization in all qPCR assays. All primers used in this study and their respective sources or design are listed in Table 2.1.

#### *NRF2 and KEAP1 Knockdown by siRNA Transfection*

NRF2 and KEAP1 are knocked down using short interfering RNA (siRNA). All siRNAs were obtained from Sigma: non-targeting control (SIC001), si-NRF2-1 (SASI\_Hs01\_00182393), si-NRF2-2 (SASI\_Hs02\_00341015) and si-KEAP1 (SASI\_Hs01\_00080908). Transfection is performed using 25 mM siRNA and 0.1% Dharmafect 1 (ThermoFisher) for 12 hours following manufacturer's protocol. Knockdown and resulting transcription changes are determined by qPCR 48 hours post-transfection.

#### *SPP1 Protein Quantification by Western Blot and ELISA*

SPP1 levels were assessed in whole cell extract using standard Western Blot procedures with 1:1000 Anti-Osteopontin antibody (EPR3688, abcam). Relative protein concentrations were quantified in Image-J (NIH). SPP1 levels in cell culture media was assessed using the Human Osteopontin (OPN) Quantikine ELISA Kit (DOST00, R&D Systems). For ELISA of Pb exposed cells, media was sampled at 20, 40 and 60 hours of exposure. For ELISA of si-KEAP1 cells, cells were transfected for 12 hours at which

**Table 2.1 Summary of primer sequences used in this study.** Target refers to the gene transcript or genetic loci primers are designed to amplify. Primers are self designed in primer3 (Whitehead Institute) using transcript/genetic sequences; obtained from a database such as PrimerBank (Massachusetts General Hospital/Harvard) or Roche Universal Primer Design (Roche); taken from a paper; supplied by a company; or are part of a general lab stock.

Target	Application	Source	Forward	Reverse
DIO3OS	qPCR	Designed in Primer3	GCTACAGGCTTCAGCAGGTC	CCCCAGACAGCAGAGAAATC
F2RL2	qPCR	PrimerBank - 375151566c1	GCAAAGCCAACCTTACCCATT	GAGGTAGATGGCAGGTATCAGT
FTL	qPCR	PrimerBank - 56682960c1	CAGCCTGGTCAATTTGTACCT	GCCAATTCGCGAAGAAGTG
HMOX1	qPCR	Roche Universal Primer Design - #15, cat.no. 04685148001	GGCAGAGGGTGATAGAAGAGG	AGCTCTGCAACTCCTCAAA
LUCAT1	qPCR	Roche Universal Primer Design - #10, cat.no. 04685091001	CGATCTCGTTCACTGCAA	AGCAAACAGCAAGTTGGATTC
NQO1	qPCR	PrimerBank - 70995395c1	GAAGAGCACTGATCGTACTGGC	GGATACTGAAAGTTCGCAGGG
OSGIN1	qPCR	PrimerBank - 118572592c2	CGAGGGTGTGCGAGAAGGTG	TCCACGTCAATGGGGTTCCT
SPP1	qPCR	Designed in Primer3	GGAGTTGAATGGTGATACAAGG	CCACGGCTGTCCCAATCAG
KEAP1	qPCR	PrimerBank - 45269144c1	CTGGAGGATCATACCAAGCAGG	GGATACCCTCAATGGACACCAC
NRF2	qPCR	Designed in Primer3	TCCAGTCAGAAACCAGTGGAT	GAATGTCTGCGCCAAAAGCTG
SPP1-a	qPCR	Mirza et al. (2008)	ATCTCCTAGCCCCACAGAAT	CATCAGACTGGTGAGAATCAT
SPP1-b	qPCR	Mirza et al. (2008)	ATCTCCTAGCCCCAGAGAC	AAAATCAGTGACCAGTTCATCAG
SPP1-c	qPCR	Mirza et al. (2008)	TGAGGAAAAGCAGAATGCTG	GTCAATGGAGTCTCGGCTGT
RPL30 Exon 3	ChIP- qPCR	Cell Signaling Technology, Inc	N/A	N/A
NQO1 ARE	ChIP- qPCR	Chorley et al. 2012	CCCTTTTAGCCTTGGCACGAAA	TGCACCCAGGGAAGTGTGTTGTAT
SPP1 ARE	ChIP- qPCR	Designed in Primer3	ATAGGTAGGCTGGGCGATTT	GTGGTTCTGAATCCGCTGT
GAPDH	QPCR	Lab stock	GGAAACTGTGGCGTGATG	AGTGAGCTTCCCGTTCAG

point the media was replaced. Media was sampled 60 hours post-transfection, after 48 hours of contact with the cells.

#### *Identification and Confirmation of a Putative Antioxidant Response Element (ARE)*

A position weight matrix describing AREs (Wang et al., 2007) was used to score putative AREs 2kb upstream of the SPP1 transcript start site using the Biostrings software package implemented in R (Pages et al.). AREs are scored by the sum of their  $\log_2$  probability ratios across all 22 bases and compared to the scores of experimentally validated AREs (Wang et al., 2007). The high scoring *SPP1* ARE was confirmed by NRF2 Chromatin Immunoprecipitation followed by Quantitative Polymerase Chain Reaction (ChIP-PCR). NSCs were expanded to approximately 8 million cells, of which half were enriched for NRF2 using siRNA knockdown of NRF2's negative regulator KEAP1 and the other half were transfected with a non-targeting siRNA control. Samples were prepared following the SimpleChIP Plus Enzymatic Chromatin IP Kit protocol supplied by the manufacturer (Cell Signaling Technology). Briefly, 48 hours post si-KEAP1 transfection, NRF2 was cross-linked to DNA using 1.5% formaldehyde. Chromatin is digested with Micrococcal Nuclease for 18 minutes into 150-900 bp fragments. Nuclei are collected and lysed by sonication. An aliquot of lysate is analyzed for appropriate digestion by gel electrophoresis. Nuclear extracts are incubated overnight with NRF2 antibody (cat. #12721, Cell Signaling Technology) and antibody-bound complexes are captured by protein G magnetic beads. Bound DNA is purified and undergoes quantitation by PCR using primers flanking the bioinformatically-identified putative SPP1 ARE, the NQO1 ARE (Chorley et al., 2012) and RPL30-exon 3 (Cell Signaling Technology). The NQO1 ARE serves as a positive control and RPL30-exon 3 as a negative control. Primers for amplifying the SPP1 ARE were designed in Primer 3 (Whitehead Institute) to target the 50 base pair regions flanking the 21 base pair ARE. Primers are shown in Table 2.1.

## 2.4. Results

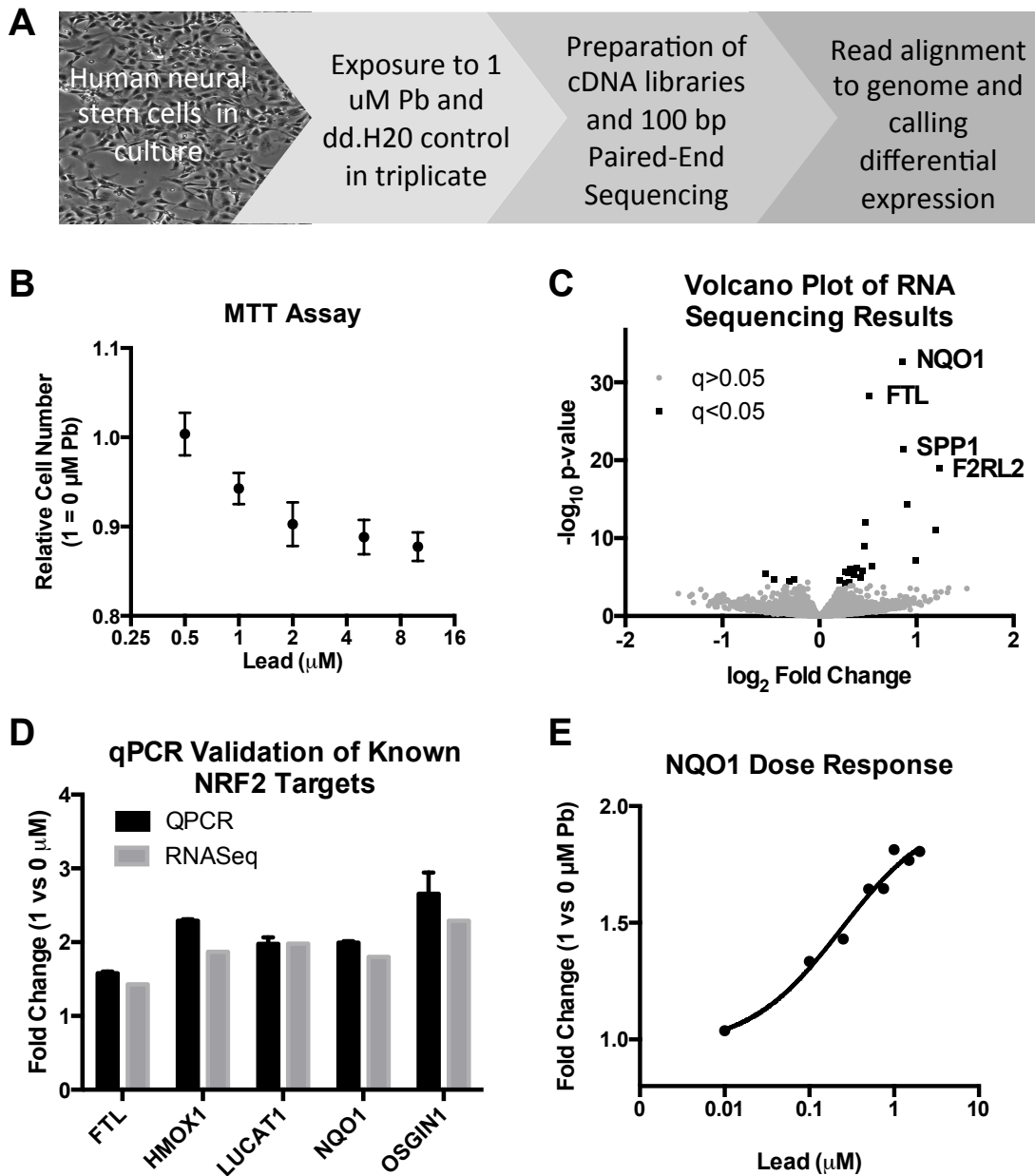
### *RNA Sequencing identifies gene expression changes in NSCs exposed to Pb*

The critical period of Pb exposure overlays with the activity of NSCs in the developing brain. To better understand the effects of Pb on this early and affected cell type, we have performed RNA sequencing of Pb-exposed human NSCs. The sensitivity of RNA sequencing allows for the use of a physiologic concentration of Pb exposure. The human NSCs used in this study were initially generated from the H09 human embryonic stem cell line and have a normal karyotype. To determine the effects of Pb on gene expression, we exposed NSCs to Pb at 1 $\mu$ M and vehicle double distilled water control (Figure 2.1A). The concentration used in this study, 1  $\mu$ M, is roughly 20  $\mu$ g/dl or four times the current CDC level of concern of Pb in blood. It is a reasonable blood Pb concentration to expect in exposed populations (Pirkle et al., 1994, Zheng et al., 2008). A mild 5.7% drop in viable cell number is observed at 1  $\mu$ M after 24 hours, indicating a mild effect of the exposure on either cell growth or apoptosis over the course of the exposure (Figure 2.1B).

Sequencing yielded an average of 38.2 million reads per sample ( $\sigma$ =8.9 million). We then tested for differential expression of 17,938 GRCh37 ensembl-annotated gene regions after eliminating very lowly expressed transcripts, which are defined as transcripts having an average of fewer than one read per million mapped reads. Following a Benjamini-Hochberg multiple testing correction, a total of 19 genes were identified as statistically significantly ( $\alpha$ <0.05) differentially expressed by more than  $\pm 0.2 \log_2$  fold change (Table 2.2, Figure 2.1C). Of the 19 genes, whose expression is perturbed by Pb exposure, 3 were down-regulated and 16 upregulated.

### *Pb induces the expression of NRF2 target genes*

The top two most statistically significant differentially expressed genes in the RNA sequencing dataset are *NQO1* and *HMOX1*, which are known NRF2 target genes involved in the cellular response to oxidative stress. A dose-response relationship is shown for top hit *NQO1*, which shows an alarming 33% induction at only 0.1  $\mu$ M Pb exposure (Figure 2.1E). Therefore, NRF2 activation may be particularly sensitive to Pb exposure in NSCs. Upon close examination of the other upregulated genes, we found that ten of the sixteen upregulated genes have been previously shown to be direct targets of NRF2 (Table



**Figure 2.1 Differential expression in human NSCs exposed to 1  $\mu\text{M}$  Pb and vehicle by RNA sequencing.** (A) Schema of the RNA sequencing study. (B) MTT assay showing relative numbers of NSCs after 24 hours of various Pb concentrations. Error bars represent standard error of the mean of 8 replicates. (C) Volcano plot of RNA Sequencing results with top 4 genes annotated; ■ differentially expressed genes defined by greater than  $\pm 0.2$   $\log_2$  fold change and FDR-adjusted  $q$ -value  $< 0.05\%$ ; ● genes that do not meet significance thresholds. (D) QPCR validation of known NRF2 targets identified by RNA Sequencing. QPCR error bars refer to standard error of three biologic replicates. (E) Pb dose response of bona fide NRF2 target gene NQO1 following 24 hours of exposure in NSCs.



**Table 2.2 Differential expression of human NSCs exposed to 1 vs 0  $\mu\text{M}$  Pb by RNA sequencing.** Differential expression of triplicate pairs was performed in edgeR, and statistically significantly differentially expressed transcripts are defined by greater than  $\pm 0.2 \log_2$  fold change and FDR-adjusted q-value  $< 0.05\%$ . Annotation shows known NRF2 target genes according to: a. Chorley et al. (2012), b. Wang et al. (2007), c. Cho et al. (2005), d. Lee et al. (2003), e. Li et al. (2002), f. Malhotra et al. (2010) and g. Thai et al. (2013).

HGNC Name	Gene Description	Fold Change	p-value	FDR q-value	NRF2 Target
<b>F2RL2</b>	<i>coagulation factor II (thrombin) receptor-like 2</i>	2.35	$1.05 \times 10^{-19}$	$4.62 \times 10^{-16}$	
<b>OSGIN1</b>	<i>oxidative stress induced growth inhibitor 1</i>	2.29	$8.21 \times 10^{-12}$	$2.06 \times 10^{-8}$	a
<b>LUCAT1</b>	<i>lung cancer associated transcript 1 (non-protein coding)</i>	1.98	$6.60 \times 10^{-8}$	$1.16 \times 10^{-4}$	g
<b>HMOX1</b>	<i>heme oxygenase (decycling) 1</i>	1.87	$4.63 \times 10^{-15}$	$1.63 \times 10^{-11}$	a,b,d,e
<b>SPP1</b>	<i>secreted phosphoprotein 1</i>	1.82	$3.27 \times 10^{-22}$	$1.92 \times 10^{-18}$	
<b>NQO1</b>	<i>NAD(P)H dehydrogenase, quinone 1</i>	1.8	$2.24 \times 10^{-33}$	$3.93 \times 10^{-29}$	a,b,c,f
<b>EGF</b>	<i>epidermal growth factor</i>	1.45	$4.07 \times 10^{-7}$	$6.50 \times 10^{-4}$	
<b>FTL</b>	<i>ferritin, light polypeptide</i>	1.43	$5.00 \times 10^{-29}$	$4.39 \times 10^{-25}$	a,b
<b>VGF</b>	<i>VGF nerve growth factor inducible</i>	1.39	$8.65 \times 10^{-13}$	$2.53 \times 10^{-9}$	b
<b>TXNRD1</b>	<i>thioredoxin reductase 1</i>	1.38	$1.22 \times 10^{-9}$	$2.68 \times 10^{-6}$	a,b,f
<b>SERPINE1</b>	<i>serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1</i>	1.36	$1.53 \times 10^{-6}$	$1.92 \times 10^{-3}$	b,c,f
<b>SLC7A11</b>	<i>solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11</i>	1.34	$1.20 \times 10^{-5}$	$1.11 \times 10^{-2}$	a
<b>SLC7A8</b>	<i>solute carrier family 7 (amino acid transporter light chain, L system), member 8</i>	1.31	$6.88 \times 10^{-7}$	$1.01 \times 10^{-3}$	
<b>GREM1</b>	<i>gremlin 1, DAN family BMP antagonist</i>	1.28	$5.19 \times 10^{-6}$	$5.07 \times 10^{-3}$	
<b>PIR</b>	<i>pirin (iron-binding nuclear protein)</i>	1.25	$8.54 \times 10^{-7}$	$1.15 \times 10^{-3}$	a
<b>F13A1</b>	<i>coagulation factor XIII, A1 polypeptide</i>	1.24	$5.05 \times 10^{-5}$	$3.55 \times 10^{-2}$	
<b>B3GALT2</b>	<i>UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2</i>	0.81	$3.00 \times 10^{-5}$	$2.29 \times 10^{-2}$	
<b>MIR503HG</b>	<i>MIR503 host gene (non-protein coding)</i>	0.73	$2.14 \times 10^{-5}$	$1.79 \times 10^{-2}$	
<b>DIO3OS</b>	<i>DIO3 opposite strand/antisense RNA</i>	0.68	$3.35 \times 10^{-6}$	$3.47 \times 10^{-3}$	

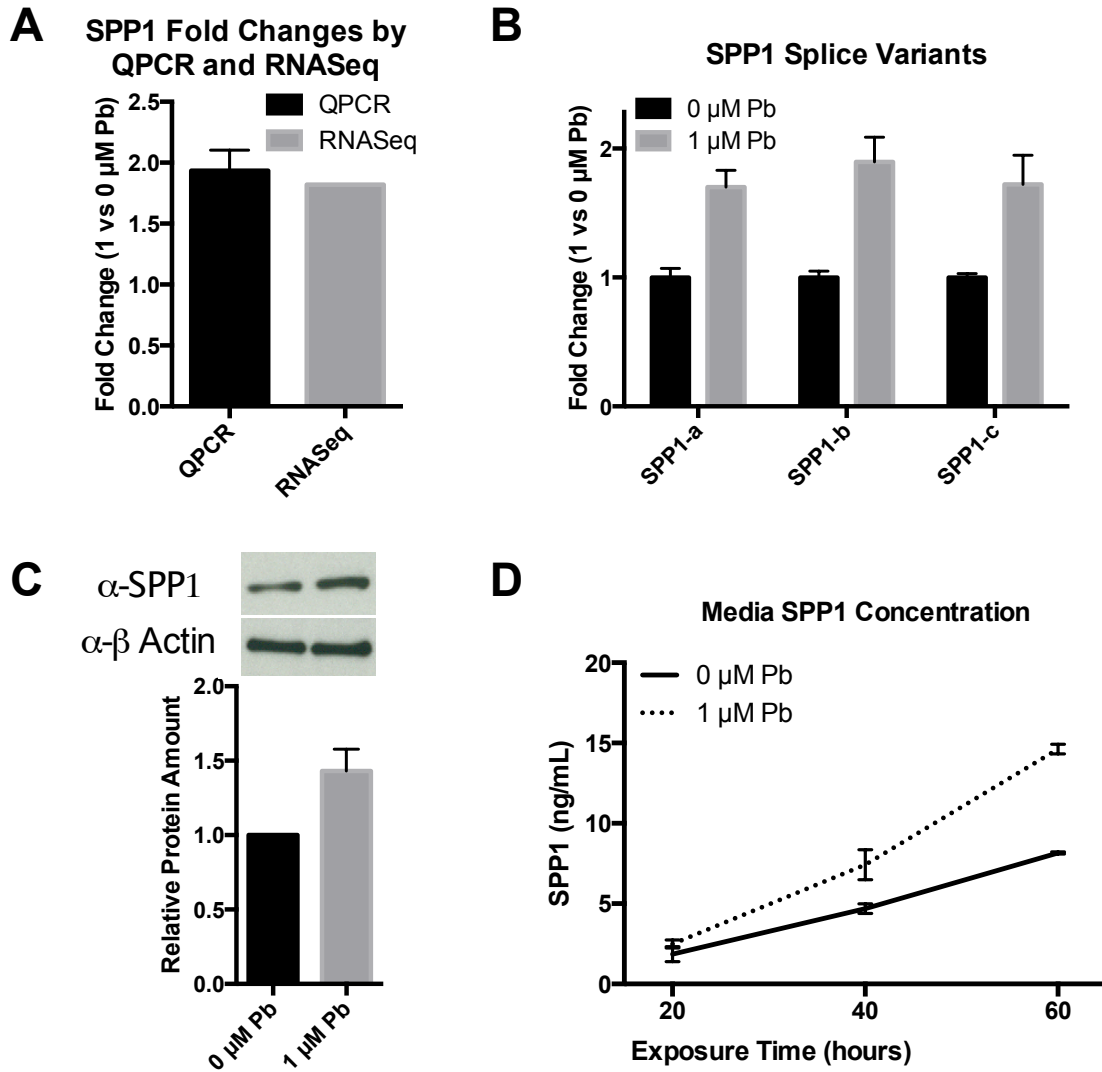
2.2). The expression changes of many of these genes induced by Pb were confirmed by qPCR (Figure 2.1D). Importantly, gene expression changes by RNA sequencing analyses were confirmed by almost identical results from qPCR. The induction of *NQO1*, *HMOX1* and the other known NRF2 target genes strongly suggests that Pb elicits oxidative stress and activates NRF2 in NSCs.

#### *Pb Induces SPP1 Expression*

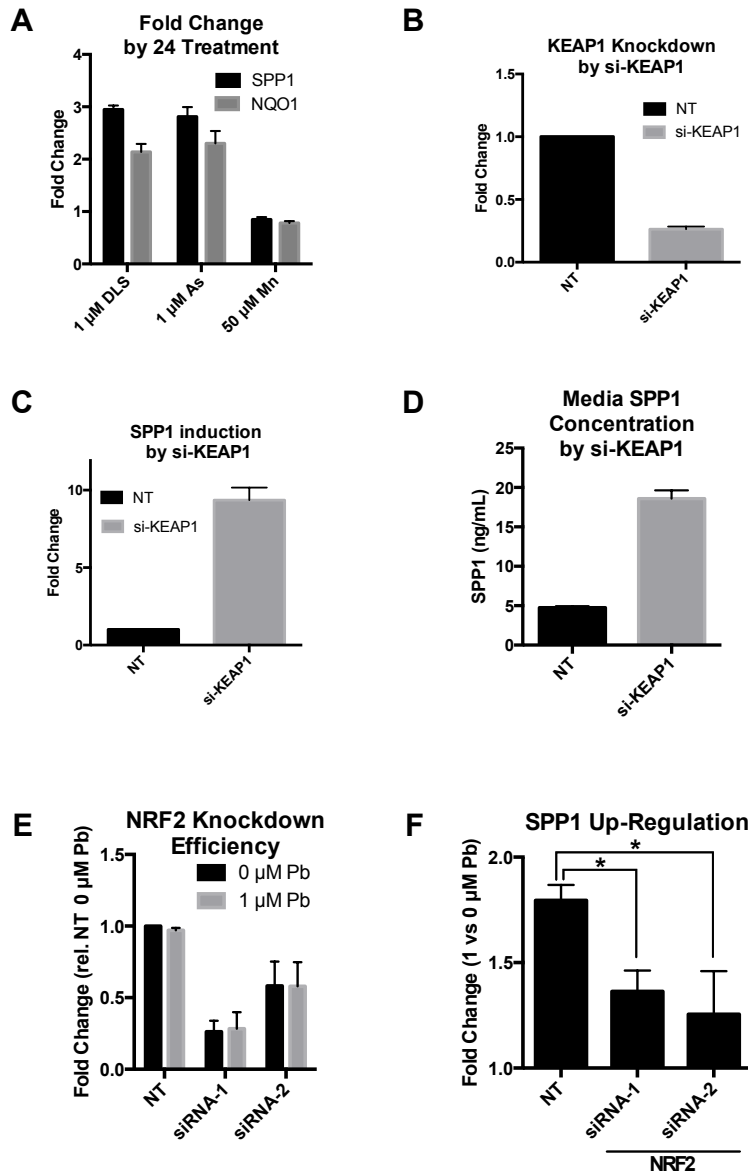
*SPP1* is among the top five Pb-upregulated genes and is one of the few not previously identified as a target of NRF2 (Figure 2.1C and Table 2.2). *SPP1*, also known as Osteopontin (OPN), is an extracellular matrix protein first described as an organic component of bone (Kiefer et al., 1989). However, *SPP1* is pleiotropic and is emerging as a potent neuroprotectant. To look into its role in Pb neurotoxicity, it was chosen for further study. Primers common to all *SPP1* splice variants in QPCR showed an almost identical upregulation after 24 hours of Pb exposure (1.93-fold) when compared to the RNA sequencing-derived value (1.82-fold, Figure 2.2A). The *SPP1* gene has three splice variants, all of which are upregulated upon Pb exposure (Figure 2.2B), indicating that the mechanism involved in *SPP1* upregulation by Pb is not splice variant specific. Western blotting showed that *SPP1* is also upregulated at the protein level in whole cell extracts of Pb-treated NSCs (Figure 2.2C). Since *SPP1* is secreted protein, we also examined the amount of *SPP1* in the cell culture medium of exposed cells (Figure 2.2D). As shown in Figure 2.2D, the concentration of *SPP1* in the media of NSCs cell culture increased in a time-dependent manner following Pb exposure. At 60 hours of exposure, the level of *SPP1* in cell culture media of Pb-exposed cells was 1.79-fold higher.

#### *SPP1 is upregulated via an NRF2-mediated Mechanism*

NRF2 is normally sequestered and degraded in the cytoplasm by its negative regulator KEAP1. NRF2 dissociates from KEAP1 in periods of oxidative stress, in what is called NRF2 activation. Upon activation, NRF2 accumulates in the cytoplasm and translocates to the nucleus. To test whether *SPP1* is upregulated in an NRF2-dependent manner, we exposed cells to the canonical NRF2 activator DL-Sulforaphane. 1  $\mu$ M DL-Sulforaphane induced *SPP1* expression by 2.9-fold (Figure 2.3A). *SPP1* was also induced by arsenic, known to cause oxidative stress (Figure 2.3A). *SPP1* was not induced by Manganese, but lack of *NQO1* upregulation indicates a potential lack of Manganese-induced oxidative stress in NSCs



**Figure 2.2 All *SPP1* splice variants are upregulated following Pb exposure and upregulation is confirmed at the protein level.** (A) Primers common to all *SPP1* isoforms reveal a close match between RNA sequencing-derived fold changes and those from qPCR. (B) All three major splice variants of *SPP1* are upregulated after 24 hours exposure using splice-variant specific primers. (C) Western Blot in whole cell extract shows a normalized relative SPP1 protein amount of 1.41x in cells exposed to 1  $\mu\text{M}$  Pb compared to control after 24 hours. (D) Cell culture media concentration of SPP1 determined by ELISA in cells exposed for 20, 40 and 60 hours to Pb and vehicle H<sub>2</sub>O. All error bars represent the standard error of the mean of three biologic replicates.



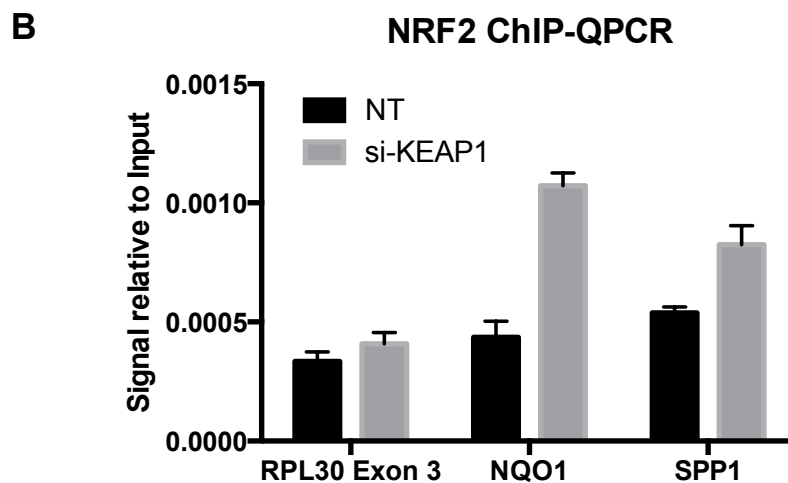
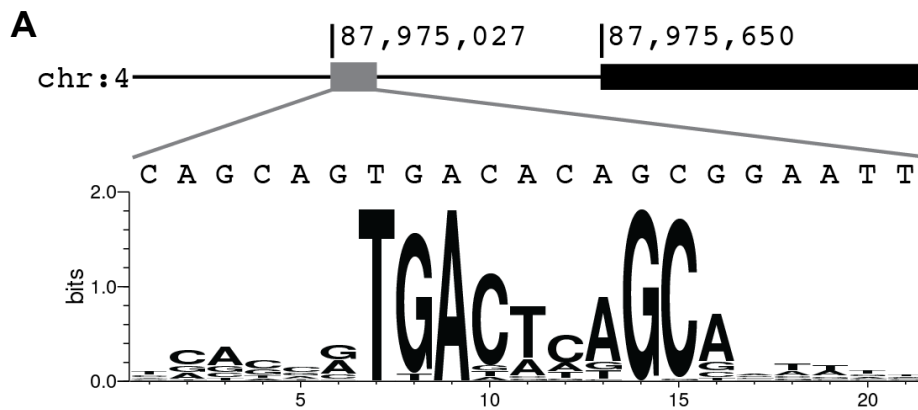
**Figure 2.3 SPP1 is upregulated in an NRF2-dependent mechanism.** (A) *SPP1* and *NQO1* expression fold changes in cells exposed to canonical NRF2 inducer 1  $\mu$ M DL-Sulforaphane (DLS), 1  $\mu$ M Arsenic (As) and 50  $\mu$ M Manganese (Mn) for 24 hours. *SPP1* expression changes mirror those of bona fide NRF2 target gene *NQO1* expression, suggesting common regulation. (B) Efficiency of siRNA knockdown of NRF2's negative regulator KEAP1 48 hours post transfection is 73.7% as assessed by qPCR. (C) Transcriptional *SPP1* upregulation following siRNA-mediated knockdown of KEAP1 at 48 hours post transfection is 9.6-fold. (D) Cell culture media concentration of *SPP1* was 3.9-fold higher at 60 hours post transfection, after 48 hours of contact with the cells (fresh media is added after a 12 hour si-KEAP1 transfection.) (E) Efficiency of siRNA knockdown of NRF2 using two siRNAs 48 hours post transfection. Pb and vehicle are added to cells 24 hours post transfection for a 24-hour exposure. NRF2 knockdown no affected by Pb exposure. (F) *SPP1* upregulation after 24 hours of Pb exposure in si-NRF2 knockdown cells is attenuated compared to NT-siRNA transfected cells. All figure error bars represent standard error of the mean of three biologic replicates.

(Figure 2.3A). The expression trends of *SPP1* among these three exposures mirrored those of the known NRF2 target gene *NQO1* (Figure 2.3A). NRF2 can also be activated by siRNA-mediated knockdown of its negative regulator KEAP1. The qPCR-assessed 73.7% KEAP1 knockdown (Figure 2.3 B) led to a *SPP1* transcriptomic change of 9.6-fold and a secreted protein change of 3.9-fold (Figure 2.3C and D). Along with the chemical exposures, these results show that activation of NRF2 lead to increased *SPP1* transcription and protein expression.

We next tested whether NRF2 is required for *SPP1* upregulation by knocking down cellular NRF2 and subsequently exposing NSCs to Pb. While some evidence suggests NRF2 is positively self-regulating (Jain et al., 2010), Pb exposure itself did not affect either the baseline NRF2 expression or the siRNA-mediated knockdown efficiency (Figure 2.3E). Knockdown of NRF2 significantly attenuates *SPP1* upregulation using two NRF2-targeting siRNAs (Figure 2.3F). The effect is likely specific as two different NRF2 siRNAs produced similar attenuation on *SPP1* upregulation. The attenuation of the response following siRNA-mediated knockdown is evidence that NRF2 is required for upregulation of *SPP1* following 24 hours of Pb exposure.

#### *SPP1 is a direct target of NRF2*

NRF2 controls its target gene expression by binding to the specific DNA sequences known as Antioxidant Response Elements (AREs) within the promoters of target genes. Analyses of AREs in the canonical NRF2 target gene promoters have identified a consensus sequence motif of 5'-RTKAYnnnGCR-3' that is required for NRF2 binding (Erickson et al., 2002). Using an 21-base pair ARE position weight matrix (Wang et al., 2007), we examine the promoter of *SPP1* and identified a putative ARE with the sequence of CAGCAGTGACACAGCGGAATT starting 623 bases upstream of the *SPP1* transcription start site (Figure 2.4A). The putative ARE has a score of 15.5, which can be compared to known ARE scores that have an average of 15.1 and a standard deviation of 5.0 according to data from Wang et al. (2007). To test whether NRF2 interacts with the putative *SPP1* ARE, we performed a ChIP (chromatin immunoprecipitation) assay. The NRF2 protein is immunoprecipitated and bound DNA fragments containing the *SPP1* ARE are probed using PCR. Because baseline NRF2 is usually low in unstimulated cells, we used KEAP1 knockdown NSCs to boost the NRF2 signal and compared the si-KEAP1 treated cells to NT-treated



**Figure 2.4 NRF2 binds directly to an ARE in the *SPP1* promoter.** (A) A putative high scoring ARE beginning 623 base pairs upstream of *SPP1* transcription start site was found using a predictive ARE position weight matrix adapted here from Wang et al. (2007). The ARE is on chromosome 4 at base pair position 87,975,027 and the *SPP1* transcription start site is 87,975,650. Positions are taken from GRCh37/hg19. (B) Chromatin Immunoprecipitation (ChIP) of NRF2 followed by PCR amplification validates the bioinformatically-identified ARE upstream of *SPP1*. NRF2 was activated in cells by KEAP1 knockdown and NT-siRNA was transfected into control cells. Upon NRF2 activation, increased binding is seen at the validated *NQO1* ARE included here as a positive control and at the *SPP1* ARE. No change in NRF2 binding is observed at *RPL30 Exon 3*, included here as a negative control.

cells. As shown in Figure 2.4B, a significant increase in the PCR signal specific to the *SPP1* ARE is observed in si-KEAP1 immunoprecipitates compared to control NT-treated immunoprecipitates. A similar increase was also observed for a canonical ARE located upstream of *NQO1*, which is included as a positive control. No significant increase is observed at a non-NRF2 target sequence, *RPL30-exon 3*, upon NRF2 activation by KEAP1 knockdown, which indicates the NRF2-ChIP-qPCR assay is specific.

## 2.5. Discussion

RNA sequencing of NSCs exposed to a physiologic level of Pb reveals upregulation of many NRF2 hits. One of the top genes, *SPP1*, was not previously shown to be a NRF2 target. We show that activation of NRF2 leads to *SPP1* upregulation and that Pb-induced *SPP1* upregulation is NRF2-dependent. Using a ChIP-PCR assay, we also show that *SPP1* is a direct target of NRF2. *SPP1* is, in fact, the only known secreted protein whose gene is targeted by NRF2. NRF2 activation by Pb is cell type dependent (Simmons et al., 2011), and further study is needed to determine the exact mechanisms of Pb's activation of NRF2. Our study supports many reports of NRF2 activation and upregulation of NRF2 targets by Pb (Simmons et al., 2011, Korashy and El-Kadi, 2006, Zeller et al., 2010, Yang et al., 2007). Notably, however, some transcription profiling of Pb-exposed animals (Peterson et al., 2011, Schneider et al., 2012) do not include several NRF2 targets among the top hits. This is likely due to the numerous secondary effects of Pb in animals undergoing long-term exposure. These secondary effects may crowd out the primary cellular transcriptional response. Furthermore, compensatory regulation of the NRF2 pathway may bring down the level of NRF2 activation upon long-term exposure.

*SPP1* is a pleiotropic extracellular glycoprotein with emerging roles as a potent neuroprotectant. *SPP1* in the brain is upregulated in several morphological stress conditions including hypoxic ischemia (Albertsson et al., 2014, Chen et al., 2009, Meller et al., 2005), cortical lesion (Chan et al., 2014) and subarchnoid hemorrhage (Topkoku et al., 2013). Environmental exposures also induce *SPP1* in a variety of tissues, including cigarette smoke (Shan et al., 2012) and ozone (Bass et al., 2013) in the lung, perinatal polybrominated diphenyl ether DE-71 exposure in offspring gonads (Blake et al., 2011) and ethanol in human primary neurospheres in culture (Vangipuram et al., 2008). Stimuli known to regulate

*SPP1* expression include pro-inflammatory mediators, hormones, growth factors and mechanical stressors reviewed in Mazzali et al. (2002). NRF2 activation is consistent with many of these conditions and exposures. However, it is important to note that transcription factor binding sites for AP1 and NF- $\kappa$ B have also been validated in the promoter of *SPP1* (Zhao et al., 2011), which are also consistent with upregulation upon many stress conditions. *SPP1* appears to be a hub for a number of conditions and understanding its effect on the developing brain is important to understand its putative roles in modulating Pb neurotoxicity and other stress responses.

*SPP1* has intracellular and extracellular forms, but is better studied in its extracellular form. *SPP1* through binding with the Integrin  $\beta$ 3 receptor leads to Focal Adhesion Kinase (FAK) phosphorylation, which has many downstream effects such as phosphorylation of Protein Kinase B (Akt) (Kalluri and Dempsey, 2012, Fong et al., 2009, Meller et al., 2005, Topkuru et al., 2013) and the Mitogen-activated protein kinases (MAPK) Extracellular Receptor Kinase 1 and 2 (ERK1/2) (Chen et al., 2009). While *SPP1* has not been previously shown to be upregulated by Pb, gene networks related to MAPKs, extra-cellular matrix receptor and focal adhesion are enriched in a microarray study of perinatally exposed mice (Kasten-Jolly et al., 2012). Enrichment of these networks is consistent with the involvement of Pb in the *SPP1*-FAK signaling axes. In an animal model of hypoxic ischemia, *SPP1* treatment through Akt signaling decreased apoptosis via a decrease cleaved caspase 3, and improved animals' swim distances compared to control animals following injury (Meller et al., 2005). In subarchnoid hemorrhage, intranasal *SPP1* treatment also lowers apoptosis through the same FAK-Akt axis resulting in improved neurological scores and smaller edema size (Topkuru et al., 2013). Vasospasm, which is a common complication of subarchnoid hemorrhage thought to be responsible for much of the condition's associated morbidity and mortality, is also avoided following *SPP1* treatment (He et al., 2015). Following unilateral entorhinal cortical lesion, *SPP1* is a pro-synaptogenic factor in the recovery phase and *SPP1* knockout animals show delayed cognitive recovery as measured by a novel object recognition test (Chan et al., 2014).

In rat neural progenitor cells, exogenous recombinant *SPP1* increases proliferation via Akt signaling (Kalluri and Dempsey, 2012). *SPP1*-coated plates support neurite growth *in vitro* via integrin signaling, leading to an even higher number of primary neurites than standard laminin coating (Plantman,



2012). Brain SPP1 protein levels are high in newborn animals and fall post-natally in mice and rats. From post natal day 3 to 60, there is a decrease of approximately 65% in whole brain SPP1 levels in mice (Albertsson et al., 2014). Similarly, in rats SPP1 decreases from birth to post natal day 21 by approximately 95% (Chen et al., 2009). Therefore, *SPP1* may have a role in the developing brain in addition to its roles in stress responses.

The data supporting SPP1 as a neuroprotective factor in stress conditions is consistent with the paradigm of NRF2 activation as a cytoprotective response to oxidative stress. SPP1 is the only secreted protein identified as a target of NRF2, and is a possible mechanism of cell-to-cell communication of oxidative stress conditions. Activation of the Akt and MAPK-ERK1/2 pathways would lead to compensatory growth and survival in tissues affected by oxidative stress. However, the benefits of SPP1 signaling are not without exceptions. In conflict with other studies on the subject, one report shows no benefit in a mouse preterm brain injury model with recombinant SPP1 treatment and more severe injury using thrombin-cleaved SPP1 (Albertsson et al., 2014). In other settings, the pro-survival signaling leads to greater morbidity. For instance, SPP1 enhances survival of activated T cells, which induces relapse and progression of multiple sclerosis lesions (Hur et al., 2007). SPP1 is also upregulated and associated with poor prognosis in many cancers. For instance, SPP1 levels are correlated with poor prognosis among glioma patients (Zhao et al., 2015).

The implications of NRF2 targeting of SPP1 are of broader interest than Pb exposure, since the NRF2 response is central to many environmental stressors, neurodegenerative diseases and cancers. For instance, in Figure 2.3A we show arsenic exposure upregulates SPP1 in a manner consistent with NRF2 activation. How the SPP1 protein and its downstream signaling contribute to the progression or recovery of these insults will be important to understanding the potential role of SPP1, NRF2 and antioxidants in therapies for exposed individuals.

## **2.6. Acknowledgements**

We would like to thank Rory Kirchner in the Center for Health Bioinformatics of Harvard T.H. Chan School of Public Health for all his work and advice in handling the RNA sequencing data set. Peter Wagner was

supported by the Joseph D. Brain and Jere Meade Fellowship Funds as well as the Training In Interdisciplinary Pulmonary Sciences Grant (NIH 5T32HL007118). Project was supported by the Harvard NIEHS center (ES000002), the Harvard Superfund Research Program (P42ES16454) and the NIEHS RO1 (RO1022230).

## 2.7. References

- ALBERTSSON, A. M., ZHANG, X., LEAVENWORTH, J., BI, D., NAIR, S., QIAO, L., HAGBERG, H., MALLARD, C., CANTOR, H. & WANG, X. 2014. The effect of osteopontin and osteopontin-derived peptides on preterm brain injury. *J Neuroinflammation*, 11, 197.
- BASS, V., GORDON, C. J., JAREMA, K. A., MACPHAIL, R. C., CASCIO, W. E., PHILLIPS, P. M., LEDBETTER, A. D., SCHLADWEILER, M. C., ANDREWS, D., MILLER, D., DOERFLER, D. L. & KODAVANTI, U. P. 2013. Ozone induces glucose intolerance and systemic metabolic effects in young and aged Brown Norway rats. *Toxicol Appl Pharmacol*, 273, 551-60.
- BLAKE, C. A., MCCOY, G. L., HUI, Y. Y. & LAVOIE, H. A. 2011. Perinatal exposure to low-dose DE-71 increases serum thyroid hormones and gonadal osteopontin gene expression. *Exp Biol Med (Maywood)*, 236, 445-55.
- BREIER, J. M., RADIO, N. M., MUNDY, W. R. & SHAFER, T. J. 2008. Development of a high-throughput screening assay for chemical effects on proliferation and viability of immortalized human neural progenitor cells. *Toxicological Sciences*, 105, 119-33.
- CHAN, J. L., REEVES, T. M. & PHILLIPS, L. L. 2014. Osteopontin expression in acute immune response mediates hippocampal synaptogenesis and adaptive outcome following cortical brain injury. *Exp Neurol*, 261, 757-71.
- CHEN, Y. J., WEI, Y. Y., CHEN, H. T., FONG, Y. C., HSU, C. J., TSAI, C. H., HSU, H. C., LIU, S. H. & TANG, C. H. 2009. Osteopontin increases migration and MMP-9 upregulation via  $\alpha$ 3 $\beta$ 1 integrin, FAK, ERK, and NF- $\kappa$ B-dependent pathway in human chondrosarcoma cells. *J Cell Physiol*, 221, 98-108.
- CHO, H. Y., REDDY, S. P., DEBIASE, A., YAMAMOTO, M. & KLEEBERGER, S. R. 2005. Gene expression profiling of NRF2-mediated protection against oxidative injury. *Free Radical Biology and Medicine*, 38, 325-43.
- CHORLEY, B. N., CAMPBELL, M. R., WANG, X., KARACA, M., SAMBANDAN, D., BANGURA, F., XUE, P., PI, J., KLEEBERGER, S. R. & BELL, D. A. 2012. Identification of novel NRF2-regulated genes by ChIP-Seq: influence on retinoid X receptor  $\alpha$ . *Nucleic Acids Res*, 40, 7416-29.
- DIETRICH, K. N., WARE, J. H., SALGANIK, M., RADCLIFFE, J., ROGAN, W. J., RHOADS, G. G., FAY, M. E., DAVOLI, C. T., DENCKLA, M. B., BORNSCHEIN, R. L., SCHWARZ, D., DOCKERY, D. W., ADUBATO, S. & JONES, R. L. 2004. Effect of chelation therapy on the neuropsychological and behavioral development of Pb-exposed children after school entry. *Pediatrics*, 114, 19-26.
- ERICKSON, A. M., NEVAREA, Z., GIPP, J. J. & MULCAHY, R. T. 2002. Identification of a variant antioxidant response element in the promoter of the human glutamate-cysteine ligase modifier subunit gene. Revision of the ARE consensus sequence. *J Biol Chem*, 277, 30730-7.

- FONG, Y. C., LIU, S. C., HUANG, C. Y., LI, T. M., HSU, S. F., KAO, S. T., TSAI, F. J., CHEN, W. C., CHEN, C. Y. & TANG, C. H. 2009. Osteopontin increases lung cancer cells migration via activation of the alphavbeta3 integrin/FAK/Akt and NF-kappaB-dependent pathway. *Lung Cancer*, 64, 263-70.
- GILBERT, M. E., KELLY, M. E., SAMSAM, T. E. & GOODMAN, J. H. 2005. Chronic developmental Pb exposure reduces neurogenesis in adult rat hippocampus but does not impair spatial learning. *Toxicol Sci*, 86, 365-74.
- HE, J., LIU, M., LIU, Z. & LUO, L. 2015. Recombinant osteopontin attenuates experimental cerebral vasospasm following subarachnoid hemorrhage in rats through an anti-apoptotic mechanism. *Brain Res*.
- HU, H., TELLEZ-ROJO, M. M., BELLINGER, D., SMITH, D., ETTINGER, A. S., LAMADRID-FIGUEROA, H., SCHWARTZ, J., SCHNAAS, L., MERCADO-GARCIA, A. & HERNANDEZ-AVILA, M. 2006. Fetal Pb exposure at each stage of pregnancy as a predictor of infant mental development. *Environmental Health Perspectives*, 114, 1730-5.
- HUANG, F. & SCHNEIDER, J. S. 2004. Effects of Pb exposure on proliferation and differentiation of neural stem cells derived from different regions of embryonic rat brain. *Neurotoxicology*, 25, 1001-12.
- HUR, E. M., YOUSSEF, S., HAWS, M. E., ZHANG, S. Y., SOBEL, R. A. & STEINMAN, L. 2007. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat Immunol*, 8, 74-83.
- JAIN, A., LAMARK, T., SJOTTEM, E., LARSEN, K. B., AWUH, J. A., OVERVATN, A., MCMAHON, M., HAYES, J. D. & JOHANSEN, T. 2010. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J Biol Chem*, 285, 22576-91.
- KALLURI, H. S. & DEMPSEY, R. J. 2012. Osteopontin increases the proliferation of neural progenitor cells. *Int J Dev Neurosci*, 30, 359-62.
- KASTEN-JOLLY, J., PABELLO, N., BOLIVAR, V. J. & LAWRENCE, D. A. 2012. Developmental Pb effects on behavior and brain gene expression in male and female BALB/cAnNTac mice. *Neurotoxicology*, 33, 1005-20.
- KIEFER, M. C., BAUER, D. M. & BARR, P. J. 1989. The cDNA and derived amino acid sequence for human osteopontin. *Nucleic Acids Res*, 17, 3306.
- KORASHY, H. M. & EL-KADI, A. O. 2006. Transcriptional regulation of the NAD(P)H:quinone oxidoreductase 1 and glutathione S-transferase ya genes by mercury, Pb, and copper. *Drug Metab Dispos*, 34, 152-65.
- LEE, J. M., CALKINS, M. J., CHAN, K., KAN, Y. W. & JOHNSON, J. A. 2003. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. *Journal of Biological Chemistry*, 278, 12029-38.
- LI, J., LEE, J. M. & JOHNSON, J. A. 2002. Microarray analysis reveals an antioxidant responsive element-driven gene set involved in conferring protection from an oxidative stress-induced apoptosis in IMR-32 cells. *Journal of Biological Chemistry*, 277, 388-94.
- MALHOTRA, D., PORTALES-CASAMAR, E., SINGH, A., SRIVASTAVA, S., ARENILLAS, D., HAPPEL, C., SHYR, C., WAKABAYASHI, N., KENSLER, T. W., WASSERMAN, W. W. & BISWAL, S. 2010. Global mapping

of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Res*, 38, 5718-34.

MARTIN, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet*, 17, 10-12.

MAZZALI, M., KIPARI, T., OPHASCHAROENSUK, V., WESSON, J. A., JOHNSON, R. & HUGHES, J. 2002. Osteopontin--a molecule for all seasons. *QJM*, 95, 3-13.

MELLER, R., STEVENS, S. L., MINAMI, M., CAMERON, J. A., KING, S., ROSENZWEIG, H., DOYLE, K., LESSOV, N. S., SIMON, R. P. & STENZEL-POORE, M. P. 2005. Neuroprotection by osteopontin in stroke. *J Cereb Blood Flow Metab*, 25, 217-25.

MIRZA, M., SHAUGHNESSY, E., HURLEY, J. K., VANPATTEN, K. A., PESTANO, G. A., HE, B. & WEBER, G. F. 2008. Osteopontin-c is a selective marker of breast cancer. *Int J Cancer*, 122, 889-97.

OPLER, M. G., BROWN, A. S., GRAZIANO, J., DESAI, M., ZHENG, W., SCHAEFER, C., FACTOR-LITVAK, P. & SUSSER, E. S. 2004. Prenatal Pb exposure, delta-aminolevulinic acid, and schizophrenia. *Environmental Health Perspectives*, 112, 548-52.

OPLER, M. G., BUKA, S. L., GROEGER, J., MCKEAGUE, I., WEI, C., FACTOR-LITVAK, P., BRESNAHAN, M., GRAZIANO, J., GOLDSTEIN, J. M., SEIDMAN, L. J., BROWN, A. S. & SUSSER, E. S. 2008. Prenatal exposure to Pb, delta-aminolevulinic acid, and schizophrenia: further evidence. *Environmental Health Perspectives*, 116, 1586-90.

PAGES, H., ABOYOUN, P., GENTLEMAN, R. & DEBROY, S. Biostrings: String objects representing biological sequences, and matching algorithms. R package version 2.34.1. ed.

PETERSON, S. M., ZHANG, J., WEBER, G. & FREEMAN, J. L. 2011. Global gene expression analysis reveals dynamic and developmental stage-dependent enrichment of Pb-induced neurological gene alterations. *Environmental Health Perspectives*, 119, 615-21.

PIRKLE, J. L., BRODY, D. J., GUNTER, E. W., KRAMER, R. A., PASCHAL, D. C., FLEGAL, K. M. & MATTE, T. D. 1994. The decline in blood Pb levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). *JAMA*, 272, 284-91.

PLANTMAN, S. 2012. Osteopontin is upregulated after mechanical brain injury and stimulates neurite growth from hippocampal neurons through beta1 integrin and CD44. *Neuroreport*, 23, 647-52.

ROBINSON, M. D., MCCARTHY, D. J. & SMYTH, G. K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26, 139-40.

ROGAN, W. J., DIETRICH, K. N., WARE, J. H., DOCKERY, D. W., SALGANIK, M., RADCLIFFE, J., JONES, R. L., RAGAN, N. B., CHISOLM, J. J., JR. & RHOADS, G. G. 2001. The effect of chelation therapy with succimer on neuropsychological development in children exposed to Pb. *New England Journal of Medicine*, 344, 1421-6.

SCHNEIDER, J. S., ANDERSON, D. W., TALSANIA, K., METTIL, W. & VADIGEPALLI, R. 2012. Effects of developmental Pb exposure on the hippocampal transcriptome: influences of sex, developmental period, and Pb exposure level. *Toxicological Sciences*, 129, 108-25.

- SCHNEIDER, J. S., ANDERSON, D. W., WADE, T. V., SMITH, M. G., LEIBRANDT, P., ZUCK, L. & LIDSKY, T. I. 2005. Inhibition of progenitor cell proliferation in the dentate gyrus of rats following post-weaning Pb exposure. *Neurotoxicology*, 26, 141-5.
- SHAN, M., YUAN, X., SONG, L. Z., ROBERTS, L., ZARINKAMAR, N., SERYSHEV, A., ZHANG, Y., HILSENBECK, S., CHANG, S. H., DONG, C., CORRY, D. B. & KHERADMAND, F. 2012. Cigarette smoke induction of osteopontin (SPP1) mediates T(H)17 inflammation in human and experimental emphysema. *Sci Transl Med*, 4, 117ra9.
- SIMMONS, S. O., FAN, C. Y., YEOMAN, K., WAKEFIELD, J. & RAMABHADRAN, R. 2011. NRF2 Oxidative Stress Induced by Heavy Metals is Cell Type Dependent. *Curr Chem Genomics*, 5, 1-12.
- THAI, P., STATT, S., CHEN, C. H., LIANG, E., CAMPBELL, C. & WU, R. 2013. Characterization of a novel long noncoding RNA, SCAL1, induced by cigarette smoke and elevated in lung cancer cell lines. *Am J Respir Cell Mol Biol*, 49, 204-11.
- TOPKORU, B. C., ALTAY, O., DURIS, K., KRAFFT, P. R., YAN, J. & ZHANG, J. H. 2013. Nasal administration of recombinant osteopontin attenuates early brain injury after subarachnoid hemorrhage. *Stroke*, 44, 3189-94.
- TRAPNELL, C., PACHTER, L. & SALZBERG, S. L. 2009. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics*, 25, 1105-11.
- VANGIPURAM, S. D., GREVER, W. E., PARKER, G. C. & LYMAN, W. D. 2008. Ethanol increases fetal human neurosphere size and alters adhesion molecule gene expression. *Alcohol Clin Exp Res*, 32, 339-47.
- VERINA, T., ROHDE, C. A. & GUILARTE, T. R. 2007. Environmental Pb exposure during early life alters granule cell neurogenesis and morphology in the hippocampus of young adult rats. *Neuroscience*, 145, 1037-47.
- WANG, L., WANG, S. & LI, W. 2012. RSeQC: quality control of RNA-seq experiments. *Bioinformatics*, 28, 2184-5.
- WANG, X., TOMSO, D. J., CHORLEY, B. N., CHO, H. Y., CHEUNG, V. G., KLEEBERGER, S. R. & BELL, D. A. 2007. Identification of polymorphic antioxidant response elements in the human genome. *Human Molecular Genetics*, 16, 1188-200.
- WRIGHT, J. P., DIETRICH, K. N., RIS, M. D., HORNUNG, R. W., WESSEL, S. D., LANPHEAR, B. P., HO, M. & RAE, M. N. 2008. Association of prenatal and childhood blood Pb concentrations with criminal arrests in early adulthood. *PLoS Med*, 5, e101.
- YANG, L., KEMADJOU, J. R., ZINSMEISTER, C., BAUER, M., LEGRADI, J., MULLER, F., PANKRATZ, M., JAKEL, J. & STRAHLE, U. 2007. Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo. *Genome Biol*, 8, R227.
- ZELLER, I., KNOFLACH, M., SEUBERT, A., KREUTMAYER, S. B., STELZMULLER, M. E., WALLNOEFER, E., BLUNDER, S., FROTSCHNIG, S., MESSNER, B., WILLEIT, J., DEBBAGE, P., WICK, G., KIECHL, S., LAUFER, G. & BERNHARD, D. 2010. Pb contributes to arterial intimal hyperplasia through nuclear factor erythroid 2-related factor-mediated endothelial interleukin 8 synthesis and subsequent invasion of smooth muscle cells. *Arterioscler Thromb Vasc Biol*, 30, 1733-40.
- ZHAO, M., XU, H., LIANG, F., HE, J. & ZHANG, J. 2015. Association of osteopontin expression with the prognosis of glioma patient: a meta-analysis. *Tumour Biol*, 36, 429-36.

ZHAO, W., WANG, L., ZHANG, M., WANG, P., ZHANG, L., YUAN, C., QI, J., QIAO, Y., KUO, P. C. & GAO, C. 2011. NF-kappaB- and AP-1-mediated DNA looping regulates osteopontin transcription in endotoxin-stimulated murine macrophages. *J Immunol*, 186, 3173-9.

ZHENG, L., WU, K., LI, Y., QI, Z., HAN, D., ZHANG, B., GU, C., CHEN, G., LIU, J., CHEN, S., XU, X. & HUO, X. 2008. Blood Pb and cadmium levels and relevant factors among children from an e-waste recycling town in China. *Environmental Research*, 108, 15-20.

## **CHAPTER 3: Association of Secreted Phosphoprotein 1 (*SPP1*) SNPs with Mental and Psychomotor Development in a Lead-Exposed Population**

This work is currently unpublished.

Peter Wagner performed all analyses for Tables 3.1 and 3.2, and Figure 3.1

Cohort was collected and genotyped by ELEMENT Investigators Robert Wright, David Christiani, Mauricio Hernandez and Hakon Hakonarson

Laboratory quality control performed by Li Su and Yongyue Wei

Data quality control and principle component analysis Xihong Lin's research group

Lead measurements performed by Trace Metals Laboratory at Harvard T.H. Chan School of Public Health

### 3.1. Abstract

Lead (Pb) has an early critical period of toxicity and emerging evidence shows that prenatal Pb exposure has disproportional and lasting effects. Through the second trimester in pregnancy, neural stem cells (NSCs) grow and differentiate into the terminal cell types of the central nervous system. In the previous Chapter, we identified Secreted Phosphoprotein 1 (*SPP1*) as a novel NRF2 target gene upregulated in NSCs upon exposure to Pb. Here we evaluate the importance of *SPP1* in a prospective birth cohort by analyzing *SPP1*-associated SNPs in models of main effect and interaction with second trimester maternal blood Pb. In total 16 SNPs were genotyped in the *SPP1* transcribed locus or its 10kb flanking regions. The two neurological phenotypes used as outcomes were the Mental Development Index (MDI) and the Psychomotor Development Index (PDI) of the Bayley Scales of Infant Development. No statistically significant association was observed for *SPP1* SNPs with MDI, but a preponderance of SNPs closely approaching significance suggests a possible association. In the PDI analysis, one statistically significant interaction was found with SNP rs4693923 and second trimester maternal blood Pb ( $p=0.004$ ,  $p$ -value cut off 0.00568). As such rs4693923 is an effect modifier of Pb with A-allele carriers experiencing dose-dependent increases in PDI with Pb exposure. Therefore, these epidemiological data support the RNA sequencing data from the previous chapter, identifying a critical role for *SPP1* in modulating prenatal Pb neurotoxicity.



### 3.2. Introduction

In the previous chapter, Secreted Phosphoprotein 1 (*SPP1*) was identified as a novel NRF2 target gene upregulated in human neural stem cells following Pb exposure. NRF2 activation and the upregulation of its target gene products are cytoprotective responses, which help cells detoxify exposures and recover from their secondary effects. As the only known NRF2-targeted secreted protein, the role of *SPP1* in the NRF2 cytoprotective paradigm is interesting and complex. *SPP1* is an extracellular matrix protein with putative signaling properties by binding  $\beta$ 3-integrin and thereby phosphorylating Focal Adhesion Kinase (FAK). FAK phosphorylation by *SPP1* is shown to increase activation of two major cell growth and survival pathways: Mitogen Activated Protein Kinases (MAPK) (Chen et al., 2009) and Protein Kinase B (Akt) (Kalluri and Dempsey, 2012, Fong et al., 2009). Therefore, NRF2-mediated *SPP1* upregulation and secretion under oxidative stress may be involved in compensatory activation of growth and survival pathways in neighboring cells. To assess if variants in *SPP1* were important for neural development and Pb-affected neural development, we examined *SPP1* SNPs in a prospective birth cohort in outcomes of Mental Development Index (MDI) and Psychomotor Development Index (PDI) of the Bayley Scales of Infant Development.

Evidence of gene variant main effect and gene variant-Pb interactions in describing cognitive outcomes in human populations provides strong evidence for the role of *SPP1* in human neurodevelopment. In addition, such evidence could help explain the observed inter-individual susceptibility to Pb exposure (Bellinger, 2004). Comprehensive understanding of gene-environment interactions in the future could allow for identification of highly susceptible individuals and subpopulations, which would allow for more effective use of limited public health resources.

Several epidemiological studies have identified genetic variants as possible effect modifiers of neurocognitive outcomes related to Pb exposure, including variation in the genes: Delta-Aminolevulinic Acid Dehydratase (*ALAD*) (Bellinger et al., 1994, Pawlas et al., 2012), Vitamin D Receptor (*VDR*) (Krieg et al., 2010, Pawlas et al., 2012), Dopamine receptor D2 (*DRD2*) (Roy et al., 2011), Dopamine receptor D4 (*DRD4*) (Froehlich et al., 2007) and Apolipoprotein E (*APOE*) (Wright et al., 2003). *ALAD* is a main Pb-binding protein in blood, and the polymorphism associated in interaction with Pb exposure is a variant

known to influence blood Pb levels by altering the protein's Pb binding efficiency (Scinicariello et al., 2007). Dopamine receptors D2 and D4 are regulators of the dopamine system and Apolipoprotein E is a fatty acid metabolism regulator. Together these associations indicate a wide variety of pathways implicated in Pb neurotoxicity.

While the case for interaction studies is clear, their implementation is encumbered by statistical power considerations. Generally speaking, a sample size required to detect a multiplicative interaction between two variables is roughly at least four times the size of the sample size needed to detect a main effect of equivalent magnitude in each of the variables (Smith and Day, 1984). Therefore, false-negative findings and replication failure will be a substantial problem in the foreseeable future with epidemiological approaches alone. For instance, while the Pb-ALAD interaction in cognitive outcomes is observed in two papers, it could not be replicated in one large study (n=842) (Krieg et al., 2009). Failure to replicate should not completely annul a previously demonstrated association. In addition to power considerations, differences in study design and populations' genetic linkage structures may be responsible for lack of replication. Because comprehensive and detailed replication of many epidemiological findings is costly and impractical, integration of data across disciplines may be a more fruitful strategy to discover true associations and susceptibility variants.

In Chapter 2 of this thesis, we took a hypothesis-free transcriptomic approach to identify a new potential mediator of Pb neurotoxicity, *SPP1*. In this chapter, we selected SNPs within 10kb of the *SPP1* transcript for main effect and interaction analyses. By identifying a candidate gene prior to analyzing the genome-wide SNP array data, the burden of multiple testing on the small subset of SNPs is greatly reduced and the likelihood of a true positive signal in a cohort of limited size is enhanced.

### **3.3. Materials and Methods**

#### *ELEMENT Cohort Collection*

The cohort of infants analyzed in this study is a subset of the Early Life Exposures in MEXico and NeuroToxicology (ELEMENT) prospective birth cohort, which was designed to assess the roles of environmental and social stressors in birth outcomes as well as infant and child development. Between

2007 and 2011, mothers were recruited during pregnancy and only one child for each mother was included in the study. Detailed information on the study design and data collection procedures has been published previously (Gonzalez-Cossio et al., 1997, Ettinger et al., 2009, Hernandez-Avila et al., 2002). Briefly, healthy pregnant women between 12 and 24 weeks' gestation living in Mexico City were enrolled at prenatal clinics and maternity hospitals affiliated with the Instituto Mexicano del Seguro Social, the Mexican social security system. The Instituto Mexicano del Seguro Social provides healthcare to 34.3% of Mexico City residents (according to 2010 National Census), predominantly serving middle-class workers and their families. Eligible participants were at least 18 years of age, had telephone access, lived within the Mexico City metropolitan area, and planned to remain in the area for at least 3 years. Mothers past 20 weeks gestation, having a history of heart or kidney disease, consuming alcohol daily and using steroids or anti-epilepsy drugs were excluded from the study.

1,054 women were informed of the study and provided written consent to participate.

Participants gave birth to 948 singleton infants, of which 541 were followed until 2 years of age for neurodevelopmental assessment. Low birth weight infants (<1500 g; n=7), premature births (<32 weeks gestation; n=1) and infants lacking birth outcomes data (n=8) were excluded from the study. The human subjects committees of the National Institutes of Public Health in Mexico, Harvard T.H. Chan School of Public Health, Icahn School of Medicine at Mt. Sinai, and participating hospitals approved all study materials and procedures.

#### *Prenatal Pb Exposure Assessed in Second Trimester Maternal Blood*

In each expecting mother's second trimester, maternal venous blood was collected in trace element-free tubes and frozen. Samples were shipped at 4°C to the Trace Metals Laboratory at the Harvard T.H. Chan School of Public Health, Boston, MA. Samples were processed in a dedicated trace metal clean room outfitted with a Class 100 clean hood and using glassware cleaned for 24 hours in 10% HNO<sub>3</sub> and rinsed several times with 18Ω Milli-Q water. Approximately 1 g of blood from each mother was digested in 2 ml concentrated nitric acid for 24 hours, and subsequently overnight in 30% hydrogen peroxide (1 ml per 1 g of blood). Samples were diluted to 10 ml with deionized water. Acid-digested samples were analyzed for total Pb using dynamic reaction cell-inductively coupled plasma mass

spectrometry (DRC-ICP-MS, Perkin Elmer). Final values are the average of five replicate measurements for each individual sample.

#### *Assessment Using the Bayley Scales of Infant and Toddler Development*

Infant neurodevelopment was assessed at 24 months of age using a Spanish version of the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley, 2005). Two primary outcome indices are derived from the assessment: the Mental Development Index (MDI), which is a composite variable of test scores pertaining to cognition, expressive language, and receptive language; and the Psychomotor Development Index (PDI) score, which is a composite variable of tests pertaining to fine and gross motor skills.

#### *Genotyping of Infants using an Illumina SNP Chip*

Umbilical cord blood samples were collected at time of delivery from infants using an umbilical cord catheter and Paxgene DNA collection tubes. DNA was extracted from whole blood samples using the Puregene DNA isolation kit (Gentra Systems) and stored at -20°C. Samples were genotyped using the high density Illumina 1 Million Duo at the Center for Applied Genomics Core of the Children's Hospital of Philadelphia. Raw genotyping data underwent quality control following standard procedures (Anderson et al., 2010). Samples were excluded in quality control following the criteria: overall genotype completion rates <95%; gender discrepancies; unexpected duplicates or probable relatives (based on pairwise identity by state value, PI\_HAT in PLINK >0.185); or heterozygosity rates >6 times the standard deviation from the mean. SNPs were excluded if they fit the following criteria: a call rate <95% in all samples; an allelic distribution deviating from Hardy-Weinberg equilibrium ( $p < 1.0 \times 10^{-6}$ ); or monoallelic in the cohort. To adjust for population stratification, we used EigenCorr, a principal component (PC) analysis that selects principal components based on both their eigenvalues and their correlation with phenotype (Lee et al., 2011).

#### *SNP Analyses by Main Effect and Interaction*

Following the quality control procedures outlined above, 16 SNPs within the SPP1 transcript locus or within the 10 kilobase flanking regions were available in 462 infants. SNPs were evaluated in linear regression for both main effect and interaction in PLINK. For main effect values, linear regression

analyses of minor allele copy number on MDI and PDI were adjusted for sex, gestational age, maternal age, marital status, presence of siblings, maternal education (high school vs no high school), age at Bayley Scale assessment and genome-wide principle components 1 and 2. For interaction analyses, natural log of second trimester maternal blood Pb and a multiplicative interaction term of minor allele copy number and natural log of second trimester maternal blood Pb were included. The p-value cut off for statistical significance was determined using the method proposed by Li and Ji (2005), which takes into account that each SNP test is not an independent test given the linkage disequilibrium (LD) among neighboring SNPs. A LD map and haplotypes were generated using the genotyped data in LDPlus (Vanderbilt University). In order to better describe the interaction, data were divided into equal tertiles by Pb exposure and two-sided t-tests were used to determine differences between groups. Because of the low minor allele frequency of rs4693923, minor allele homozygotes and heterozygotes are grouped together in data visualization. Smoothed spline functions were fit to the data by genotype to visualize the differences in response by genotype groups in PRISM (GraphPad).

### **3.4. Results**

#### *SPP1 genetic variants nominally associate with Mental Development Index in infants*

SPP1 was found upregulated by Pb exposure in human neural stem cells, which are a major component of the brain through the second trimester. Maternal blood Pb was measured in the second trimester in the ELEMENT cohort, providing a relevant exposure time-point for all downstream analyses. In order to assess how SPP1 may be affecting children exposed to Pb in early life, we performed SNP main effect and SNP by second trimester maternal blood Pb interaction regression analyses. A total of 16 common SNPs (minor allele frequency > 5%) were genotyped in the cohort within the *SPP1* transcript locus or the 10kb flanking regions (relative SNP locations are shown in Figure 3.1A). Due to the LD among the SNPs, the total number of effective tests is 9.0001 and the resulting p-value cut off for this analysis to maintain a 5% Type 1 error rate is 0.00568. The LD correction is done using the method of Li and Ji (2005).

**Table 3.1 Summary of SPP1 SNP association effect sizes and p-values in determining MDI and PDI.** Models were adjusted for sex, gestational age, maternal age, marital status, presence of siblings, maternal education, genome-wide principle components 1 and 2, and log<sub>e</sub> of second trimester maternal blood Pb level. Alleles are given in the format minor allele / major allele. According to the method proposed by Li and Ji (2005), the p-value cut off for this analysis of SNPs in LD to maintain a 5% Type 1 error rate is 0.00568; a single significant result is marked with an asterisk.

rs id	chr4 bp	Alleles	MAF	Mental Development Index (MDI)				Psychomotor Development Index (PDI)			
				Main Effect		SNP*logPb		Main Effect		SNP*logPb	
				β	p-value	β	p-value	β	p-value	β	p-value
<b>rs4693923</b>	88888113	A / G	0.11	0.37	0.698	2.47	0.122	-0.33	0.495	-2.92	0.004*
<b>rs12641001</b>	88888940	T / C	0.10	2.25	0.024	-0.46	0.765	0.40	0.426	1.87	0.070
<b>rs6833161</b>	88889605	T / C	0.32	0.45	0.487	0.78	0.464	0.24	0.452	-0.64	0.366
<b>rs6813526</b>	88894235	C / T	0.13	1.90	0.038	-0.01	0.996	0.32	0.490	1.90	0.049
<b>rs2728127</b>	88895115	G / A	0.14	1.99	0.022	-0.08	0.950	0.57	0.194	1.82	0.046
<b>rs2853744</b>	88896248	T / G	0.07	2.81	0.013	-1.57	0.371	0.45	0.432	2.35	0.048
<b>rs11730582</b>	88896421	T / C	0.46	0.70	0.250	1.18	0.253	-0.03	0.912	-0.99	0.126
<b>rs11728697</b>	88898941	C / T	0.44	0.54	0.374	0.67	0.514	-0.03	0.910	-0.65	0.309
<b>rs6811536</b>	88902405	T / C	0.18	-1.25	0.096	0.05	0.969	-0.33	0.388	0.21	0.795
<b>rs4754</b>	88902692	C / T	0.43	1.27	0.031	-0.60	0.520	0.40	0.177	1.11	0.065
<b>rs1126616</b>	88903853	T / C	0.43	1.26	0.030	-0.71	0.440	0.40	0.177	1.20	0.046
<b>rs1126772</b>	88904186	G / A	0.17	1.34	0.084	-1.81	0.204	0.25	0.527	0.62	0.501
<b>rs9138</b>	88904342	C / A	0.43	1.36	0.022	-0.57	0.544	0.40	0.178	1.11	0.066
<b>rs10012150</b>	88905795	C / T	0.17	1.35	0.075	-1.71	0.221	0.35	0.360	0.70	0.429
<b>rs7685225</b>	88906458	C / T	0.11	0.17	0.861	0.03	0.981	0.14	0.782	0.61	0.536
<b>rs7675246</b>	88908998	A / G	0.18	-1.58	0.035	0.28	0.821	-0.26	0.489	0.41	0.603

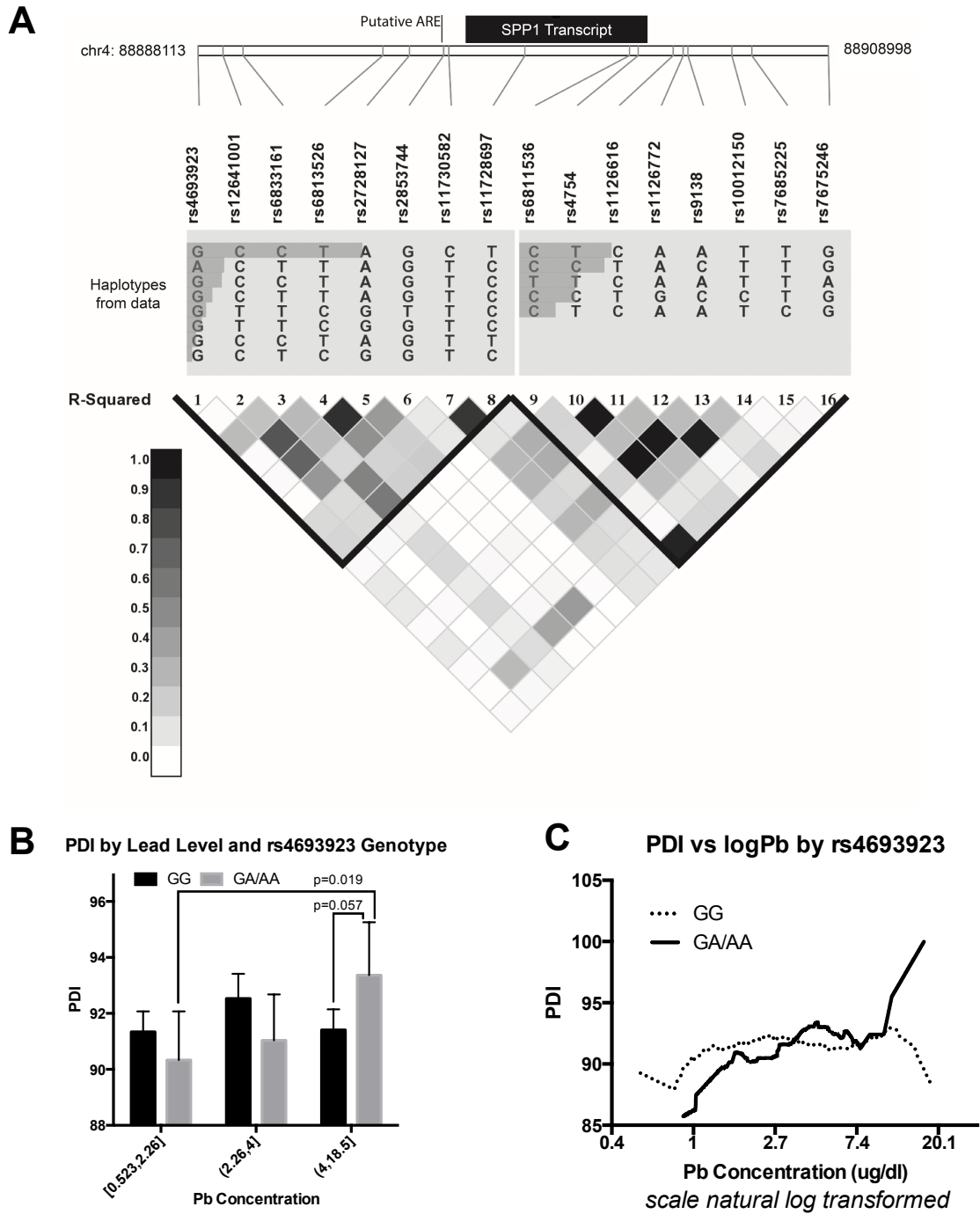
No single statistically significant association with MDI was detected among any of the SNPs with respect to main effect or Pb interaction after correction for multiple testing (Table 3.1). However, there is an enrichment of nominally significant SNPs in the gene with respect to their main effect on MDI. In 16 SNPs, a p-value of 0.05 would be expected in 0.8 SNPs. However, here 8 of 16 SNPs are below this threshold, which constitutes a 10-fold enrichment. The results here provide suggestive evidence that SPP1 has a main effect in modulating MDI scores, but further study is needed to explore this possible association.

#### *A SPP1 SNP significantly interacts with Pb in psychomotor development in children*

Psychomotor impairment is not a well-established consequence of low-dose Pb exposure. SPP1, however, has been linked to several measures of dexterity (Yamamoto et al., 2013) and its levels are correlated with many neurological diseases with motor deficits including multiple sclerosis and Parkinson's disease (Iaffaldano et al., 2014, Hur et al., 2007, Iczkiewicz et al., 2006). We thus examined the association of SPP1 SNPs with PDI. Although there is no significant association of the SNPs with PDI in terms of main effect (Table 3.1), one SNP (rs4693923) has a statistically significant interaction with Pb in determining PDI (Table 3.1). The SNP rs4693923 is located approximately 8 kb upstream of the SPP1 transcriptional start site. Upon examining the LD map (Figure 3.1A), we found the A-allele of rs4693923 tags a single haplotype that spans the SPP1 promoter region and the first part of the transcript. When divided into tertiles by Pb exposure, carriers of the A-allele of rs4693923 show statistically significantly higher PDI in the highest tertile compared to the lowest tertile (Figure 3.1B). The linear relationship among A-carriers is seen in smooth kernel regression (Figure 3.1C). At high levels of Pb, A-carriers tend to have a higher PDI than GG homozygotes (Figures 3.1B and C). No relationship is observed between second trimester Pb exposure and PDI among GG homozygotes.

#### *Little Evidence of Confounding Among Genotype Groups*

MDI and PDI models were adjusted for sex, gestational age, maternal age, maternal marital status, presence of siblings, maternal education (high school vs no high school) and genome-wide principle components 1 and 2. The summary statistics for the whole cohort and each genotype group is



**Figure 3.1 LD patterns around *SPP1* in the study population and analysis of the rs4693923\* $\log_e$ Pb interaction.** (A) Schema of SNPs in relation to the *SPP1* transcribed locus and the ARE discovered in Chapter 2. The relative abundances of haplotypes are shown above the LD plot. LD plot reflects pairwise  $R^2$  among SNPs. (B) Average PDI by tertiles of Pb concentration and rs4693923 A-allele carrier status. AA homozygotes and GA heterozygotes are grouped together due to the low number of AA homozygotes. (C) Smoothed spline PDI dose-response by  $\log_e$ Pb and genotype group.



**Table 3.2 Summary of variables in the ELEMENT birth cohort.** Traits are given for the cohort as a whole and by genotype of rs4693923. Individuals of genotypes GA and AA are added together as a group due to the low abundance of AA homozygotes.

<b>Trait</b>	<b>Cohort</b>	<b>GG Genotype</b>	<b>GA/AA Genotype</b>
n	462	317	93
Sex (male/female)	245/217	202/167	43/50
Mean gestational age in weeks (sd)	38.38 (1.65)	38.36 (1.66)	38.46 (1.59)
Mean maternal age in years at birth (sd)	27.81 (5.51)	28.00 (5.69)	27.08 (4.76)
Percentage of mothers married	80.3%	80.5%	79.6%
Percentage of children with siblings	53.0%	53.7%	50.5%
Percentage of mothers who completed high school education	58.4%	59.6%	53.8%
Mean second trimester maternal blood Pb level in ug/dL (sd)	3.76 (2.62)	3.77 (2.66)	3.76 (2.52)
Mean Raw Mental Development Index at ~24 months (sd)	108.32 (9.58)	108.16 (9.41)	108.91 (10.27)
Mean Raw Psychomotor Development Index at ~24 months (sd)	91.73 (4.56)	91.75 (4.45)	91.61 (4.99)

given in Table 3.2. In addition we checked two-year loss-to-follow-up measures, which were 63% for the entire cohort, 64% among GG homozygotes and 59% for A-allele carriers.

### 3.5. Discussion

SNPs in and around *SPP1* were interrogated for main effect and effect modification of Pb exposure on two neurodevelopmental outcomes in infants aged 24 months in Mexico City. Pb levels tend to be higher in Mexico City due to old infrastructure more likely to contain Pb piping and leaded paint, an abundance of Pb-glazed pottery and a recent change from leaded gasoline. A statistically significant interaction was detected for rs4693923 in PDI, indicating effect modification of the PDI response to Pb. Due to the low minor allele frequency of the SNP, 11% (Table 3.1), the interaction is visualized in Figures 3.1B and 3.1C by grouping AA homozygotes and GA heterozygotes for this SNP together. The A-allele carriers have lower PDI's at low Pb levels and higher PDI at higher Pb levels. A greater than expected number of suggestively significant SNPs is associated with MDI in main effect. While promising, a larger sample size or replication population is needed to determine if these suggestive associations are true signals.

*SPP1* has emerging roles in the brain relating to stress response, cancer, neurodegenerative diseases and dexterity. *SPP1* is upregulated following several stressors including: in the lungs following exposure to ozone (Bass et al., 2013) and cigarette smoke (Shan et al., 2012) and in the brain after hypoxic ischemia (Chen et al., 2011) and lesion (Shin et al., 2005). NRF2 activation is consistent with all three of these scenarios, but it is important to note that the transcription factors AP1 and NF- $\kappa$ B have validated binding sites in the promoter of *SPP1* (Zhao et al., 2011). Since the transcription factors AP1 and NF- $\kappa$ B are also involved in various stress responses, it is possible that *SPP1* is involved in stress resolution in many scenarios. Understanding the consequences of *SPP1* upregulation early in life will be important to understanding its effect on the developing brain and its role in neurotoxicology.

The association between Pb and lower PDI scores is not well established, which could result from underlying confounding of genetic factors like rs4693923. A correlational study (Yamamoto et al., 2013) has linked *SPP1* to measures of dexterity in three ways: (1) there are more *SPP1*-positive neurons in the

corticospinal tract of species with highly developed corticospinal systems (rhesus macaque, capuchin monkey, and humans) compared to animals with less developed corticospinal systems (squirrel monkey, marmoset, and rat); (2) the number of *SPP1*-positive neurons increases logarithmically during postnatal development concurrent with the increase in conduction velocity in the corticospinal tract; and (3) after a lesion of the lateral corticospinal tract at the mid-cervical level, *SPP1*-positive neurons increase in layer V of the ventral premotor cortex in a manner positively correlated with the extent of recovery in finger dexterity. Therefore, *SPP1* upregulation in the presence of Pb with its cytoprotective and pro-growth properties may enhance dexterity among those exposed to Pb. The A-allele of rs4693923 and its haplotype may encode a *SPP1* promoter more responsive to Pb exposure, and that the increase in *SPP1* transcription resulting from Pb exposure in early life contributes to an increase in PDI scores. Therefore, the A-allele of rs4693923 could be considered a resistance allele to Pb exposure with respect to PDI. Interestingly, *SPP1* is also upregulated in the frontal cortex of non-human primates exposed to manganese (Guilarte, 2010). Manganese toxicity manifests in loss of motor function, and thus *SPP1* regulation could be a compensatory mechanism upon manganese exposure as well.

The interacting SNP rs4693923 is not likely to encode the causative variant for the interaction, but is instead in LD with a causative variant. Finding the exact causative variant would entail sequencing of the promoter and fine-mapping within the region to pinpoint the effector variant. Because the rs4693923 A-allele appears in only one haplotype that spans the promoter region and transcription start site, a functional variant may be anywhere in the space. However, the causative variant may also be further away from the transcription start site than the rs4693923 SNP itself. The only known expression quantitative trait locus (eQTL) for *SPP1* is rs4693905, which lies a further 65,664 bp upstream of rs4693923 (Schadt et al., 2008). SNPs rs4693923 and rs4693905 are in limited LD with each other, having pairwise  $r^2=0.055$ .

The confluence of in vitro data from Chapter 2 and the epidemiological data here in Chapter 3 presents a new approach to the study of chronic environmental toxicant exposure. Chapter 2 focused on the discovery of *SPP1* as a novel NRF2 target gene in NSCs following a hypothesis-free transcriptomic screen using next-generation RNA Sequencing. In Chapter 3, we identify SNP rs4693923 upstream of

*SPP1* as an effect modifier of the PDI response to second trimester Pb exposure. Haplotype analysis reveals the SNP may be tagging a causative variant in the promoter region. In looking more closely at the interaction, the minor allele confers a positive correlation between PDI and Pb exposure not seen among major allele homozygotes. The positive correlation between Pb exposure and PDI may be the result of increased *SPP1* expression at higher levels of Pb exposure, as a compensatory response against Pb toxicity. If increased *SPP1* expression is tied to increased PDI without detriment to other health outcomes, targeting *SPP1* expression may be useful in finding new treatments for psychomotor diseases and for children exposed to toxicants that pose a risk to psychomotor functioning.

### **3.6. Acknowledgements**

First of all we would like to thank all ELEMENT Study Participants. Peter Wagner was supported by the Joseph D. Brain and Jere Meade Fellowship Funds as well as the Training In Interdisciplinary Pulmonary Sciences Grant (NIH 5T32HL007118). Project was supported by the Harvard Superfund Research Program (P42ES16454) and the NIEHS RO1 (ES015533-04).

### **3.7. References**

- ANDERSON, C. A., PETERSSON, F. H., CLARKE, G. M., CARDON, L. R., MORRIS, A. P. & ZONDERVAN, K. T. 2010. Data quality control in genetic case-control association studies. *Nat Protoc*, 5, 1564-73.
- BASS, V., GORDON, C. J., JAREMA, K. A., MACPHAIL, R. C., CASCIO, W. E., PHILLIPS, P. M., LEDBETTER, A. D., SCHLADWEILER, M. C., ANDREWS, D., MILLER, D., DOERFLER, D. L. & KODAVANTI, U. P. 2013. Ozone induces glucose intolerance and systemic metabolic effects in young and aged Brown Norway rats. *Toxicol Appl Pharmacol*, 273, 551-60.
- BAYLEY, N. 2005. Bayley Scales of Infant and Toddler Development, San Antonio, TX, Pearson.
- BELLINGER, D., HU, H., TITLEBAUM, L. & NEEDLEMAN, H. L. 1994. Attentional correlates of dentin and bone lead levels in adolescents. *Archives of Environmental Health*, 49, 98-105.
- BELLINGER, D. C. 2004. Lead. *Pediatrics*, 113, 1016-22.
- CHEN, W., MA, Q., SUZUKI, H., HARTMAN, R., TANG, J. & ZHANG, J. H. 2011. Osteopontin reduced hypoxia-ischemia neonatal brain injury by suppression of apoptosis in a rat pup model. *Stroke*, 42, 764-9.
- CHEN, Y. J., WEI, Y. Y., CHEN, H. T., FONG, Y. C., HSU, C. J., TSAI, C. H., HSU, H. C., LIU, S. H. & TANG, C. H. 2009. Osteopontin increases migration and MMP-9 up-regulation via  $\alpha$ v $\beta$ 3 integrin, FAK, ERK, and NF- $\kappa$ B-dependent pathway in human chondrosarcoma cells. *J Cell Physiol*, 221, 98-108.

- ETTINGER, A. S., LAMADRID-FIGUEROA, H., TELLEZ-ROJO, M. M., MERCADO-GARCIA, A., PETERSON, K. E., SCHWARTZ, J., HU, H. & HERNANDEZ-AVILA, M. 2009. Effect of calcium supplementation on blood lead levels in pregnancy: a randomized placebo-controlled trial. *Environ Health Perspect*, 117, 26-31.
- FONG, Y. C., LIU, S. C., HUANG, C. Y., LI, T. M., HSU, S. F., KAO, S. T., TSAI, F. J., CHEN, W. C., CHEN, C. Y. & TANG, C. H. 2009. Osteopontin increases lung cancer cells migration via activation of the alphavbeta3 integrin/FAK/Akt and NF-kappaB-dependent pathway. *Lung Cancer*, 64, 263-70.
- FROELICH, T. E., LANPHEAR, B. P., DIETRICH, K. N., CORY-SLECHTA, D. A., WANG, N. & KAHN, R. S. 2007. Interactive effects of a DRD4 polymorphism, lead, and sex on executive functions in children. *Biological Psychiatry*, 62, 243-9.
- GONZALEZ-COSSIO, T., PETERSON, K. E., SANIN, L. H., FISHBEIN, E., PALAZUELOS, E., ARO, A., HERNANDEZ-AVILA, M. & HU, H. 1997. Decrease in birth weight in relation to maternal bone-lead burden. *Pediatrics*, 100, 856-62.
- GUILARTE, T. R. 2010. APLP1, Alzheimer's-like pathology and neurodegeneration in the frontal cortex of manganese-exposed non-human primates. *Neurotoxicology*, 31, 572-4.
- HERNANDEZ-AVILA, M., PETERSON, K. E., GONZALEZ-COSSIO, T., SANIN, L. H., ARO, A., SCHNAAS, L. & HU, H. 2002. Effect of maternal bone lead on length and head circumference of newborns and 1-month-old infants. *Arch Environ Health*, 57, 482-8.
- HUR, E. M., YOUSSEF, S., HAWS, M. E., ZHANG, S. Y., SOBEL, R. A. & STEINMAN, L. 2007. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat Immunol*, 8, 74-83.
- IAFFALDANO, P., RUGGIERI, M., VITERBO, R. G., MASTRAPASQUA, M. & TROJANO, M. 2014. The improvement of cognitive functions is associated with a decrease of plasma Osteopontin levels in Natalizumab treated relapsing multiple sclerosis. *Brain Behav Immun*, 35, 96-101.
- ICZKIEWICZ, J., JACKSON, M. J., SMITH, L. A., ROSE, S. & JENNER, P. 2006. Osteopontin expression in substantia nigra in MPTP-treated primates and in Parkinson's disease. *Brain Res*, 1118, 239-50.
- KALLURI, H. S. & DEMPSEY, R. J. 2012. Osteopontin increases the proliferation of neural progenitor cells. *Int J Dev Neurosci*, 30, 359-62.
- KRIEG, E. F., JR., BUTLER, M. A., CHANG, M. H., LIU, T., YESUPRIYA, A., DOWLING, N. & LINDEGREN, M. L. 2010. Lead and cognitive function in VDR genotypes in the third National Health and Nutrition Examination Survey. *Neurotoxicology and Teratology*, 32, 262-72.
- KRIEG, E. F., JR., BUTLER, M. A., CHANG, M. H., LIU, T., YESUPRIYA, A., LINDEGREN, M. L. & DOWLING, N. 2009. Lead and cognitive function in ALAD genotypes in the third National Health and Nutrition Examination Survey. *Neurotoxicology and Teratology*, 31, 364-71.
- LEE, S., WRIGHT, F. A. & ZOU, F. 2011. Control of population stratification by correlation-selected principal components. *Biometrics*, 67, 967-74.
- LI, J. & JI, L. 2005. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity (Edinb)*, 95, 221-7.

- PAWLAS, N., BROBERG, K., OLEWINSKA, E., PROKOPOWICZ, A., SKERFVING, S. & PAWLAS, K. 2012. Modification by the genes ALAD and VDR of lead-induced cognitive effects in children. *Neurotoxicology*, 33, 37-43.
- ROY, A., HU, H., BELLINGER, D. C., MUKHERJEE, B., MODALI, R., NASARUDDIN, K., SCHWARTZ, J., WRIGHT, R. O., ETTINGER, A. S., PALANIAPAN, K. & BALAKRISHNAN, K. 2011. Hemoglobin, lead exposure, and intelligence quotient: effect modification by the DRD2 Taq IA polymorphism. *Environmental Health Perspectives*, 119, 144-9.
- SCHADT, E. E., MOLONY, C., CHUDIN, E., HAO, K., YANG, X., LUM, P. Y., KASARSKIS, A., ZHANG, B., WANG, S., SUVER, C., ZHU, J., MILLSTEIN, J., SIEBERTS, S., LAMB, J., GUHATHAKURTA, D., DERRY, J., STOREY, J. D., AVILA-CAMPILLO, I., KRUGER, M. J., JOHNSON, J. M., ROHL, C. A., VAN NAS, A., MEHRABIAN, M., DRAKE, T. A., LUSIS, A. J., SMITH, R. C., GUENGERICH, F. P., STROM, S. C., SCHUETZ, E., RUSHMORE, T. H. & ULRICH, R. 2008. Mapping the genetic architecture of gene expression in human liver. *PLoS Biol*, 6, e107.
- SCINICARIELLO, F., MURRAY, H. E., MOFFETT, D. B., ABADIN, H. G., SEXTON, M. J. & FOWLER, B. A. 2007. Lead and delta-aminolevulinic acid dehydratase polymorphism: where does it lead? A meta-analysis. *Environ Health Perspect*, 115, 35-41.
- SHAN, M., YUAN, X., SONG, L. Z., ROBERTS, L., ZARINKAMAR, N., SERYSHEV, A., ZHANG, Y., HILSENBECK, S., CHANG, S. H., DONG, C., CORRY, D. B. & KHERADMAND, F. 2012. Cigarette smoke induction of osteopontin (SPP1) mediates T(H)17 inflammation in human and experimental emphysema. *Sci Transl Med*, 4, 117ra9.
- SHIN, T., AHN, M., KIM, H., MOON, C., KANG, T. Y., LEE, J. M., SIM, K. B. & HYUN, J. W. 2005. Temporal expression of osteopontin and CD44 in rat brains with experimental cryolesions. *Brain Res*, 1041, 95-101.
- SMITH, P. G. & DAY, N. E. 1984. The design of case-control studies: the influence of confounding and interaction effects. *Int J Epidemiol*, 13, 356-65.
- WRIGHT, R. O., HU, H., SILVERMAN, E. K., TSAIH, S. W., SCHWARTZ, J., BELLINGER, D., PALAZUELOS, E., WEISS, S. T. & HERNANDEZ-AVILA, M. 2003. Apolipoprotein E genotype predicts 24-month bayley scales infant development score. *Pediatric Research*, 54, 819-25.
- YAMAMOTO, T., OISHI, T., HIGO, N., MURAYAMA, S., SATO, A., TAKASHIMA, I., SUGIYAMA, Y., NISHIMURA, Y., MURATA, Y., YOSHINO-SAITO, K., ISA, T. & KOJIMA, T. 2013. Differential expression of secreted phosphoprotein 1 in the motor cortex among primate species and during postnatal development and functional recovery. *PLoS One*, 8, e65701.
- ZHAO, W., WANG, L., ZHANG, M., WANG, P., ZHANG, L., YUAN, C., QI, J., QIAO, Y., KUO, P. C. & GAO, C. 2011. NF-kappaB- and AP-1-mediated DNA looping regulates osteopontin transcription in endotoxin-stimulated murine macrophages. *J Immunol*, 186, 3173-9.

## **CHAPTER 4: Conclusions and Perspectives**

## ***SPP1* is a Mediator of Lead (Pb) Neurotoxicity in Early Neurodevelopment**

While the mechanisms of Pb's effects on late synaptogenesis and neuron functions are increasingly well established, the effects of Pb on neural stem cells (NSCs) are not well studied. NSCs are the original multipotent progenitor cells of the central nervous system. NSCs are present, proliferating and differentiating during an emerging prenatal critical window of Pb neurotoxicity (Hu et al., 2006). Pb slows the growth of NSCs (Gilbert et al., 2005, Schneider et al., 2005, Verina et al., 2007, Breier et al., 2008, Huang and Schneider, 2004), but the molecular and cellular mechanisms underlying this effect are not known. To better understand how Pb affects NSCs, we performed transcriptomic profiling of Pb-exposed NSCs and unexposed controls. The differential expression profile of Pb-exposed NSCs was consistent with a primary transcriptional response predominantly regulated by the transcription factor NRF2. Alongside known NRF2 targets among the top differentially expressed genes, however, was *SPP1*. *SPP1* is a pleiotropic molecule originally discovered as a component of bone mineralization, but has an emerging role in the brain as a potent neuroprotectant. *SPP1* affects signaling through a  $\beta 3$ -integrin ( $\beta 3$ ) – Focal Adhesion Kinase (FAK) – Protein Kinase B (Akt) signaling axis, which is anti-apoptotic and pro-proliferative (Kalluri and Dempsey, 2012, Fong et al., 2009, Meller et al., 2005, Topkuru et al., 2013). Therefore, *SPP1* upregulation and secretion may induce a compensatory growth and survival response among neighboring cells and tissues.

To determine if *SPP1* is important in the context of early life neurotoxicity, we evaluated the significance of variants in *SPP1* in predicting infants' neurocognitive outcomes in a longitudinal birth cohort with heterogeneous prenatal Pb exposure. In total, sixteen single nucleotide polymorphisms (SNPs) were genotyped within the *SPP1* transcribed locus and its ten kilobase flanking regions. Eight SNPs had a significant ( $p < 0.05$ ) main effect association with the Mental Development Index (MDI), but none of the associations passed multiple testing correction. In analyses of the Psychomotor Development Index (PDI), a statistically significant interaction was observed between a SNP upstream of *SPP1*, rs4693923, and second trimester maternal blood Pb. Carriers of the minor allele, the A-allele, had a positive correlation between second trimester *in utero* Pb exposure and PDI. Comparing the highest tertile of second trimester Pb exposure to the lowest among A-allele carriers, a statistically significant



higher PDI is observed in the higher tertile ( $p=0.019$ ). Among the highest tertile of Pb-exposed individuals, A-allele carriers had a higher average PDI than major allele homozygotes of the genotype GG ( $p=0.057$ ). No relationship between Pb exposure and PDI was observed among GG homozygotes.

The improved psychomotor functioning at high Pb exposure among rs4693923 A-allele carriers was unexpected, because we anticipated identification of susceptibility variants in our analyses. One possible explanation of the improved PDI among A-allele carriers at high Pb levels relates back to our discovery of *SPP1* as a gene upregulated upon Pb exposure. According to one study, *SPP1* expression is closely correlated with measures of dexterity and motor function (Yamamoto et al., 2013). Therefore, the improved PDI may be an auxiliary benefit of the cytoprotective *SPP1* upregulation and NRF2 response. In the context of the interacting SNP, it is possible that the A-allele confers a promoter more responsive to Pb, leading to the observed interaction. If the Pb-responsive promoter were the result of greater NRF2 affinity, stimulating NRF2 activation using antioxidants in early life should lead to similar benefits without the related consequences of Pb exposure.

A great deal of work remains on linking the gene expression changes observed *in vitro* to the interaction observed in the longitudinal birth cohort. Logical next steps include tracing the expression dynamics of *SPP1* over time *in utero* by Pb exposure. Using animal models can better pinpoint the brain regions and time points when the *SPP1* response is most relevant. In addition, *SPP1* knockout mice (International Knockout Mouse Consortium) can be used to evaluate differences in Pb susceptibility using a battery of neurocognitive assays following Pb exposure at various time points. Further epidemiological studies can shed light on the possible association of *SPP1* SNPs with MDI and may be able to replicate the PDI interaction finding. Sequencing a cohort's *SPP1* promoter regions and subsequent fine mapping of variants' association with MDI and PDI is needed to identify causative variants. Evidence of *SPP1*'s importance from both our *in vitro* transcriptomics and our candidate gene study provides a compelling case for the role of *SPP1* in Pb neurotoxicity, which warrants further study.

### ***SPP1* is a Novel Target of NRF2**

NRF2 is activated in oxidative stress conditions and leads to the upregulation of target genes involved in the detoxification of toxicants and reactive oxygen species (ROS), and in the management of their secondary effects. The intermediates and temporal nature of the NRF2 response to Pb are not well understood, and they are likely cell type dependent. Pb-induced glutathione depletion and the subsequent buildup of endogenous ROS from routine oxidative phosphorylation is a reasonable explanation for Pb-derived oxidative stress and NRF2 activation. Modification of the KEAP1 Cys151 residue by ROS releases NRF2 and is the canonical mechanism of NRF2 activation. However, another non-redox cycling metal, arsenic, activates NRF2 via an autophagy-mediated mechanism (Lau et al., 2013b), which indicates potential alternative activation pathways of NRF2 among non-redox cycling metals. In our study, a dose-response curve shows that Pb is a potent activator of the NRF2 response with noticeable upregulation already at a tenth of the physiological dose we used for our RNA Sequencing discovery dataset.

In the first chapter of this thesis, we hypothesized *SPP1* was a novel target of NRF2 based on its presence among known NRF2 target genes in the top hits of our differential expression dataset. We first demonstrated that the antioxidant DL-Sulforaphane and the heavy metal arsenic, both known NRF2 activators, also upregulate *SPP1* transcriptionally. Dramatic *SPP1* upregulation is also observed upon siRNA-mediated knockdown of KEAP1, another strategy of NRF2 activation. In placing NRF2 between Pb exposure and *SPP1* upregulation, we showed that siRNA-mediated knockdown of NRF2 attenuated the *SPP1* upregulation response to Pb exposure. With this preponderance of evidence suggesting direct targeting of *SPP1* by NRF2, we bioinformatically identified a putative Antioxidant Response Element (ARE) in the promoter of *SPP1* and validated it using an NRF2 chromatin immunoprecipitation (ChIP) assay.

NRF2 biology is increasingly well studied, especially in the fields of toxicology and oncology. Independent of Pb neurotoxicity, the targeting of *SPP1* by NRF2 is a significant finding. Aberrant and sustained NRF2 activation is common to many cancers (see Moon and Giaccia (2015) for review), and *SPP1* is independently shown to be upregulated in tumor tissue (see Rangaswami et al. (2006) for review). Therefore, NRF2 targeting of *SPP1* may be consistent with the dysregulation observed in cancer

cells. The role of NRF2 in *SPP1*'s upregulation is also relevant to the biological response to a number of oxidative stressors. For instance, here we showed arsenic also upregulates *SPP1*. In addition, a microarray study demonstrated increased *SPP1* transcription among manganese-exposed nonhuman primates (Guilarte, 2010). The way *SPP1* affects the brain in response to metals and toxicants as a whole may be valuable to understanding the biologic processes and consequences underlying real-world environmental exposure mixtures.

The existence and nature of mechanisms that desensitize NRF2 to long-term Pb or oxidative stress exposures are not known. Antioxidant NRF2 inducers, like DL-Sulforaphane, are short-term NRF2 activators leading to transient cytoprotective responses. It is argued for arsenic, however, that exposure leads to persistent NRF2 activation resulting in pro-cancerous cytoprotective conditions (Lau et al., 2013a). Because cancer is not a common risk outcome of Pb exposure and long-term Pb exposure in animals does not cause a sustained NRF2-mediated response, Pb's NRF2 induction may be transient. However, NSCs exposed to Pb for a week *in vitro* maintained higher *SPP1* transcriptional levels compared to unexposed controls (data not shown). While the response may be transient, proteins resulting from an initial NRF2 activation event may not be cleared for a long time. Furthermore, lasting secondary effects elicited by an early NRF2 response are possible due to the enhanced activation of the  $\beta$ 3-FAK-Akt growth and survival axis by *SPP1*.

*SPP1* is correlated with many stress conditions. In cancer, increasing *SPP1* levels are often associated with growth, migration and metastasis. For instance, higher *SPP1* levels are correlated with poor prognosis among glioma patients (Zhao et al., 2015). Circulating *SPP1* is correlated with Multiple Sclerosis (MS) severity, and Natalizumab MS treatment lowers *SPP1* levels over a timeline consistent with the return of cognitive functioning (Iaffaldano et al., 2014). Recently, the FDA has written a first-of-its-kind biomarker letter of support for evaluating the use of urinary *SPP1* as a biomarker for drug-induced acute nephrotoxicity in nonclinical studies, and for exploring *SPP1*'s use in clinical drug development (Woodcock, 2014). The strong correlations observed previously with *SPP1* and the ongoing initiative with respect to kidney toxicity would suggest utility for *SPP1* as a biomarker. However, due to the

heterogeneity of SPP1 sources and regulation, interpretation of the biomarker measurement may prove difficult.

SPP1 use as a therapeutic may also be complicated. SPP1 undergoes a great deal of post-translational modifications being a heavily phosphorylated and glycosylated protein. In addition, SPP1 has protein cleavage sites, including a thrombin cleavage site that is thought to change its signaling properties. Such modifications make production *in vitro* more challenging and differences in post-translational modifications likely underlie some of the protein's pleiotropic effects. Despite these challenges, recombinant SPP1 is shown to be an effective treatment or pretreatment in animal models of hypoxic ischemia (Meller et al., 2005), subarachnoid hemorrhage (Topkoru et al., 2013), vasospasm following subarachnoid hemorrhage (He et al., 2015) and unilateral entorhinal cortical lesion (Chan et al., 2014). On the other hand, in one study, thrombin-cleaved SPP1 treatment is observed to have negative effects in a mouse preterm brain injury model and no effect in the non-thrombin cleaved form (Albertsson et al., 2014).

### **More Opportunities in the Transcriptomic Profile of NSCs**

While *SPP1* was chosen from our RNA sequencing dataset for follow-up in this thesis, other differentially regulated hits also merit further study. For instance, *F2RL2* (coagulation factor II (thrombin) receptor-like 2) was the top differentially expressed gene in the dataset. *F2RL2* is a G protein-coupled receptor that is a member of the protease activated receptor family. *F2RL2* was also upregulated by other methods of NRF2 activation, and its induction by Pb was attenuated by NRF2-knockdown (data not shown). Unfortunately, *F2RL2* upregulation could not be confirmed at the protein level due to a lack of an effective antibody. We were also unable to identify a high scoring ARE in the *F2RL2* promoter, although one or more lower scoring AREs may be functional in *F2RL2* promoter or an alternative ARE such as that proposed by Chorley et al. (2012) may be present. *F2RL2* is activated by thrombin cleavage, an event that leads to activation of pathways related to proliferation and migration in NSCs (Guo et al., 2013). Both *F2RL2* and *SPP1* have thrombin cleavage sites, indicating that NRF2 activation may alter NSCs' sensitivity to thrombin signaling.

One reason for opting to perform transcriptomic profiling by RNA sequencing was to identify long non-coding RNAs and other transcripts not typically included on commercial microarrays. Here we report differential expression of the long non-coding RNA Lung Cancer Associated Transcript 1 (*LUCAT1*). *LUCAT1* is an NRF2 target induced by cigarette smoke extract both *in vitro* and *in vivo*, and is upregulated in numerous lung cancer cell lines (Thai et al., 2013). Knockdown of *LUCAT1* by siRNA in human bronchial epithelial cells aggravates cigarette smoke extract cytotoxicity *in vitro* (Thai et al., 2013). The exact mechanisms by which this non-protein coding RNA is cytoprotective are not understood. This is only the second study to identify this transcript, and the presence, NRF2 regulation and cytoprotective properties of the transcript with respect to Pb and other environmental exposures in the developing brain may be critical to NSC function.

### **An Integrative Approach to Study Environmental Exposures**

This study provides a model for studying environmental exposures in an omics-integrative approach. This is the first single study to link cell-based assays with genetic epidemiology in the field of heavy metal and Pb neurotoxicity. While Pb is among the most studied and canonical neurotoxicants, the exact molecular and cell biology mechanisms underlying its toxicity are still emerging. Due to the complex etiology of neurological phenotypes, relating outcomes to specific molecular and cellular phenomena is challenging. Similarly, due to the imprecise quantification of neurological phenotypes and the statistical burdens of both genome-wide screening and interaction studies, gene-environment interactions are hard-found with statistical approaches alone.

Within the confines of the data gathered in ELEMENT, we identified an appropriate cell model for a discovery-driven approach *in vitro*. Using computational tools supported by laboratory verification, we identified the regulatory mechanism of a transcript that we could then use as a candidate gene for a genetic epidemiology study. Because the statistical burden of gene by environment interaction studies is far greater than a study of main effects alone, targeted candidate gene approaches may be more reasonable until larger cohorts with accurate genetic and environmental exposure assessments can be gathered. Furthermore, the characterization of *SPP1* prior to the SNP-Pb interaction study provided a

potential functional mechanism of the gene's involvement in the PDI outcome. As the ELEMENT cohort is profiled for other environmental and metal toxicants, like arsenic and manganese, cell-based assays can be applied to identify new candidate genes for statistical analysis in ELEMENT.

This study was made possible by relatively recent technological advances in both transcriptomic profiling and SNP genotyping. As these technologies revolutionize biology, it is important to understand how to integrate their outputs since each approach, model system and technology has inherent limitations. One of the most effective ways of showing biological effect is providing lines of evidence from many different approaches. Various funding bodies, including the National Institutes of Health, now favor inter- and trans-disciplinary approaches for biological discovery. Genetic epidemiology provides a useful link between molecular and cellular biology-based approaches and epidemiological studies. Molecular and cellular biology is useful to determine the underlying biology of toxicant exposures, and potentially help identify therapies to assist in recovery. Genetic epidemiology provides a translational opportunity for what is found in the laboratory. In addition, in the advent of personalized medicine, genetic epidemiology can identify susceptible individuals and subpopulations for specialized intervention. New technologies on the horizon, such as genomic screening using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and whole-genome sequencing, will provide new opportunities to further address the biology underlying toxicant exposures. Such new technologies offer the opportunity to strengthen our current understanding of gene-Pb interactions as well as further identify new and important modulators of Pb neurotoxicity in children. A better understanding of Pb's effects on the developing brain will help identify better interventions for Pb exposure in early life, which otherwise has lasting effects on the brain.

## **References**

BREIER, J. M., RADIO, N. M., MUNDY, W. R. & SHAFER, T. J. 2008. Development of a high-throughput screening assay for chemical effects on proliferation and viability of immortalized human neural progenitor cells. *Toxicological Sciences*, 105, 119-33.

CHAN, J. L., REEVES, T. M. & PHILLIPS, L. L. 2014. Osteopontin expression in acute immune response mediates hippocampal synaptogenesis and adaptive outcome following cortical brain injury. *Exp Neurol*, 261, 757-71.

- CHORLEY, B. N., CAMPBELL, M. R., WANG, X., KARACA, M., SAMBANDAN, D., BANGURA, F., XUE, P., PI, J., KLEEBERGER, S. R. & BELL, D. A. 2012. Identification of novel NRF2-regulated genes by ChIP-Seq: influence on retinoid X receptor alpha. *Nucleic Acids Res*, 40, 7416-29.
- FONG, Y. C., LIU, S. C., HUANG, C. Y., LI, T. M., HSU, S. F., KAO, S. T., TSAI, F. J., CHEN, W. C., CHEN, C. Y. & TANG, C. H. 2009. Osteopontin increases lung cancer cells migration via activation of the alphavbeta3 integrin/FAK/Akt and NF-kappaB-dependent pathway. *Lung Cancer*, 64, 263-70.
- GILBERT, M. E., KELLY, M. E., SAMSAM, T. E. & GOODMAN, J. H. 2005. Chronic developmental lead exposure reduces neurogenesis in adult rat hippocampus but does not impair spatial learning. *Toxicol Sci*, 86, 365-74.
- GUILARTE, T. R. 2010. APLP1, Alzheimer's-like pathology and neurodegeneration in the frontal cortex of manganese-exposed non-human primates. *Neurotoxicology*, 31, 572-4.
- GUO, H., ZHAO, Z., YANG, Q., WANG, M., BELL, R. D., WANG, S., CHOW, N., DAVIS, T. P., GRIFFIN, J. H., GOLDMAN, S. A. & ZLOKOVIC, B. V. 2013. An activated protein C analog stimulates neuronal production by human neural progenitor cells via a PAR1-PAR3-S1PR1-Akt pathway. *J Neurosci*, 33, 6181-90.
- HE, J., LIU, M., LIU, Z. & LUO, L. 2015. Recombinant osteopontin attenuates experimental cerebral vasospasm following subarachnoid hemorrhage in rats through an anti-apoptotic mechanism. *Brain Res*.
- HU, H., TELLEZ-ROJO, M. M., BELLINGER, D., SMITH, D., ETTINGER, A. S., LAMADRID-FIGUEROA, H., SCHWARTZ, J., SCHNAAS, L., MERCADO-GARCIA, A. & HERNANDEZ-AVILA, M. 2006. Fetal lead exposure at each stage of pregnancy as a predictor of infant mental development. *Environmental Health Perspectives*, 114, 1730-5.
- HUANG, F. & SCHNEIDER, J. S. 2004. Effects of lead exposure on proliferation and differentiation of neural stem cells derived from different regions of embryonic rat brain. *Neurotoxicology*, 25, 1001-12.
- IAFFALDANO, P., RUGGIERI, M., VITERBO, R. G., MASTRAPASQUA, M. & TROJANO, M. 2014. The improvement of cognitive functions is associated with a decrease of plasma Osteopontin levels in Natalizumab treated relapsing multiple sclerosis. *Brain Behav Immun*, 35, 96-101.
- KALLURI, H. S. & DEMPSEY, R. J. 2012. Osteopontin increases the proliferation of neural progenitor cells. *Int J Dev Neurosci*, 30, 359-62.
- LAU, A., WHITMAN, S. A., JARAMILLO, M. C. & ZHANG, D. D. 2013a. Arsenic-mediated activation of the Nrf2-Keap1 antioxidant pathway. *J Biochem Mol Toxicol*, 27, 99-105.
- LAU, A., ZHENG, Y., TAO, S., WANG, H., WHITMAN, S. A., WHITE, E. & ZHANG, D. D. 2013b. Arsenic inhibits autophagic flux, activating the Nrf2-Keap1 pathway in a p62-dependent manner. *Mol Cell Biol*, 33, 2436-46.
- MELLER, R., STEVENS, S. L., MINAMI, M., CAMERON, J. A., KING, S., ROSENZWEIG, H., DOYLE, K., LESSOV, N. S., SIMON, R. P. & STENZEL-POORE, M. P. 2005. Neuroprotection by osteopontin in stroke. *J Cereb Blood Flow Metab*, 25, 217-25.
- MOON, E. J. & GIACCIA, A. 2015. Dual roles of NRF2 in tumor prevention and progression: possible implications in cancer treatment. *Free Radic Biol Med*, 79, 292-9.

RANGASWAMI, H., BULBULE, A. & KUNDU, G. C. 2006. Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol*, 16, 79-87.

SCHNEIDER, J. S., ANDERSON, D. W., WADE, T. V., SMITH, M. G., LEIBRANDT, P., ZUCK, L. & LIDSKY, T. I. 2005. Inhibition of progenitor cell proliferation in the dentate gyrus of rats following post-weaning lead exposure. *Neurotoxicology*, 26, 141-5.

THAI, P., STATT, S., CHEN, C. H., LIANG, E., CAMPBELL, C. & WU, R. 2013. Characterization of a novel long noncoding RNA, SCAL1, induced by cigarette smoke and elevated in lung cancer cell lines. *Am J Respir Cell Mol Biol*, 49, 204-11.

TOPKORU, B. C., ALTAY, O., DURIS, K., KRAFFT, P. R., YAN, J. & ZHANG, J. H. 2013. Nasal administration of recombinant osteopontin attenuates early brain injury after subarachnoid hemorrhage. *Stroke*, 44, 3189-94.

VERINA, T., ROHDE, C. A. & GUILARTE, T. R. 2007. Environmental lead exposure during early life alters granule cell neurogenesis and morphology in the hippocampus of young adult rats. *Neuroscience*, 145, 1037-47.

WOODCOCK, J. 2014. Biomarker Letter of Support. In: SERVICES, D. O. H. A. H. (ed.). Food and Drug Administration.

YAMAMOTO, T., OISHI, T., HIGO, N., MURAYAMA, S., SATO, A., TAKASHIMA, I., SUGIYAMA, Y., NISHIMURA, Y., MURATA, Y., YOSHINO-SAITO, K., ISA, T. & KOJIMA, T. 2013. Differential expression of secreted phosphoprotein 1 in the motor cortex among primate species and during postnatal development and functional recovery. *PLoS One*, 8, e65701.