

In Vitro and In Vivo Models to Assess Kidney Toxicity of Environmental Carcinogens

Michelle Carin DeSimone

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Toxicology.

Chapel Hill
2011

Approved by:

David W. Threadgill, Ph.D

Kimryn Rathmell, M.D., Ph.D

John E. French, Ph.D

William Coleman, Ph.D

Thomas O'Connell, Ph.D

©2011
Michelle Carin DeSimone
ALL RIGHTS RESERVED

ABSTRACT

MICHELLE CARIN DESIMONE: *In Vitro* and *In Vivo* Models to Assess Kidney Toxicity of Environmental Carcinogens
(Under the direction of David W. Threadgill, Ph.D)

The etiology of sporadic kidney cancer in humans is unclear. However, it is known that *VHL* tumor suppressor gene mutations are found in the majority of human renal tumors, even in the earliest stages, and play an important role in kidney cancer development. Evidence suggests that *VHL* may be a target for environmental carcinogens such as trichloroethylene (TCE), which is associated with a specific point mutation in the *VHL* gene. Co-exposures to elevated levels of TCE and inorganic arsenic through groundwater and dietary sources are frequently found in human populations and are thought to cause multiple organ toxicities. We hypothesize that there is a mechanistic link between environmental co-exposures to TCE- and arsenic-induced renal toxicity that can be elucidated using genetically engineered model systems. Here we have generated both an *in vitro* model system of the TCE-associated *VHL* mutation in embryonic stem cells, as well as a unique F₃ mouse population derived from the cross between FVB/N-*Abcb1a/1b*^{-/-}, a multi- drug resistant transporter knockout, and CAST/EiJ, a wild-derived strain from a different subspecies of *Mus musculus*. We have employed these systems to elucidate the genetic and environmental components critical for

the development of renal cell carcinoma due to environmental co-exposures to TCE and arsenic.

DEDICATION

To my parents, John and Eileen DeSimone, for their love and support.

To my husband, Edward Flynn, and our puppy Reeses, for all else.

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation to my mentor, Dr. David Threadgill, for his unyielding support, and my clinical co-mentor Dr. Kimryn Rathmell, for her insightful conversations and guidance. I am greatly appreciative for the knowledgeable advice and recommendations contributed by my committee: Dr. William Coleman, Dr. John E. French, Dr. Thomas O'Connell. I am indebted to the members of Threadgill Lab, both present and past: Mrs. Francis Haire, Mrs. Shawn Ballard, Ms. Emily Spence, Dr. Rachel Lynch, Mrs. Megan Garlapow, Dr. Drew Hillhouse, Dr. David Bautz, Dr. Chevonne Eversley, Dr. Erica Rinella, and Dr. Delia Barrick, and to the members of the Rathmell Lab: Mrs. Leslie Kennedy, Ms. Alex Arreola, Mr. Neil Rassmussen, and Mrs. Shufen Chen.

I would like to recognize the following friends and colleagues:

- Dr. Christine Powell, for her guided inspiration.
- Dr. Katherine Horvath, for her friendship and love of buttons.
- Drs. Tricia Wright and Rose Brannon, for their ability to make each lab day better with laughter and cake.

I would not have been able to complete the work presented here without the assistance from the undergraduate students I was so fortunate to have mentored. It is my great pleasure to recognize the following individuals:

- Mr. Garrison Glavich, Mr. Alan Bohn, and Mr. Zachary Mashburn

TABLE OF CONTENTS

ABSTRACT	iii
DEDICATION	v
ACKNOWLEDGEMENTS	vi
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xv
Chapter One: INTRODUCTION	1
A. Genetic alterations in renal cancer	2
1) Kidney cancer and the <i>VHL</i> tumor suppressor gene.....	2
2) Effects of mutations on pVHL function	3
3) Modeling <i>VHL</i> mutations and their disease phenotypes.....	4
B. Environmental exposures are associated with renal cancer	6
1) Trichloroethylene exposure and <i>VHL</i> mutations	6
2) Bioactivation as a mechanism in TCE toxicity	7
3) Arsenic as a co-carcinogen.....	8
4) Mechanisms of co-carcinogenesis	9
C. Susceptibility to renal toxicants.....	10
1) Xenobiotic metabolism implicated in susceptibility	10

2) Arsenic susceptibility in mice	11
3) Dietary factors can modulate susceptibility	12
4) Modeling genetic susceptibility using inbred mouse strains	13
D. Specific aims	15
REFERENCES	22
Chapter Two: THE TCE-ASSOCIATED <i>VHL</i> MUTATION DISRUPTS HIF REGULATION AND PROMOTES TUMOR GROWTH	28
A. Abstract.....	29
B. Introduction	30
C. Methods.....	32
Cell lines and growth conditions.....	32
Construct generation.....	32
Site-directed mutagenesis.....	33
Electroporation into <i>Vhl</i> ^{-/-} stem cells.....	33
Hypoxia treatments.	34
Immunoprecipitation of E3-complex.....	34
Western blot analysis.....	35
RNA isolation.....	36
Quantitative RT-PCR.....	36
<i>In vivo</i> teratoma formation assay.....	36
Immunohistochemistry.....	37
Hypoxia measurements.....	38
Microarray analysis.....	38

Pathway analysis and statistics.....	39
D. Results.....	40
P81S pVHL expression in <i>Vhl</i> ^{-/-} embryonic stem cells.....	40
<i>VHL</i> P81S mutation alters E3-complex and disrupts HIF regulation in vitro.....	41
<i>VHL</i> P81S mutant continues to suppress HIF1 α targets in vitro.	42
<i>VHL</i> P81S mutant displays a marked growth advantage and differentiation in the teratoma assay.	42
<i>VHL</i> P81S teratoma growth is not due to increased proliferation but decreased apoptosis.	43
Mitochondrial biogenesis as mechanism of apoptotic aversion and cell survival in the <i>VHL</i> P81S mutant.....	44
E. Discussion	45
REFERENCES	56
Chapter Three: DIETARY FATTY ACIDS AND MICRONUTRIENTS MODULATE LOW DOSE ARSENIC-INDUCED KIDNEY TOXICITY AND GENE EXPRESSION.....	59
A. Abstract.....	60
B. Introduction	61
C. Methods.....	63
Animals and treatments.	63
RNA isolation.	64
Microarray analysis.	64
Pathway analysis and statistics.....	65
D. Results.....	66

High fat diet exacerbates kidney toxicity due to arsenic exposure.	66
High fat diet alone increased genes involved in fatty acid metabolism and arsenic metabolism.	67
Hierarchical clustering reveals a diet effect on low dose exposures.....	67
Dose-response signatures evident on both diets.	68
Upregulation of cancer-related pathways in high fat fed mice.	69
E. Discussion	70
REFERENCES	78
Chapter Four: A POPULATION-LEVEL GENETIC MODEL REVEALS AN EARLY MARKER OF KIDNEY INJURY AND A GENETIC-BASIS FOR SUSCEPTIBILITY	82
A. Abstract.....	83
B. Introduction	84
C. Methods.....	86
Mouse population generation and design.	86
Dietary treatment: a dose-ratio approach.....	87
Serum collection.....	87
Urine collection.....	88
Clinical chemistry.	88
NGAL ELISA.	89
Serum cytokine analysis.	89
D. Results.....	90
Serum ALT and BUN are non-sensitive markers of injury	90

Urinary NGAL is a sensitive marker of early renal injury and reveals a genetic basis for susceptibility.	91
Serum cytokine analysis indicates elevated NGAL is not due to systemic inflammation.	92
E. Discussion	92
REFERENCES	103
Chapter Five: DISCUSSION	106
A. Conclusions and perspectives	107
1. Gene mutation signatures of environmental exposures.....	108
2. Exploiting genetic variation for toxicant susceptibility studies.....	110
3. Environmentally-relevant doses and diets in toxicological studies	112
B. Challenges and limitations	114
1. Study limitations	114
2. Limitations in co-carcinogenesis studies	116
2.1 <i>Overcoming exposure-data deficits</i>	116
2.2 <i>Chemical mixture evaluations require population-level models</i>	117
C. Future directions.....	118
D. Summary	119
REFERENCES	121

LIST OF TABLES

Table 1.1	Classification of VHL Disease Mutations.....	20
Table 4.1	Diet Formulations.....	96

LIST OF FIGURES

Figure 1.1	Regulation of pVHL in Normoxia and Hypoxia.....	19
Figure 1.2	Metabolic Activation of Trichloroethylene.....	21
Figure 2.1	Site-Directed Mutagenesis of pVHL cDNA.....	49
Figure 2.2	Effect of P81S Mutation on E3-Complex Formation and Function.....	50
Figure 2.3	P81S <i>VHL</i> Mutant Displays Growth Advantage And Differentiation <i>in vivo</i>	51
Figure 2.4	Effect of P81S Mutation on <i>in vivo</i> HIF Stability and Transcriptional Activity.....	52
Figure 2.5	Effect of P81S <i>VHL</i> Mutation on Teratoma Proliferation and Apoptosis.....	53
Figure 2.6	Unsupervised Hierarchical Clustering of ES Cell Teratomas.....	54
Figure 2.7	Clustering and Biological Pathway Analysis of P81S Teratomas.....	55
Figure 3.1	Arsenic Dose and Diet Effect of LDH and Kidney Weights.....	74
Figure 3.2	Unsupervised Hierarchical Clustering of LFD and HFD-fed Mouse Kidneys.....	75
Figure 3.3	Effect of Diet on Gene Expression with Low Dose Arsenic Exposure.....	76
Figure 3.4	Effect of Diet on Gene Expression with High Dose Arsenic Exposure.....	77
Figure 4.1	Determination of Environmentally-Relevant Dosage of TCE and iAs.....	97
Figure 4.2	F ₃ Population Generation and Dosing Groups.....	98
Figure 4.3	Timeline of the 52-week Study and Sample Collections.....	99

Figure 4.4	Effect of Co-Exposures of Serum Clinical Chemistry.....	100
Figure 4.5	Effect of Co-Exposures on Urinary Marker of Kidney Injury.....	101
Figure 4.6	Biochemical Interactions of TCE and iAs in Renal Tubule Cells.....	102

LIST OF ABBREVIATIONS

ALT	Alanine amino transferase
ANOVA	Analysis of variance
ARNT	Arylhydrocarbon nuclear translocator
BUN	Blood urea nitrogen
ccRCC	Clear cell renal cell carcinoma
CREB	Cyclic AMP-response element binding protein
DCA	Dichloroacetic acid
DCVC	Dichlorovinylcysteine
DCVG	Dichlorovinylglutathione
ES	Embryonic stem
EPA	Environmental Protection Agency
FDR	False discovery rate
GST	Glutathione-S-transferase
HFD	High fat western diet
HIF	Hypoxia inducible factor
HRE	Hypoxia responsive elements
iAs	Inorganic arsenic
LDH	Lactate dehydrogenase
LFD	Low fat diet
LSDP	Laboratory strain diversity panel
mTOR	Mammalian target of rapamycin
MRP-1	Multi-drug resistant protein 1

MDR	Multi-drug resistant transporter
NCI	National Cancer Institute
NRF2	Nuclear factor-erythroid 2 p45-related factor 2
PAS	Per-arnt-sim
PUFA	Polyunsaturated fatty acids
pVHL	von Hippel-Lindau tumor suppressor protein
QTL	Quantitative trait loci
<i>RASSF1A</i>	Ras-associated family 1A gene
RXR	Retinoic acid receptor
RCC	Renal cell carcinoma
SAM	S-adenosylmethionine
TCA	Trichloroacetic acid
TCE	Trichloroethylene
TCS2	Tuberous sclerosis 2 gene
<i>VHL</i>	von Hippel-Lindau tumor suppressor gene
VXR	Vitamin D3 receptor
WT	Wild-type
ω -3	Omega 3
ω -6	Omega 6

Chapter One:
INTRODUCTION

A. Genetic alterations in renal cancer

1) Kidney cancer and the *VHL* tumor suppressor gene

The National Cancer Institute (NCI) estimates that over 54,000 new cases of kidney cancer were diagnosed in 2010, and that incidence increases 2% each year [1]. Despite this upward trend, the underlying cause of this continued increase is still unclear. Several risk factors have been identified that may predispose an individual to the development of renal cell carcinoma (RCC) that can be described as both environmental and genetic, including cigarette smoking, obesity, hypertension, and exposure to occupational chemicals [1]. Recent advances in our understanding of the genetics underlying the predisposition to RCC development enable certain susceptible individuals to be identified, despite their lack of common environmental risk factors. One example is the identification of the von-Hippel Lindau (*VHL*) tumor suppressor gene and the predisposition to develop *VHL* disease, a hereditary cancer syndrome predisposing to RCC as well as other tumors. *VHL* mutations and chromosomal deletions in the 3p13-p26 region, which harbors the *VHL* gene and other tumor suppressor genes, have been observed in both sporadic and hereditary clear cell RCC [2].

VHL is considered the “gatekeeper” of human kidney cancer and plays an important role in the renal tumorigenesis [3]. As one would expect, many RCC tumors have mutations in the *VHL* gene that are not inherited, but are somatic in nature [2]. Clear cell renal cell carcinoma (ccRCC) is the most common form of sporadic kidney cancer and epidemiological studies have shown 60-80% of these

tumors have a *VHL* mutation [4]. The descriptive “clear cell” histological phenotype of these tumors results from the upregulation of glycolytic gene pathways. This increase in intracellular glycogen storage appears as clear spaces with hematoxylin and eosin histological staining [5]. Importantly, VHL protein (pVHL) functions as a substrate recognition protein in an E3-ubiquitin ligase complex which targets hypoxia-regulated transcription factors for degradation under normoxia, or normal oxygen conditions [6, 7]. Its ability to act in this complex is thought to be critical for its tumor suppressor function.

2) Effects of mutations on pVHL function

To understand the consequence of *VHL* mutations, it is important to understand the functional role of VHL protein as a tumor suppressor. pVHL in association with Elongin B and C, Cullin 2, and RBX1, exerts its tumor suppressor function by acting as an E3-ubiquitin ligase complex (E3-complex) which targets hypoxia inducible transcription factors (HIFs) for degradation during normoxia (Figure 1.1) [6,7,8]. pVHL also maintains interactions with cell cycle regulatory proteins and transcriptional machinery [9,10]. The three major isoforms of HIF α , HIF1, HIF2 and HIF3, are conditionally regulated basic helix-loop-helix transcription factors that share Per-Arnt-Sim (PAS) homology domains for dimerization with the aryl hydrocarbon nuclear translocator (ARNT or HIF1 β) [11]. ARNT dimerization is considered necessary for HIF transactivation [12]. During low oxygen conditions, the failure of HIFs to be hydroxylated allows them to escape recognition and subsequent degradation, which allows them to bind to the hypoxia responsive elements (HRE) on target genes, some of which are

involved in cellular glycolysis, growth factor production, and angiogenesis [7,8,11]. Mutations in *VHL* that disrupt this complex formation even partially can allow accumulation of nuclear HIF and transcriptional upregulation of hypoxia-inducible genes under normoxic conditions [12, 13].

HIF1 and HIF2 play dynamic roles in the progression of *VHL*-mediated renal tumorigenesis [7, 12, 13]. Both HIF1 and HIF2 are capable of binding the same HRE target genes, however, HIF2 is thought to bind with greater affinity to *TGF- α* , *EPO*, and *VEGF* [12]. While HIF1a is believed to be mainly responsible for the pro-glycolytic phenotype of ccRCC tumors, HIF2 stabilization is thought to contribute to the progression of tumor growth [12]. Molecular studies have shown that introduction of a stabilized HIF1a to RCC cells was not able to induce a tumorigenic phenotype [14]. However, when all three HIF isoforms are stabilized, tumorigenesis is restored. Further investigating this opposing role of HIF1 and HIF2, Kondo et al. developed a cell line with constitutive expression of stable HIF2 in the presence of normal pVHL. Since pVHL and E3-complex was functional, HIF1 was suppressed while HIF2 remained stable. Stable expression of HIF2 alone was able to promote cell proliferation and tumorigenesis when grown as a xenograft [15]. This indicates that stabilization of HIF1, and importantly HIF2, during normoxic conditions is an important indicator of the pathogenesis of a particular *VHL* mutation.

3) Modeling *VHL* mutations and their disease phenotypes

A strong genotype-phenotype correlation exists in the type of *VHL* mutation and the *VHL* disease phenotype observed in affected patients [16]. *VHL*

disease subtypes, and their representing mutations, have been classified based on their risk of developing pheochromocytoma and RCC (Table 1.1) [17]. For example, Type 1 mutations result in loss of pVHL expression that results in a high risk for renal cell carcinoma. Type 2 mutations are subdivided into 2A, 2B, and 2C. Type 2A mutations result in low risk for renal cell carcinoma while Type 2B mutations result in high risk for renal cell carcinoma. The difference in renal cancer risk between type 2A and type 2B mutations can be inferred from its E3 complex interactions and HIF regulation; 2A maintains E3 complex formation, while 2B does so partially [20]. Finally, 2C mutations do not result in any increased risk for renal cancer and maintain interactions with the E3-ubiquitin ligase complex [17]. Taken together, the risk for renal cell carcinoma associated with a particular *VHL* mutation can be inferred by molecularly classifying *VHL* mutations based on their ability to destabilize the E3-ubiquitin ligase complex formation and regulate HIFs.

Genetically engineered mouse embryonic stem cell systems have been a good model for determining the molecular alterations associated with particular *VHL* mutations [18, 19, 20, 21]. *Vhl*^{-/-} embryonic stem cells are unable to regulate the hypoxia response pathway and have elevated levels of stabilized HIF1 and HIF2 under normal oxygen conditions [19, 20]. The predicted upregulation of HRE-responsive genes is also observed [19]. Introduction of the wild-type human pVHL into these cells, rescues the constitutive hypoxia signaling phenotype [20]. Cell lines harboring type 2A and type 2B *VHL* mutations, respectively named Y112H and R167Q, have been created and characterized for their ability to

interact and function with the E3-complex, as well as degrade HIF1 and HIF2 under normoxic conditions [20]. Consistent with human data, the 2A mutant retains E3-complex formation, reflecting a low risk for RCC mutation, while the 2B mutant shows disrupted complex formation due to a diminished interaction with Elongin C. However, it was observed that the 2B mutant still retains interactions with other complex members, Ringbox1 and Cullin-2, which explains its ability to partially ubiquitinate HIF1 during normoxia [21]. Interestingly, when these cells are grown in a teratoma model system, they are highly vascular due to aberrant HIF signaling [20]. These studies confirm that *VHL* mutations can be successfully modeled using genetically modified *Vhl*^{-/-} ES cells to study the molecular effects of *VHL* mutations both *in vivo* and *in vitro*.

B. Environmental exposures are associated with renal cancer

1) Trichloroethylene exposure and *VHL* mutations

The *VHL* gene has been proposed to be a target of environmental carcinogens, such as trichloroethylene and N-nitrosodimethylamine [22, 23]. TCE is an aliphatic chlorinated hydrocarbon which was once used heavily as an industrial solvent, fire-retardant, and dry cleaning agent [24]. Due to the ubiquitous use and improper disposal of TCE from a multitude of industries over the past fifty years, TCE can be found in drinking water at low levels as well as concentrated at high levels in underground plumes [24]. TCE is considered a likely human carcinogen and national priority site compound by the Environmental Protection Agency, restricting its current use and disposal to reduce human exposure [25]. An epidemiological study by Brauch *et al.*

conducted at an industrial site in Germany, put forth evidence to suggest that occupational exposure to trichloroethylene resulted in a specific hotspot mutation in the *VHL* gene, and that multiplicity of *VHL* mutations in kidney tumors of this population positively correlated with their TCE exposure level [26]. The nucleotide 454 hotspot mutation resulted in a proline to serine amino acid change at codon 81, thus referred to as the P81S missense mutation. Despite the discovery of this specific hotspot mutation in *VHL* over a decade ago, the functional consequences of this mutation remain unknown.

2) Bioactivation as a mechanism in TCE toxicity

The mechanisms governing kidney toxicity due to TCE exposure remain unclear due to its complex metabolism and extensive bioactivation (Figure 1.2). TCE is metabolized preferentially by the cytochrome p450 pathway to trichloroacetic acid (TCA), dichloroacetic acid (DCA) and trichloroethanol, and as a secondary pathway, TCE can also be conjugated to glutathione forming dichlorovinylglutathione (DCVG) [27]. DCVG, once in the blood stream, can be taken up into the proximal tubule epithelium of the kidney and is further hydrolyzed to dichlorovinylcysteine (DCVC). The metabolism of DCVC by beta-lyase within the cytoplasm and mitochondria to a reactive thiol, is thought to be the reactive metabolite that contributes to the development of kidney toxicity [29]. However, the argument against a mutagenic mode of action is that this metabolite represents less than 0.1% of the total metabolite formation, and is unlikely to form at a concentration large enough to overwhelm DNA repair to result in mutagenesis of the *VHL* gene [27, 29].

Further complicating our understanding is a discord between rodent species in the development of renal toxicity following TCE exposure. Carcinogenesis bioassays in Fisher 344 rats and B6C3F1 mice conducted at the National Toxicology Program have been inconclusive, with 3/50 rats and 0/50 mice developing kidney tumors after a 2-year maximum tolerated dose bioassay [28]. Rodent genetics has been taken into consideration to find a more susceptible strain for testing. The Eker rat, a rat harboring a dominant mutant tuberous sclerosis (TSC2) allele, is more susceptible to renal carcinogens but did not show increased susceptibility to kidney tumor formation with TCE exposure [29]. In addition, haploinsufficient *Vhl*^{+/-} mice have also been tested for increased susceptibility to known nephrotoxics with the intention to later test the ability of TCE to mutate and inactivate the remaining *Vhl* allele if successful. However, no increase in susceptibility was observed [30]. Finally, conditional inactivation of *Vhl* without further challenge does not promote tumor formation, indicating that in mice, loss of *Vhl* is insufficient for the development of tumors [31]. The lack of RCC animal models of both VHL disease and trichloroethylene toxicity has thwarted an understanding of the mechanisms governing the role of TCE exposure in the development of renal carcinogenesis, as well as the possible role of *VHL* mutations in that development.

3) Arsenic as a co-carcinogen

There is increasing evidence that environmental exposure to inorganic arsenic can act as a co-carcinogen [32]. Inorganic arsenic is a naturally occurring element found in the Earth's crust, thus it can be found in most water sources

and food items grown from arsenic-contaminated earth. It was discovered as one of the first known human carcinogens, causing tumors of the skin, lungs, bladder, kidney and liver [33]. Inorganic arsenic is metabolized to more toxic trivalent metabolites, dimethyl- and monomethyl- arsenic, which are excreted by the kidney into urine [34]. Inorganic arsenic is tightly monitored in public water systems with a federal EPA standard of 10ppb or 10µg/L being set. However, these limits are not well monitored in private wells or in food sources [35]. This has led to an increased exposure to certain populations where arsenic is abundant in soils, such as the “slate belt” of North Carolina, New Hampshire, India, Asia, Thailand and Mexico [36, 37]. A recent study has shown that arsenic is taken up and concentrated in rice at concentrations upwards of 50-450ug/kg rice [38], ten-times more than other grains and leading to a significant dietary exposure. The U.S. currently imports several varieties of basmati and jasmine rice, sake, a rice wine, and products made from rice from these endemic areas of the world without regulations on the arsenic level resulting in a potentially increased exposure to the population.

4) Mechanisms of co-carcinogenesis

It is well established that arsenic can act to decrease DNA repair, increase HIF stabilization and alter DNA methylation by sequestering methyl donors [32]. A mechanism of carcinogenesis identified for environmental metals involves the disruption of pVHL-mediated regulation of oxygen sensing through the stabilization of HIFs [32]. However, it remains unclear and untested whether low

dose arsenic exposure could result in chronic stabilization of HIFs or lead to aberrant methylation of the *VHL* tumor suppressor gene to induce ccRCC.

One of the caveats of exploring this hypothesis is that mice are not sensitive to the loss of *VHL* and do not develop renal tumors upon *VHL* loss. However, it was found that chronic oral exposure of A/J mice to inorganic arsenic resulted in the development of lung tumors with loss of *p16INK4a* and *RASSF1A* tumor suppressor gene expression due to promoter hypermethylation [39]. In 2001, the RAS association family 1 gene (*RASSF1A*), located at 3p21.3, was identified as a candidate lung tumor suppressor gene in humans as well [40]. Due to the genomic proximity of *RASSF1A* to chromosome 3p25, harboring the *VHL* gene, the methylation state of *RASSF1A* using bisulfite sequencing in human renal cell carcinomas was examined, and *RASSF1A* was found to be hypermethylated in both *VHL*-mutated and non-mutated ccRCC tumors [41]. It has been proposed that loss of both *RASSF1A* and *VHL* is required for the development of renal tumors in the mouse due to a functional redundancy in tumor suppression [42]. Until a mouse model of human ccRCC is developed, this only suggests that co-exposure to arsenic may result in epigenetic silencing of tumor suppressor genes, which may act to induce renal neoplasms.

C. Susceptibility to renal toxicants

1) Xenobiotic metabolism implicated in susceptibility

Genetic polymorphisms in drug metabolizing genes were found to increase renal cancer risk in TCE and arsenic exposed human populations [57, 58]. TCE and inorganic arsenic are both conjugated to glutathione by glutathione-

s-transferases during metabolism, and it is thought that these metabolites lead to the formation of nephrotoxic species [29]. Several epidemiological studies examining renal cancer risk associated with occupational exposure to TCE found a significant association among TCE-exposed subjects with at least one intact *GSTT1* allele (active genotype; OR = 1.88) but not among subjects with two deleted alleles (null genotype; OR = 0.93) [59]. Similarly, with inorganic arsenic exposed individuals, a significantly increased risk (OR = 3.9) was observed in study subjects with specific genotypes of polymorphic genes *GSTO1* and *GSTO2* [60]. Further, genes involved in other mechanisms, such as antioxidant defense, DNA repair, folate one-carbon metabolism and in arsenic transport across the cell membrane have all been shown to affect an individual's susceptibility [61]. Together, we can infer from these studies that genetic variants play a role in increasing renal susceptibility to both TCE and arsenic exposure, and that this is likely due to differences in the formation of, or protection from, nephrotoxic metabolites.

2) Arsenic susceptibility in mice

Mice are exceedingly efficient at metabolizing and extruding arsenic. For this reason, mice are relatively resistant to the effects of arsenic, unlike humans [43]. Inbred mouse strains have been used to examine the dependence of arsenic susceptibility on genetic background. Kimura et al. acutely exposed BALB/c and C57BL/6 mice to sodium arsenite and found that BALB/c mice showed elevated levels of BUN, creatinine, severe hemorrhage, tubular necrosis, and the disappearance of the PAS-positive brush border, while the C57BL/6 mice

had markedly attenuated injury, including a decreased intra-renal arsenic concentration. This difference in strain susceptibility was found to be due to an increased renal expression of heavy metal inducible proteins, like multidrug resistance-associated protein-1 (MRP-1), a renal transporter that acts to extrude arsenic from the kidney. Using a specific inhibitor of MRP-1, they were able to augment renal injury in the C57BL/6 strain [44]. Subsequent studies using the MDR p-glycoprotein ABC transporter knockout mouse showed that these mice accumulate arsenic in the kidney and are more susceptible to renal injury [45]. This suggests that inhibition of renal transport of arsenic can increase susceptibility to the development of renal injury in mice, and that the use of a transgenic transporter knockout mouse could be critical for the investigation of arsenic co-exposure in mice.

3) Dietary factors can modulate susceptibility

Obesity, hypertension, and Type 2 diabetes are diseases that increase the risk for the development of renal cancer in humans, and are also associated with an improper diet and high body mass index [46]. Epidemiological studies have reported a significant association with the intake of saturated fat, red meat, cholesterol, and dietary starch with an increased risk for renal cell cancer, while intake of fats from vegetable sources, rich in ω -3 polyunsaturated fatty acids was associated with decreased risk [47, 48, 49]. Dietary modifications such as increased saturated fat are thought to contribute to the increase in carcinogenic potential of toxicants by altering baseline gene expression in histologically normal tissues and increasing serum lipids, hormones, and other metabolic factors [50].

The “western diet” is a chow formulated for rodents which mimics the fat and calorie consumption of the western world, and has proven to be a useful tool in animal models of breast cancer, cardiovascular disease and colon cancer [51, 52, 53]. Dietary modification studies using FVB/N, C57BL/6, and ICR mice on a control (10% fat) or high fat diet (60% fat) regimen found that relative to the control diet, mice on the high fat diet showed increased circulating insulin-like growth factor-I (IGF-1) and activation/phosphorylation of Akt and mTOR, two pathways frequently dysregulated in ccRCC. They also show that this effect was not dependant on genetic background [54]. These findings suggest that dietary fat consumption can modulate signaling through cell-surface receptors, like IGF-I, and in turn activate downstream pathways including Akt and mTOR. Interestingly, molecular evaluation of human kidney tumors has shown that expression levels of activated mTOR and Akt were significantly higher in tumor tissue when compared to normal tissue and further, enhanced Akt/mTOR and ERK/MAPK signaling was shown to be independent of *VHL* status, but instead dependent on HIF1 and HIF2 relative expression [55]. These studies indicate that diet is an environmental factor that plays an important role in the development of cancer in humans through the modulation of cell signaling, and that dietary formulation should be given serious consideration during the design of animal studies of carcinogenesis.

4) Modeling genetic susceptibility using inbred mouse strains

Inbred mouse strains are the foundation of mammalian genetics research and continue to be a powerful tool in toxicological research [62]. Individuals of a

given strain are virtually homozygous and isogenic. In addition, with the sequencing of the mouse genome and many inbred strains, the naturally occurring genetic variation that drives their phenotypic differences can now be discovered at the gene level. Phenotypic variation between mouse lines, such as their toxicity and disease susceptibility traits, can be used to model certain human populations [63]. As such, the Laboratory Strain Diversity Panel (LSDP), a panel of inbred strains, has recently been used to identify susceptible and resistant strains to xenobiotic toxicity, as well as identification of biomarker genes whose expression strongly correlates with toxicity [64]. Many toxicity phenotypes are not controlled by only one gene, but are polygenetic, a product of many genes and their environment, and as such, are quantitative traits.

Quantitative trait loci (QTLs) are defined as segments of DNA containing the genes that underlie a quantitative trait. QTL mapping techniques are frequently used to identify genomic regions associated with variation in a phenotype of interest. The mapping of quantitative traits, such as susceptibility to renal injury, can be achieved through generation of a mouse mapping population, by controlled breeding of two inbred strains [65]. F_2 or F_3 intercross populations from diverse inbred strains are predicted to will exhibit variation in response to toxicant injury that can be then be mapped to the gene variants underling the response [65]. QTL mapping can be a powerful tool for the identification of genomic loci that confer susceptibility, potentially identifying human populations most at risk for developing renal injury due to exposure.

D. Specific aims

An unfortunate gap in toxicological research is the lack of experimental studies that explain how chronic, low dose exposures to a mixture of environmental pollutants, such as groundwater contaminants and dietary carcinogens, can modulate disease susceptibility and outcome. Co-exposures to elevated levels of TCE and inorganic arsenic through groundwater and dietary sources are frequently found in human populations and are thought to cause multiple organ toxicities in susceptible individuals. However due to conflicting species-specific differences in metabolism and detoxification, the interpretation of human relevance from rodent bioassays is subject to great debate. An example is the unclear etiology of kidney cancer in humans. It is known that *VHL* tumor suppressor gene mutations are found in the majority of human renal cell carcinomas, even in the earliest stages, and that *VHL* plays an important role in kidney cancer development. Epidemiological evidence suggests that *VHL* may be a target for environmental carcinogens such as TCE, which is thought to induce specific point mutations in the *VHL* gene. Furthermore, despite this known co-exposure, there is no data examining the role of arsenic as a possible co-carcinogen in the kidney. ***We hypothesize that there is a mechanistic link between environmental co-exposures to TCE- and arsenic-induced renal toxicity that can be elucidated using genetically engineered model systems.*** Several deficiencies have been highlighted in TCE and arsenic research including 1) lack of experimental models with which to investigate the effects of the TCE-induced *VHL* mutations, 2) poor understanding of the linearity

of the dose response relationship, 3) lack of studies examining the effect of nutritional deficiencies, and 4) lack of consideration for susceptible populations in estimating risks [27]. There is, therefore, a pressing need for the development of *in vitro* and *in vivo* model systems with which to address these research deficiencies. Towards this end, the *Specific Aims* of this project were:

Specific aim 1: To develop an *in vitro* model to functionally characterize the TCE-associated P81S missense mutation on *VHL* tumor suppressor function to determine its relevance to renal tumorigenesis. It remains undetermined whether the TCE-associated point mutation in the *VHL* gene is sufficient to alter pVHLs function and tumor suppressor capacities. The hypothesis is that the P81S *VHL* mutation will disrupt the tumor suppressor function of *VHL* and lead to phenotypic characteristics similar to *VHL* disease-causing mutations. To investigate a mouse embryonic stem cell system was used to model the P81S *VHL* missense mutation seen in human patients exposed to TCE. The P81S *VHL* mutant was compared to other clinically relevant *VHL* disease mutations for hypoxia-inducible transcription factor stabilization and activity, E3-complex interactions, and *in vivo* growth and differentiation characteristics.

Specific aim 2: To establish whether dietary factors influence kidney toxicity due to low dose inorganic arsenic exposure through gene expression analysis. Dietary deficiency in micronutrients as well as an imbalance in dietary fatty acids can play a large part by increasing susceptibility to environmental toxicants [34]. This becomes especially true in the case of

inorganic arsenic, where dietary co-factors play a role in the metabolism and detoxification [35]. As such, nutritionally deficient populations represent a unique and vulnerable subset of the population that is likely to be at increased risk for kidney toxicity due to arsenic exposure [36]. It is hypothesized that a nutrient poor western diet will increase arsenic toxicity in the kidney corresponding to altered gene expression pathways. To investigate this, FVB/N-*Abcb1a/1b*^{-/-} mice, a multidrug resistant ABC transporter knockout model, will be exposed to environmentally-relevant concentrations of inorganic arsenic either on a standard rodent chow or a nutrient poor western diet for 10 weeks. Serum clinical chemistry, histopathology, and kidney gene expression by microarray analysis will be analyzed.

Specific aim 3: To develop an *in vivo* population-level model of TCE and arsenic co-exposure that can address genetic variation and dose-response linearity. It is well known that drug metabolism and transport, as well as genetic variation and diet play roles in rodent-to-human differences in response to kidney injury. To address these three issues, we have a unique F₃ mouse intercross population derived from an intercross between the FVB/N-*Abcb1a/1b*^{-/-} strain, a multidrug resistant ABC transporter knockout mouse, and CAST/EiJ, a wild-derived strain from a different subspecies of *Mus musculus*. FVB/N-*Abcb1a/1b*^{-/-} mice show decreased clearance of drug metabolites, including arsenic, and are more susceptible to injury in the kidney, while CAST/EiJ allows examination of genetic variability as this strain carries a completely different set of allele combinations, including *VHL*. It is hypothesized

that co-exposure to trichloroethylene and arsenic will act in an additive or synergistic fashion to induce renal toxicity, which can be modulated by genetically determined factors. To model human exposure, mice were administered TCE and arsenic via drinking water and chow, respectively, at environmentally-relevant concentrations for 52 weeks. After 16 weeks of exposure, serum and urine will be examined for markers of early renal injury and general organ toxicity, and observed whether the response to injury is attributed to genetic variation within the population.

Figure 1.1

Regulation of Hypoxia Inducible Factors by pVHL in Normoxia and Hypoxia

Illustration depicts the regulation of HIF α by pVHL through the formation of an E3-ubiquitin ligase complex. During normal oxygen, HIF α becomes hydroxylated at specific proline residues whereby allowing pVHL in association with Elongin B, Elongin C, Ring-box1 (Rbx1) and Cullin2 (Cul2) to recognize it as a substrate. HIF α is then polyubiquitinated and targeted for proteosomal degradation. Under hypoxic conditions, the lack of molecular oxygen prevents hydroxylation of HIF, allowing it to escape recognition by pVHL. HIF α then dimerizes with HIF- β and transcriptional co-activators CBP/p300 to transcriptionally activate hypoxia-responsive genes (i.e., *Epo*, *VEGF*, *Glut1*) involved in energy metabolism, angiogenesis and a host of other cellular processes.

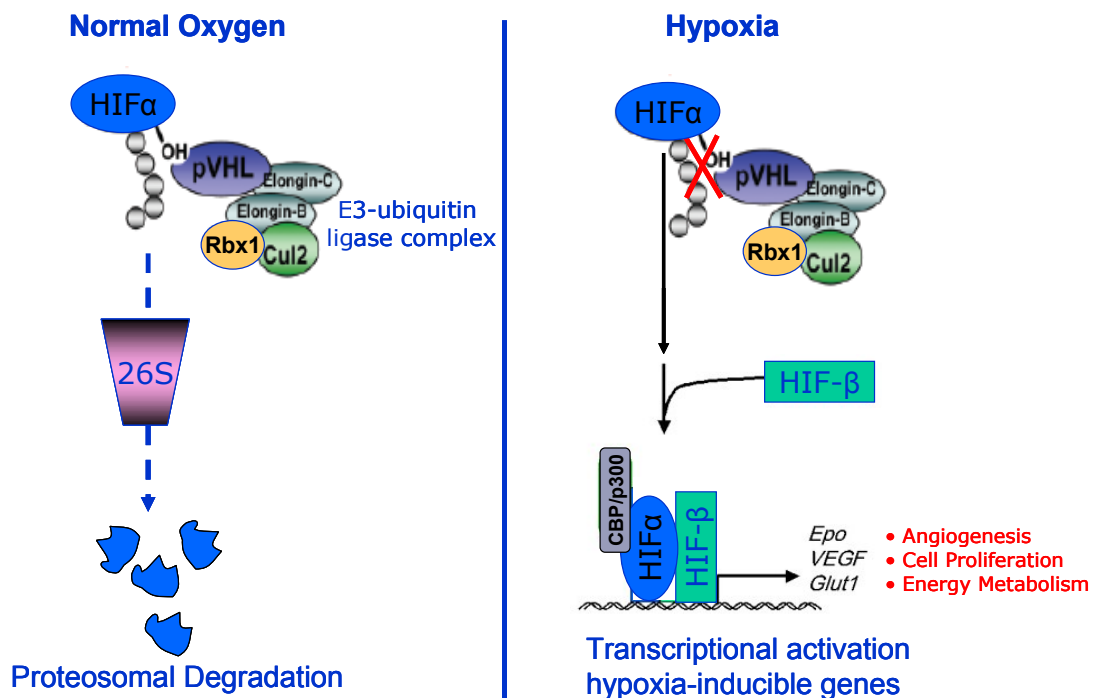


Table 1.1**Classification of VHL Disease Mutations**

VHL mutations identified in VHL disease patients reveal genotype-phenotype correlations with disease presentation. There are two subtypes, classified by no risk, low risk (type 1) or high risk (type 2) for pheochromocytoma, a neuroendocrine tumor of the adrenal glands. In each case a representative *VHL* missense mutations was identified to correlate with disease presentation [20]. *VHL* mutations also correlate with risk for development of renal cell carcinoma (RCC). Type 1 mutations are frameshift or truncating mutations which result in loss of VHL protein expression and have a high risk of RCC. Type 2A, 2B and 2C are point mutations with varying abilities to disrupt the VHL-HIF axis corresponding to RCC risk. In the case of the R200W mutation, there is not risk of tumor formation, only Chuvash Polycythemia.

VHL Disease Subtype	Representing Mutation	Pheochromocytoma	Renal Cell Carcinoma
Type 1	Loss of VHL	Low	High
Type 2A	Y112H	High	Low
Type 2B	R167Q	High	High
Type 2C	L188V	High	none
Chuvash Polycythemia	R200W	none	none

Metabolic Activation of Trichloroethylene

The diagram illustrates the metabolic pathways of Trichloroethylene (TCE) and their potential for causing nephrotoxicity. TCE (1,1,2-trichloroethene) is shown at the top. Two primary metabolic routes are depicted:

- GSH Transferase Pathway:** TCE reacts with Glutathione (GSH) to form an S-Glutathione Conjugate (1,2-DCVG). This intermediate is then converted to S-1,2-Dichlorovinylcysteine. This pathway leads to the excretion of Mercapturic Acids.
- CYP450 Pathway:** TCE is oxidized by CYP450 to form an epoxide intermediate. This epoxide is then converted to Chloral Hydrate. Chloral Hydrate can be further metabolized by ADH to Trichloroethanol, which is then converted to Glucuronide Conjugates for excretion. Alternatively, Chloral Hydrate can be converted by ALDH to Trichloroacetic Acid, which is then converted to Dichloroacetic Acid.

A box labeled "B-Lyase" highlights the conversion of S-1,2-Dichlorovinylcysteine to a Reactive Thiol (S=C=CHCl). This Reactive Thiol is shown to lead to Nephrotoxicity.

REFERENCES

- 1: American Cancer Society. 2010 "Facts and Figures" <http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-026238.pdf>. Accessed March 5, 2011.
- 2: Gnarr JR, Glenn GM, Latif F, Anglard P, Lerman MI, Zbar B, Linehan WM. Molecular genetic studies of sporadic and familial renal cell carcinoma. *Urol Clin North Am*. 1993;20(2):207-16.
- 3: Kaelin WG Jr. The von Hippel-Lindau tumor suppressor gene and kidney cancer. *Clin Cancer Research* 2004;10:6290S-5S.
- 4: Zbar B. Von Hippel-Lindau disease and sporadic renal cell carcinoma. *Cancer Surv*. 1995;25:219-32.
- 5: Steinberg P, Störkel S, Oesch F, Thoenes W. Carbohydrate metabolism in human renal clear cell carcinomas. *Lab Invest*. 1992;67(4):506-11.
- 6: Gordan JD, Lal P, Dondeti VR, et al. HIF- α effects on c-Myc distinguish two subtypes of sporadic VHLdeficient clear cell renal carcinoma. *Cancer Cell*. 2008;14(6):435-46.
- 7: Kaelin WG Jr. The von Hippel-Lindau tumour suppressor protein: O₂ sensing and cancer. *Nat Rev Cancer*. 2008;8(11):865-73.
- 8: Tyers M, Rottapel R. VHL: a very hip ligase. *Proc Natl Acad Sci U S A*. 1999;96(22):12230-2.
- 9: Zatyka M, da Silva NF, Clifford SC, et al. Identification of cyclin D1 and other novel targets for the von Hippel-Lindau tumor suppressor gene by expression array analysis and investigation of cyclin D1 genotype as a modifier in von Hippel-Lindau disease. *Cancer Res*. 2002;62(13):3803-11.
- 10: Mikhaylova O, Ignacak ML, Barankiewicz TJ, et al. The von Hippel-Lindau tumor suppressor protein and Egl-9-Type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress. *Mol Cell Biol*. 2008;28(8):2701-17.
- 11: Hu CJ, Sataur A, Wang L, Chen H, Simon MC. The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1 α and HIF-2 α . *Mol Biol Cell*. 2007;18(11):4528-42.
- 12: Gordan JD, Simon MC. Hypoxia-inducible factors: central regulators of the tumor phenotype. *Curr Opin Genet Dev*. 2007;17(1):71-7.

- 13: Maynard MA, Evans AJ, Shi W, Kim WY, Liu FF, Ohh M. Dominant-negative HIF-3 alpha 4 suppresses VHL-null renal cell carcinoma progression. *Cell Cycle*. 2007;6(22):2810-6.
- 14: Maranchie JK, Vasselli JR, Riss J, Bonifacino JS, Linehan WM, Klausner RD. The contribution of VHL substrate binding and HIF1-alpha to the phenotype of VHL loss in renal cell carcinoma. *Cancer Cell*. 2002;1(3):247-55.
- 15: Kondo K, Kim WY, Lechpammer M, Kaelin WG Jr. Inhibition of HIF2alpha is sufficient to suppress pVHLdefective tumor growth. *PLoS Biol*. 2003;1(3):E83
- 16: Crossey PA, Richards FM, Foster K, et al. Identification of intragenic mutations in the von Hippel-Lindau disease tumour suppressor gene and correlation with disease phenotype. *Hum Mol Genet*. 1994;3(8):1303-8.
- 17: Clifford SC, Cockman ME, Smallwood AC, et al. Contrasting effects on HIF-1alpha regulation by disease-causing pVHL mutations correlate with patterns of tumourigenesis in von Hippel-Lindau disease. *Hum Mol Genet*. 2001;10(10):1029-38.
- 18: Mack FA, Rathmell WK, Arsham AM, Gnarr J, Keith B, Simon MC. Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. *Cancer Cell*. 2003;3(1):75-88.
- 19: Lee CM, Hickey MM, Sanford CA, McGuire CG, Cowey CL, Simon MC, Rathmell WK. VHL Type 2B gene mutation moderates HIF dosage in vitro and in vivo. *Oncogene*. 2009;28(14):1694-705.
- 20: Rathmell WK, Hickey MM, Bezman NA, Chmielecki CA, Carraway NC, Simon MC. In vitro and in vivo models analyzing von Hippel-Lindau disease-specific mutations. *Cancer Res*. 2004;64(23):8595-603.
- 21: Hacker KE, Lee CM, Rathmell WK. VHL type 2B mutations retain VBC complex form and function. *PLoS ONE*. 2008;3(11):e3801.
- 22: Shiao YH, Rice JM, Anderson LM, Diwan BA, Hard GC. von Hippel-Lindau gene mutations in N-nitrosodimethylamine-induced rat renal epithelial tumors. *J Natl Cancer Inst*. 1998;90(22):1720-3.
- 23: Brauch H, Weirich G, Brieger J, et al. VHL alterations in human clear cell renal cell carcinoma: association with advanced tumor stage and a novel hot spot mutation. *Cancer Res*. 2000;60(7):1942-8.
- 24: Kumar P, Prasad AK, Dutta KK. Steroidogenic alterations in testes and sera of rats exposed to trichloroethylene (TCE) by inhalation. *Hum Exp Toxicol*. 2000;19(2):117-21.

- 25: Chiu WA, Caldwell JC, Keshava N, Scott CS. Key scientific issues in the health risk assessment of trichloroethylene. *Environ Health Perspect.* 2006;114(9):1445-9.
- 26: Brauch H, Weirich G, Hornauer MA, Storkel S, Wohl T, Bruning T. Trichloroethylene exposure and specific somatic mutations in patients with renal cell carcinoma. *J Natl Cancer Inst.* 1999; 91(10):854-61.
- 27: National Research Council (U.S.), National Research Council (U.S.). Committee on Human Health Risks of Trichloroethylene. Assessing the human health risks of trichloroethylene: key scientific issues ;National Academies Press, 2006.
- 28: National Toxicology Program. NTP Carcinogenesis Studies of Trichloroethylene (Without Epichlorohydrin)(CAS No. 79-01-6) in F344/N Rats and B6C3F1 Mice (Gavage Studies). *Natl Toxicol Program Tech Rep Ser.* 1990; 243:1-174.
- 29: Mally A, Walker CL, Everitt JI, Dekant W, Vamvakas S. Analysis of renal cell transformation following exposure to trichloroethene in vivo and its metabolite S-(dichlorovinyl)-L-cysteine in vitro. *Toxicology.* 2006;224(1-2):108-18.
- 30: Kleymenova E, Everitt JI, Pluta L, Portis M, Gnarr JR, Walker CL. Susceptibility to vascular neoplasms but no increased susceptibility to renal carcinogenesis in Vhl knockout mice. *Carcinogenesis.* 2004;25(3):309-15.
- 31: Rankin EB, Tomaszewski JE, Haase VH. Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. *Cancer Res.* 2006;66(5):2576-83.
- 32: Salnikow K, Zhitkovich A. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem Res Toxicol.* 2008;21(1):28-44.
- 33: Guha Mazumder DN. Chronic arsenic toxicity & human health. *Indian J Med Res.* 2008;128(4):436-47.
- 34: Tseng CH. A review on environmental factors regulating arsenic methylation in humans. *Toxicol Appl Pharmacol.* 2009;235(3):338-50.
- 35: Frumkin H, Thun MJ. EPA re-establishes lower limit for drinking water; New standard fully in effect by 2006. *CA Cancer J Clin* 2001;51: 254-262
- 36: Tchounwou PB, Wilson B, Ishaque A. Important considerations in the development of public health advisories for arsenic and arsenic-containing compounds in drinking water. *Rev Environ Health.* 1999;14(4):211-29.

- 37: Kumar A, Adak P, Gurian PL, Lockwood JR. Arsenic exposure in US public and domestic drinking water supplies: A comparative risk assessment. *J Expo Sci Environ Epidemiol*. 2009.
- 38: Stone R. Food safety. Arsenic and paddy rice: a neglected cancer risk?. *Science*. 2008;321(5886):184-5.
- 39: Cui X, Wakai T, Shirai Y, Hatakeyama K, Hirano S. Chronic oral exposure to inorganic arsenate interferes with methylation status of p16INK4a and RASSF1A and induces lung cancer in A/J mice. *Toxicol Sci*. 2006;91(2):372-81.
- 40: Dammann R, Takahashi T, Pfeifer GP. The CpG island of the novel tumor suppressor gene RASSF1A is intensely methylated in primary small cell lung carcinomas. *Oncogene*. 2001;20(27):3563-7.
- 41: Dreijerink K, Braga E, Kuzmin I, et al. The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. *Proc Natl Acad Sci U S A*. 2001; 98(13):7504-9.
- 42: Peters I, Rehmet K, Wilke N, Kuczyk MA, Hennenlotter J, Eilers T, Machtens S, Jonas U, Serth J. RASSF1A promoter methylation and expression analysis in normal and neoplastic kidney indicates a role in early tumorigenesis. *Mol Cancer*. 2007;6:49.
- 43: Clewell HJ, Thomas RS, Gentry PR, et al. Research toward the development of a biologically based dose response assessment for inorganic arsenic carcinogenicity: a progress report. *Toxicol Appl Pharmacol*. 2007;222(3):388-98.
- 44: Kimura A, Ishida Y, Wada T, Yokoyama H, Mukaida N, Kondo T. MRP-1 expression levels determine strain-specific susceptibility to sodium arsenic-induced renal injury between C57BL/6 and BALB/c mice. *Toxicol Appl Pharmacol*. 2005;203(1):53-61.
- 45: Liu J, Liu Y, Powell DA, Waalkes MP, Klaassen CD. Multidrug-resistance *mdr1a/1b* double knockout mice are more sensitive than wild type mice to acute arsenic toxicity, with higher arsenic accumulation in tissues. *Toxicology*. 2002; 170(1-2):55-62.
- 46: Decastro GJ, McKiernan JM. Epidemiology, clinical staging, and presentation of renal cell carcinoma. *Urol Clin North Am*. 2008;35(4):581-92.
- 47: Bidoli E, Talamini R, Zucchetto A, et al. Macronutrients, fatty acids, cholesterol and renal cell cancer risk. *Int J Cancer*. 2008;122(11):2586-9.

- 48: Lee JE, Spiegelman D, Hunter DJ, Albanes D et al., Fat, protein, and meat consumption and renal cell cancer risk: a pooled analysis of 13 prospective studies. *J Natl Cancer Inst.* 2008;100(23):1695-706.
- 49: Brock KE, Gridley G, Chiu BC, Ershow AG, Lynch CF, Cantor KP. Dietary fat and risk of renal cell carcinoma in the USA: a case-control study. *Br J Nutr.* 2009;101(8):1228-38.
- 50: Yang K, Yang W, Mariadason J, Velcich A, Lipkin M, Augenlicht L. Dietary components modify gene expression: implications for carcinogenesis. *J Nutr.* 2005;135(11):2710-4.
- 51: Akiyama TE, Lambert G, Nicol CJ, et al. Peroxisome proliferator-activated receptor beta/delta regulates very low density lipoprotein production and catabolism in mice on a Western diet. *J Biol Chem.* 2004;279(20):20874-81.
- 52: Gordon RR, Hunter KW, La Merrill M, Sørensen P, Threadgill DW, Pomp D. Genotype X diet interactions in mice predisposed to mammary cancer: II. Tumors and metastasis. *Mamm Genome.* 2008;19(3):179-89.
- 53: Chen H, Hansen MJ, Jones JE, Vlahos R, Anderson GP, Morris MJ. Detrimental metabolic effects of combining long-term cigarette smoke exposure and high-fat diet in mice. *Am J Physiol Endocrinol Metab.* 2007;293(6):E1564-71.
- 54: Moore T, Beltran L, Carbajal S, Strom S, Traag J, Hursting SD, DiGiovanni J. Dietary energy balance modulates signaling through the Akt/mammalian target of rapamycin pathways in multiple epithelial tissues. *Cancer Prev Res* 2008;1(1):65.
- 55: Gordan JD, Lal P, Dondeti VR, et al. HIF-alpha effects on c-Myc distinguish two subtypes of sporadic VHL deficient clear cell renal carcinoma. *Cancer Cell.* 2008;14(6):435-46.
- 56: Hernández-Zavala A, Matoušek T, Drobná Z, et al. Speciation analysis of arsenic in biological matrices by automated hydride generation-cryotrapping-atomic absorption spectrometry with multiple microflame quartz tube atomizer. *J Anal At Spectrom.* 2008;23:342.
57. Brüning T, Lammert M, Kempkes M, Thier R, Golka K, Bolt HM. Influence of polymorphisms of GSTM1 and GSTT1 for risk of renal cell cancer in workers with long-term high occupational exposure to trichloroethene. *Arch Toxicol.* 1997; 71(9):596-9.
- 58: Colin P, Koenig P, Ballereau C, et al. Sporadic upper urinary tract urothelial cell carcinomas: identification of interaction between toxic carcinogens and individuals genetic susceptibility. *Prog Urol.* 2010;20(1):1-10.

- 59: Moore LE, Boffetta P, Karami S, Brennan P et al., Occupational trichloroethylene exposure and renal carcinoma risk: evidence of genetic susceptibility by reductive metabolism gene variants. *Cancer Res.* 2010; 70(16):6527-36.
- 60: Wang YH, Yeh SD, Shen KH, et al. A significantly joint effect between arsenic and occupational exposures and risk genotypes/diplotypes of CYP2E1, GSTO1 and GSTO2 on risk of urothelial carcinoma. *Toxicol Appl Pharmacol.* 2009;241(1):111-8.
- 61: Hernández A, Marcos R. Genetic variations associated with inter-individual sensitivity in the response to arsenic exposure. *Pharmacogenomics.* 2008; 9(8):1113-32.
- 62: Beck JA, Lloyd S, Hafezparast M, Lennon-Pierce M, Eppig JT, Festing MF, Fisher EM. Genealogies of mouse inbred strains. *Nat Genet.* 2000;24(1):23.
- 63: Threadgill DW, Hunter KW, Williams RW. Genetic dissection of complex and quantitative traits: from fantasy to reality via a community effort. *Mamm Genome.* 2002;13(4):175-8. Review. PubMed PMID: 11956758.
- 64: Harrill AH, Ross PK, Gatti DM, Threadgill DW, Rusyn I. Population-based discovery of toxicogenomics biomarkers for hepatotoxicity using a laboratory strain diversity panel. *Toxicol Sci.* 2009;110(1):235-43.
- 65: Xiao J, Liang Y, Li K, et al. A novel strategy for genetic dissection of complex traits: the population of specific chromosome substitution strains from laboratory and wild mice. *Mamm Genome.* 2010;21(7-8):370-6.
- 66: Zou F, Gelfond JA, Airey DC, Lu L, Manly KF, Williams RW, Threadgill DW. Quantitative trait locus analysis using recombinant inbred intercrosses: theoretical and empirical considerations. *Genetics.* 2005 ;170(3):1299-311.

Chapter Two:

**THE TCE-ASSOCIATED *VHL* MUTATION DISRUPTS
HIF REGULATION AND PROMOTES TUMOR GROWTH**

A. Abstract

Trichloroethylene (TCE) is a suspect kidney carcinogen that has been associated with a specific missense mutation (P81S) in the von-Hippel Lindau (*VHL*) tumor suppressor gene. Although *VHL* is commonly mutated in sporadic renal cell carcinoma, little is known about the functional consequences of the P81S *VHL* mutation in the context of tumor suppressor function and disease relevance. pVHL plays a critical role in regulating cellular response to hypoxia, and while some mutations in *VHL* disrupt its function as an E3 ubiquitin ligase targeting hypoxia-inducible factors (HIF) for degradation during normoxia, others have been shown not to affect the HIF axis. Consequently, we hypothesize that the P81S *VHL* mutation may alter pVHL function through disruption of complex formation, leading to loss of tumor suppressor activity. In this study, we have modeled the TCE-associated *VHL* gene mutation using an embryonic stem cell system where the endogenous wild-type mouse *Vhl* gene was replaced with the human P81S *VHL* mutant gene. This allows an evaluation of the consequences of this mutation on pVHL function, HIF degradation and *in vivo* teratoma formation. We found that the P81S mutant has partial loss of E3-complex formation allowing HIF stabilization during normal oxygen conditions. When grown *in vivo*, the P81S teratomas also display phenotypic evidence of increased HIF signaling, as well as a novel anti-apoptotic phenotype. Transcript analysis further suggested a mitochondrial-mediated mechanism of apoptosis avoidance. We purport that the P81S mutation represents a unique disease-relevant mutation, which contributes to both initiation as well as promoting tumor growth.

B. Introduction

TCE is an organic solvent that was once used heavily in industry and is a suspect kidney carcinogen in both animals and humans. Human studies have linked exposure to TCE with a specific missense mutation *C454T* in the von Hippel-Lindau tumor suppressor gene (*VHL*) [1, 2, 3]. This mutation results in a single amino acid change from proline to serine at codon 81 of the VHL protein (pVHL), but it remains undetermined whether this mutation has a functional consequence on tumor suppressor activity. While the findings linking TCE exposure to kidney cancer are of great relevance to public health, our lack of understanding of the tumorigenic potential of the P81S mutation in the kidney has hampered risk assessment of TCE.

VHL is mutated in over 80% of sporadic clear cell renal cell carcinomas (ccRCC), and for this reason, it is thought to be an early and key event in the development of kidney cancer in humans [4]. Some missense mutations in *VHL* disrupt its function as part of an E3 ubiquitin ligase complex targeting hypoxia-inducible factor transcription factors (HIF) for degradation during normoxia [5, 6]. The transcriptional upregulation of HIF target genes is thought to mediate the majority of the aberrant phenotypes associated with ccRCC [7]. pVHL also plays a role in cell cycle arrest by stabilizing the cyclin-dependent kinase inhibitor p27 [8]. Loss of normal pVHL function is thought to initiate and drive the tumorigenic process however it has been observed that not all mutations have the same tumorigenic potential [9].

The lack of an experimental animal model with which to investigate the effects of the TCE-associated mutation in *VHL* and kidney tumor development has hindered mechanistic studies. Unlike what is seen in humans, *VHL* loss does not appear to be sufficient for tumorigenesis in mice [10, 11] and heterozygosity does not predispose to carcinogen-induced neoplasia [12]. As such, it remains debatable whether alterations in *VHL* alone are sufficient to trigger tumorigenic processes in the kidney. *In vitro* embryonic stem cell systems have been promising in characterizing the genotype-phenotype correlations of specific mutations found in VHL disease families, and thus have given us insight into the tumor suppressor functions of pVHL [9].

VHL was first identified as an important tumor suppressor for its role in VHL disease, a hereditary cancer syndrome resulting in a variety of tumors in affected patients [13]. Among VHL disease family members, strong genotype-phenotype correlations have been observed between type and location of the mutation and the resulting tumor spectrum [9, 14]. As such, not all mutations in the gene predispose to renal cell cancer in affected families. As an example, the R167Q *VHL* missense mutation is frequently found in VHL disease families that develop ccRCC, and has been found to result in partial loss of HIF regulation and a decrease in tumor suppressor activity [15, 16]. In a separate example, the germline mutation R200W is not correlated with the occurrence of renal cell carcinomas in familial VHL disease, but only predisposes patients to Chuvash polycythemia, further confirming that not all mutated *VHL* bases contribute to tumorigenesis in the kidney [17].

In the investigation presented here, we have utilized an *in vitro* system to characterize the *VHL* P81S mutation to examine both the functional consequence on tumor suppressor activity as well as to understand its possible role in the progression of renal tumorigenesis.

C. Methods

Cell lines and growth conditions.

Vhl^{-/-} embryonic stem (ES) cells were generated as previously described [14]. In this study, *Vhl*^{-/-} ES cells expressing wild-type human VHL protein (pVHL) as well as the R167Q clones were used for comparison. ES cells were grown in high glucose DMEM without sodium pyruvate, supplemented with 2mM L-glutamine, 1x non-essential amino acids (all from Gibco Invitrogen), 0.1mM betamercaptoethanol (Sigma), and 150uL LIF. All cells were grown in a 37°C incubator under 5% CO₂ and were passaged at 3-day intervals. Cells were used in experiments during a 4-month period at approximately 70% confluence.

Construct generation.

cDNA from the human *VHL* gene was ligated into the multiple cloning site of pBluescript II KS⁺ vector using *Hind* III. For mammalian expression constructs, the hemagglutinin epitope tag (GKPIP_NPLLGLDST) and PolyA tail was added to the coding region by sequential PCR along with *Bam* HI and *Hind* III sites allowing the resultant PCR product to be cloned into pStationII vector under control of the EF1 promoter and 4F2 enhancer, also containing the hygromycin resistance gene for clonal selection.

Site-directed mutagenesis.

Site-directed mutagenesis of the pST-HA-VHL parental construct to mutate the proline at nucleotide position 454 to a serine residue was performed using the Quikchange II XL site-directed mutagenesis kit as per the manufacturer's protocol (Stratagene). The mutagenesis primer sets used are listed with the specific base changes underlined: Forward 5'-GCAATCGCAGTTCGCGCGTCG; Reverse 5'-CGACGCGCGAACTGCGATTGC. The following sequencing primer was used for verification of the nt454 VHL mutation: 5'-TCGAAGAGTACGCCCCTGAAGAA-3'.

Electroporation into *Vhl*^{-/-} stem cells.

Vhl^{-/-} cells were seeded at 1×10^7 cells/well onto gelatin coated 100mm dishes and propagated overnight. Cells were trypsinized and washed twice using electroporation medium (serum-free, unsupplemented DMEM). The cells were resuspended in 0.8mL electroporation medium at 2×10^7 cells/mL. Cell suspensions were mixed with 10ug of DNA at 25°C for 10min before electroporation. The cells were pulsed at 250 V with 0.68-0.72 time constant using a Bio-Rad GenePulser (Bio-Rad, Hercules,CA). The treated cells were kept at 25°C for another 10 min before re-plating. At 24h, the plates were rinsed with PBS once before adding the new culture medium containing 200uM hygromycin selection media. Selection media was replaced daily for 14 days before the selection of clones. Clones were tested for HA-VHL expression via western blot.

Hypoxia treatments.

ES cells expressing the P81S mutation, as well as previously generated pVHL cell lines: R167Q, WT and null were plated on gelatin-coated 60mm cell culture dishes and allowed to adhere overnight. Cells were subsequently exposed to 100uM CoCl₂, a hypoxia mimic, while normoxic controls received media replacement. Exposure duration was 16hrs at 37°C and 5% CO₂ in a humidified incubator. After treatment, cell monolayers were washed twice with PBS and were either scraped into 175µL cold PBS and snap frozen for RNA isolation or lysed in the dish by the addition of 175µL lysis buffer (900mM NaCl, 0.5% Nonidet P-40, 10 µg/ml leupeptin, and 20 µg/ml aprotinin for western blot analysis. Cell suspensions were immediately incubated on ice for 5min and centrifuged at 10,000rpm at 4°C for 10 min and protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard. Samples were prepared using 35µg total protein mixed with 5x sample buffer containing 1M DTT and heated in boiling water bath for 10min. Samples were then run on a 8% SDS-PAGE gel.

Immunoprecipitation of E3-complex.

Based on the predicted interaction domains for wild-type pVHL, we tested the effect the P81S mutation on the protein-protein interactions with the E3-complex, and compared those interactions with the other clinically relevant VHL mutations, Y112H and R167Q. IP experiments were performed as described using the Pierce Mammalian HA-tag Co-IP kit (Pierce, Rockford, IL). Cells were gently lysed using supplied IP buffer supplemented with protease inhibitors

(20µg/ml). 700ug protein extract was incubated with 1ug HA-tag IgG conjugated to 6ul Protein A/G agarose beads (Pierce) for 24 hours at 4°C. Columns were washed with 500 ul TTBS three times for 10 minutes at 4°C and protein eluted by boiling in 30 ul 1X non-denaturing sample buffer. Samples were centrifuged through supplied column to remove IgG beads and reboiled with 1mM DTT for 10min. Supernatants were resolved by SDS-PAGE and western blotting as described below.

Western blot analysis.

To evaluate the ability of the P81S mutant to alter HIF degradation as well as E3-complex interactions under normoxic and hypoxic conditions, we quantified protein levels by western blot. Protein samples were resolved by denaturing electrophoresis using polyacrylamide gels (SDS-PAGE) and were electrophoretically transferred to nitrocellulose membrane. Immunochemical staining was carried out with varying concentrations of primary antibody diluted in 1% milk/TBST buffer incubated at times ranging from 2 hours to overnight at 4°C. Specific primary antibodies against pVHL (Cell Signaling), HIF1α (Cayman), HIF2 (Genetex), HA-tag (Abcam), Cul2 (Abcam), and Elongin C (Santa Cruz) were purchased from commercially available vendors. HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were washed with three changes of TBST for a total of 30 min. The blot was then incubated in 1% milk/TBST buffer containing a 1:10,000 dilution of goat anti-rabbit, donkey anti-goat or goat anti-mouse-HRP secondary antibodies for 2 h at 24°C and washed in TTBS or PBST. Before detection, the blots were rinsed

PBS for 1 min. Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer (Amersham Biosciences, Piscataway, NJ). Multiple exposures of each membrane were produced using X-ray film.

RNA isolation.

To quantify changes in gene expression, total RNA from teratomas and cell pellets was isolated using RNeasy® Mini Kits (Qiagen, Valencia, CA) per manufacturer's protocol. Total RNA was quantified with a NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA quality was determined with an Agilent BioAnalyzer (Agilent Tech. Inc., Santa Clara, CA).

Quantitative RT-PCR.

cDNA was prepared from total RNA using Applied Biosystems cDNA Archive kit, with an initial concentration of RNA between 10-40ng/uL. For quantitative RT-PCR, validated primer/probe sets were purchased from Applied Biosystems. Samples were prepared with 20ng cDNA per reaction according to manufacturer's instructions (ABI, Carlsbad CA). Samples were run in a 96-well plate using the Stratagene Mx2000 qRT-PCR machine. Data was normalized to loading control beta-glucuronidase or beta-actin and results were calculated using $\Delta\Delta C_t$ method.

***In vivo* teratoma formation assay.**

To evaluate the effect of the P81S *VHL* mutation on tumor growth, vascularization, hypoxic regions, HIF transcriptional activity and cellular differentiation, we performed an *in vivo* teratoma assay. Briefly, the teratoma

assay was performed using two clones of the P81S ES cell line, P81S.4 and P81S.12, as well as wild-type cells and R167Q mutant lines. Cells were grown to 70% confluency under normal conditions. Female nude mice (n = 4) between 6-8 weeks of age were injected subcutaneously in bilateral flanks with 1×10^6 cells of each HA-VHL cell line suspended in saline. Tumor growth was measured three times a week using calipers until tumors reached a maximum dimension of 1cm. To examine tumor hypoxia, mice were injected with 60mg/kg Hypoxyprobe™ (HPI, Inc., Burlington MA) one hour before sacrifice by CO₂. Tumors were excised within 5 minutes and samples were either flash frozen for gene expression and protein analysis or formalin fixed and paraffin embedded for histology and immunohistochemistry.

Immunohistochemistry.

To evaluate teratomas for proliferation, apoptosis, and DNA damage, IHC was performed. Formalin-fixed, paraffin-embedded sections (6µm) were mounted on glass slides. Sections were deparaffinized in xylene, rehydrated in a series of graded alcohol concentrations, and placed in PBS with 1% Tween 20. Immunostaining was performed using standard laboratory procedures with primary antibody dilutions 1:1000 H2AX; 1:500 Ki67 (Abcam). Secondary detection was done with biotinylated rabbit, or goat specific antibodies, ABC enhancement kit (Vector, Burlingame CA), and DAB detection reagent (Vector). Apoptosis Staining was performed using the ApopTag® In Situ Apoptosis Detection Kit (Millipore) according to manufacturer's protocol. Slides were counterstained with hematoxylin to facilitate cell counts. In order to ensure the

quantitative measurement of each immunoreaction, all teratomas were compared were processed in parallel. Quantitative analysis of immunostained sections was performed using BIOQUANT software (BIOQUANT Image Analysis, Nashville TN) by averaging percent positively stained cells to total cells within 6 random fields at 200 \times .

Hypoxia measurements.

Hypoxyprobe or pimonidazole hydrochloride forms adducts with protein thiol groups under low oxygen conditions (<10mm Hg). To examine tumor hypoxia, mice were injected with 60mg/kg Hypoxyprobe™ one hour before sacrifice by CO₂. Tumors were excised within 5 minutes and were sliced, formalin fixed and paraffin embedded for immunohistochemical detection of protein adducts. Anti-Hypoxyprobe-1 is a monoclonal antibody that detects protein adducts of Hypoxyprobe™ in hypoxic cells. Staining was performed according to manufacturer's protocol (HPI, Inc. Burlington MA). Three serial sections per teratoma were quantified by choosing evaluating 5 fields per 6 μ M slice.

Microarray analysis.

Microarray experiments were performed using GeneChip® Mouse ST Gene 1.0 whole genome arrays from Affymetrix (Santa Clara, CA) at the UNC Genomics Core Facility. Total RNA samples were processed following a standard one-cycle eukaryotic target preparation protocol from Affymetrix. Briefly, total RNA was first reverse-transcribed using T7-oligo(dT) promoter primer in the first-strand cDNA synthesis reaction. Following second-strand cDNA synthesis,

the double stranded cDNA was purified and served as a template in the subsequent *in vitro* transcription (IVT) reaction. The IVT reaction was carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. Biotinylated cRNA targets were then purified, fragmented, and hybridized to GeneChip® array during the overnight incubation at 45°C in a rotating hybridization oven. After hybridization, the arrays were stained with streptavidin-phycoerythrin in the GeneChip® Fluidics station and then scanned using Affymetrix GeneChip® Scanner (laser filter set at 570 nm; pixel size 2.5 µm). The array image data were acquired, and the fluorescent signal intensities were quantified using Affymetrix® GCOS software with following settings of quantitation parameters: Alpha1 = 0.05, Alpha2 = 0.065, Tau = 0.015, Gamma1H = 0.0045, Gamma1L = 0.0045, Gamma2H = 0.006, Gamma2L = 0.006, Perturbation = 1.1, Target Intensity = 150.

Pathway analysis and statistics.

The Affymetrix Mouse ST Gene 1.0 array has approximately 27 probes spread across each of the 28,853 genes represented on the array, providing a more complete and more accurate picture of gene expression than 3'-based expression array designs. The array probe set sequences were derived from GenBank®, Ensembl, dbEST, and RefSeq. Gene expression data (CEL files) were analyzed using Partek® Genomic Suite, version 6.3 (Partek Inc., St. Louis MO). Gene expression data were normalized by robust multi-array analysis (RMA) and analysis of variance was performed using Partek® software. We

identified differentially expressed genes between classes using the non-parametric rank-product method (p-value <0.05 and 0.01) and \log_2 expression values for each gene were generated. Canonical pathway analysis was generated through the use of Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). Canonical pathway analysis was measured in two ways: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway, and (2) Fischer's exact test to calculate a p-value.

D. Results

P81S pVHL expression in *Vhl*^{-/-} embryonic stem cells.

Site-directed mutagenesis was performed on plasmid constructs containing HA-tagged human *VHL* cDNA to generate the C454T missense mutation at codon 81 of pVHL, representing the TCE-exposure associated P81S mutation. Forty colonies were isolated and the *VHL* cDNA sequenced to verify the *C454T* mutation and to ensure no other mutations in the gene were introduced (Figure 2.1). Then, *Vhl*^{-/-} embryonic stem cells generated as described previously [14] were transfected with plasmid encoding the HA-tagged human *VHL* P81S cDNA. Twenty-eight transgenic clones were selected for pVHL P81S expression by western blot, and two representative clones were selected that matched the expression levels of the *VHL* WT and *VHL* R167Q cell lines previously described [9] to avoid effects of overexpression.

***VHL* P81S mutation alters E3-complex and disrupts HIF regulation *in vitro*.**

pVHL plays an important role in the regulation of HIF during normal oxygen conditions via its activity as a substrate recognition protein in the E3-complex [9]. As a first step, we examined the ability of the P81S mutant to interact with the E3-complex and promote ubiquitination of HIF under normoxia. Unlike *VHL* WT cells which retain their interaction with Elongin C and Cullin 2, extracts from the *VHL* P81S mutant and *VHL* R167Q disease mutant did not pull down Elongin C but retained interaction with Cullin 2 (Figure 2.2A). Our findings are consistent with previous findings that the *VHL* R167Q disease mutant undergoes partial abrogation of the E3-complex through loss of interaction with Elongin C, and is only partially able to regulate HIF [16].

We then examined the ability of the pVHL mutants to degrade HIF protein during normoxic and hypoxic conditions. Two representative clones of the *VHL* P81S mutant as well as the *Vhl*^{-/-}, *VHL* WT, *VHL* R167Q cell lines were analyzed for HIF1a protein expression after 16hrs of growth in the presence of normal oxygen (21% O₂) or hypoxia mimetic (CoCl₂) (Figure 2.2B). As a positive control, the lack of pVHL expression in *Vhl*^{-/-} cells allowed HIF1 to be stabilized under normoxic conditions with no further increase during hypoxia. When *Vhl*^{-/-} cells were complimented with the wild-type human pVHL, HIF1 ubiquitination and degradation was restored during normoxia. In contrast, when we examined the TCE-associated *VHL* P81S mutant and the *VHL* R167Q disease mutant, we found that HIF1 was partially stabilized during normoxia, consistent with the idea that loss of Elongin C binding perturbed E3-complex function.

***VHL* P81S mutant continues to suppress HIF1 α targets *in vitro*.**

Given the observation that HIF1 protein was stabilized in both the TCE-associated P81S mutant and the R167Q disease mutant, we next determined whether the increase corresponded to an increase in HIF1 transcriptional activity. We performed quantitative RT-PCR on the ES cell lines to examine HIF transcriptional target gene expression. Despite the partial stabilization of HIF1 during normoxia (Figure 2.2B) we were unable to observe HIF transcriptional activity during normoxic conditions (not shown). This may be due to the limited targets examined, as only four target genes were quantified. On the other hand, the R167Q cells showed an increase in HIF target expression consistent with HIF protein stability, suggesting that the P81S pVHL mutant may be binding other HIF targets.

***VHL* P81S mutant displays a marked growth advantage and differentiation in the teratoma assay.**

One disadvantage of the embryonic stem cell system is the inability to examine the contribution of HIF2 transcriptional activity to the overall phenotype of pVHL mutants [9]. To examine the 3D growth and differentiation of the *VHL* stem cell lines, we performed an *in vivo* teratoma assay in nude mice, measuring tumor growth over 4 weeks followed by histopathological examination (Figure 2.3A). Strikingly, we found that the *VHL* P81S mutant displayed a 3-fold increase in tumor volume over *VHL* WT cells and a 6-fold increase over *VHL* R167Q cells (Figure 2.3B). We also observed an increase in differentiation of the P81S teratomas over the WT and R167Q (Figure 2.3C). However, no significant

increase in HIF2 protein stabilization was observed for P81S when compared to WT, as both of these teratomas are likely hypoxic (Figure 2.4A). We did see increases in HIF target gene expression likely contributed by both HIF1 and HIF2 protein stability (Figure 2.4B). HIF1 plays a role in cellular differentiation through Notch signaling [18], so while the P81S and R167Q mutants show similar dysregulation of HIF, their response to the tumor microenvironment may be re-directing their HIF target expression. The relative growth rates of WT and R167Q teratomas as well as the histological features were consistent with previous studies [9]. Therefore, the remarkable growth advantage displayed by the P81S mutant suggests that the mutation may be affecting alternate functions of pVHL or altering HIF activity.

VHL P81S teratoma growth is not due to increased proliferation but decreased apoptosis.

To explore the growth phenotype of the P81S, we analyzed the WT, R167Q and P81S ES cell teratomas for proliferative activity using the marker Ki67. The relative proliferation index between WT and P81S teratomas was not significantly different (Figure 2.5A), suggesting that a proliferative phenotype was not the reason for the increased tumor volume. We then investigated whether an apoptotic defect could be the source of the increased growth using an apoptosis specific stain based on modification the 3'-OH end fragments utilizing terminal deoxynucleotidyl transferase (TdT) and fluorescent detection. The results of the apoptosis stain showed there was a significant reduction in the percent of apoptotic cells compared to WT or R167Q teratomas (Figure 2.5B). This anti-

apoptotic phenotype is a novel finding for a VHL disease mutant and suggests the TCE-exposure associated mutant may be capable of evading apoptotic signals, allowing teratocarcinoma growth.

Mitochondrial biogenesis as mechanism of apoptotic aversion and cell survival in the *VHL* P81S mutant.

Investigations into pVHL tumor suppressor activity suggest that there are both HIF-dependent and HIF-independent mechanisms of apoptotic regulation [19]. To evaluate the role of the P81S mutation in the anti-apoptotic phenotype, we performed a whole genome microarray analysis on the ES cell teratomas. We performed unsupervised hierarchical clustering of four replicate P81S teratomas and three replicate WT and R167Q teratomas followed by analysis of variance of all genes (Figure 2.6). The dendrogram shows that samples segregated based on genotype, with the WT and R167Q mutant teratomas appearing more similar to each other compared to the P81S mutant, ~2622 genes were differentially expressed ($p \leq 0.001$) across all three genotypes. Performing a t-test analysis to characterize genes differentially expressed by genotype ($p \leq 0.001$) further confirmed the striking differences in gene expression between the P81S and the WT teratomas: ~2293 genes were found to be differentially regulated (Figure 2.7A). Canonical pathway analysis revealed that the up-regulation of DNA damage/G2 checkpoint, G1/S Checkpoint, and p53 signaling in the WT teratomas, suggestive of increased apoptosis and DNA damage. On the other hand, in the P81S teratomas an up-regulation of HIF signaling and the Vitamin D3 pathway suggests cellular growth and HIF target gene expression. These

pathway networks are consistent with our histological findings, as the WT teratomas displayed an increase in H2AX foci formation (not shown) and an increase in apoptosis (Figure 2.5B) compared to P81S teratomas, which showed an increase in HIF target gene expression (Figure 2.4B).

Of importance to the apoptotic phenotype, a pathway identified to be significantly up-regulated in the P81S teratomas, but not in WT and R167Q teratomas, was mitochondrial biogenesis (Figure 2.7B). Mitochondrial biogenesis is characterized by the up-regulation of *Ppargc1a* (peroxisome proliferator gamma coactivator-1 transcription factor) and downstream targets such as *mtTfa* and *Creb* [20]. Biogenesis signaling increases mitochondrial proliferation in response to environmental stress [21]. Cancer cells often up-regulate this intrinsic stress response system during tumor hypoxia or oxidative stress to avoid apoptotic signals and aid in survival [22]. The data suggest that the P81S mutants may have the ability to alter the intrinsic stress response to avoid apoptosis through the up-regulation of mitochondrial biogenesis, a hallmark of many cancer cell types. This hypothesis is currently under validation using additional biochemical measurements.

E. Discussion

Since the identification of the TCE-associated P81S mutation over a decade ago, the disease relevance of this mutation in the alteration of *VHL* tumor suppressor gene function has gone unexplored. The lack of an animal model with which to study the role of *VHL* mutations in the initiation and progression of renal carcinogenesis has hindered this assessment [12]. In the present study, we have

utilized a novel mouse embryonic stem cell model system [9] and transcriptomics to characterize cells expressing the P81S pVHL mutant in comparison to cells expressing wild-type pVHL, as well as missense mutant R167Q pVHL, a mutation subtype which predisposes to renal cancer [9]. Through this comparison, we were able to show that the P81S mutant behaves similarly to the R167Q mutant in the E3-complex formation and dysregulation of HIF. However, unique to P81S was an anti-apoptotic phenotype which allowed the teratomas to grow exponentially faster. This lack of a balance between proliferation and apoptosis is characteristic of an aberrant phenotype as one might find in the transformation of cancer cells [22]. We have suggested that the upregulation of mitochondrial biogenesis may be a survival mechanism to avoid apoptotic signals due to the intrinsic stress response.

Dysregulated proliferation alone is not sufficient for tumor promotion, but also requires the support of anti-apoptotic signals [23]. This finding has been observed in studies where oncogene overexpression actually sensitized the cells to apoptosis, rather than growth, and suggests that either inactivation of pro-apoptotic signals or expression of anti-apoptotic signals would be additionally required for progression [24]. In addition, *in vitro* and *in vivo* studies where pVHL had been acutely inactivated also found that this inactivation resulted in a senescent-like phenotype that was independent of p53 and HIF, but dependent on Rb inactivation and upregulation of p27 [25]. These studies suggest that when cells are faced with inactivation of pVHL, tumor suppressive mechanisms prevent further progression. In our study, the P81S mutation in *VHL* did not result in

protein inactivity or loss of translation, but rather suppressed the normal apoptotic signals required for growth regulation. It is unclear from this evaluation if the apoptotic affect was due to a HIF-dependent or a HIF-independent evasion pathway through mitochondrial biogenesis, as HIFs play a dynamic role in regulating many cellular processes including mitochondrial respiration and response to oxidative stress [26, 27, 28].

There is not a clear understanding of how mitochondrial biogenesis leads to respiratory dysfunction, or vice versa, and the Warburg Effect. The Warburg Effect, or aerobic glycolysis, occurs when cancer cells convert glucose to lactic acid even when there is sufficient oxygen present [29]. This adaptive pro-survival response in cancer cells, while inefficient at producing ATP, allows these cells to suppress apoptosis signals through the upregulation of PI3K/Akt signaling [30]. Interestingly, to overcome the lack of ATP production, these cells may increase mitochondrial biogenesis as yet another adaptive response, which also plays an important role in mediating tumor hypoxia [31]. During conditions of tumor hypoxia, the upregulation of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (*PGC-1alpha*), which increases mitochondrial biogenesis, leads to increased expression of HIF-regulated genes through stabilization of HIF protein [32]. The relationship between the pVHL-Biogenesis-HIF axes is unclear, yet the missense mutation of *VHL* at codon 81 appears to result in a pro-survival phenotype, implicating mutant pVHL or its effector functions in promoting tumor growth.

In the epidemiological studies linking *VHL* mutations to TCE exposure, there were tumors with multiple *VHL* mutations as well as many with only the single P81S mutation [1, 2]. Based on the evidence that can be obtained from an *in vitro* system, the P81S pVHL mutant has strong implications as an RCC disease mutation. Many of the shared characteristics that describe the R167Q mutant, which confers a high risker for RCC, also apply to the P81S mutant. Our most striking finding was the anti-apoptotic phenotype, which was unique to this missense mutation, and allowed the promotion of teratocarcinoma growth by either suppressing or averting apoptotic signals. While the mechanism is yet unclear, this investigation into the P81S *VHL* mutation strongly suggests that certain mutations in *VHL* tumor suppressor gene may allow it to function in both the initiation and promotion of tumorigenesis.

Figure 2.1

Site Directed Mutagenesis of pVHL cDNA

Sequencing of pBluescript II construct containing *VHL* cDNA to confirm CCG > TCG point mutation at *C454T* of the *VHL* gene. Site-directed mutagenesis was performed on the pBluescript II construct harboring wild-type *VHL* cDNA to induce a missense mutation at codon 81 changing the amino acid from proline to serine.

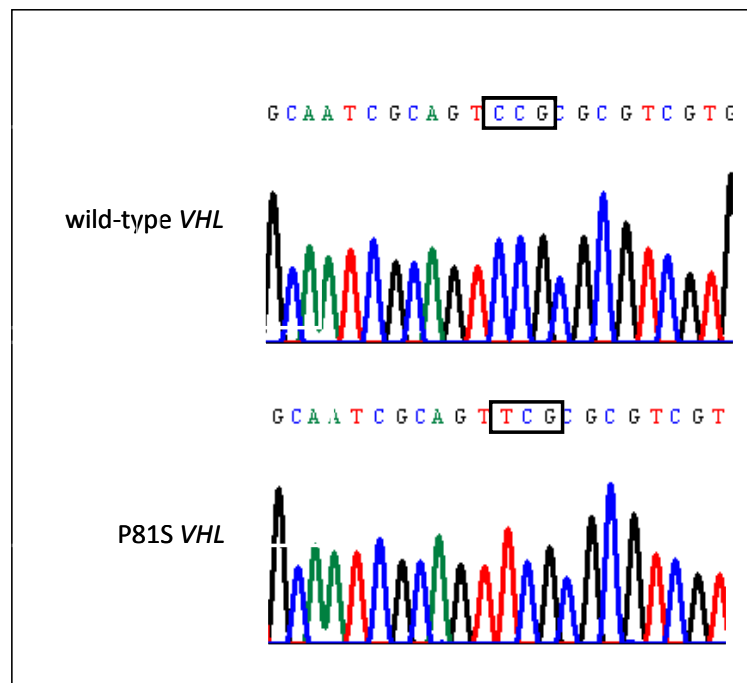
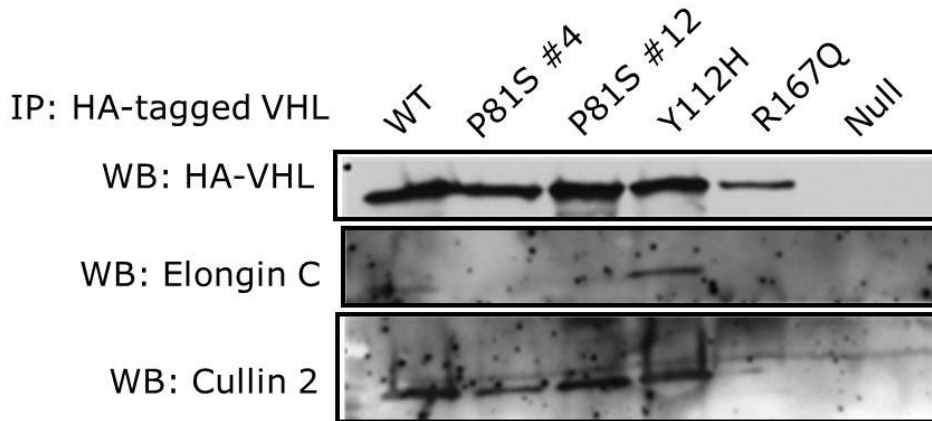


Figure 2.2

Effect of P81S *VHL* Mutation of E3-complex Formation and Function

Co-immunoprecipitation of HA-pVHL and associated E3-complex members in transgenic ES cell clones (A) and Western Blot analysis of HIF1a Stabilization during normoxic and hypoxic conditions (B). Stably-transfected *Vhl*^{-/-} murine ES cells expressing wild-type HA-pVHL or mutant HA-pVHL were grown under normoxic conditions till 70% confluency, lysed under non-denaturing conditions and subject to co-immunoprecipitation with an antibody against the HA-tag. Anti-HA IP products were probed for successful pull-down of WT or mutant HA-pVHL and for co-IP of the E3-complex members Elongin C and Cullin2 (A). Whole-cell protein extracts were prepared from stably-transfected *Vhl*^{-/-} murine ES cells expressing wild-type HA-pVHL or mutant HA-pVHL under normoxic and hypoxic conditions using 100uM CoCl₂ as a hypoxia mimic. Western blot shows HIF1a (*upper band) and loading control (B).

A.



B.

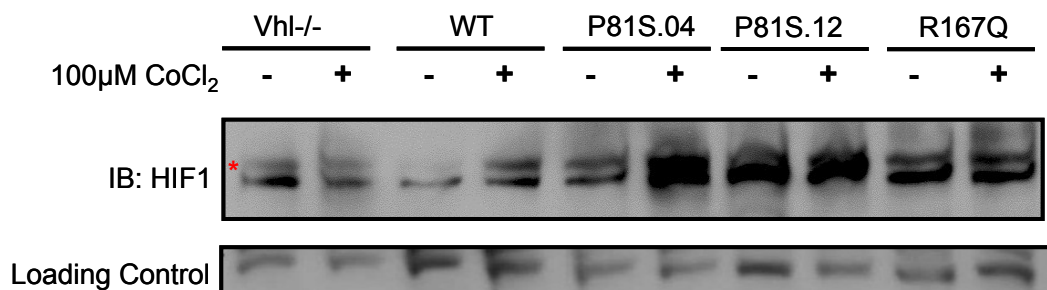
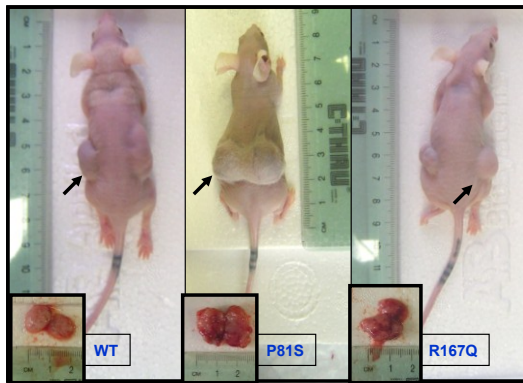


Figure 2.3

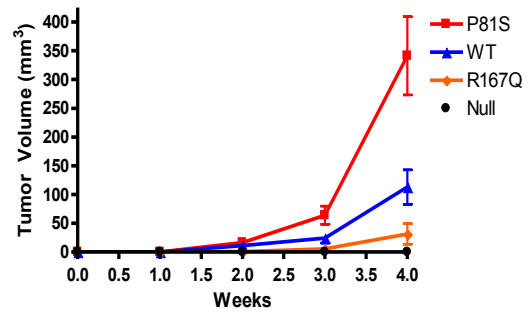
P81S *VHL* Mutant Displays Growth Advantage and Differentiation *In Vivo*

In vivo teratoma assay grown from ES cell clones. Stably-transfected *Vhl*^{-/-} murine ES cells expressing wild-type, P81S or R167Q pVHL were injected subcutaneously into the flank region of nude mice and allowed to grow for 4 weeks. Mice were sacrificed and teratomas removed, measured and photographed (A). Tumor volume was measured weekly using calipers (B). Hematoxylin and eosin staining of teratomas (C) shows areas of differentiation and vasculature, as indicated by a black arrow.

A.



B.



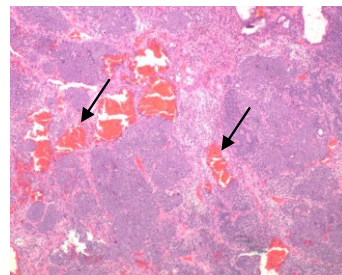
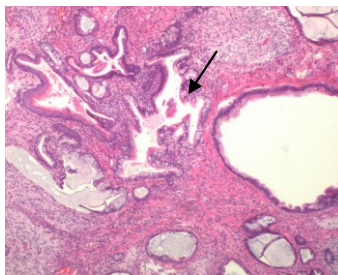
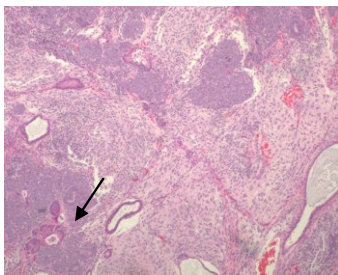
C.

H&E Staining of ES Teratomas

WT

P81S

R167Q



40x magnification

Figure 2.4

Effect of P81S Mutation on *In Vivo* HIF Stability and Transcriptional Activity

Immunoblot of HIF2 protein in ES cell pVHL teratomas (A) and HIF target gene expression by qRT-PCR (B). Stably-transfected *Vhl*^{-/-} murine ES cells expressing wild-type, P81S or R167Q pVHL were injected subcutaneously into the flank region of nude mice and allowed to grow for 4 weeks. Teratomas were removed and either (A) whole cell lysates prepared for immunoblotting for HIF2 protein and loading control beta-tubulin, or (B) RNA was extracted and qRT-PCR was performed on HIF targets (glucose transporter; *Glut1*, vascular endothelial growth factor; *Vegf*, erythropoietin; *Epo* and phosphofructokinase; *Pfk*) to examine gene expression. Statistical analysis by ANOVA *p≤0.05.

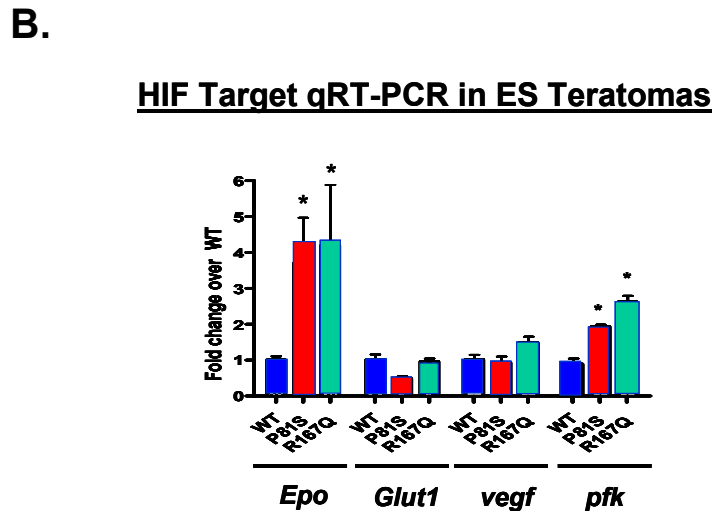
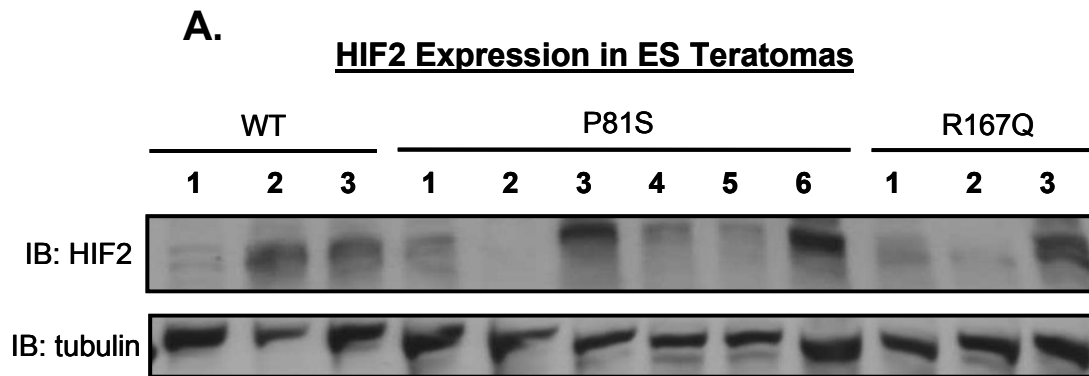


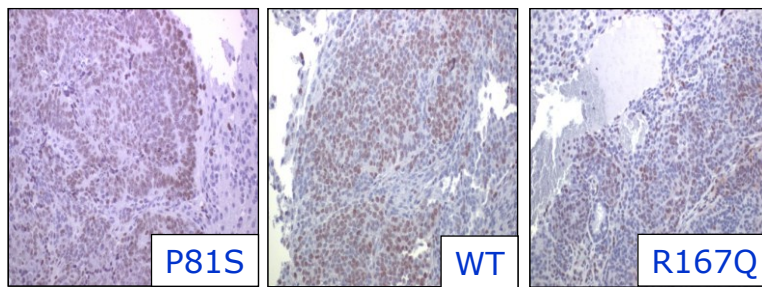
Figure 2.5

Effect of the P81S *VHL* Mutation on Teratoma Proliferation and Apoptosis

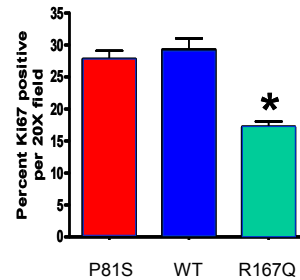
Immunohistochemistry of proliferation (A,B) and apoptosis (C,D) markers in ES cell pVHL teratomas. Stably-transfected *Vhl*^{-/-} murine ES cells expressing wild-type, P81S or R167Q pVHL were injected subcutaneously into the flank region of nude mice and allowed to grow for 4 weeks. Teratomas were removed, paraffin-embedded and sectioned for immunohistochemical analysis. 5µM sections were stained with proliferation marker Ki67 (A) or apoptosis (C) Quantification of percent positive cells for each mutant was performed (B, D). N = 4 with 5-6 sections scored. Statistical significance by ANOVA, *p≤0.05.

A.

Staining for Teratoma Proliferation

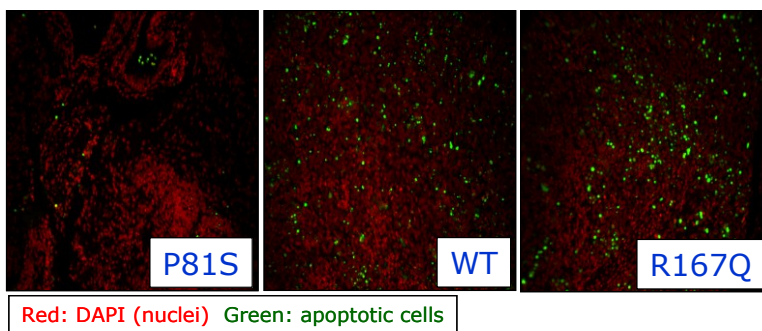


B.



C.

Staining for Teratoma Apoptosis



D.

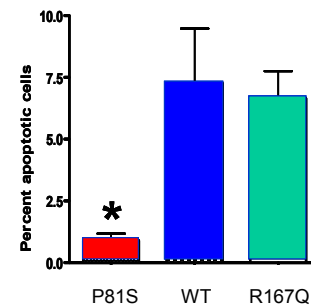


Figure 2.6

Unsupervised Hierarchical Clustering of ES Cell Teratomas

Hierarchical clustering of ES cell teratomas for differentially expressed genes. Stably-transfected *Vhl*^{-/-} murine ES cells expressing wild-type, P81S or R167Q pVHL were injected subcutaneously into the flank region of nude mice and allowed to grow for 4 weeks. RNA was isolated and used to perform microarray analysis using the Affymetrix Mouse Gene ST 1.0 whole genome array. Differentially expressed genes were determined using Partek® Genomics Suite. Statistical significance by ANOVA with a FDR = 5%, * $p \leq 0.001$. Gene list of 2,622 genes was then clustered unsupervised by expression. Red – upregulated, Blue-downregulated.

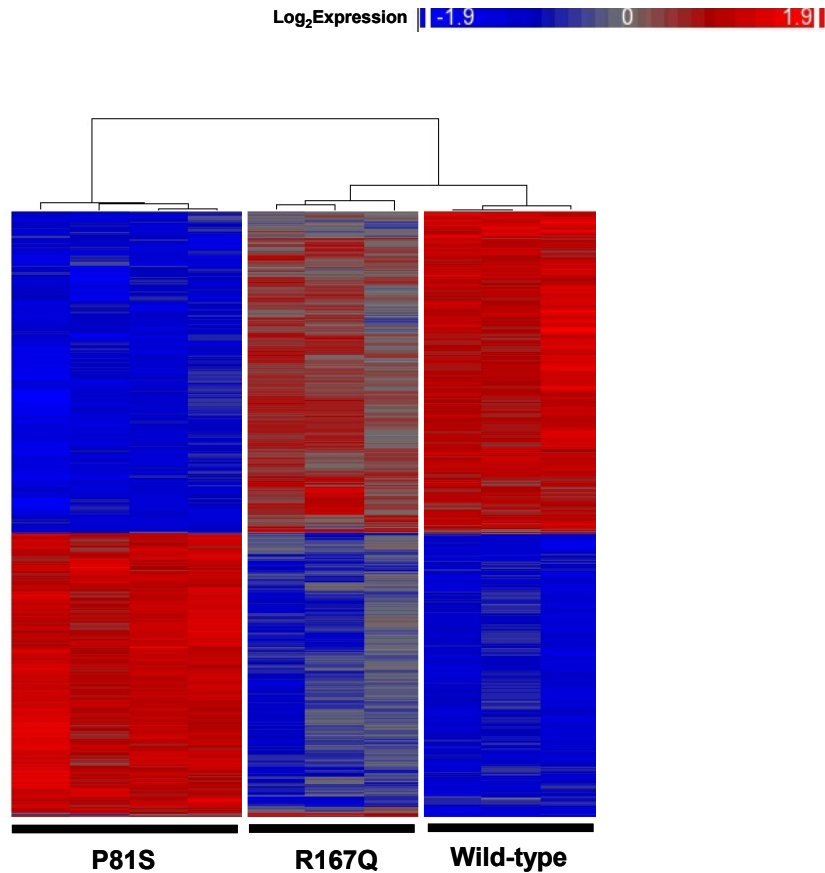
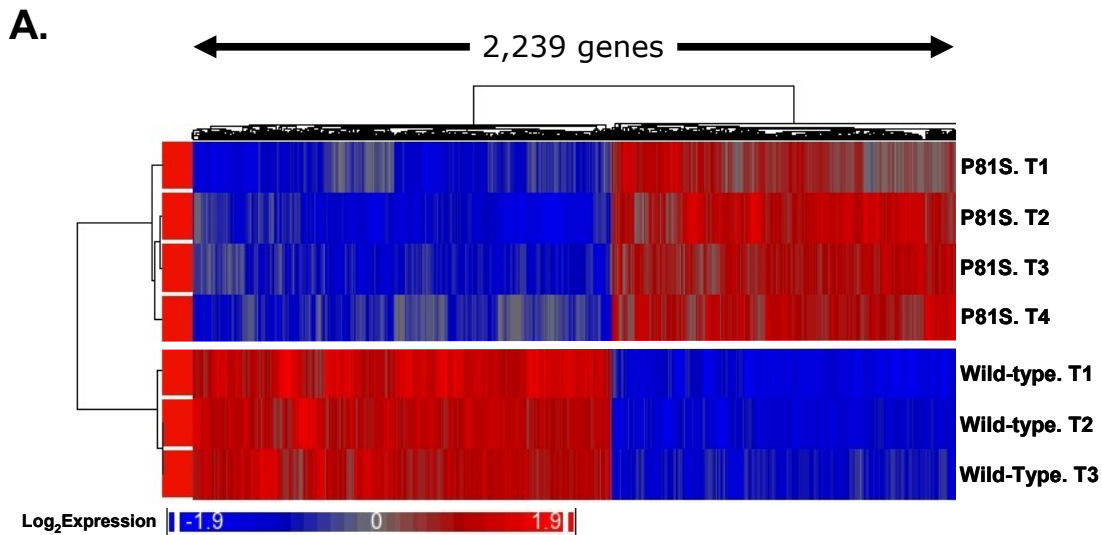


Figure 2.7

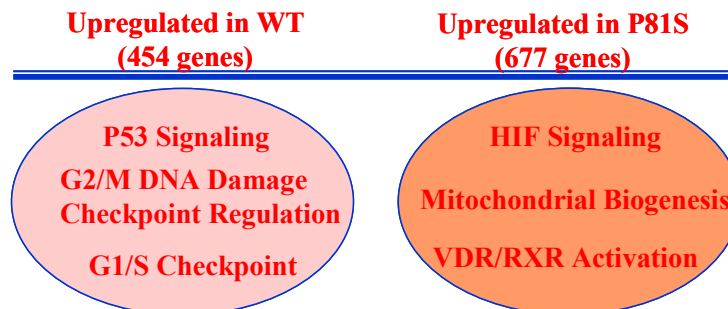
Clustering and Biological Pathway Analysis of P81S Teratomas

Hierarchical clustering of P81S and WT ES cell teratomas for differentially expressed genes. Stably-transfected *Vhl*^{-/-} murine ES cells expressing wild-type, P81S pVHL were injected subcutaneously into the flank region of nude mice and allowed to grow for 4 weeks. RNA was isolated from 4 replicate P81S and 3 replicate WT teratomas, and used to perform microarray analysis using the Affymetrix Mouse Gene ST 1.0 whole genome array. Differentially expressed genes were determined using Partek by two-sample t-test, $p \leq 0.001$. Gene list was clustered unsupervised by expression (A). Biological pathway analysis was performed on genes that were differentially upregulated by either WT or P81S teratomas (B). Red – upregulated, Blue- downregulated



B.

**Biological Pathway Analysis of Genes
with > 2-fold Increase in *VHL* Mutant Teratomas**



REFERENCES

- 1: Brüning T, Weirich G, Hornauer MA, Höfler H, Brauch H. Renal cell carcinomas in trichloroethene (TRI) exposed persons are associated with somatic mutations in the von Hippel-Lindau (VHL) tumour suppressor gene. *Arch Toxicol.* 1997;71(5):332-5.
- 2: Brauch H, Weirich G, Hornauer MA, Störkel S, Wöhl T, Brüning T. Trichloroethylene exposure and specific somatic mutations in patients with renal cell carcinoma. *J Natl Cancer Inst.* 1999;91(10):854-61.
- 3: Brauch H, Weirich G, Klein B, Rabstein S, Bolt HM, Brüning T. VHL mutations in renal cell cancer: does occupational exposure to trichloroethylene make a difference? *Toxicol Lett.* 2004;151(1):301-10.
- 4: Foster K, Prowse A, van den Berg A, et al. Somatic mutations of the von Hippel-Lindau disease tumour suppressor gene in non-familial clear cell renal carcinoma. *Hum Mol Genet.* 1994;3(12):2169-73.
- 5: Iwai K, Yamanaka K, Kamura T, Minato N, Conaway RC, Conaway JW, Klausner RD, Pause A. Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proc Natl Acad Sci U S A.* 1999;96(22):12436-41.
- 6: Lisztwan J, Imbert G, Wirbelauer C, Gstaiger M, Krek W. The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. *Genes Dev.* 1999;13(14):1822-33.
- 7: Gordan JD, Bertout JA, Hu CJ, Diehl JA, Simon MC. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. *Cancer Cell.* 2007;11(4):335-47.
- 8: Pause A, Lee S, Lonergan KM, Klausner RD. The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. *Proc Natl Acad Sci U S A.* 1998;95(3):993-8.
- 9: Rathmell WK, Hickey MM, Bezman NA, Chmielecki CA, Carraway NC, Simon MC. In vitro and in vivo models analyzing von Hippel-Lindau disease-specific mutations. *Cancer Res.* 2004;64(23):8595-603.
- 10: Rankin EB, Tomaszewski JE, Haase VH. Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. *Cancer Res.* 2006;66(5):2576-83.

- 11: Haase VH, Glickman JN, Socolovsky M, Jaenisch R. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. *Proc Natl Acad Sci U S A*. 2001;98(4):1583-8.
- 12: Kleymanova E, Everitt JI, Pluta L, Portis M, Gnarr JR, Walker CL. Susceptibility to vascular neoplasms but no increased susceptibility to renal carcinogenesis in Vhl knockout mice. *Carcinogenesis*. 2004;25(3):309-15.
- 13: Latif F, Tory K, Gnarr J, et al. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science*. 1993;260(5112):1317-20.
- 14: Mack FA, Rathmell WK, Arsham AM, Gnarr J, Keith B, Simon MC. Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. *Cancer Cell*. 2003;3(1):75-88.
- 15: Hacker KE, Lee CM, Rathmell WK. VHL type 2B mutations retain VBC complex form and function. *PLoS One*. 2008;3(11):e3801.
- 16: Lee CM, Hickey MM, Sanford CA, McGuire CG, Cowey CL, Simon MC, Rathmell WK. VHL Type 2B gene mutation moderates HIF dosage in vitro and in vivo. *Oncogene*. 2009;28(14):1694-705.
- 17: Huang JS, Huang CJ, Chen SK, Chien CC, Chen CW, Lin CM. Associations between VHL genotype and clinical phenotype in familial von Hippel-Lindau disease. *Eur J Clin Invest*. 2007;37(6):492-500.
- 18: Sainson RC, Harris AL. Hypoxia-regulated differentiation: let's step it up a Notch. *Trends Mol Med*. 2006;12(4):141-3.
- 19: Li M, Kim WY. Two sides to every story: the HIF-dependent and HIF-independent functions of pVHL. *J Cell Mol Med*. 2011;15(2):187-195.
- 20: Won JC, Park JY, Kim YM, et al. Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha overexpression prevents endothelial apoptosis by increasing ATP/ADP translocase activity. *Arterioscler Thromb Vasc Biol*. 2010;30(2):290-7.
- 21: Lee HC, Wei YH. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int J Biochem Cell Biol*. 2005;37(4):822-34.
- 22: Formentini L, Martínez-Reyes I, Cuezva JM. The mitochondrial bioenergetics capacity of carcinomas. *IUBMB Life*. 2010;62(7):554-60.
- 23: Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature*. 2001 May 17;411(6835):342-8.

- 24: Jiang X, Tsang YH, Yu Q. c-Myc overexpression sensitizes Bim-mediated Bax activation for apoptosis induced by histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) through regulating Bcl-2/Bcl-xL expression. *Int J Biochem Cell Biol.* 2007;39(5):1016-25.
- 25: Young AP, Schlisio S, Minamishima YA, Zhang Q, Li L, Grisanzio C, Signoretti S, Kaelin WG Jr. VHL loss actuates a HIF-independent senescence programme mediated by Rb and p400. *Nat Cell Biol.* 2008;10(3):361-9.
- 26: Zhang H, Gao P, Fukuda R, Kumar G, Krishnamachary B, Zeller KI, Dang CV, Semenza GL. HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. *Cancer Cell.* 2007;11(5):407-20.
- 27: Hervouet E, Simonnet H, Godinot C. Mitochondria and reactive oxygen species in renal cancer. *Biochimie.* 2007;89(9):1080-8.
- 28: Semenza GL. HIF-1 mediates the Warburg effect in clear cell renal carcinoma. *J Bioenerg Biomembr.* 2007;39(3):231-4.
- 29: Pedersen PL. Warburg, me and Hexokinase 2: Multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg Effect", i.e., elevated glycolysis in the presence of oxygen. *J Bioenerg Biomembr.* 2007;39(3):211-22.
- 30: Katoh Y, Katoh M. Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. *Curr Mol Med.* 2009;9(7):873-86.
- 31: Samper E, Morgado L, Estrada JC, Bernad A, Hubbard A, Cadenas S, Melov S. Increase in mitochondrial biogenesis, oxidative stress, and glycolysis in murine lymphomas. *Free Radic Biol Med.* 2009;46(3):387-96.
- 32: O'Hagan KA, Cocchiglia S, Zhdanov AV, et al. PGC-1alpha is coupled to HIF-1alpha-dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. *Proc Natl Acad Sci U S A.* 2009;106(7):2188-93.

Chapter Three:

**DIETARY FATTY ACIDS AND MICRONUTRIENTS MODULATE LOW DOSE
ARSENIC-INDUCED KIDNEY TOXICITY AND GENE EXPRESSION**

A. Abstract

Inorganic arsenic is a common co-contaminant in both food and water sources worldwide, and is thought to contribute to the increased risk of kidney cancer. Dietary fat and micronutrients have been shown to profoundly affect genomic and toxicological studies, implicating a role for nutritional status in disease outcome. Given the increase in obesity and poor diet in the U.S., the role of dietary fatty acids and micronutrients has not been adequately considered in genomic studies of inorganic arsenic. In this study, we compared arsenic-induced gene expression changes in the kidney between mice fed either a standard purified lab chow or a high fat western diet. Male and female FVB/N-*Abcb1a/1b*^{-/-} (Mdr^{-/-}) knockout mice were divided into 6 groups (n=6) with free access to either a standard fat (7% fat) or western diet (34% fat) containing 0, 10, or 150ug/kg sodium arsenite for 10 weeks. At sacrifice, kidneys were removed for histopathological examination as well as examined for changes in gene expression. Subchronic exposure to low dose arsenic on a high fat western diet significantly reduced kidney weights and increased serum lactate dehydrogenase with arsenic exposure. This effect was not seen with arsenic exposure on a low fat diet. Microarray analysis of the kidneys revealed a clear dose-response signature in both diets. Moreover, diet had a significant effect on the type of signaling pathways altered by arsenic exposure. The results indicate that arsenic exposure in the context of a high fat diet can induce signaling pathways commonly overexpressed in most human kidney tumors, and further suggests

that dietary composition may play a critical role in modifying toxicity to low dose arsenic in chronic studies.

B. Introduction

Inorganic arsenic is a global water and food contaminant and is associated with the development of kidney cancer in susceptible human populations [1]. The nutritional state of an individual is known to modify susceptibility to toxicity and disease risk [2]. This is especially true in the case of inorganic arsenic, where essential nutrients are important co-factors in the metabolism and detoxification of arsenic [3]. Given the variability in nutritional intake among human populations, there are significant gaps in toxicological studies examining the role of dietary fat and micronutrients in the modulation of renal toxicity due to arsenic exposure. Since standard rodent chow diets are balanced for optimal nutrition, it seems critical to examine arsenic toxicity in the context of altered nutritional intake to explore the role of diet in the development of arsenic-induced disease pathologies.

The amount and type of dietary fat is known to alter a variety of physiological and pathophysiological functions critical to the outcome of toxicological studies [4]. ω -6 and ω -3 polyunsaturated fatty acids (PUFAs), which are derived from animal and plant based lipids, have been shown to induce a pro-oxidant state within cellular compartments when their intake ratios are imbalanced [5,]. These two types of fatty acids compete directly with one another for incorporation into cellular compartments, where they can influence cell signaling pathways and receptor function [6]. In the U.S. the ω -6 to ω -3 intake

ratio of PUFAs has changed dramatically from an average 7:1 to an imbalanced ratio of 15:1 [7]. This change is likely due to an increased consumption of highly processed foods, and a decreased consumption of fresh fish and natural oils. Despite this shift in dietary intake, it remains undetermined how an imbalanced ratio of PUFAs modulate susceptibility to arsenic-induced renal toxicity.

Essential micronutrients obtained through diet play a direct role in arsenic metabolism and toxicity. Micronutrients such as methionine, folic acid and choline interact with arsenic by altering its binding to target proteins, metabolism and tissue retention [8]. Diets deficient in folic acid have been shown to reduce the urinary elimination of arsenic metabolites, allowing increased tissue retention [9]. Additionally, diets low in choline and methionine decrease levels of S-adenosylmethionine, a universal methyl donor, which reduces the detoxification of arsenic as well as alters the normal methylation of DNA resulting in epigenetic alterations [10]. We can infer that individuals consuming a diet deficient in micronutrients may be predisposed to arsenic-induced renal toxicity and epigenetic alterations in gene expression.

The average American adult ingests less than 10 μ g inorganic arsenic per day, however this varies greatly by lifestyle and environmental exposures through groundwater, grains, and cigarette smoke [11]. The increased rate of metabolism and elimination of arsenic in mice hinders the examination of low doses, relevant to human exposure [12]. This has greatly hampered our ability to explore the role of diet arsenic-induced toxicity. Recently, the use of knockout or humanized mouse models has captured the inherent species differences in the metabolism,

distribution, and excretion of inorganic arsenic that is under genetic control [13, 14]. The multi-drug resistant transporter knockout mouse (*Mdrb1a/1b*^{-/-}) accumulates arsenic within the mouse kidney as well as other tissues, mimicking the delay in elimination of arsenic in humans [14]. These models have proven effective in generating organ-specific arsenic toxicity due to sequestration. In this study, we evaluated the role of dietary fat and micronutrients following a subchronic exposure to inorganic arsenic at environmentally-relevant doses using the *Mdr*^{-/-} knockout mouse. Kidney gene expression patterns and pathway analysis reveal both a dose- and diet-dependent role in modulating cellular response to inorganic arsenic.

C. Methods

Animals and treatments.

All animal studies were conducted in accordance with AALAC approved guidelines using a protocol approved by IACUC at North Carolina State University Biological Resources Facility. At six to eight-weeks of age FVB/N-*Abcb1a/1b*^{-/-} knockout mice (Taconic, Rockville MD) were randomly assigned to either a custom purified high fat western diet, low in choline and folate (HFD) or standard low fat AIN-93M chow (LFD) that had been specially formulated for this study by the manufacturer with the addition of 0, 10 or 150ug/kg sodium arsenite (Harlan Teklad, Madison, WI). Mice were acclimated to the HFD or LFD for 2 weeks prior to initiation of the iAs study to allow for washout of residual diet. Male and female mice were housed in same-sex cages, 3 mice per cage, with ad libitum access to food and water. To preserve quality, all diets were kept in

refrigerated, vacuum sealed-pouches, and were replaced biweekly. Animals were weighed and euthanized using a single overdose injection of Avertin anesthetic. Serum was collected for routine clinical chemistry. Kidneys were removed, weighed, and either fixed in 10% neutral buffered formalin solution for histological sections or flash frozen and stored at -80°C for molecular analyses.

RNA isolation.

Frozen kidney samples were homogenized using a tissue bead-lyser and RNA isolated using RNeasy® Mini Kits (Qiagen, Valencia, CA) per manufacturer's protocol. Total RNA was quantified with a NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA quality was determined with the Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA).

Microarray analysis.

Microarray experiments were performed using GeneChip® Mouse ST Gene 1.0 whole genome arrays from Affymetrix (Santa Clara, CA) at the UNC Genomics Core Facility. Total RNA samples were processed following a standard one-cycle eukaryotic target preparation protocol from Affymetrix. Briefly, total RNA was first reverse-transcribed using T7-oligo(dT) promoter primer in the first-strand cDNA synthesis reaction. Following second-strand cDNA synthesis, the double stranded cDNA was purified and served as a template in the subsequent in vitro transcription (IVT) reaction. The IVT reaction was carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide

analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. Biotinylated cRNA targets were then purified, fragmented, and hybridized to array during the overnight incubation at 45°C in a rotating hybridization oven. After hybridization, the arrays were stained with streptavidin-phycoerythrin in the GeneChip® Fluidics station and then scanned using Affymetrix GeneChip® Scanner (laser filter set at 570 nm; pixel size 2.5 µm). The array image data were acquired, and the fluorescent signal intensities were quantified using Affymetrix® GCOS software with following settings of quantitation parameters: Alpha1 = 0.05, Alpha2 = 0.065, Tau = 0.015, Gamma1H = 0.0045, Gamma1L = 0.0045, Gamma2H = 0.006, Gamma2L = 0.006, Perturbation = 1.1, Target Intensity = 150.

Pathway analysis and statistics.

The Affymetrix Mouse ST Gene 1.0 array has approximately 27 probes spread across each of the 28,853 genes represented on the array, providing a more complete and more accurate picture of gene expression than 3'-based expression array designs. The sequences from which the array probe sets were derived from GenBank®, Ensembl, dbEST, and RefSeq. Gene expression data (CEL files) were analyzed using Partek® Genomic Suite, version 6.3 (Partek Inc., St. Louis MO). Gene expression data were normalized by robust multi-array analysis (RMA) and analysis of variance was performed using Partek® software. We identified differentially expressed genes between classes using the non-parametric rank-product method (p-value <0.05 and 0.01) and log2 expression values for each gene were generated. Canonical pathway analysis was

generated through the use of Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). Canonical pathway analysis was measured in two ways: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway, and (2) Fischer's exact test to calculate a p-value.

D. Results

High fat diet exacerbates kidney toxicity due to arsenic exposure.

Male and female *Mdrb1a/1b*^{-/-} knockout mice were divided into 6 groups (n=6) with free access to either AIN-93M (10%) or Americanized high fat (34% fat) chow containing 0, 10, or 150ug/kg sodium arsenite for 10 weeks. At sacrifice, kidneys were removed, weighed and sectioned for histopathological examination. Food intake of the mice was not affected by the type of diet or by dose of arsenic (data not shown). While overall kidney morphology was unchanged between diets and doses, kidney to body weight ratios (Figure 3.1B) significantly decreased in mice fed a low dose of arsenic on a high fat, micronutrient poor diet. The mice fed a high fat diet given a low dose of arsenic for 10 weeks lost nearly one-third of their kidney weight over control animals by 10 weeks. In addition, serum lactate dehydrogenase, a general marker of tissue injury, was significantly increased in the HFD-fed mice with arsenic (Figure 3.1A). This effect was consistent across sexes but was not observed in LFD-fed mice treated with arsenic. This indicates that a high fat, micronutrient poor diet increases kidney-specific organ toxicity.

High fat diet alone increased genes involved in fatty acid metabolism and arsenic metabolism.

We performed full genome array analysis on the kidney tissues of male and female Mdr^{-/-} knockout mice given either LFD or HFD alone for 10 weeks to investigate the gene expression changes that would occur due to diet alone. We performed a two sample t-test to identify differentially expressed genes ($p < 0.05$) between the two control groups and identified 1408 genes (Figure 3.2A). Of them, 797 genes were specifically upregulated in the HFD-fed mice. Canonical pathway analysis of upregulated genes of high fat diet treated mice revealed expression of genes involved in fatty acid metabolism (*Acyl-Co synthase: Acsl6*, *aldo-keto reductases: AKR1A1, AKR1C1*) and Cytochrome p450-xenobiotic metabolism of sterols such as cholesterol (*Cyp11a1, Cyp17a1, Cyp2b13, Cyp2c44, Cyp2d13, Cyp2s1, Cyp3a43*) (Figure 3.2B). We also found a significant upregulation of *Gsto2* (glutathione-s-transferase omega 2) which is involved in glutathione conjugation during arsenic metabolism [15]. Several polymorphisms in the *Gsto2* gene have been associated with increased urothelial cancer risk due to arsenic exposure [16]. This suggests that a high fat diet alone increases the expression of arsenic metabolism genes which may increase its metabolism and toxicity *in situ*.

Hierarchical clustering reveals a diet effect on low dose exposures.

To understand how this diet-induced change in baseline gene expression would affect the same exposure-level to inorganic arsenic, we first performed unsupervised hierarchical clustering of the low dose groups with their respective

control groups (low fat and high fat). As seen in Figure 3.3A, a multiple comparisons ANOVA with FDR of less than 5%, found 1365 genes differentially expressed by diet and dose. Clustering analysis was performed on this gene set, and the dendrogram shows that the samples segregated based on dietary treatment. This suggests that the dietary composition is able to alter the types of genes expressed during a response to low dose arsenic exposure.

To further examine the gene clusters identified, we performed a two sample t-test on each low dose group compared to their respective diet control. Exposure to a low dose of arsenic on a LFD significantly induced 695 genes, whereas the same dose on a HFD induced 833 genes ($p < 0.05$). Canonical pathway analysis on both genes sets showed that fibroblast growth factor signaling, steroid biosynthesis and PTEN signaling were upregulated on a LFD, while mitochondrial dysfunction, AMPK signaling, Type II diabetes mellitus signaling (*Pparg*, *Adipo*, *Akt*) and NRF2-mediated oxidative stress were induced in mice fed a HFD (Figure 3.3B). Together this suggests that renal cells from mice fed a LFD respond to low dose arsenic by increasing angiogenesis, growth, apoptosis and biosynthesis, while mice on a HFD have a more aberrant response of mitochondrial dysfunction, compensatory AMPK signaling to account for the loss of ATP, increased oxidative stress and upregulation of genes involved in insulin resistance.

Dose-response signatures evident on both diets.

To compare the dose-response signatures across both diets, we performed an unsupervised clustering of the control, low dose, and high dose

groups on each of their respective diets. (Figure 3.4A and B) We performed analysis of variance ($p < 0.05$) and found 1297 genes differentially expressed by dose on a LFD (Figure 3.4A) and 1505 genes differentially expressed by dose on a HFD (Figure 3.4B). The dendrograms indicate a clear dose-response gene expression signature for all doses on both diet backgrounds, suggesting that even at low environmentally-relevant doses of inorganic arsenic exposure, a unique expression pattern can characterize the magnitude of exposure. This confirms the use of gene expression analysis in the evaluation of environmentally-relevant exposures to inorganic arsenic.

Upregulation of cancer-related pathways in high fat fed mice.

To examine the effect of high dose arsenic compared to their respective dietary controls, we performed two-sample t-test ($p < 0.05$). We identified 565 genes differentially expressed by high dose arsenic on LFD and 902 genes on a HFD. Canonical pathway analysis (Figure 3.4C) revealed bladder cancer signaling, colorectal cancer metastasis signaling, CREB signaling and RXR activation were induced in mice given a high dose of arsenic on a HFD background. In contrast, mitochondrial dysfunction, cholesterol metabolism, RXR activation and cytochrome p450 metabolism of steroids were induced on a LFD background. Interestingly, the LFD + high dose pathways closely overlap with the HFD alone without arsenic exposure suggesting that metabolic perturbations may be playing a role in the altered gene expression. The upregulation of genes associated with cancer-related pathways was only observed in HFD fed mice,

and suggests that altered dietary fats and micronutrient deficiency exacerbates arsenic toxicity on the kidney.

E. Discussion

The standard of practice for inorganic arsenic administration in laboratory mouse experiments ranges from 1 to 50 ppm in drinking water, which is several thousand-fold greater than the average daily intake for an American adult [17, 18]. In addition, a nutritionally poor diet, high in ω -6 PUFA, refined sugars, and vitamin deficiency, is well known to contribute to the rising incidence of obesity and cancer in the U.S. [19]. However, little attention has been given to the nutritionally poor as a susceptible population in toxicological studies of arsenic exposure. In this study, we have evaluated the role of dietary fatty acids and micronutrients in arsenic-induced kidney gene expression changes following a subchronic exposure to environmentally-relevant doses of inorganic arsenic using the Mdr-/- knockout mouse. Kidney gene expression patterns and pathway analysis reveal both a dose- and diet-dependent role in modulating renal toxicity and response to inorganic arsenic. Importantly, we found that altering the diet composition to mimic human consumption of vitamins and dietary fats, was able to increase several cancer-related signaling pathways in the kidney.

Reducing dietary folic acid in the Americanized HFD diet has mechanistic ties to increasing arsenic toxicity. Much of what we currently know about the nutritional epigenetics of arsenic is that human susceptibility to certain arsenic-related cancers is modified by dietary folate intake [20]. Folate plays an essential role in one-carbon metabolism and production of S-adenosylmethionine (SAM),

the primary methyl group donor for most biological methylation reactions [10]. DNA methylation is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, chromosomal modifications, and in the development of mutations [10]. Aberrant patterns and dysregulation of DNA methylation at CpG islands are mechanistically related to the development of renal carcinogenesis through the silencing or mutation of key tumor suppressor genes such as the *VHL* tumor suppressor gene [21]. This suggests that a potential mechanism of altered gene expression in this study may be due, in part, to epigenetic changes.

We found that a folate reduced-high fat diet, without arsenic, induced the basal expression of *Gsto2*, glutathione-S-transferase omega 2, which conjugates arsenic to glutathione prior to methylation by SAM [15]. In human studies, polymorphisms in *GSTO2* that increase transferase activity showed a positive correlation to renal cancer risk [22]. We can infer then that driving the conjugation and subsequent methylation of arsenic increases arsenic toxicity. Several studies examining the methylated metabolites of arsenic agree that the metabolism of arsenic increases its toxicity *in vitro* and *in vivo* [23, 24]. This has been postulated to occur through several mechanisms: SAM depletion and epigenetic alterations, a direct interaction of methylated arsenic with proteins, as well as an indirect mechanism of inhibition of DNA repair [25]. Certainly, the limitation of gene expression data is that without mechanistic studies, it is impossible to know if the rate of metabolism of arsenic was increased in our HFD treated mice. It

does suggest, however, that dietary factors could be playing a role in increasing tissue susceptibility through the upregulation of arsenic metabolism genes.

We also found that the administration of arsenic on the HFD background was able to increase mammalian target of rapamycin (mTOR) signaling, a signaling pathway commonly dysregulated in renal cell carcinoma [26]. mTOR plays an important role in cellular growth, differentiation, angiogenesis, and regulation of mitogenesis, and is thought to act as a nutrient and environmental sensor [27]. mTOR has been shown to increase hypoxia inducible factor (*HIF1*) levels and promote angiogenesis through the expression of vascular endothelial growth factor (*VEGF*) [28, 29]. In several studies examining arsenic-induced alterations in gene expression using human cell lines, *HIF1 α* , *VEGF* and *STAT1* were all upregulated in response to arsenic exposure [29, 30], suggesting that mTOR may be a central player. While our results are consistent with the data obtained from human cells lines, this report is the first to show this effect through an *in vivo* exposure in the mouse kidney, suggesting that both dietary modifications and low dose exposure is a valid approach to study the effects of chronic arsenic exposure on kidney toxicity.

Oxidative stress and mitochondrial dysfunction have also been shown to mediate the cytotoxic effects of arsenic [31]. Inorganic arsenic competes with molecular phosphate, which can perturb the production of ATP and affect the mitochondria [31]. In our pathway analysis, NRF2-mediated oxidative stress was found to be upregulated with arsenic exposure, and suggests a pro-oxidant state may be induced with arsenic exposure [32]. *In vivo* experiments have shown that

folic acid supplementation can alleviate this toxicity by reducing oxidative stress generated by free radicals [33]. Our results showed that a folate reduced-high fat diet can exacerbate oxidative stress by lowering the dose of arsenic necessary to see such an effect, implicating a dietary role in the toxicity of arsenic at very low doses.

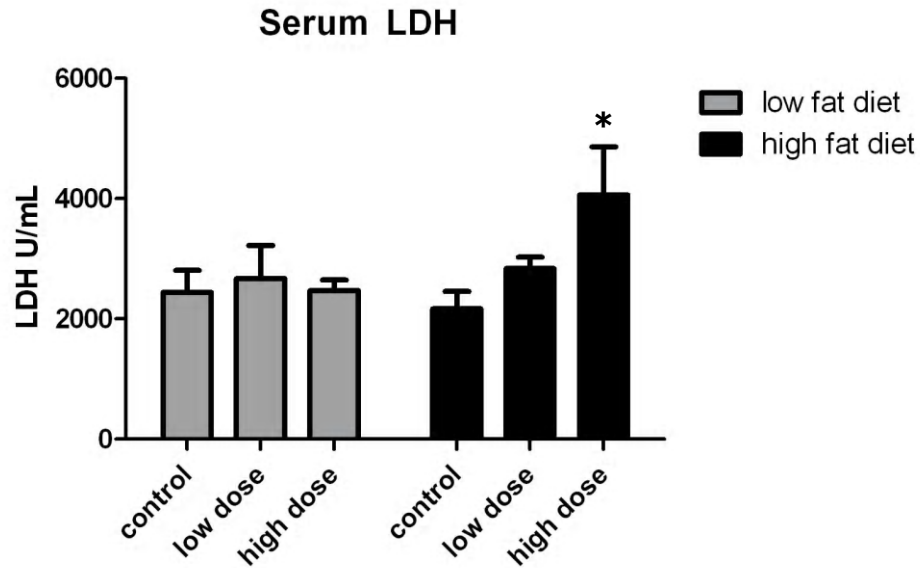
Nutritional state as a modifier of susceptibility has been clearly described in human epidemiological studies as well as in several mouse models of cancer susceptibility [34, 35]. However, nutritional state is rarely accounted for in toxicological studies in rodents. In this study we have shown that a nutritionally poor diet alters tissue response to inorganic arsenic and has revealed an increase in cancer-related signaling pathways. Such modifications were observed not only at environmentally-relevant doses, but also in the appropriate target tissue which aids to strengthen the utility of mechanistic studies of chronic exposure and the translation to human relevance.

Figure 3.1

Arsenic Dose and Diet Effect on LDH and Kidney Weight

FVB *Mdr*^{-/-} knockout mice were divided into 6 groups (N = 6) with free access to either AIN-93 (low fat diet) or an Americanized (high fat diet) chow containing 0, 10, or 150ug/kg (control, low dose, high dose) sodium arsenite for 10 weeks. Serum was analyzed for lactate dehydrogenase (LDH) as a measure of tissue injury (A). Kidneys were harvested and weighed and compared to body weights (B). Statistical Analysis by ANOVA compared to respective controls, *p≤0.05.

A.



B.

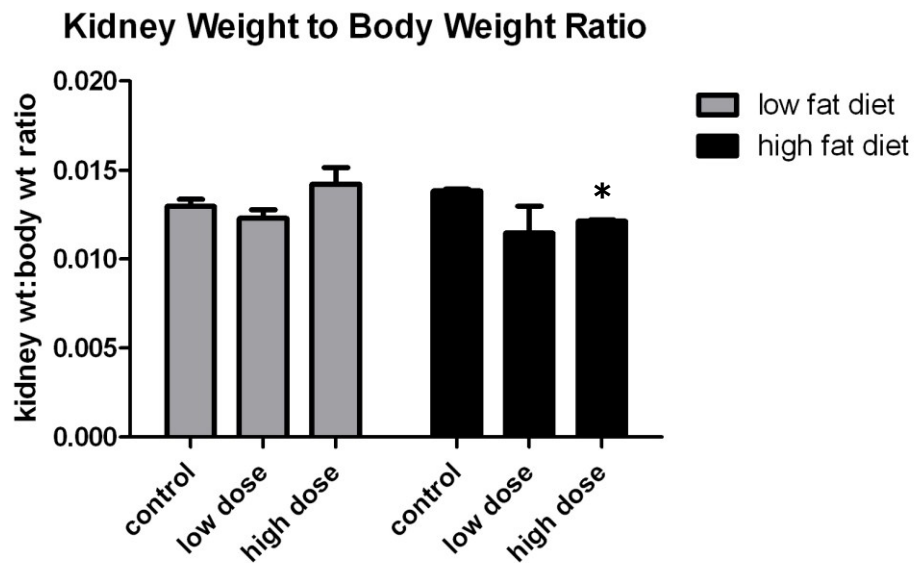
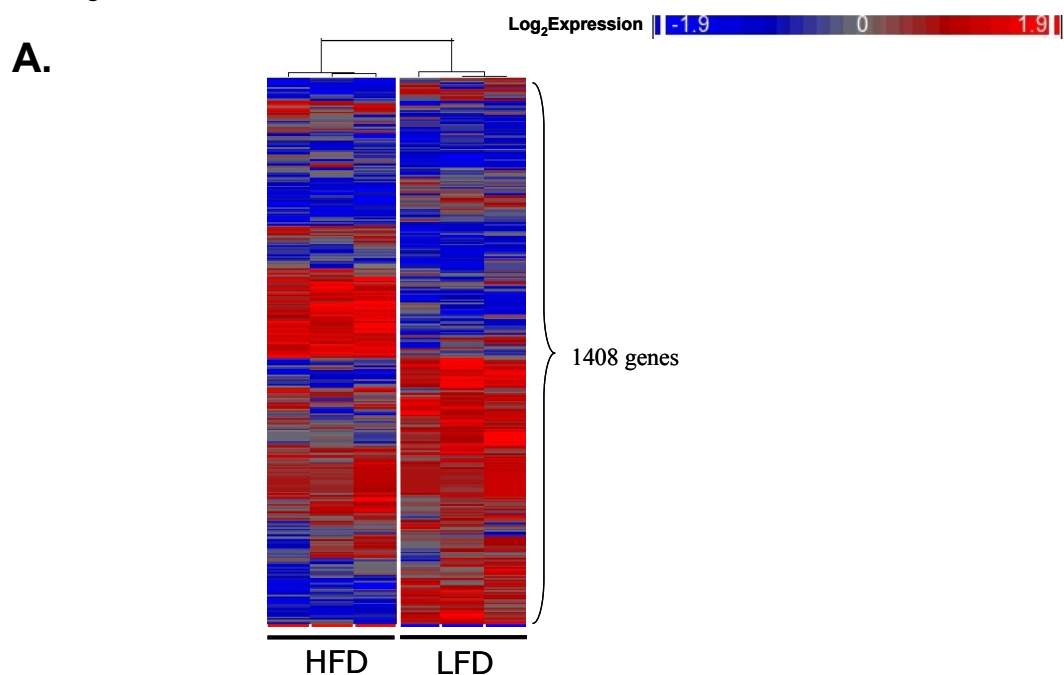


Figure 3.2

Unsupervised Hierarchical Clustering of LFD and HFD-fed Mouse Kidneys

Hierarchical clustering of LFD and HFD-fed mouse kidneys for differentially expressed genes. FVB *Mdr*^{-/-} knockout mice were divided into 6 groups (N = 6) with free access to either AIN-93 (low fat diet) or an Americanized (high fat diet) chow containing 0, 10, or 150ug/kg (control, low dose, high dose) sodium arsenite for 10 weeks. RNA was isolated and used to perform microarray analysis using the Affymetrix Mouse Gene ST 1.0 whole genome array. Differentially expressed genes were determined using Partek® Genomics Suite. Statistical significance by t-test with a FDR = 5%, $p \leq 0.05$. Gene list of 1,408 genes was then clustered unsupervised by expression (A). Biological pathway analysis was performed on genes that were differentially upregulated in HFD (B). Red – upregulated, Blue- downregulated



B.

Biological Pathway Analysis of Genes with > 2-fold Increase in HFD-fed mice

Upregulated in HFD (797 genes)

Cytochrome P450 Metabolism of Sterols

Cyp11a1, Cyp17a1, Cyp2b13, Cyp2c44, Cyp2d13, Cyp2s1, Cyp3a43

Fatty Acid Metabolism

Acl6, Akrla1, Akrlc1

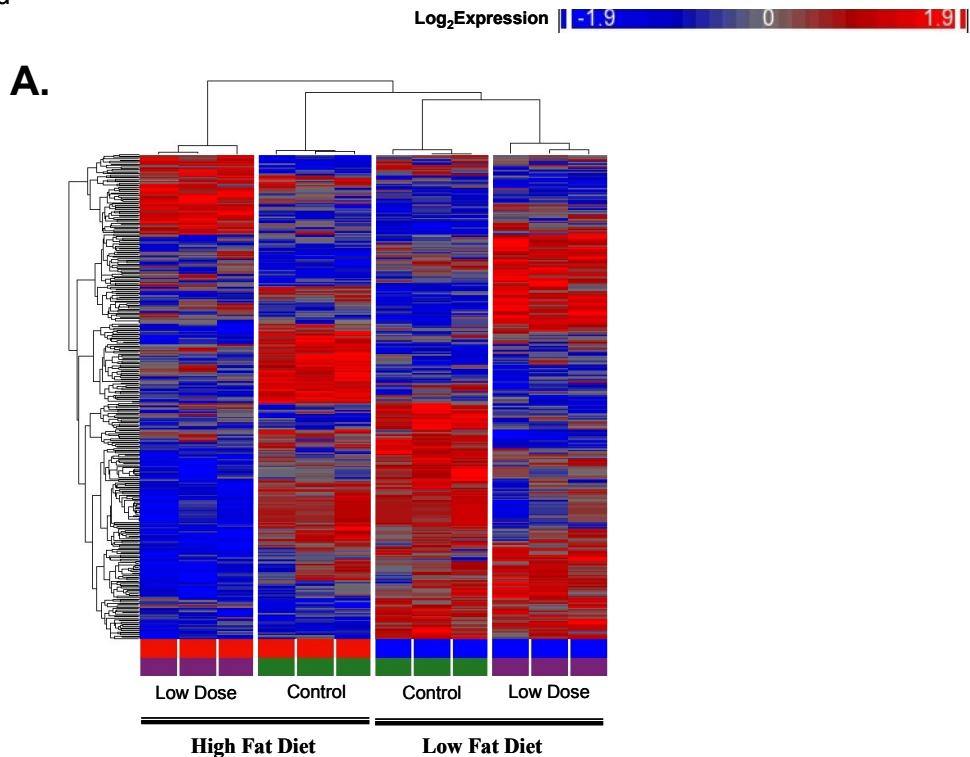
Cytochrome P450 Metabolism

Gsto2

Figure 3.3

Effect of Diet on Gene Expression with Low Dose Arsenic Exposure

Hierarchical clustering of Low Dose and Control (LFD and HFD) mouse kidneys for differentially expressed genes. FVB *Mdr*^{-/-} knockout mice were divided into 4 groups (N = 3) with free access to either AIN-93 (low fat diet) or an Americanized (high fat diet) chow containing 0 or 10ug/kg (control, low dose) sodium arsenite for 10 weeks. RNA was isolated and used to perform microarray analysis using the Affymetrix Mouse Gene ST 1.0 whole genome array. Differentially expressed genes were determined using Partek® Genomics Suite. Statistical significance by ANOVA with a FDR = 5%, $p \leq 0.05$. Gene lists were then clustered unsupervised by expression (A). Biological pathway analysis was performed on genes that were differentially upregulated by diet (B). Red – upregulated, Blue-downregulated



B.

Biological Pathway Analysis of Genes
with > 2-fold Increase in Low Dose Arsenic

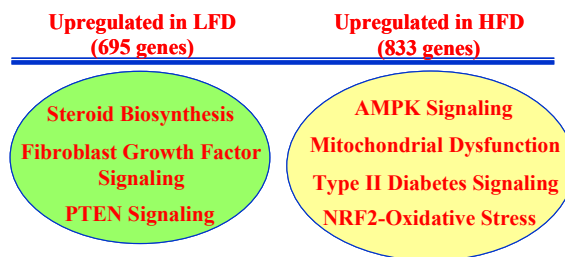
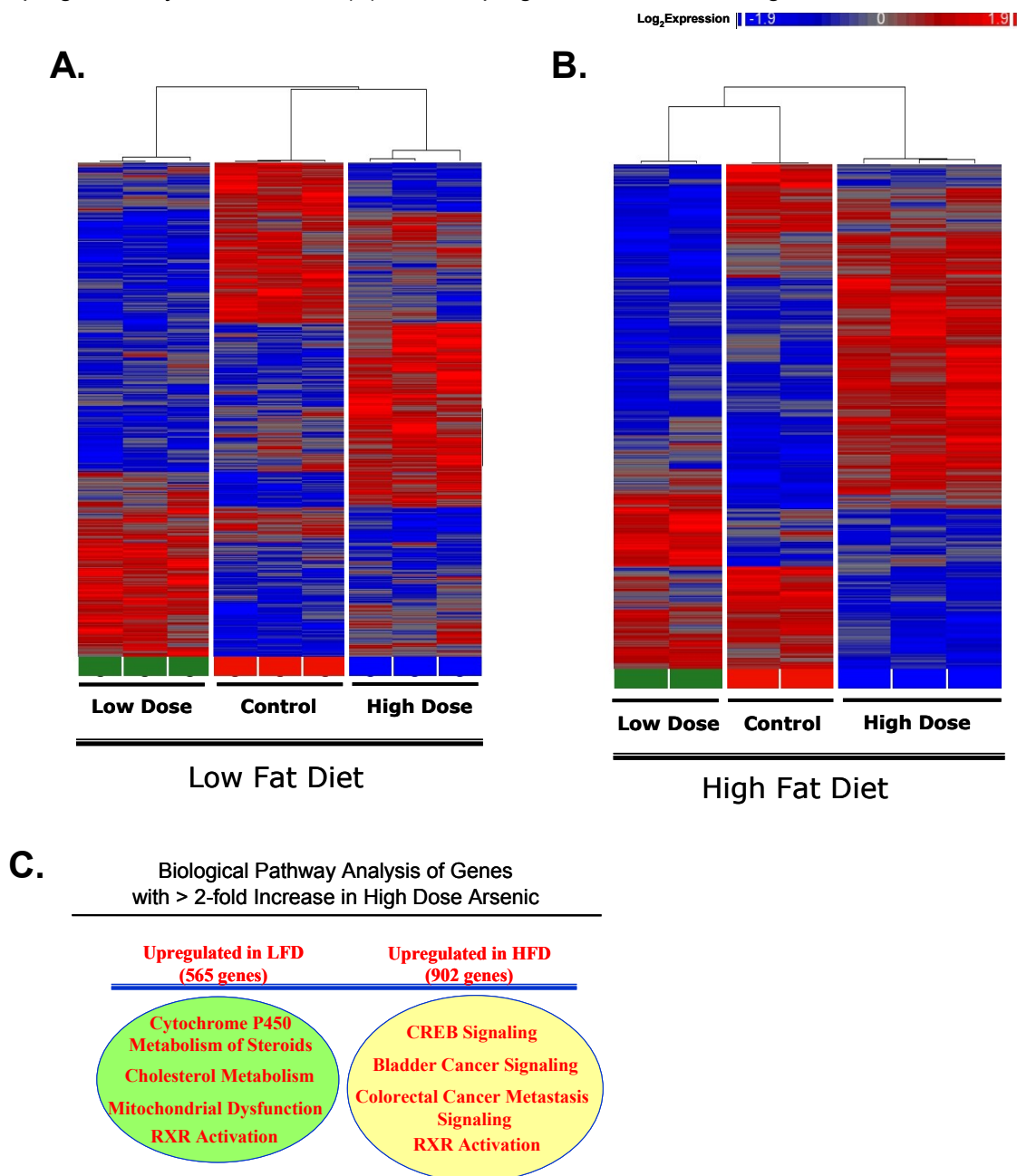


Figure 3.4

Effect of Diet on Gene Expression with High Dose Arsenic Exposure

Hierarchical clustering of differentially expressed genes in Control, Low and High Dose treated mice on either a LFD or HFD. FVB *Mdr*^{-/-} knockout mice were divided into 6 groups (N = 2-3) with free access to either AIN-93 (low fat diet) or an Americanized (high fat diet) chow containing 0, 10 or 150ug/kg (control, low dose, high dose) sodium arsenite for 10 weeks. RNA was isolated and used to perform microarray analysis using the Affymetrix Mouse Gene ST 1.0 whole genome array. Differentially expressed genes between control and high dose were determined using Partek® Genomics Suite. Statistical significance by two sample t-test with a FDR = 5%, $p \leq 0.05$. Gene lists were then clustered unsupervised by expression (A). Biological pathway analysis was performed on genes that were differentially upregulated by diet and dose (B). Red – upregulated, Blue- downregulated



REFERENCES

- 1: Yuan Y, Marshall G, Ferreccio C, Steinmaus C, Liaw J, Bates M, Smith AH. Kidney cancer mortality: fifty-year latency patterns related to arsenic exposure. *Epidemiology*. 2010;21(1):103-8.
- 2: Palmer S, Bakshi K. Diet, nutrition, and cancer: interim dietary guidelines. *J Natl Cancer Inst*. 1983;70(6):1151-70.
- 3: Maharjan M, Watanabe C, Ahmad SA, Umezaki M, Ohtsuka R. Mutual interaction between nutritional status and chronic arsenic toxicity due to groundwater contamination in an area of Terai, lowland Nepal. *J Epidemiol Community Health*. 2007;61(5):389-94.
- 4: Konkel A, Schunck WH. Role of cytochrome P450 enzymes in the bioactivation of polyunsaturated fatty acids. *Biochim Biophys Acta*. 2011;1814(1):210-22.
- 5: Escrich E, Solanas M, Moral R, Escrich R. Modulatory Effects and Molecular Mechanisms of Olive Oil and Other Dietary Lipids in Breast Cancer. *Curr Pharm Des*. [published online ahead of print] Accessed 2011 Mar 28.
- 6: Hulbert AJ, Turner N, Storlien LH, Else PL. Dietary fats and membrane function: implications for metabolism and disease. *Biol Rev Camb Philos Soc*. 2005;80(1):155-69.
- 7: Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century.[published online ahead of print] *Am J Clin Nutr*. 2011 Mar 2.
- 8: Heck JE, Gamble MV, Chen Y, Graziano JH, Slavkovich V, Parvez F, Baron JA, Howe GR, Ahsan H. Consumption of folate-related nutrients and metabolism of arsenic in Bangladesh. *Am J Clin Nutr*. 2007;85(5):1367-74.
- 9: Spiegelstein O, Lu X, Le XC, Troen A, Selhub J, Melnyk S, James SJ, Finnell RH. Effects of dietary folate intake and folate binding protein-1 (Folbp1) on urinary speciation of sodium arsenate in mice. *Toxicol Lett*. 2003;145(2):167-74.
- 10: Loenen WA. S-adenosylmethionine: jack of all trades and master of everything? *Biochem Soc Trans*. 2006;34(Pt 2):330-3.
- 11: Janković S, Radosavljević V. Risk factors for bladder cancer. *Tumorigenesis* 2007;93(1):4-12.

- 12: Cohen SM, Arnold LL, Eldan M, Lewis AS, Beck BD. Methylated arsenicals: the implications of metabolism and carcinogenicity studies in rodents to human risk assessment. *Crit Rev Toxicol*. 2006;36(2):99-133
- 13: Yokohira M, Arnold LL, Pennington KL, et al. Effect of Sodium Arsenite Dose Administered in the Drinking Water on the Urinary Bladder Epithelium of Female Arsenic (+3 oxidation state) Methyltransferase Knockout Mice. [published online ahead of print] *Toxicol Sci*. 2011. Accessed Mar 7, 2011.
- 14: Liu J, Liu Y, Powell DA, Waalkes MP, Klaassen CD. Multidrug-resistance *mdr1a/1b* double knockout mice are more sensitive than wild type mice to acute arsenic toxicity, with higher arsenic accumulation in tissues. *Toxicology*. 2002;170(1-2):55-62.
- 15: Chung CJ, Hsueh YM, Bai CH, Huang YK, Huang YL, Yang MH, Chen CJ. Polymorphisms in arsenic metabolism genes, urinary arsenic methylation profile and cancer. *Cancer Causes Control*. 2009;20(9):1653-61.
- 16: Chung CJ, Pu YS, Su CT, Huang CY, Hsueh YM. Gene polymorphisms of glutathione S-transferase omega 1 and 2, urinary arsenic methylation profile and urothelial carcinoma. *Sci Total Environ*. 2011;409(3):465-70.
- 17: Tokar EJ, Diwan BA, Ward JM, Delker DA, Waalkes MP. Carcinogenic effects of "whole-life" exposure to inorganic arsenic in CD1 mice. *Toxicol Sci*. 2011;119(1):73-83.
- 18: Boellmann F, Zhang L, Clewell HJ, et al. Genome-wide analysis of DNA methylation and gene expression changes in the mouse lung following subchronic arsenate exposure. *Toxicol Sci*. 2010;117(2):404-17.
- 19: Nieters A, Becker N, Linseisen J. Polymorphisms in candidate obesity genes and their interaction with dietary intake of n-6 polyunsaturated fatty acids affect obesity risk in a sub-sample of the EPIC-Heidelberg cohort. *Eur J Nutr*. 2002;41(5):210-21.
- 20: Vahter ME. Interactions between arsenic-induced toxicity and nutrition in early life. *J Nutr*. 2007;137(12):2798-804.
- 21: Arai E, Kanai Y. Genetic and epigenetic alterations during renal carcinogenesis. *Int J Clin Exp Pathol*. 2010;4(1):58-73.
- 22: Wang YH, Yeh SD, Shen KH, et al. A significantly joint effect between arsenic and occupational exposures and risk genotypes/diplotypes of CYP2E1, GSTO1 and GSTO2 on risk of urothelial carcinoma. *Toxicol Appl Pharmacol*. 2009;241(1):111-8.

- 23: Wang TC, Jan KY, Wang AS, Gurr JR. Trivalent arsenicals induce lipid peroxidation, protein carbonylation, and oxidative DNA damage in human urothelial cells. *Mutat Res*. 2007;615(1-2):75-86.
- 24: Jomova K, Jenisova Z, Feszterova M, Baros S, Liska J, Hudecova D, Rhodes CJ, Valko M. Arsenic: toxicity, oxidative stress and human disease. *J Appl Toxicol*. 2011;31(2):95-107.
- 25: Salnikow K, Zhitkovich A. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem Res Toxicol*. 2008;21(1):28-44.
- 26: Banumathy G, Cairns P. Signaling pathways in renal cell carcinoma. *Cancer Biol Ther*. 2010;10(7):658-64.
- 27: Borders EB, Bivona C, Medina PJ. Mammalian target of rapamycin: biological function and target for novel anticancer agents. *Am J Health Syst Pharm*. 2010;67(24):2095-106.
- 28: Land SC, Tee AR. Hypoxia-inducible factor 1 α is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *J Biol Chem*. 2007;282(28):20534-43.
- 29: Jiang BH, Liu LZ. PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochim Biophys Acta*. 2008;1784(1):150-8.
- 30: Duyndam MC, Hulscher ST, van der Wall E, Pinedo HM, Boven E. Evidence for a role of p38 kinase in hypoxia-inducible factor 1-independent induction of vascular endothelial growth factor expression by sodium arsenite. *J Biol Chem*. 2003;278(9):6885-95.
- 31: Lu TH, Su CC, Chen YW, Yang CY, et al. Arsenic induces pancreatic β -cell apoptosis via the oxidative stress-regulated mitochondria-dependent and endoplasmic reticulum stress-triggered signaling pathways. *Toxicol Lett*. 2011;201(1):15-26.
- 32: Fu J, Woods CG, Yehuda-Shnaidman E, et al. Low-level arsenic impairs glucose-stimulated insulin secretion in pancreatic beta cells: involvement of cellular adaptive response to oxidative stress. *Environ Health Perspect*. 2010;118(6):864-70.
- 33: Majumdar S, Mukherjee S, Maiti A, et al. Folic acid or combination of folic acid and vitamin B(12) prevents short-term arsenic trioxide-induced systemic and mitochondrial dysfunction and DNA damage. *Environ Toxicol*. 2009;24(4):377-87.

34: Kim YI. Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. *J Nutr.* 2005;135(11):2703-9.

35: Song J, Medline A, Mason JB, Gallinger S, Kim YI. Effects of dietary folate on intestinal tumorigenesis in the apcMin mouse. *Cancer Res.* 2000;60(19):5434-40.

Chapter Four:

A POPULATION-LEVEL GENETIC MODEL REVEALS AN EARLY MARKER OF KIDNEY INJURY AND A GENETIC-BASIS FOR SUSCEPTIBILITY

A. Abstract

Co-exposure to low levels of trichloroethylene (TCE) and inorganic arsenic (iAs) through groundwater and dietary contamination occurs in many localities and is thought to result in multiple organ toxicities in susceptible human populations. The interpretation of disease associations with TCE and iAs is under great debate in part due to the lack of an appropriate population-level experimental model with which to test the effect of inter-individual variation in metabolism, detoxification, and transport. To model the genetic heterogeneity of exposed human populations, we developed a unique intercross population derived from FVB/N-*Abcb1a/1b*^{-/-}, a multi-drug resistant p-glycoprotein knockout mouse model, and CAST/EiJ a wild-derived strain that is genetically distinct from the FVB/N background. By modulating drug transport and genetic variation in the mouse to model human biology, including using a western diet, environmentally-relevant doses of TCE and iAs can be evaluated for their effects on toxicity susceptibility. A study cohort of 900 FVB/N-*Abcb1a/1b*^{-/-} x CAST/EiJ F₃ mice was divided into nine dose groups, each containing 50 female and 50 males, and was administered TCE and sodium arsenite via the drinking water and chow at environmentally-relevant concentrations for 52 weeks using a dose-ratio approach. We collected serum and urine for evaluation of biomarkers of kidney and other organ injury. Levels of classical biochemical markers serum alanine aminotransferase and blood urea nitrogen showed no significant differences at 16 weeks. However, urinary neutrophil gelatinase-associated lipocalin-2, a sensitive biomarker of early renal injury, was increased 3- to 7-fold over controls

at week 16 in mice treated with both TCE and iAs. This data suggests that TCE and iAs may cooperate to produce renal toxicity that may be modulated by genetic determinants.

B. Introduction

Human exposure to environmental carcinogens often occurs as complex mixtures at low doses, and such interactions between individual compounds may contribute significantly towards human cancer risk [1]. Trichloroethylene (TCE) and inorganic arsenic (iAs) are suspect kidney carcinogens commonly found in contaminated drinking water worldwide [1]. Although epidemiological evidence shows an association between dietary exposures and an increased risk for kidney cancer in humans [2], animal models of TCE or iAs-induced kidney toxicity and carcinogenesis have been largely absent [3]. Further, few efforts have addressed potential modifiers of response such as species-differences in drug metabolism and transport, and nutritional status that play roles in an individual's response to kidney toxicants at low doses. This lack of biologically-based models has hindered both mechanistic studies as well as identification of biomarkers of effect that have clinical translation.

The genetic basis of susceptibility to environmental carcinogens has been well established in human populations, but remains poorly modeled in toxicological studies. Inbred mouse lines provide a fixed genotype within a particular strain and capture an enormous genetic diversity across strains due to the number and distribution of genetic polymorphisms [4]. As such, inbred mouse diversity panels have recently been exploited in toxicological studies for the

determination of susceptible and resistant strains as well as identification of biomarker genes whose expression strongly correlates with toxicity [5]. However, the identification of genetic loci that both control and modify a specific trait, i.e. susceptibility to kidney injury, requires a mapping population obtained through controlled crosses [6].

The development of a population-based genetic model for use in the identification of susceptibility genes requires selection of parental strains that exhibit variation at the DNA sequence and phenotype levels [6]. In this study, we examined the hypothesized mechanisms of cytotoxicity for TCE and iAs as a basis for selecting critical genes of interest that may modify the resulting toxicity phenotype. We then examined the kidney gene expression of DNA repair enzymes *Ogg1* as well as drug transporters *Abcb1a/1b* across 37 inbred strains to identify two strains with differential expression, in addition to extensive polymorphic variants. Here, we developed a unique F₃ mouse population derived from FVB/N-*Abcb1a/1b*^{-/-} mice, a multidrug resistant ABC transporter knockout, and CAST/EiJ a wild-derived strain from a different subspecies of *Mus musculus*. The *Mdr*^{-/-} mice show decreased clearance of drug metabolites, including arsenic, and are more susceptible to injury in the kidney [7], while CAST/EiJ allows us to examine genetic variability as this strain carries a completely different set of allele combinations, including *VHL*. In the present study, we hypothesized that co-exposure to TCE and iAs will act in an additive fashion to induce renal toxicity and that the use of an F₃ mouse population model will reveal

a genetic basis for renal toxicity due to environmentally-relevant dose mixtures of trichloroethylene and inorganic arsenic.

C. Methods

Mouse population generation and design.

All breeding was performed in house at North Carolina State University Biological Resource Facility. Female FVB/N-*Abcb1a/1b*^{-/-} knockout mice (Taconic, Rockville MD) and male CAST/EiJ (Jackson Laboratory, Bar Harbor ME) were intercrossed for three generations to generate a population of nine hundred FVB/N-*Abcb1a/1b*^{-/-} x CAST/EiJ F₃ mice. To avoid litter effects in the study, mice were weaned to one of nine dose groups, with one same-sex pup per litter per group at 3 weeks of age. Maximum litter size never exceeded more than 9 pups of each gender. Each same-sex cage contained 10 mice, which remained together throughout the duration of the study. Each of nine dose groups included 100 mice (50 females and 50 males). At 6 weeks, mice were switched from the purified AIN-93G diet (Harlan Teklad, Madison WI) to a custom purified high fat Americanized diet (Harlan Teklad, Madison WI) (Table 4.1) and were allowed to acclimate for 2 weeks before entering the study. We staggered entry dates for F₃ mice from all crosses, to minimize any confounding by calendar time of procedures. Approximately 200-300 F₃ mice entered the study protocol every four to five weeks over a 16-week window. Furthermore, all sample collections were performed within narrow time windows during the day to minimize the impact of circadian effects on the data.

Dietary treatment: a dose-ratio approach.

Dose-equivalent quantitation was used to determine dosage. Trichloroethylene and inorganic arsenic were added to the drinking water and chow, respectively, at environmentally relevant concentrations (Figure 4.1). A low and high dose for each compound was selected to be no more than 2 to 6-fold greater than an actual human exposure dose-equivalent. To incorporate an assessment of a two-compound mixture as well as a dose-response, a dose-ratio approach was used to form nine dose groups (Figure 4.2).

Nine hundred FVB/N-*Abcb1a/1b*^{-/-} x CAST/EiJ F₃ mice were assigned to one of nine dosing groups at weaning as described above. At 6 weeks, all mice were acclimated to the custom high fat Americanized diet for 10-14 days before a baseline serum and urine measurement was collected from each animal. Mice were then switched to Nanopure drinking water containing 0, 5, or 2850ppb TCE and a custom high fat chow with the addition of 0, 10 or 150µg sodium arsenite per kilogram diet. To preserve freshness and minimize degradation, TCE water was prepared fresh, replaced weekly and administered in UV-light protected water bottles. Arsenic containing chow was kept in vacuum-sealed refrigerated pouches and replaced weekly. Animals remained on this custom dietary treatment for the duration of the 52 week study.

Serum collection.

For evaluation of biochemical markers of tissue injury, whole blood was collected at week 0, 16, 32 and 52 using the submandibular bleed method. Briefly, a 5mm lancet (GoldenRod, Mineola NY) was used to puncture the

submandibular vein and whole blood was collected in EDTA-free capillary tubes (Sarstedt, Newton NC). Blood was allowed to coagulate 30min at room temperature before being centrifuged at 10,000 x g for 20min. The serum layer was placed into a clean 1.5mL tube and stored at -80°C until future analysis. All serum collections were performed in the morning hours, within a narrow time window to minimize the impact of circadian effects on the data.

Urine collection.

Urine was collected at week 0, 16, 32, and 52 for endpoints such as arsenic metabolite measurement, NMR-based metabolomics and evaluation of urinary biomarkers. Briefly, mice were individually housed in custom-designed metabolic cages (Hatteras Instruments Inc., Cary NC) for 16 hours with *ad libitum* access to drinking water only. Each cage collects the freshly voided urine using a stainless steel funnel to channel the urine, and not feces, into a chilled 2mL collection tube, maintaining the urine sample at 34° to 42°C. Urine samples were then placed on ice while color, clarity and volume were recorded. Samples were stored at -80°C until future analysis.

Clinical chemistry.

Measurement of serum alanine aminotransferase and blood urea nitrogen as biochemical markers of tissue injury was performed at the UNC Animal Pathology Clinical Chemistry Core Facility. Results are reported as mean \pm standard error with n = 20-30 in each group. Treatment groups were compared

using one-way ANOVA analysis. A $p < 0.05$ was selected before the study to determine statistical differences between groups.

NGAL ELISA.

Neutrophil gelatinase-associated lipocalin (NGAL) was measured in urine samples by ELISA assay as a marker of early renal injury (R&D Systems, Minneapolis MN). Urine samples were thawed on ice and an aliquot removed, centrifuged and diluted 1:10 in the provided assay buffer. The assay was performed per manufacturer's protocol. Samples were measured in duplicate using a microplate reader set at 540nm/450nm within 30min of addition of stop buffer. Concentrations of NGAL were determined based on a standard curve. Results are reported as mean \pm standard error with $n = 20-30$ in each group. Treatment groups were compared using one-way ANOVA analysis followed by Tukey's multiple comparison post-hoc test, where appropriate. A $p < 0.05$ was selected before the study to determine statistical differences between groups.

Serum cytokine analysis.

Serum cytokines were quantified as a measure of systemic inflammation. The flow-cytometry based fluorescent assay simultaneously measures 31 cytokines in a single serum sample (BioPlex, Hercules CA). Briefly, fluorescent beads conjugated with antibodies against: eotaxin, G-CSF, GM-CSF, IFN γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IP-10, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1 α , MIP-1 β , MIP-2, RANTES, TNF α , VEGF, were incubated with a 25ul aliquot of serum overnight at 4°C. The assay was

completed according to the manufacturer's protocol (Millipore, Thousand Oaks CA). Samples were analyzed using a BioRad 200 Multiplex Reader (BioRad, Hercules CA) and quantified using a standard curve provided.

D. Results

Serum ALT and BUN are non-sensitive markers of injury

Male and female FVB/N-*Abcb1a/1b*^{-/-} x CAST/EiJ F₃ mice were divided into 9 groups with free access to drinking water containing 0, 5, or 2,850ppb TCE and a high fat (34% fat) chow containing 0, 10, or 150ug/kg sodium arsenite for 16 weeks. Food and water intake of the mice was not affected by diet or by dose (data not shown). Serum samples collected from mice after 16 weeks of treatment were analyzed for ALT and BUN, both classical injury markers. An increase in ALT would most likely indicate moderate to severe liver injury, but is non-specific for kidney. An increase in BUN would indicate incomplete removal of urea nitrogen, a function of the kidney [8]. The analysis revealed no significant difference between treatment groups in either of these two markers (Figure 4.2). Furthermore, despite the genetic variation present in the F₃ population, the ALT and BUN remain stable in both control and treated animals. This is likely due to the small degree of variation in ALT or BUN between the two parental strains FVB/N and CAST/EiJ (phenome.jax.org) as well as the lack of sensitivity of the measurement itself [9].

Urinary NGAL is a sensitive marker of early renal injury and reveals a genetic basis for susceptibility.

Historically, ALT and BUN elevations are seen in cases of moderate to severe tissue injury and may not reflect minor chronic injury to the kidney that we expect in a low dose study. Recently, several clinical markers of early kidney injury in humans have been shown to also be predictive in rodents [10]. Of them, neutrophil gelatinase-associated lipocalin (NGAL) was found to be the most promising marker of acute kidney injury [11, 12]. Here, we collected urine samples after 16 weeks of dietary administration of TCE, arsenic, or control diet to examine urinary NGAL concentrations. Mice exposed to either high dose arsenic alone, or in combination with low or high dose TCE showed a 2 to 7-fold increase in urinary NGAL, respectively (Figure 4.3). Interestingly, the analysis reveals a striking increase in variation of NGAL concentrations within the groups receiving low dose combinations of TCE and arsenic as well as TCE alone. This degree of variation was not seen in the control animals, indicating that inherent variation in basal NGAL concentrations between individual F₃ animals is unlikely to explain this increase. Rather, this indicates that the increased NGAL variation in response to the treatment is genetically controlled. Such an observation suggests a genetic basis for susceptibility to early kidney injury due to TCE and arsenic exposure.

Serum cytokine analysis indicates elevated NGAL is not due to systemic inflammation.

Neutrophil gelatinase-associated lipocalin (NGAL) is found in both the renal tubule as well as in neutrophils. An increase in serum NGAL can indicate a state of inflammation due to its release from neutrophils [13]. To verify that the increase in urinary NGAL we observed was not due to a general state of increased inflammation, we performed a multiplex assay which quantitatively measures 31 different cytokines, chemokines, and adipokines in the serum of the control group as well as the highest NGAL response group exposed to both high dose TCE and high dose arsenic. The results of all 31 cytokines measured showed no significant difference between control and treated mice, confirming that our NGAL increase in urine is not due to a systemic increase in inflammation (data not shown).

E. Discussion

Several deficiencies have been highlighted in TCE and arsenic research including (1) lack of experimental models with which to investigate renal toxicity, (2) poor understanding of the linearity of the dose-response relationship at low doses, (3) lack of studies examining the effect of nutritional deficiencies, and (4) lack of consideration for genetic susceptibility in estimating risks [14]. In the present study, we addressed each of these four issues by investigating whether the use of an FVB/N-*Abcb1a/1b*^{-/-} x CAST/EiJ F₃ mouse population model could reveal a genetic basis for renal toxicity due to environmentally-relevant dose mixtures of TCE and inorganic arsenic on a nutritionally-modified background.

Here we show data from week 16 of our 52-week study that combinatorial exposure to TCE and iAs increases renal injury and that susceptibility to toxicity may be genetically controlled.

Susceptibility to exposure-associated increases in renal cancer risk has been investigated in a limited number of human population studies, with most results focus on polymorphic genes in drug metabolism and transport [15, 16]. In our F₃ mice, we have modeled the population-wide genetic variation in drug metabolism and transport, as well as DNA repair capacity by intercrossing the two genetically diverse strains of mice, FVB/N and CAST/EiJ, that represent two different subspecies of *Mus musculus*. Urinary NGAL concentrations in the control group reveal that there is little effect of genetics or nutrition on the basal release of NGAL into urine. However, within groups treated with TCE and/or iAs, there was considerable variation in the response to treatment. This within-group variation in response can be attributed to the genetic variation captured in the F₃ cross, as each individual mouse represents a unique genetic recombination. Because we have held the exposure within each group constant and only varied the genetics, this data strongly suggests that the toxicity response is a multigenic trait, representing the complex interplay of polymorphic genes derived from the two parental strains [17].

We have considerable evidence that diet plays an important role in modifying toxicant response through the regulation of gene expression and intracellular signaling [18]. One important consideration in the interpretation of our data is the gene x diet interaction and how that may be affecting the variation

in toxicity response within treatment groups. Dietary factors in this study included both modifications in dietary fatty acids as well as reduction of choline and folic acid. Uptake and utilization of dietary nutrients such as folic acid are partially under genetic control [19] and represent a source of variation in addition to TCE and iAs treatment. While adding complexity to our analysis, the gene x diet x toxicant interaction will ultimately improve our understanding of how nutrients modulate susceptibility to kidney carcinogens, and may lead to the development of nutritional intervention strategies in the prevention of disease.

The NGAL data also suggests that TCE and iAs may cooperate to increase renal toxicity. Environmental metals such as arsenic can act to enhance toxicity of organic compounds by targeting common biological pathways [20]. For both TCE and iAs, the metabolism and “detoxification” results in bioactivation within the kidney to active and more toxic metabolites [21]. Both compounds have been shown to interact with protein sulfhydryl groups and increase intracellular oxidative stress [21], as well as alter the methylation capacity of DNA, leading to epigenetic changes [22] (Figure 4.5). The relative significance of this biological overlap remains unexplored pending completion of our 52-week study and biochemical evaluation of kidney tissues. However, important for risk assessment, individuals exposed to these agents that carry polymorphisms in genes that are important in the metabolism and detoxification of these chemicals show an increased renal cancer risk, which provides biological plausibility of the association in humans [23].

Translation of animal study data to potential clinical and human applications has hampered biomedical research of chronic diseases [24]. Here we have presented a novel biologically-based animal model which addresses key research deficiencies in the toxicological evaluation of carcinogen-induced renal toxicity. While traditional risk assessments examine the effect of treatment independent of genetics and nutrition, our approach has the combined benefit of not only evaluating response to treatment but has the potential to identify the genetic variants underlying susceptibility, and biomarkers that predict response as well as an understanding of gene-nutrient interactions in the modification of toxicity. Consequently, our model has the potential to become a new paradigm in the evaluation of chemical risk assessments by improving the clinical translation of animal study data.

Table 4.1**Diet Formulations**

Diet Ingredient	AIN-93M, Low Fat (soybean oil)	Americanized, High Fat (soybean, safflower, and coconut oil, lard)
%kcal Fat	10.2	34.4
%kcal Protein	13.8	15.1
%kcal Carbohydrates	76.0	50.6
Folic Acid	1.5 mg/kg	0.2 mg/kg
Choline	2.5 g/kg	0.496 g/kg
Vitamin mix	10 g/kg	11.5 g/kg
Mineral Mix	35 g/kg	40.35 g/kg
% Saturated Fat	12.8	23.6
Omega 6:Omega 3	6.6:1	14.7:1

Figure 4.1

Determination of Environmentally-Relevant Dosage for TCE and iAs

The determination of environmentally-relevant dosage was based on human exposure data. Tables depict the tissue dose-equivalent calculations of TCE (A) and inorganic arsenic (B) in both human and mouse for selection of both a low and high dose for use in this study. Parameters such as body weight, food intake and water intake were based on previous studies in our lab. Dose equivalents for inorganic arsenic do not exceed 2 to 6-fold the exposure found in human populations. *[26], #[25]

A.

TCE (drinking water)	Water Concentration (ppb = $\mu\text{g/L}$)	Dose Equivalent ($\mu\text{g/kg}$ body wt/day)	Body Weight	Intake based on human blood values
Human - Low	42 ppb	1.20 $\mu\text{g/kg/day}$	70kg (~150lbs)	0.017 $\mu\text{g/L}$ blood
Human - High	25,000 ppb	714.3 $\mu\text{g/kg/day}$	70kg (~150lbs)	Based on water well measurements
TCE (drinking water)	Water Concentration (ppb = $\mu\text{g/L}$)	Dose Equivalent ($\mu\text{g/kg}$ body wt/day)	Body Weight	Water Intake
Mouse - Low	5 ppb	1.25 $\mu\text{g/kg/day}$	0.02kg (~20g)	5 mL/day
Mouse - High	2,850 ppb	712.5 $\mu\text{g/kg/day}$	0.02kg (~20g)	5 mL/day

B.

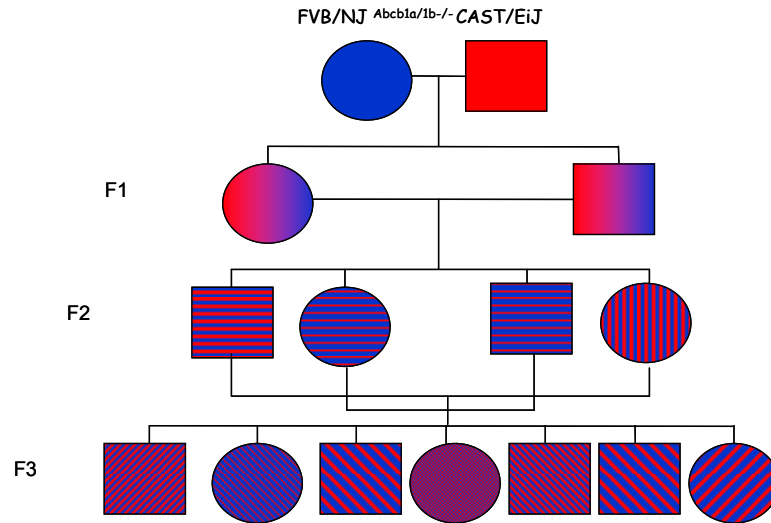
Arsenic (dietary)	Average Intake ($\mu\text{g/day}$)	Dose Equivalent ($\mu\text{g/kg}$ body wt/day)	Body Weight	Intake based on Consumption
Human - Low	50 $\mu\text{g/day}$	0.71	70kg (~150lbs)	*WHO limit: 10ppb and 2L/person/day
Human - High	350 $\mu\text{g/day}$	5.0	70kg (~150lbs)	#1.7mg/kg in rice and 200g/person/day
Arsenic (dietary)	Chow Concentration ($\mu\text{g/kg}$)	Dose Equivalent ($\mu\text{g/kg}$ body wt/day)	Body Weight	Food Intake
Mouse - Low	10 $\mu\text{g/kg}$	2.0	0.02kg (~20g)	4g/day
Mouse - High	150 $\mu\text{g/kg}$	30	0.02kg (~20g)	4g/day

Figure 4.2

F₃ Population Generation and Dosing Groups

Female FVB/N-*Abcb1a/1b*^{-/-} knockout mice and male CAST/EiJ were intercrossed for three generations to generate an F₃ population of nine hundred *FVB/N-Abcb1a/1b*^{-/-} x *CAST/EiJ* F₃ mice (A). Mice were then assigned to one of nine dose groups.(B) Dose groups were created using a dose-ratio approach to assess a two-compound mixture at three dose levels (0, low and high).

A.



B.

Dose Group	Dose Ratio iAs:TCE	As concentration (chow: ug/kg)	TCE concentration (water: ppb)
1	0 : 0	0	0
2	0 : low	0	5
3	0 : high	0	2850
4	low : 0	10	0
5	low : low	10	5
6	low : high	10	2850
7	high : 0	150	0
8	high: low	150	5
9	high : high	150	2850

Figure 4.3

Timeline of the 52-week Study and Sample Collections

Figure shows a time line of the 52-week study design. Nine hundred *FVB/N-Abcb1a/1b*^{-/-} x *CAST/EiJ* F₃ mice were assigned to one of nine dose groups and weaned onto Americanized high fat diet at Week 3. All mice were allowed to acclimate to the Americanized diet for 14 days before a baseline serum and urine measurement was collected from each animal at Week 6. Mice were then switched to Nanopure drinking water containing 0, 5, or 2850ppb TCE and a custom chow with the addition of 0, 10 or 150ug sodium arsenite per kilogram diet at Week 7. Animals remained on this custom dietary treatment for the duration of the 52 week study. Collection dates and samples types collected are depicted in boxes. Highlighted black box represents the analysis of urinary NGAL and serum clinical chemistry after 16 weeks on the dietary treatments that is shown in this paper.

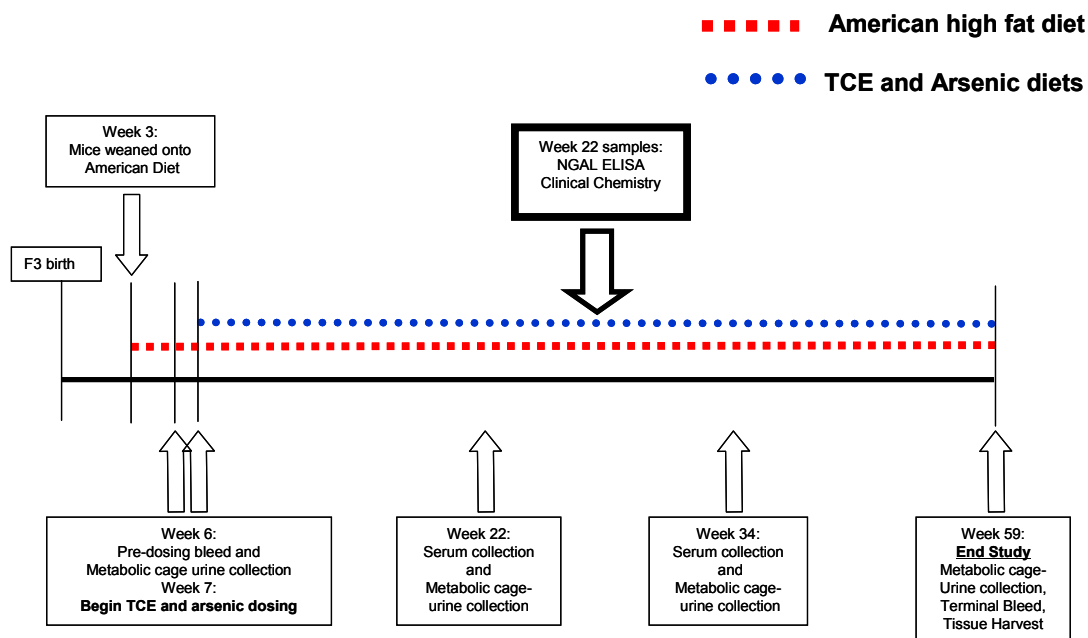
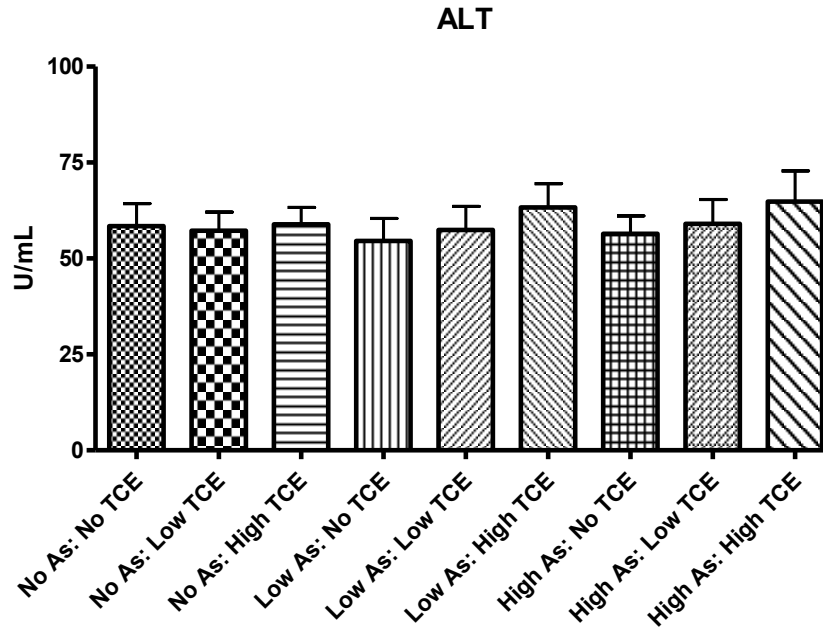


Figure 4.4

Effect of Co-Exposures on Serum Clinical Chemistry

FVB/N-Abcb1a/1b^{-/-} x *CAST* F₃ mice were given a combinatorial dietary treatment containing a dose-ratio of 0, 5 or 2,850ppb TCE (No, Low, High) and 0, 10, 150 ug/kg As (No, Low, High). Serum ALT (A) and BUN (B) were measured after 16 weeks of exposure to assess classical markers of tissue injury. Data are represented as mean \pm standard error from 20-30 animals per group. Statistical difference measured by one-way ANOVA, $p \leq 0.05$.

A.



B.

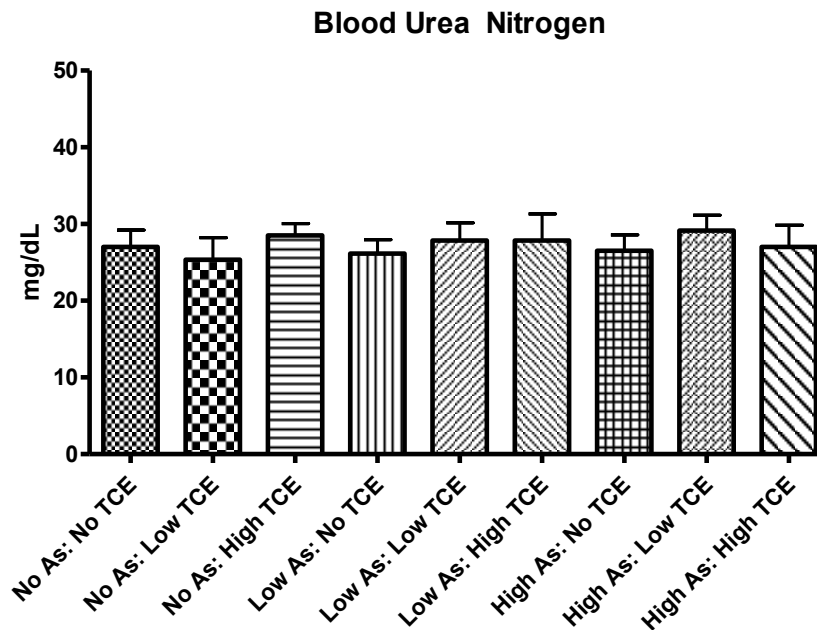


Figure 4.5

Effect of Co-Exposures on Urinary Marker of Kidney Injury

FVB/N-Abcb1a/1b^{-/-} x CAST F₃ mice were given a combinatorial dietary treatment containing a dose-ratio of 0, 5 or 2,850ppb TCE (No, Low, High) and 0, 10, 150 ug/kg As (No, Low, High). Urinary concentrations of NGAL, neutrophil gelatinase-associated lipocalin, an early marker of renal injury, were measured after 16 weeks of exposure. Data are represented in a box and whisker plot representing group mean \pm standard deviation from 30 animals per group. Statistical difference compared to corresponding control group by ANOVA and multiple comparisons test (* $p \leq 0.05$).

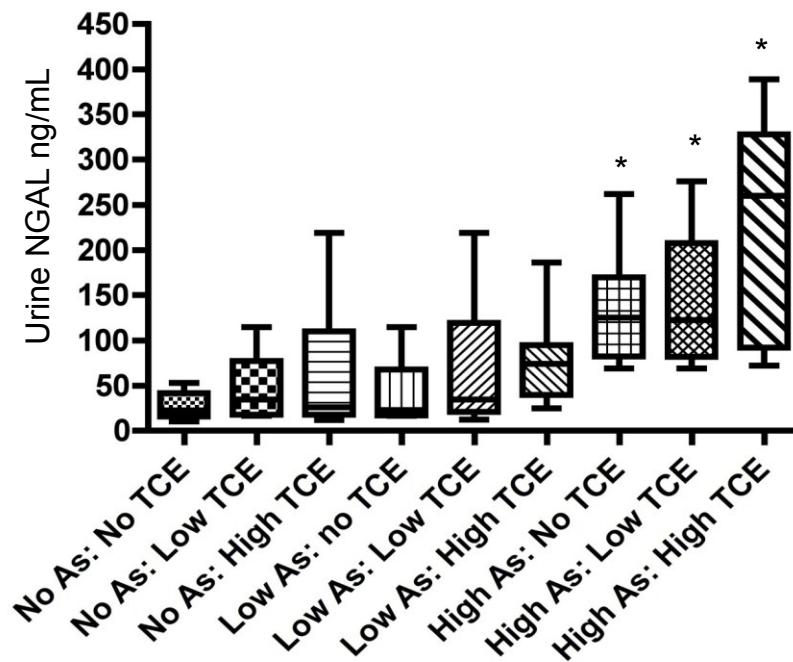
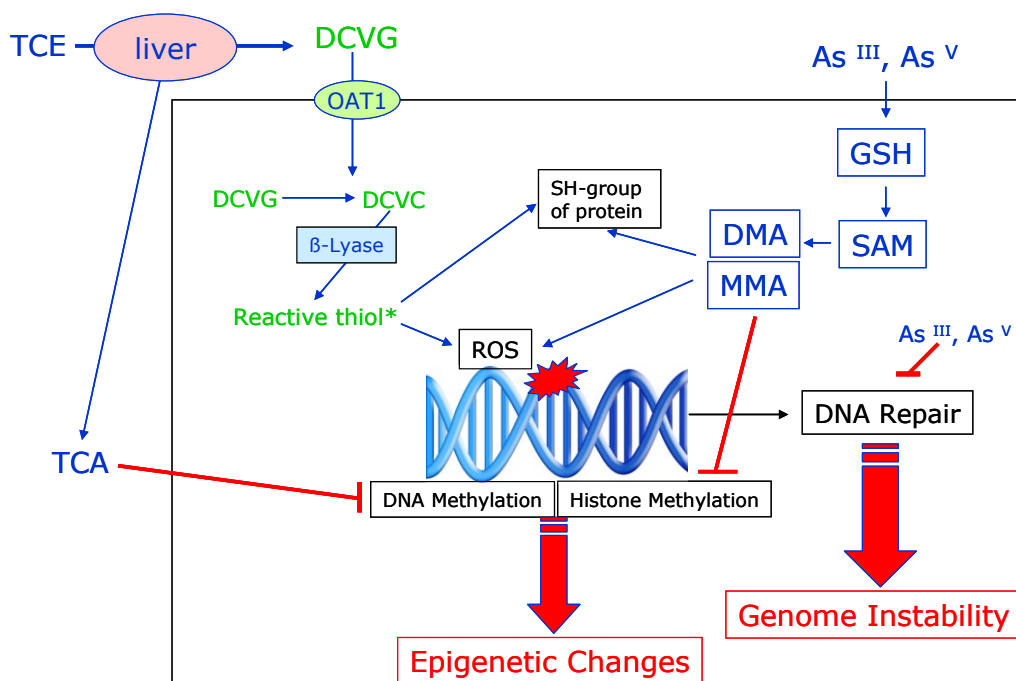


Figure 4.6

Biochemical Interactions of TCE and iAs in Renal Tubule Cells

Scheme depicts the proposed metabolism and mechanism of action of TCE and iAs in the renal epithelial cell. TCE is primarily metabolized within the liver to a glutathione conjugate (DCVG) which is transported across the cell membrane further metabolized by a renal-specific enzyme, beta-lyase, to a reactive thiol (*), the primary nephrotoxic metabolite of TCE. This reactive thiol is thought to interact with protein sulfhydryl groups (SH-) as well as induce oxidative stress (ROS). In addition, another metabolite of TCE, trichloroacetic acid (TCA), has been shown to alter DNA methylation through an unknown mechanism [21]. iAs is transported into the renal cell via an unknown mechanism and is conjugated by glutathione (GSH) and further methylated by s-adenosylmethionine (SAM) to a di- and mono- arsenical (DMA, MMA) which can also interact with protein SH-groups and induce ROS. The metabolism on iAs depletes the cellular SAM and GSH levels over chronic exposure leading to alterations in DNA methylation and epigenetic changes. Further iAs has been shown to inhibit DNA repair through an indirect mechanism which can lead to genomic instability if DNA lesions are not repaired before replication [27].



REFERENCES

- 1: Ma TH, Sandhu SS, Peng Y, Chen TD, Kim TW. Synergistic and antagonistic effects on genotoxicity of chemicals commonly found in hazardous waste sites. *Mutat Res.* 1992;270(1):71-7.
- 2: Scott CS, Chiu WA. Trichloroethylene cancer epidemiology: a consideration of select issues. *Environ Health Perspect.* 2006;114(9):1471-8.
- 3: Rossman TG, Uddin AN, Burns FJ, Bosland MC. Arsenite cocarcinogenesis: an animal model derived from genetic toxicology studies. *Environ Health Perspect.* 2002;110 Suppl 5:749-52.
- 4: Beck JA, Lloyd S, Hafezparast M, Lennon-Pierce M, Eppig JT, Festing MF, Fisher EM. Genealogies of mouse inbred strains. *Nat Genet.* 2000;24(1):23-5.
- 5: Harrill AH, Ross PK, Gatti DM, Threadgill DW, Rusyn I. Population-based discovery of toxicogenomics biomarkers for hepatotoxicity using a laboratory strain diversity panel. *Toxicol Sci.* 2009;110(1):235-43.
- 6: Darvasi A. Experimental strategies for the genetic dissection of complex traits in animal models. *Nat Genet.* 1998;18(1):19-24.
- 7: Liu J, Liu Y, Powell DA, Waalkes MP, Klaassen CD. Multidrug-resistance *mdr1a/1b* double knockout mice are more sensitive than wild type mice to acute arsenic toxicity, with higher arsenic accumulation in tissues. *Toxicology.* 2002;170(1-2):55-62.
- 8: Sekine M, Monkawa T, Morizane R, et al. Selective depletion of mouse kidney proximal straight tubule cells causes acute kidney injury.[published online ahead of print] *Transgenic Res.* 2011. Accessed March 7, 2011
- 9: Lock EA. Sensitive and early markers of renal injury: where are we and what is the way forward? *Toxicol Sci.* 2010 Jul;116(1):1-4.
- 10: Endre ZH, Pickering JW, Walker RJ, et al. Improved performance of urinary biomarkers of acute kidney injury in the critically ill by stratification for injury duration and baseline renal function.[published online ahead of print] *Kidney Int.* 2011. Accessed March 7, 2011.
- 11: Wagener G, Minhaz M, Mattis FA, Kim M, Emond JC, Lee HT. Urinary neutrophil gelatinase-associated lipocalin as a marker of acute kidney injury after orthotopic liver transplantation. [published online ahead of print] *Nephrol Dial Transplant.* 2011. Accessed March 7, 2011.

- 12: Paragas N, Qiu A, Zhang Q, et al. The Ngal reporter mouse detects the response of the kidney to injury in real time. *Nat Med*. 2011;17(2):216-22.
- 13: Hoffmann D, Fuchs TC, Henzler T, et al. Evaluation of a urinary kidney biomarker panel in rat models of acute and subchronic nephrotoxicity. *Toxicology*. 2010;277(1-3):49-58.
- 14: Chiu WA, Caldwell JC, Keshava N, Scott CS. Key scientific issues in the health risk assessment of trichloroethylene. *Environ Health Perspect*. 2006;114(9):1445-9.
- 15: Moore LE, Boffetta P, Karami S, et al. Occupational trichloroethylene exposure and renal carcinoma risk: evidence of genetic susceptibility by reductive metabolism gene variants. *Cancer Res*. 2010;70(16):6527-36.
- 16: Chung CJ, Pu YS, Su CT, Huang CY, Hsueh YM. Gene polymorphisms of glutathione S-transferase omega 1 and 2, urinary arsenic methylation profile and urothelial carcinoma. *Sci Total Environ*. 2011;409(3):465-70.
- 17: Rocha JL, Eisen EJ, Van Vleck LD, Pomp D. A large-sample QTL study in mice:II. Body composition. *Mamm Genome*. 2004;15(2):100-13.
- 18: van Beelen VA, Aarts JM, Reus A, et al. Differential induction of electrophile-responsive element-regulated genes by n-3 and n-6 polyunsaturated fatty acids. *FEBS Lett*. 2006;580(19):4587-90.
- 19: Thuesen BH, Husemoen LL, Ovesen L, Jørgensen T, Fenger M, Linneberg A. Lifestyle and genetic determinants of folate and vitamin B12 levels in a general adult population. *Br J Nutr*. 2010;103(8):1195-204.
- 20: Nordberg GF, Andersen O. Metal interactions in carcinogenesis: enhancement, inhibition. *Environ Health Perspect*. 1981;40:65-81.
- 21: Lock EA, Reed CJ. Trichloroethylene: mechanisms of renal toxicity and renal cancer and relevance to risk assessment. *Toxicol Sci*. 2006;91(2):313-31.
- 22: Jomova K, Jenisova Z, Feszterova M, Baros S, Liska J, Hudecova D, Rhodes CJ, Valko M. Arsenic: toxicity, oxidative stress and human disease. *J Appl Toxicol*. 2011;31(2):95-107.
- 23: Reichard JF, Puga A. Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. *Epigenomics*. 2010;2(1):87-104.
- 24: Wendler A, Wehling M. The translatability of animal models for clinical development: biomarkers and disease models. *Curr Opin Pharmacol*. 2010;10(5):601-6.

25: Stone R. Food safety. Arsenic and paddy rice: a neglected cancer risk?.*Science*. 2008;321(5886):184-5.

26: National Research Council (U.S.), National Research Council (U.S.). Committee on Human Health Risks of Trichloroethylene. Assessing the human health risks of trichloroethylene: key scientific issues ;National Academies Press, 2006.

27: Salnikow K, Zhitkovich A. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem Res Toxicol*. 2008;21(1):28-44.

Chapter Five:

DISCUSSION

A. Conclusions and perspectives

The amount and degree of useful data that can be obtained through experimental studies is limited by the available models. Carcinogen risk assessments attempt to predict cancer rates and risk for disease in humans using mathematical models that are often based upon limited experimental data in rodents. They do not adequately incorporate inter-individual variation, the possibility of co-exposures, or nutritional deficiencies in the assessment of carcinogenicity. This oversight in the evaluation of sensitive and resistant populations, limits the accuracy of current risk assessment models. Thus to establish experimental models that accurately capture the human condition, both in host susceptibility factors and environmental exposures, holds with it the promise to improve not only the data obtained, but our ability to use that data to make better risk predictions.

The risk assessment of trichloroethylene with regards to kidney cancer has been under debate for the past three decades. This is due, in part, to the lack of biologically-based models with which to examine TCE-induced renal toxicity [1]. In addition, the ban on household use of TCE and improvement in occupational standards, has limited our assessment in human population studies [2]. It was the goal of the work herein, to evaluate several mechanistic gaps identified in the risk assessment of TCE-induced renal carcinogenesis using novel genetic approaches to design a best-fit model for our questions.

1. Gene mutation signatures of environmental exposures

The mutation or inactivation of tumor suppressor genes is considered to be one of the main molecular events in the multistep process of carcinogenesis [3]. Environmental carcinogens can interact with DNA structure both directly and indirectly, with heritable change in DNA sequence constituting mutational effects [4]. With the development of improved molecular tools for the evaluation of genetic mutations, a push has been made to link specific chemical exposures to unique genetic mutation signatures found in tumors [5]. The study of mutational spectra of cancer-related tumor suppressor genes can give us clues about carcinogen-DNA interactions, functions of gene products and mechanisms of carcinogenesis in specific tissues [6].

The link between exposure and mutation is best exemplified by mutations found in the *TRP53* tumor suppressor gene. Several studies have demonstrated a significant correlation between *TRP53* mutational spectra and exposure to various types of carcinogens. Cigarette smoking has been associated with *TRP53* mutational hotspots are codons 157, 158, 248, 249, and 273 in lung tumors [7]. Similarly, liver tumors in populations co-exposed to aflatoxin B1 contaminated grain and hepatitis B virus, which are risk factors for hepatocellular carcinoma, have the *TRP53* AGG to AGT mutation in codon 249 [8, 9]. Further, it was shown that *in vitro* exposure to benzo(a)pyrene, a carcinogen found in tobacco smoke, generates strong and selective adduct formation at guanine positions in codons 157, 248, and 273 [10]. This type of mutation profiling has

been beneficial in cases where we have both human tumors and exposure data, which has been a limitation for trichloroethylene.

As of October 2010, 388 mutations in the *VHL* gene were identified in sporadic kidney cancers [11]. However, identifying specific exposure “signatures” has not been undertaken. This is likely due to several factors including (1) poor exposure histories taken at time of surgery, and (2) lack of experimental models to confirm disease phenotype. It was the goal of this study to molecularly evaluate the P81S mutation using a novel *in vitro* system to characterize how this missense mutation affects the known functions of pVHL and its downstream effector molecules. We have shown that this mutation represents a unique phenotype, not previously known to pVHL disease mutants [12]. Furthermore, the P81S mutation causes a two-fold hit on tumor suppressor activities of pVHL, first by the dysregulation of HIF signaling and secondly by the development of apoptosis avoidance. Such a mutation, if not the initiator of tumorigenesis, has the potential to cause unchecked proliferation, supporting tumor promotion [13]. This will certainly impact the risk assessment of TCE, due to the fact that mutated cells need not have access to a tumor promoter in order to stimulate proliferation.

Over a decade ago, Bruning *et al.* and Brauch *et al.* published three studies identifying the P81S *VHL* mutational hotspot in a German population with known high occupational exposure to TCE [14, 15, 16]. More recently, molecular evaluation of *VHL* gene in a patient presenting with bilateral RCC with known long-term exposure to TCE found a single cytidine deletion mutation which

resulted in a frameshift at codon 98 onwards [17]. In each case of exposure the resultant mutation identified was located at cytidine residues in exon 1. Interestingly, exon 1 contains a known CpG island [18], and further, a deamination of a methylated cytidine would result in a C > T transition or apurinic site during replication [19]. This suggests that CpG island regions of VHL may be more labile to carcinogen insult, an interesting hypothesis that will remain for future directions.

2. Exploiting genetic variation for toxicant susceptibility studies

Given the knowledge of human genetic variants and the role they play in determining toxicant susceptibility, there have been surprisingly few laboratory rodent models used in chemical risk assessment that interrogate genetic variation in a manner allowing identification of genes underlying susceptibility. Inbred mouse strains are the foundation of mammalian genetics research and continue to be a powerful tool in toxicological research [20]. Individuals of a given strain are both homozygous and isogenic, and with the sequencing of the mouse genome from many inbred strains, the naturally occurring genetic variation that drives their phenotypic differences can now be discovered at the gene level [20]. Phenotypic differences between mouse lines, such as their toxicity response and disease susceptibility, can be used to model human diseases such as cancer [21]. As such, in the past five years, the Laboratory Strain Diversity Panel (LSDP) has begun to be incorporated into the assessment of susceptible and resistant phenotypes in toxicology [22].

The mapping of complex traits, such as toxicant susceptibility, is challenging. An analysis of data from the Perlegen 15-strain resequencing project (<http://www.mouse.perlegen.com/mouse/download.html>) revealed that most commonly used laboratory mouse strains are largely derived from the *Mus musculus* domesticus subspecies, rather than being mosaics of the three main mouse subspecies (*M. m. domesticus*, *M. m. musculus*, and *M. m. castaneus*) [23]. As a consequence, the LSDP that has largely been limited to the classical strains has not been maximized for genetic diversity, which limits association studies [24]. The uncommonly used wild-derived strains can increase the amount of variation. A more recent resource that is coming to fruition is the Collaborative Cross (CC), which was designed to be a genetic reference mouse population that controls for the randomization of a large amount of genetic variation by the structured breeding of wild-derived strains with classical strains. The CC is predicted to contain more single nucleotide polymorphisms than the human population [25, 26, 27]. Once this resource becomes available it will undoubtedly have a profound impact on human health and disease research.

Single strain risk assessment studies are limited in the information they can provide, and as such, human disease risk may be underestimated for susceptible individuals. For the work presented here, the CC resource was not available, but we were able to use what is known about the relative contribution of wild-derived mouse strain genetics to maximize the efficacy of our mapping population of renal toxicity. To design our mouse population, we had to make predictions about toxicity mechanisms using those factors we hypothesized

would play a role in TCE and arsenic co-carcinogenesis. We incorporated a knockout mouse to mimic human excretion of inorganic arsenic, altered the dietary factors to increase tissue dose and susceptibility, and properly chose a wild-derived strain counterpart, CAST/EiJ, to maximize the genetic variation in our F₃ population. We were able to show for the first time in a mouse model, that renal toxicity due to environmental co-exposures to TCE is dependent on genetically-determined factors. Moreover, we now have the ability to identify the genetic factors underlying susceptibility, discover biomarkers that predict response, and to investigate the gene-nutrient interactions in the modification of toxicity. Such an approach to chemical risk assessments is likely to reveal data that is more useful for translational studies, as well as aid the refinement of animal use in toxicological studies, as several phenotypes may be observed in the same study facilitating collaborations.

3. Environmentally-relevant doses and diets in toxicological studies

It is becoming increasingly obvious to the toxicology community that a great knowledge gap exists in our understanding of how environmental toxicants behave and interact at very low exposures. The National Institute of Environmental Health Sciences (NIEHS) along with other health agencies are leading a paradigm shift to investigate how such low doses impact human health and disease [28]. By definition, to achieve an environmentally-relevant dose, the dose-equivalents in a rodent study should be based on the internal concentration of the toxicant or its metabolites as measured in urine or serum of humans [28]. Although this dose will most likely represent an actual range for a typical human

exposure, it remains limited by the availability of actual human measurements. Recent work on endocrine-disrupters such as Bisphenol A suggested that high doses may not be appropriate to predict the safety of low doses with hormonally active or modulating compounds [29]. This becomes especially important when considering mixtures, nutrient-gene interactions, and genetic variation in chemical metabolism and transport.

There is a paucity of publications discussing the use and results of environmentally-relevant doses for both inorganic arsenic and TCE. The challenge with using very low doses of trichloroethylene and inorganic arsenic has been the inherent species differences in metabolism and transport between humans and rodents, and the lack of methodology to quantify internal dosimetry [30]. We have recently shown that there is a strong strain-dependent difference in the metabolism of TCE [31], supporting the need for increased genetic variation in a mouse study population to mimic human populations. In the present study, a drug transporter knockout mouse was used to increase the sequestration of arsenic metabolites in the kidney [32, 33]. We were able to use a toxicogenomic approach to show that environmentally-relevant doses of inorganic arsenic produce a characteristic gene expression signature, even after only 10 weeks of exposure. We have also shown that environmentally-relevant dose mixtures of inorganic arsenic and TCE result in significant renal injury, and that laboratory diet composition likely plays a role in enhancing toxicity.

Dietary deficiency in micronutrients as well as an imbalance in dietary fatty acids can increase susceptibility to environmental toxicants [34]. This becomes

especially true when dietary co-factors are needed for metabolism and detoxification [35]. As such, nutritionally deficient populations represent a unique and vulnerable subset of the population and are likely to be at increased risk for adverse health outcomes [36]. In the U.S., obesity and poor dietary habits likely puts the majority of American adults and children into this population [37]. It becomes important when designing best-fit models for toxicological risk assessment studies, to not rely on existing data, but rather to tailor models that can best answer the question(s) at hand, taking under consideration: (1) Who is most at risk? (2) Is the dose relevant to human exposure? and (3) Are there any dietary factors that can be identified using epidemiological and animal data?

B. Challenges and limitations

1. Study limitations

Carcinogenesis studies require animal models that best describe the human condition, both in the underlying tumor suppressor gene mutations and the mechanism of cellular dysregulation which occurs at each step from initiation through tumor progression [3]. In the study of the P81S mutation, the lack of a rodent model of RCC hindered our complete assessment of the relevance of the mutation in the development of tumors [38]. Nonetheless, this limitation showed the need to elucidate the yet undescribed functions of pVHL in order to fully develop an appropriate model. While gene expression studies comparing the P81S mutant teratomas to those containing wild-type VHL protein suggest that mitochondrial biogenesis may be the mechanism of apoptotic avoidance, the

quantification of such an increase as well as further molecular studies are required to prove such a mechanism.

In Aim 2, we studied the dietary influence on kidney gene expression with environmentally-relevant doses of inorganic arsenic. While transcriptome analysis is useful in determining a dietary effect on tissue response, the gene signatures and pathway analysis itself provides little information for understanding the underlying mechanism of toxicity. Thus it is important to make a connection between differentially expressed genes and phenotypic markers of toxicity, to understand how sets of genes and their products work together in generating adverse health outcomes [39]. In addition, a lack of pharmacokinetic data and tissue metabolite quantification of inorganic arsenic on both dietary regimens prevents us from knowing if the diets altered the absorption of arsenic or modified its metabolism *in situ*, and thus, the reason for altered kidney response signatures.

In our animal model of co-carcinogenesis of TCE and arsenic, we only examined a snap-shot in time at sixteen weeks after exposure. While we were successfully able to observe a marker of kidney toxicity, it is not necessarily a marker of frank carcinoma. It is generally believed that the presence of nephrotoxicity is not directly linked to carcinogenesis, but only suggests target tissue susceptibility [40]. Furthermore, without integration of genomic data from each of the study animals, it is not possible to draw specific conclusions as to the genetic interactions underlying variation in response, whether due to a gene x

toxicant or a gene x nutrient x toxicant interactions. This data will be required for the determination of kidney injury susceptibility in our mouse population model.

2. Limitations in co-carcinogenesis studies

A few of the current limitations are centered around two main areas: (1) the need for reliable quantification data of exposures in humans, and (2) integration of susceptibility into our laboratory animal models [41].

2.1 Overcoming exposure-data deficits

Exposure data is required for determining and characterizing risk, as well as for guiding the selection of environmentally-relevant doses in animal studies [42, 43]. Human exposure data for TCE and inorganic arsenic thus far have mainly been limited by the sensitivity of the methodology for quantification. For inorganic arsenic, there is a clear understanding of which global populations have been exposed through water and food sources [44]. The challenge is that exposure estimates have relied heavily on toenail or urinary concentrations of total arsenic [45]. These measurements are not accurate assessments of internal dose, nor do they provide information required to estimate tissue dose. Recently, the use of atomic absorption methodologies have improved the sensitivity of such measurements, enabling quantification of methylated metabolites of arsenic, which can indicate inter-individual differences in arsenic metabolism [46]. In the U.S., the exposure to arsenic is likely to be far lower than endemic areas of the world which needs to be taken into consideration in estimating exposure to mixtures.

Historical studies of TCE-associated kidney cancer have relied primarily on questionnaire data and estimates of occupational exposure based on workplace variables that have been characterized as low, medium or highly exposed [14]. Unfortunately, these assessments often neglect personal information such as body mass index and other risk factors associated with renal cancer such as smoking [15]. This has made exposure-association studies challenging despite the number of archived tumor tissues available. Gene mutation signatures may not be a realistic goal for determination of exposure, especially if the exposure is in a mixture at very low levels. Instead, collection of biological fluids such as urine and serum at time of tumor harvest may provide insight into exposure in the future. While highly sensitive mass spectrometry methods to quantify chemicals in a single drop of blood are currently under evaluation and validation, the availability of these fluids in the future may aid in exposure assessment [47]. Thus, it remains that there is a great need to discover biomarkers of exposure that have a strong correlation to tissue dosimetry and disease outcome for prediction of human health risk.

2.2 Chemical mixture evaluations require population-level models

Environmental exposures are typically complex mixtures of compounds often seen at ambient levels. The chemicals in mixtures are often poorly characterized, the mixture ratios may vary by location, exposure data is uncertain and the toxicological data may be limited [48]. This makes the implementation of methodology a challenge for estimating health risks. Under the current mixture assessment guidelines, several different methods are discussed to estimate the

risk of “common mechanism” chemicals with different potencies in a combined exposure [49]. These are the hazard index (HI), toxicity equivalence factor (TEF), and combined margin of exposure (MOET) procedures, as well as the point of departure index (PODI) and cumulative risk index (CRI) methods [50]. At a minimum, each of these methods requires the availability of *in vivo* toxicology data. Therefore, the *in vivo* animal mixture study design and its ability to communicate possible risks through mechanistic approaches are at the foundation of these decisions. This emphasizes the importance of animal models that can capture the representative population in both biology and susceptibility, and further drives the need for rodent population-level models in toxicological studies.

C. Future directions

Kidney cancer rates continue to rise in the U.S. and while risk factors have been identified, they do not provide a clear mechanistic view of the development of the disease [51]. As highlighted in the work herein, the improvement of laboratory models that capture both human biology and exposure may greatly impact the type and usefulness of the data obtained. In the study of the exposure-associated *VHL* tumor suppressor gene mutation, it is clear that not all functions of pVHL and/or downstream effectors have been completely described to date, suggesting that studies should then be directed at continuing to fill this knowledge gap. As well, future work may examine the possible structural basis of susceptibility of *VHL* to carcinogens that act by altering epigenetic marks. Finally, there is a strong need for an animal model of RCC with which to examine

disease progression and etiology. Without such a model, many questions will go unanswered.

The potential of genetically defined inbred mouse-derived populations as a surrogate for studying genetic variation in the human population is only at its infancy in toxicological studies. It is anticipated that the results from the population-level study of TCE and arsenic co-carcinogenesis will identify genes responsible for susceptibility to kidney toxicity, as well as identify biomarkers associated with susceptibility for translation into human populations. Further integration of genomic sequencing data, biochemical and histopathological evaluation of kidney tissues, NMR analysis of urine and finally QTL mapping of toxicity phenotypes will all be required to accomplish this goal.

D. Summary

In summary, *in vitro* and *in vivo* models that best describe human biology are strongly needed in toxicological evaluations if the information gained will be of any use to risk assessment decisions. For many years, toxicologists have relied on a “single exposure, single strain” approach that has led to a lack of consideration of the influence of genetics, mixtures and nutrition on cumulative risk assessments. Our approach to designing a population-based mouse model has the combined benefit of not only evaluating response to treatment but also the potential to identify the genetics underlying susceptibility, biomarkers that predict response as well as an understanding of gene-nutrient interactions in the modification of toxicity. As such, this type of model has the potential to become a

new paradigm in the evaluation of chemical risk assessments by improving the clinical translation of animal study data.

REFERENCES

- 1: Brüning T, Bolt HM. Renal toxicity and carcinogenicity of trichloroethylene: key results, mechanisms, and controversies. *Crit Rev Toxicol*. 2000;30(3):253.
- 2: Lock EA, Reed CJ. Trichloroethylene: mechanisms of renal toxicity and renal cancer and relevance to risk assessment. *Toxicol Sci*. 2006;91(2):313-31.
- 3: Minamoto T, Mai M, Ronai Z. Environmental factors as regulators and effectors of multistep carcinogenesis. *Carcinogenesis*. 1999;20(4):519-27.
- 4: Irigaray P, Belpomme D. Basic properties and molecular mechanisms of exogenous chemical carcinogens. *Carcinogenesis*. 2010;31(2):135-48.
- 5: Parsons BL, Myers MB, Meng F, Wang Y, McKinzie PB. Oncomutations as biomarkers of cancer risk. *Environ Mol Mutagen*. 2010;51(8-9):836-50.
- 6: Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*. 1994;54(18):4855-78.
- 7: Hussain SP, Hollstein MH, Harris CC. p53 tumor suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology, and human risk assessment. *Ann N Y Acad Sci*. 2000;919:79-85.
- 8: Hernandez-Boussard TM, Hainaut P. A specific spectrum of p53 mutations in lung cancer from smokers: review of mutations compiled in the IARC p53 database. *Environ Health Perspect*. 1998;106(7):385-91.
- 9: Zhang YJ. Interactions of chemical carcinogens and genetic variation in hepatocellular carcinoma. *World J Hepatol*. 2010;2(3):94-102.
- 10: Tretyakova N, Guza R, Matter B. Endogenous cytosine methylation and the formation of carcinogen carcinogen-DNA adducts. *Nucleic Acids Symp Ser (Oxf)*. 2008;(52):49-50.
- 11: Timmer FC, Neeskens LJ, van de Hoogen FJ et al. Endolymphatic Sac Tumors: Clinical Outcome and Management in a Series of 9 Cases. *Otol Neurotol*. 2011.
- 12: Rathmell WK, Hickey MM, Bezman NA, Chmielecki CA, Carraway NC, Simon MC. In vitro and in vivo models analyzing von Hippel-Lindau disease-specific mutations. *Cancer Res*. 2004;64(23):8595-603.
- 13: Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature*. 2001;411(6835):342-8.

- 14: Brauch H, Weirich G, Hornauer MA, Störkel S, Wöhl T, Brüning T. Trichloroethylene exposure and specific somatic mutations in patients with renal cell carcinoma. *J Natl Cancer Inst.* 1999;91(10):854-61.
- 15: Brüning T, Weirich G, Hornauer MA, Höfler H, Brauch H. Renal cell carcinomas in trichloroethene (TRI) exposed persons are associated with somatic mutations in the von Hippel-Lindau (VHL) tumour suppressor gene. *Arch Toxicol.* 1997;71(5):332-5.
- 16: Wells GM, Schroth W, Brauch H, Ross EA. Bilateral renal-cell carcinoma associated with an acquired VHL mutation and long-term trichloroethylene exposure. *Clin Nephrol.* 2009;71(6):708-13.
- 17: Brauch H, Weirich G, Klein B, Rabstein S, Bolt HM, Brüning T. VHL mutations in renal cell cancer: does occupational exposure to trichloroethylene make a difference? *Toxicol Lett.* 2004;151(1):301-10.
- 18: Ma X, Yang K, Lindblad P, Egevad L, Hemminki K. VHL gene alterations in renal cell carcinoma patients: novel hotspot or founder mutations and linkage disequilibrium. *Oncogene.* 2001;20(38):5393-400.
- 19: Cooper DN, Mort M, Stenson PD, Ball EV, Chuzhanova NA. Methylation-mediated deamination of 5-methylcytosine appears to give rise to mutations causing human inherited disease in CpNpG trinucleotides, as well as in CpG dinucleotides. *Hum Genomics.* 2010;4(6):406-10.
- 20: Beck JA, Lloyd S, Hafezparast M, Lennon-Pierce M, Eppig JT, Festing MF, Fisher EM. Genealogies of mouse inbred strains. *Nat Genet.* 2000;24(1):23-5.
- 21: Liu P, Wang Y, Vikis H, Maciag A, Wang D, Lu Y, Liu Y, You M. Candidate lung tumor susceptibility genes identified through whole-genome association analyses in inbred mice. *Nat Genet.* 2006;38(8):888-95.
- 22: Harrill AH, Ross PK, Gatti DM, Threadgill DW, Rusyn I. Population-based discovery of toxicogenomics biomarkers for hepatotoxicity using a laboratory strain diversity panel. *Toxicol Sci.* 2009;110(1):235-43.
- 23: Yang H, Bell TA, Churchill GA, Pardo-Manuel de Villena F. On the subspecific origin of the laboratory mouse. *Nat Genet.* 2007;39(9):1100-7.
- 24: Roberts A, Pardo-Manuel de Villena F, Wang W, McMillan L, Threadgill DW. The polymorphism architecture of mouse genetic resources elucidated using genome wide resequencing data: implications for QTL discovery and systems genetics. *Mamm Genome.* 2007;18(6-7):473-81.

- 25: Threadgill DW, Hunter KW, Williams RW. Genetic dissection of complex and quantitative traits: from fantasy to reality via a community effort. *Mamm Genome*. 2002;13(4):175-8.
- 26: Churchill GA, Airey DC, Allayee H, Angel JM, et al.; Complex Trait Consortium. The Collaborative Cross, a community resource for the genetic analysis of complex traits. *Nat Genet*. 2004;36(11):1133-7.
- 27: Valdar W, Solberg LC, Gauguier D, Cookson WO, Rawlins JN, Mott R, Flint J. Genetic and environmental effects on complex traits in mice. *Genetics*. 2006;174(2):959-84.
- 28: Birnbaum LS. Applying research to public health questions: timing and the environmentally relevant dose. *Environ Health Perspect*. 2009;117(11):A478.
- 29: Myers JP, vom Saal FS, Akingbemi BT, Arizono K et al., Why public health agencies cannot depend on good laboratory practices as a criterion for selecting data: the case of bisphenol A. *Environ Health Perspect*. 2009;117(3):309-15.
- 30: Rhomberg LR. Dose-response analyses of the carcinogenic effects of trichloroethylene in experimental animals. *Environ Health Perspect*. 2000;108 Suppl 2:343-58.
- 31: Liu J, Liu Y, Powell DA, Waalkes MP, Klaassen CD. Multidrug-resistance *mdr1a/1b* double knockout mice are more sensitive than wild type mice to acute arsenic toxicity, with higher arsenic accumulation in tissues. *Toxicology*. 2002;170(1-2):55-62.
- 32: Bradford BU, Lock EF, Kosyk O et al. Interstrain differences in the liver effects of trichloroethylene in a multistrain panel of inbred mice. *Toxicol Sci*. 2011;120(1):206-17.
- 33: Yokohira M, Arnold LL, Pennington KL et al. Effect of Sodium Arsenite Dose Administered in the Drinking Water on the Urinary Bladder Epithelium of Female Arsenic (+3 oxidation state) Methyltransferase Knockout Mice.[available online ahead of print March 7 2011] *Toxicol Sci*. 2011. Accessed March 11, 2011.
- 34: Pilsner JR, Liu X, Ahsan H et al. Folate deficiency, hyperhomocysteinemia, low urinary creatinine, and hypomethylation of leukocyte DNA are risk factors for arsenic-induced skin lesions. *Environ Health Perspect*. 2009;117(2):254-60.
- 35: Xu Y, Wang H, Wang Y, Zheng Y, Sun G. Effects of folate on arsenic toxicity in Chang human hepatocytes: involvement of folate antioxidant properties. *Toxicol Lett*. 2010;195(1):44-50.

- 36: Dammann KW, Smith C. Factors affecting low-income women's food choices and the perceived impact of dietary intake and socioeconomic status on their health and weight. *J Nutr Educ Behav*. 2009;41(4):242-53.
- 37: Wilson TA, Adolph AL, Butte NF. Nutrient adequacy and diet quality in non-overweight and overweight Hispanic children of low socioeconomic status: the Viva la Familia Study. *J Am Diet Assoc*. 2009;109(6):1012-21.
- 38: Rankin EB, Tomaszewski JE, Haase VH. Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. *Cancer Res*. 2006;66(5):2576-83.
- 39: Luo W, Fan W, Xie H, Jing L, et al. Phenotypic anchoring of global gene expression profiles induced by N-hydroxy-4-acetylamino-biphenyl and benzo[a]pyrene diol epoxide reveals correlations between expression profiles and mechanism of toxicity. *Chem Res Toxicol*. 2005;18(4):619.
- 40: Chiu WA, Caldwell JC, Keshava N, Scott CS. Key scientific issues in the health risk assessment of trichloroethylene. *Environ Health Perspect*. 2006;114(9):1445-9.
- 41: Cohen SM, Ellwein LB. Relationship of DNA adducts derived from 2-acetylaminofluorene to cell proliferation and the induction of rodent liver and bladder tumors. *Toxicol Pathol*. 1995;23(2):136-42.
- 42: Seed J, Brown RP, Olin SS, Foran JA. Chemical mixtures: current risk assessment methodologies and future directions. *Regul Toxicol Pharmacol*. 1995;22(1):76-94.
- 43: Butterworth BE, Conolly RB, Morgan KT. A strategy for establishing mode of action of chemical carcinogens as a guide for approaches to risk assessments. *Cancer Lett*. 1995;93(1):129-46.
- 44: Khan NI, Owens G, Bruce D, Naidu R. Human arsenic exposure and risk assessment at the landscape level: a review. *Environ Geochem Health*. 2009;31 Suppl 1:143-66.
- 45: Karim MR, Salam KA, Hossain E et al. Interaction between chronic arsenic exposure via drinking water and plasma lactate dehydrogenase activity. *Sci Total Environ*. 2010;409(2):278-83.
- 46: Currier JM, Svoboda M, de Moraes DP, Matoušek T, Dědina J, Stýblo M. Direct Analysis of Methylated Trivalent Arsenicals in Mouse Liver by Hydride Generation-Cryotrapping-Atomic Absorption Spectrometry. [published online ahead of print Mar. 1 2011]. *Chem Res Toxicol*. 2011; Accessed March 11, 2011.

- 47: Kuklennyik Z, Ye X, Needham LL, Calafat AM. Automated solid-phase extraction approaches for large scale biomonitoring studies. *J Chromatogr Sci.* 2009;47(1):12.
- 48: U.S. EPA. Concepts, Methods, and Data Sources for Cumulative Health Risk Assessment of Multiple Chemicals, Exposures and Effects: A Resource Document (Final Report). U.S. Environmental Protection Agency, Washington, DC, EPA/600/R06/013F, 2007.
- 49: Stara, J., J. Patterson, K. Blackburn, R. Hertzberg, AND C. DeRosa. Health Risk Assessment of Chemical Mixtures. U.S. Environmental Protection Agency, Washington, D.C., EPA/600/D-89/028 (NTIS PB89222640).
- 50: Wilkinson CF, Christoph GR, Julien E, et al. Assessing the risks of exposures to multiple chemicals with a common mechanism of toxicity: how to cumulate? *Regul Toxicol Pharmacol.* 2000;31(1):30.
- 51: American Cancer Society. 2010 "Facts and Figures" <http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-026238.pdf>. Accessed March 5, 2011.