UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Intricacies of UGT Regulation:

Protein-Protein Interactions and Environmental Arsenic Exposure

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Chemistry

by

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Professor James Halpert
Professor Alexander Hoffmann
Professor William Trogler

2012

The Dissertation of Camille Maria Konopnicki is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012

DEDICATION

To my parents, Marek Konopnicki and Barbara Pawlowski-Konopnicki

and my family, past and present.

EPIGRAPH

Play is the highest form of research.

Albert Einstein

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LIST OF ABBREVIATIONS

ADRs	adverse drug reactions
AhR	aryl hydrocarbon receptor
ARE	antioxidant response element
ARNT	aryl hydrocarbon receptor nuclear translocator
As^{3+}	arsenite, inorganic trivalent arsenic
As^{5+}	arsenate, inorganic pentavalent arsenic
B[a]P	benzo[a]pyrene
CAR	constitutive androstane receptor
CAT	catalase
CN-I,II	Crigler-Najjar syndrome type I or II
COX-2	cyclooxygenase-2
CPT-11	irinotecan
CYP	cytochrome P450
DME	drug metabolizing enzyme
dsRNA	double-stranded RNA
ER	endoplasmic reticulum
ERK	extracellular signal-related kinase
G6PD	glucose-6-phosphate dehydrogenase
Gadd45β	growth arrest and DNA-damage-inducible gene 45β
GI	gastrointestinal tract
GPx	glutathione peroxidase
GR	glucocorticoid receptor

GSH-Re	glutathione reductase
GST	glutathione S-transferase
GSTA1,2	glutathione S-transferase A1 or 2
HCC	hepatocellular carcinoma
H&E	hematoxylin and eosin
HLM	human liver microsomes
HO-1	heme oxygenase-1
HPLC-MS/MS	high-performance liquid chromatography/tandem mass spectrometry
HRE	hormone response element
hUGT1	humanized *UGT1*
iAs	inorganic arsenic
IKK	IκB kinase
JNK	c-Jun NH2-terminal protein kinase
Keap1	Kelch-like ECH-associated protein 1
K_m	Michaelis-Menten constant; substrate concentration at half the maximum rate of the reaction
K_i	dissociation constant for an inhibitory enzyme-substrate complex
Ki-67	Ki-67 antigen
LPS	lipopolysaccharide
LXRα	liver X receptor α
MAPK	mitogen-activated protein kinase
MC	3-methylcholanthrene

mEH	microsomal epoxide hydrolase
mRNA	messenger RNA
NAC	*N*-acetylcysteine
NAPQI	*N*-acetyl-*p*-benzoquinone imine
NAT	N-acetyltransferase
NF-κB	Nuclear Factor κ B
Nrf2	NF-E2-related factor-2
NQO-1	NAD(P)H: quinone oxidoreductase 1
OPTI Only	OPTI-MEM only was used in cell treatment
PAH	polycyclic aromatic hydrocarbon
PAS	Periodic Acid Schiff
PB	phenobarbital
PBREM	phenobarbital response enhancer module
PCN	pregnenolone-16α-carbonitrile
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PPARα	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
RISC	RNA-Induced Silencing Complex
RNAi	RNA interference
ROS	reactive oxygen species
SI	small intestine
siRNA	small interfering RNA

SOD-1,2	superoxide dismutase-1 or -2
SULT	sulfotransferase
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TFF	trefoil family factor peptide
TLC	thin layer chromatography
Tg	transgenic
TR Only	the transfection reagent only was used in cell treatment
UDPGA	uridine diphosphate glucuronic acid
UGT	UDP-glucuronosyltransferase
V_{max}	the maximum rate of the reaction
XRE	xenobiotic response element

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I would first like to thank my thesis advisor, Dr. Robert Tukey for the opportunity to work in his lab and do research in a field that I have always been fascinated by. His excitement for science has inspired me and his ability to clearly identify a story that needs be told is a quality I admire and strive to achieve. I am also thankful for his patience and confidence in me. He offered me direction when I needed it, but also encouraged me to trust in my own knowledge and abilities, which helped nurture me as an independent researcher. He has always had my best interest at heart and I am grateful for the invaluable advice and guidance, especially near the end of my graduate career. In addition, thank you for the opportunity of being your teaching assistant for Pharmacology and Toxicology. This experience has not only helped to solidify my understanding of material relevant to my research, but has allowed me to excel as a teacher and that is something in which I take great pride.

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Chapter 2, in full, is a currently being prepared for submission in Archives of

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Konopnicki C.M., Dickmann L., Tracy J., Tukey R.H., Wienkers L.C., and Foti R.S. Evaluation of UGT Protein Interactions in Human Hepatocytes: Effect of siRNA Down Regulation of UGT1A9 and UGT2B7 on Propofol Glucuronidation in Human Hepatocytes.

ABSTRACTS AND PRESENTATIONS

1. "Using a P450 HRGS assay to test complex environmental toxicant mixtures along the U.S.-Mexico border" at the Superfund Basic Research Program Annual Meeting in Raleigh Durham, North Carolina, December 2007.

2. “Serum Bilirubin Levels in Humanized *UGT1A1*28* Mice Can Serve as a Marker for Toxicant Exposure” at the Superfund Basic Research Program Annual Meeting in Asilomar, California, December 2008.

3. Serum Bilirubin Levels in Humanized *UGT1A1*28* Mice Can Serve as a Marker for Toxicant Exposure” at UCSD-Pfizer Global Research and Development (DMPK Symposium), San Diego, La Jolla, California, September 2009.

4. “Arsenic Exposure Modulates UGT1A1 Expression in Humanized *UGT1* Mice” at the Superfund Basic Research Program Annual Meeting in New York City, New York, November 2009.

5. “Serum Bilirubin Levels in Humanized *UGT1A1*28* Mice Can Serve as a Marker for Heavy Metal Exposure” at the UCSD-Pfizer Global Research and Development (DMPK Symposium), San Diego, La Jolla, California, October 2010.

6. “Serum Bilirubin Levels in Humanized *UGT1A1*28* Mice Can Serve as a Marker for Heavy Metal Exposure” at the Superfund Basic Research Program Annual Meeting in Portland, Oregon, November 2010.

7. “The Regulatory Role of Oral Arsenic in Humanized *UGT1* Mice” at Pharmacology Research Discussions, San Diego, La Jolla, California, January 2011.

8. “siRNA Knockdown of UDP-Glucuronosyltransferases in Human Hepatocytes” at the Amgen Summer Intern Program Poster Session, Seattle, Washington, August 2011.

9. “siRNA Knockdown of UDP-Glucuronosyltransferases in Human Hepatocytes” at PKDM Department Meeting at Amgen, Seattle, Washington, August 2011.

10. “siRNA Knockdown of UDP-Glucuronosyltransferases in Human Hepatocytes” at the Superfund Basic Research Program Annual Meeting in Lexington, Kentucky, October 2011.

11. “Oral arsenic exposure induces *UGT1A1* expression in neonatal humanized *UDP-Glucuronosyltransferase-1* mice through changes in cellular morphology associated cytotoxicity” at the Experimental Biology: ASPET Meeting in San Diego, California, April 2012.

12. “Oral arsenic exposure induces *UGT1A1* expression in neonatal humanized *UDP-Glucuronosyltransferase-1* mice through changes in cellular morphology associated cytotoxicity” at the SCDMDG Meeting, San Diego, La Jolla, California, October 2012.

ABSTRACT OF THE DISSERTATION

The Intricacies of UGT Regulation:

Protein-Protein Interactions and Environmental Arsenic Exposure

by

Camille Maria Konopnicki

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Professor Robert H. Tukey, Chair

The human UDP-glucuronsyltransferases (UGTs) are responsible for the metabolism of many endogenous and exogenous compounds. They facilitate excretion by attaching glucuronic acid to a lipophilic parent compound, transforming it to a more water-soluble glucuronide that can be easily eliminated.

Experiments performed in recombinant systems have suggested that protein-protein interactions occur between the UGTs and may play a role in modulating activity. However, evidence of UGT interactions either *in vivo* or in more

physiologically relevant *in vitro* systems has yet to be demonstrated. UGT oligomerization and its ability to affect glucuronidation were examined by siRNA knockdown and activity studies. Selective down regulation of UGT1A9 or UGT2B7 resulted in significant decreases in their respective mRNA levels. As expected, metabolism of the UGT1A9 probe substrate propofol was abolished with UGT1A9 down regulation. UGT1A9 activity also decreased with UGT2B7 down regulation, implying potential interactions between two isoforms. This represents the first piece of evidence that UGT interactions occur in human hepatocytes and suggests that expression levels of UGT2B7 may directly impact glucuronidation of selective UGT1A9 substrates.

UGT1A1 is one of the most important UGTs because it is the primary UGT responsible for bilirubin metabolism. The *UGT1A1* gene is also regulated by almost all of the xenobiotic receptors. We have recently generated a humanized *UGT1* mouse model that exhibits elevated bilirubin levels during development. Since numerous toxicants induce *UGT1A1* through association with xenobiotic receptors, *UGT1A1* induction by environmental contaminants can alter *hUGT1* bilirubin levels, therefore serving as a sensor for toxicant exposure. We investigated association between prominent metal contaminant exposure and *UGT1A1* expression through fluctuations in bilirubin. Arsenic exposure reduced bilirubin in neonatal *hUGT1* mice and significantly induced intestinal *UGT1A1*. The prevalence of arsenic contamination throughout the world fueled investigation into the regulatory role of oral arsenic on *UGT1A1* expression. Q-PCR, Western Blot, and immunohistological analysis revealed a novel mechanism that implicates Nrf2, NF-κB and cellular proliferation as potential underlying regulators of arsenic-induced *UGT1A1* expression in *hUGT1* mice.

CHAPTER 1

Introduction to Drug Metabolism

Drug Metabolizing Enzymes from an Evolutionary Perspective

While drug metabolizing enzyme (DME) genes have existed on this planet for more than 2.5 billion years, their evolutionary purpose has not solely been for what we associate these enzymes with today – the clearance of drugs. DMEs are evolutionarily very old enzymes and it is understood that they first evolved to serve critical life functions in both prokaryotes and eukaryotes (Nebert and Dieter, 2000). These enzymes have evolved to cope with selective pressures such as adaptability, environmental impact, and diet. What we have come to know as protective processes associated with drug clearance are actually natural mechanisms to provide the body with nutrients and dispose of unwanted material. The theory that life evolved from a "primordial soup" of limited organic compounds provides some of the earliest evidence of selective pressure, requiring evolution of biosynthetic pathways in order to replace dwindling supplies (Alves *et al.*, 2002). The anaerobic conditions of the earth's environment have led researchers to believe that some of the earliest Cytochrome P450s (P450s) performed vital reductase and isomerase functions (Lechner, 1994). Later, at the advent of an oxygenated environment, mechanisms to detoxify oxygen and protect against oxidative stress became necessary for species survival (Sistonen *et al.*, 2009). The existence of numerous DMEs, even prior to the divergence of prokaryotes and eukaryotes, highlights their importance for many life sustaining processes, including membrane synthesis, calcium ion and electrolyte balance, cell division, development, and the metabolism of endogenous substrates (Nebert and Dieter, 2000).

Evolutionary divergence of DME genes in animals over the last 1,000 megaannum has been strongly influenced by necessary interactions between animals and plants at the emergence of terrestrial life (Coveney *et al.*, 2012). While plants required animals for their reproductive cycles, they also needed ways to protect themselves from being consumed. As a means of defense, plants evolved new genes and thus metabolites, such as phytoalexins (Bock, 2003), to make them less enticing or even toxic, which forced animals to evolve new DME genes to adapt to the constantly changing plants (Gonzalez and Nebert, 1990). Animal consumption of available plant life launched this co-evolutionary event known as "animal-plant warfare" and resulted in an explosion of animal DME gene duplication events, as a means to cope with new dietary constituents. The role of DMEs has more recently expanded to include metabolic bioactivation and detoxification of numerous environmental pollutants, carcinogens, and drugs that are now found in humans (Nebert, 1997). The development of defense mechanisms to detoxify plant metabolites by animal DMEs undoubtedly played a significant role in our current ability to detoxify such a vast array of substrates. As the pace of the chemical revolution has overtaken biological evolution, the prominent role of DMEs in the clearance of man-made substrates, including drugs, pesticides, carcinogens and other xenobiotics, has become apparent (Burchell and Coughtrie, 1989).

The Function of Phase I and Phase II Drug Metabolizing Enzymes

DMEs assist in the metabolism, detoxification, and elimination of many endogenous compounds, as well as chemical agents that are not dietary in origin – classified as "xenobiotics". Many of these compounds are lipophilic to enable diffusion through membranes to effector sites and processes to render them more water-soluble are needed to facilitate their removal from cells and the organism as a whole. The elimination of many lipophilic xenobiotic compounds can be divided into two distinct but closely linked systems: Phase I and Phase II metabolism (Williams, 1949). Phase I oxidative metabolism by cytochrome P450 enzymes is the primary method of drug metabolism. Phase I metabolism involves small molecule modifications such as oxidation, hydroxylation, peroxidation, and dealkylation that introduce functional groups to the substrate (Nebert, 2006; Guillemette, 2003). This functionalization step is often, but not always, required for subsequent conjugative Phase II metabolism to occur. Phase II enzymes utilize these reactive groups to covalently attach large, polar moieties, such as glucuronic acid, sulfate, and glutathione. The result is a more polar, conjugated metabolite that can easily be eliminated from the body (Williams, 1971; Omiecinski *et al.*, 2011). It is important to note that Phase I and Phase II metabolism can also result in active and/or toxic metabolites that must be further detoxified.

Characterization of the Glucuronidation Process

Endogenous and exogenous compounds are detoxified and eliminated from the body through concerted metabolism between Phase I and Phase II DMEs. Phase II metabolism is characterized by the conjugation of large, hydrophilic groups to reactive moieties, thus increasing the molecular weight and water solubility of the substrate. The UDP-glucuronosyltransferases (UGTs) are Phase II enzymes that catalyze the addition of the bulky sugar group, glucuronic acid, to a nucleophilic substrate, termed the "aglycone", utilizing UDP-glucuronic acid (UDPGA) as a co-substrate. This detoxification process is called glucuronidation and the resulting sugar conjugate is referred to as a glucuronide. Glucuronidation is the major pathway in Phase II metabolism and accounts for approximately 35% of drug conjugation, making the UGTs the most important Phase II enzymes for the detoxification of drugs (Guillemette, 2003).

The first compound characterized as a sugar conjugate was euxanthic acid. It was isolated in 1855, by German scientist W. Schmidt, who fed mango leaves to cows and isolated compounds from their urine (Dutton, 1966; Dutton, 1980). Throughout the 1870s, other sugar containing metabolites were isolated from urine in various drug metabolism studies (Conti and Bickel, 1977). In 1879, Schmiedeberg and Meyer were the first to isolate and characterize the sugar moiety, glucuronic acid, while studying camphor metabolism in dogs (Schmiedeberg and Meyer, 1879; Pryde and Williams, 1933). Lipschitz and Bueding demonstrated in 1939 that the major site of glucuronide production was the liver (Lipschitz and Bueding, 1939). However, very little else was known at the time about the mechanisms by which glucuronic acid was synthesized within the body or how it became incorporated into those compounds that contained it (Dutton and Storey, 1953). The year 1953 marked a milestone discovery for Dutton and Storey, who had discovered a thermostable cofactor in the liver that was required for phenol glucuronide formation in cell-free preparations (Dutton and Storey, 1954). Further characterization determined this compound to be UDPGA, the active cofactor required in the reaction to form glucuronide metabolites (Storey and Dutton, 1955) and that the transfer of glucuronic acid to a substrate occurred by the following general mechanism: UDP Glucuronic acid + R-OH → UDP + R-O-Glucuronic acid, where UDP-Glucuronic acid, R-OH, UDP and R-O-Glucuronic acid represent the factor, substrate, uridine diphosphate, and the glucuronide, respectively (Smith and Mills, 1954).

UDP-Glucuronsyltransferases

In 1957, it was confirmed that all UGT substrates possess a nucleophilic functional group (Axelrod *et al.*, 1957). The wide range of substrates capable of being glucuronidated led to deliberations over the existence of a single UGT enzyme with a promiscuous active site (Mulder, 1971) or a population of numerous transferases, each with its own specific substrate (Storey, 1965; Gram *et al.*, 1968; Temple *et al.*, 1968). Early purification of the UGTs was challenging, due to the relative instability of UGTs in high concentrations of detergent required for solubilization and their phospholipid dependence (Burchell and Coughtrie, 1989). Without a method to isolate these enzymes from animal tissues, it was impossible to further address the question of heterogeneity. In 1975, Del Villar *et al.* demonstrated successful UGT purification by DEAE-cellulose column chromatography and confirmed that morphine and p-nitrophenol were conjugated by separate enzymes. This was the first evidence demonstrating that glucuronsyltransferase activity was attributable to more than one enzyme (Del Villar *et al.*, 1975). Also, through the use of DEAE-cellulose column chromatography, Bock *et al.* purified and separated two rat liver UGTs that catalyzed either morphine or 1-napththol and morphine glucuronidation (Bock *et al.*, 1977). In 1977, Gorski and Kasper published a method for purifying UGTs to homogeneity that utilized affinity chromatography with UDP-hexanolamine Sepharose. The isolated enzymes had much higher specific activity (Gorski and Kasper, 1977) and several UGTs were subsequently purified by this method, including phenobarbital-inducible UGT (Burchell, 1978), oestrone-UGT and p-nitrophenol-UGT (Tukey *et al.*, 1978), and testosterone-UGT (Weatherill and Burchell, 1980). Additional findings from these purification experiments demonstrated that the catalytic activity of many membrane-bound enzymes was dependent upon the presence of phospholipids, which also validated earlier reports of UGT localization within the endoplasmic reticulum membrane. Tukey *et al.* demonstrated that partially purified, delipidated enzymes were essentially catalytically inactive, yet activities could be restored upon addition of phospholipids or phosphatidylcholine mixtures (Tukey and Tephly, 1980; Gorski and Kasper, 1977; Tukey *et al.*, 1978).

With a reliable purification method, the UGT field evolved as further characterization of purified isoforms became possible. Substrate specificity analysis revealed that the UGTs displayed distinct and overlapping substrate specificities, thus strengthening the proposal that the functional heterogeneity was due to heterogeneous population of UGTs (Falany and Tephly, 1983; Roy Chowdhury *et al.*, 1986). To determine homology between purified isoforms, physical characterization was assessed through peptide mapping and amino acid analysis. In 1986, Falany *et al.* observed that the 17-hydroxysteroid-UGT and 3-hydroxyandrogen-UGT displayed significant homology, despite their different substrate specificities and that the p-nitrophenol-UGT was less closely related to the two steroid UGTs as it displayed very different amino acid composition and peptide mapping (Falany *et al.*, 1986). These findings suggested the possibility that families of related UGTs existed and was later confirmed through comparison of nucleotide sequences of cloned cDNAs encoding for UGT isoforms (Jackson and Burchell, 1986; Mackenzie, 1986; Mackenzie, 1987; Harding *et al.*, 1987; Iyanagi *et al.*, 1986).

Advancements in homogenous UGT isolation eventually led to the production of antibodies raised against purified UGTs, which could be used to specifically identify and immunoprecipitate nascent UGT proteins translating from polysomes (Mackenzie *et al.*, 1984a). The first mouse UGT mRNAs were isolated utilizing this technique, in addition to several of the rat liver UGTs (Mackenzie *et al.*, 1984b; Jackson *et al.*, 1985). Comparison of mRNA sequences suggested the existence multiple UGT isoforms within at least two gene families, but most importantly, directly confirmed the heterogeneity of UGTs. This method led to a significant increase in isolation and characterization of UGTs from various species including rat, mouse, and human, ultimately resulting in the development of guidelines for standardized nomenclature based on evolutionary divergence (Burchell *et al.*, 1991; Mackenzie *et al.*, 1997).

The UGTs have been divided into two separate gene families, *UGT1* and *UGT2*, on the basis of their sequence homology (Burchell *et al.*, 1991; Mackenzie *et al.*, 1997). The *UGT2* family is composed of individual genes, each consisting of a promoter and 6 exons, clustered on chromosome 4 at 4q13-q21. This gene cluster encodes 7 functional UGT2B proteins, as well as 3 UGT2A proteins that have yet to be functionally characterized (Monaghan *et al.*, 1994; Beaulieu *et al.*, 1997; Chen *et al.*, 1993). The UGT2B enzymes specifically catalyze the glucuronidation of bile acids (Monaghan *et al.*, 1997), steroids (Belanger *et al.*, 1998; Hum *et al.*, 1999), and hormones (Jin *et al.*, 1997; Guillemette *et al.*, 2004; Thibaudeau *et al.*, 2006), but have also been identified in the elimination of some therapeutic agents (Yeh, 1975; Coffman *et al.*, 1997; Davies *et al.*, 2003).

The *UGT1* gene family is located on chromosome 2 at 2q37 (Harding *et al.*, 1990). The UGT1 enzymes are predominantly involved in the metabolism of exogenous compounds (Dutton, 1980) with the important exception of the endogenous compound, bilirubin. Bilirubin is the yellow breakdown product of hemoglobin and if left unbound, can potentially lead to severe hyperbilirubinemia that ultimately results in brain damage due to excessive accumulation of bilirubin in the brain (Crigler and Najjar, 1952a; Gourley, 1997). It had already been established in 1956 that conjugation to form the glucuronide was the only method of bilirubin clearance (Billing *et al.*, 1957; Schmid, 1956; Talafant, 1956). However, in 1991, Ritter *et al.* identified two UGTs that displayed activity for bilirubin, HUG-Brl and HUG-Br2 (Ritter *et al.*, 1991). Bosma *et al*. later amended these findings, having determined that solely one isoform, UGT1A1, was capable of efficiently conjugating bilirubin (Bosma *et al.*, 1994). Sequence data, from studies also performed by Ritter and his co-workers during their search for the gene coding for the bilirubin transferases, revealed that the HUG-Brl and HUG-Br2 cDNAs contained 3' ends identical to each other, as well as to the human phenol transferase cDNA, HLUG P1. These results coincidentally provided the first evidence of a novel gene locus encoding for the *UGT1* family. The organization and structure of the gene complex was determined to utilize a series of unique exon 1s with accompanying transcriptional start sites and differential splicing to commonly shared exons to generate six different mature mRNAs (Ritter *et al.*, 1992). The *UGT1* complex was later extended to 220 kb and determined to encode for a total of 13 isoforms: four psuedogenes and nine functional isoforms (Gong *et al.*, 2001). These nine UGTs are generated through the transcription process of exon sharing, where exon 1s, which appear to have evolved through gene duplication events (Mackenzie *et al.*, 2005), are spliced and joined to common exons 2 through 5 (Figure 1-1). The resulting UGT1A proteins therefore have a variable N-terminal domain of approximately 280 amino acids, and an identical, 245-amino acid, C-terminal domain (Wooster *et al.*, 1991). Although the UGT1A proteins are encoded by five exons and UGT2 proteins by six exons, the carboxyl termini are highly conserved between all UGTs. It has been established that the variable N-terminal region (exon 1 for UGT1A; exon 1-2 for UGT2) dictates isoform substrate specificity (Mackenzie, 1990), while the highly conserved C-terminal region (exons 2-5 for UGT1A; exons 3-6 for UGT2) contains the co-substrate binding site (Wooster *et al.*, 1993; Burchell and Coughtrie, 1989; Ritter *et al.*, 1992; Tephly and Burchell, 1990; Radominska-Pandya *et al.*, 1999).

The ability to further study individual isoforms from complex gene families, such as the *UGT1* family, was achieved through the application of modern molecular biological techniques. Cloning of available UGT cDNAs encoding a single enzyme and subsequent transient or stable transfection into cell types with low levels of target enzymes generated *in vitro* models heterologously expressing individual enzymes that facilitated the characterization of individual UGT1A gene products (Mackenzie, 1986; Remmel and Burchell, 1993; Wooster *et al.*, 1993; Strassburg *et al.*, 1996; Strassburg *et al.*, 1998; Strassburg *et al.*, 1999a; Remmel *et al.*, 2009). Recombinant protein harvested from cells could be incubated with specific substrates in the presence of radiolabeled UDPGA to assess UGT enzymatic activity via thin layer chromatography (Nguyen and Tukey, 1997). Characterization through cDNA expression experiments led to the identification of more than 350 individual compounds that serve as substrates for the UGT superfamily (Tukey and Strassburg, 2000). This large group of structurally divergent compounds covers many different chemical classes, including alcohols, flavones, coumarins, carboxylic acids, amines, opioids, and steroids (Tukey and Strassburg, 2001). Additionally, many daily dietary constituents and pharmaceutical drugs contain the same reactive groups associated with these classes of agents, making them ideal substrates for glucuronidation.

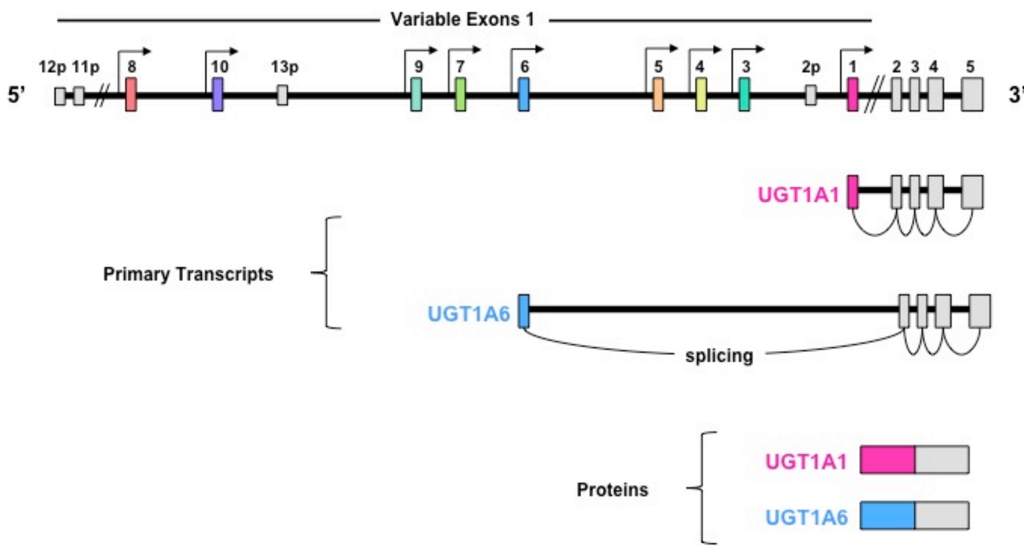

Figure 1-1. **Organization of the human *UGT1* locus.** The *UGT1* locus is located on chromosome 2 and spans 220 kb. The locus contains 13 unique exon 1 cassettes that can be spliced and joined to common exons 2-5. The nine functional UGT1A transcripts are generated through this transcription process of exon sharing. The 3' splice site of exon 1 is directly spliced to the 5' splice site of conserved exon 2. Due to absence of splices sites on their 5' ends, all intervening exons are considered intronic and spliced out. The presence of unique TATA-like elements approximately 30 bp upstream of each functional exon 1 allows for individual transcriptional regulation.

Tissue-Specific Expression of the UGTs

Human glucuronidation studies have primarily focused on hepatic tissue, because of the greater availability of the tissue source and the well-understood role of the liver in drug metabolism. However, the diverse nature of substrate specificity displayed by the UGT1 enzymes suggests that the existence of their complex gene regulation is most likely designed to account for the variable and specific glucuronidation requirements in various organs (Strassburg *et al.*, 1997b). Several studies have documented UGT activity toward bile acids, phenols, and bilirubin in human intestinal (Matern *et al.*, 1984; Pacifici *et al.*, 1986; Parquet *et al.*, 1985; Peters *et al.*, 1989; Peters *et al.*, 1991; McDonnell *et al.*, 1996) and renal tissues (Parquet *et al.*, 1985; Peters *et al.*, 1989; Pacifici *et al.*, 1988; Peters *et al.*, 1987; Peters and Jansen, 1988). It is now clear that human *UGT1* gene expression is regulated in a strict tissue-specific manner, resulting in varying levels and complementations of UGT1A proteins in each tissue (Strassburg *et al.*, 1997b; Tukey and Strassburg, 2000; Dutton, 1978). The use of RT-PCR, a method by which it was possible to distinguish single base pair differences between the highly homologous UGT sequences, advanced the study of tissue-specific expression patterns and provided a way to semi-quantify relative mRNA abundance of UGT transcripts within tissues (Strassburg *et al.*, 1997a; Strassburg *et al.*, 1997b). Expression of 17 UGT enzymes has been observed in humans. These include the nine functional UGT1A isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10), seven UGT2Bs (UGT2B4, UGT2B7, UGT2B11, UGT2B15, UGT2B17, UGT2B28), and one UGT2A enzyme (UGT2A1) (Tukey and Strassburg, 2000). Isoforms found to be expressed in liver are UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9 (Strassburg *et al.*, 1997a). UGT1A7, UGT1A8, and UGT1A10, which are notably absent in liver, are exclusively expressed extrahepatically (Strassburg *et al.*, 1997b; Strassburg *et al.*, 1998). UGT1A8 and UGT1A10 have been identified throughout the gastrointestinal (GI) tract, predominantly in the small intestine and colon, whereas UGT1A7 expression is limited to the upper GI tract, including the esophagus and stomach (Vogel *et al.*, 2002; Zheng *et al.*, 2002; Strassburg *et al.*, 1999b). Examination of non-GI extrahepatic tissues revealed that UGT1A7 was also expressed in pancreas (Ockenga *et al.*, 2003), that UGT1A9 was expressed in kidney

(McGurk *et al.*, 1998), and UGT1A6 in rat (Suleman *et al.*, 1998) and human (King *et al.*, 1999) brain. The colon exhibits an abundance of UGT1As, with UGT1A7 being the only isoform not detected (Strassburg *et al.*, 1998). *UGT2* gene expression also occurs in a tissue-specific manner. With the exception of UGT2B17, hepatic expression of the other six isoforms has been identified in human liver (Jin *et al.*, 1993; Beaulieu *et al.*, 1998; Strassburg *et al.*, 1999b; Green *et al.*, 1994; Jackson *et al.*, 1987; Chen *et al.*, 1993). UGT2B10, UGT2B11, UGT2B15, and UGT2B17 expression has been observed in steroid-sensitive tissues, including the prostate and mammary glands, as well as throughout the GI tract (Tukey and Strassburg, 2000; Beaulieu *et al.*, 1996). UGT2B11 exhibits the most extensive expression, having been identified in liver, kidney, breast, prostate, skin, adipose tissue, adrenal tissue, and lung (Beaulieu *et al.*, 1998). UGT2A1 expression is particularly tightly regulated and has been distinctly restricted to olfactory tissue. It is believed that UGT2A1 plays a role in human odorant sensing and also serves as a first line of metabolic defense for airborne toxic compounds entering the body through the nasal passage (Jedlitschky *et al.*, 1999). It is not surprising that strict tissue-specific regulation of DMEs ensures proper clearance of xenobiotics as tissues become exposed to them (Tukey and Strassburg, 2001; Strassburg *et al.*, 1999a).

Individual UGT Variability, Disease, and Adverse Drug Reactions

Individual variability in DMEs, due to environment, lifestyle and genetic influences, plays a significant role in the xenobiotic response (Burchell *et al.*, 2000). Disruption of natural metabolic processes most commonly occurs through environmental exposures, which can cause xenobiotic-, toxicant-, or dietary-initiated enzyme induction or inhibition. Enhancement or reduction in gene expression of important enzymes can ultimately lead to altered biotransformation, causing imbalances in endogenous compounds or adverse drug reactions (ADRs). In 1999, Ritter *et al.* studied UGT1A1 variation in human donor livers and their corresponding primary hepatocyte cultures. The three donors with the highest UGT1A1 levels had a history of phenytoin exposure. Phenytoin is an anticonvulsant and known UGT inducer and these findings were consistent with clinical evidence demonstrating the effectiveness of PB in inducing bilirubin clearance in patients with hyperbilirubinemia. Prototypical inducing agents, PB, phenytoin, and oltipraz also elevated UGT1A1 mRNA in isolated primary hepatocytes. However, the most significant induction was seen with 3-methylcholanthrene (MC), illustrating that exposure to polycyclic aromatic hydrocarbons (PAHs), which are potent atmospheric pollutants found in cigarette smoke, can effect UGT1A1 variation (Ritter *et al.*, 1999; Wells *et al.*, 2004).

In many cases, a decrease in expression of a specific UGT results in a compound being diverted to an unfavorable pathway that will convert it to a reactive metabolite capable of covalently binding DNA and proteins. This is known to occur with the over-the-counter pain reliever, acetaminophen (Tylenol). Glucuronidation is the major route of acetaminophen clearance and therefore reduced UGT activity leads to its accumulation. Inability to clear acetaminophen by its primary pathway diverts elimination through one of the minor elimination routes, oxidation by the CYPs, resulting in the formation of the toxic intermediate, *N*-acetyl-*p*-benzoquinone imine

(NAPQI). At normal acetaminophen doses, NAPQI can be easily detoxified by glutathione; however, glutathione becomes severely depleted at toxic levels. This leaves NAPQI free to bind protein thiols, which can affect protein activity, ultimately resulting in acute liver failure and severe renal damage (Hinson *et al.*, 1980; Uetrecht, 2010). Studies performed by de Morais *et al.* have shown that Gunn rats, which are inherently deficient in UGT1A proteins, are more susceptible to acetaminophen toxicity in comparison to normal Wistar controls, thus supporting adverse biological outcomes in the absence of UGT1A enzymes (de Morais *et al.*, 1992).

UGT1A1 plays a particularly important role in the glucuronidation of a wide array of compounds, the most notable being bilirubin. Bilirubin is an endogenous byproduct of heme derived from hemoglobin, cytochromes, catalase, peroxidase, other hemoproteins, and a small pool of free heme (Tenhunen *et al.*, 1969). It is known for its inherent antioxidant properties at low concentrations and has even been proven to decrease cardiovascular disease risk in patients with low UGT1A1 levels (Melton *et al.*, 2011). In the blood circulation, bilirubin is bound to albumin in order to prevent toxicity caused by the unbound form. Despite high-affinity binding to albumin, bilirubin is rapidly and selectively taken up from the blood circulation into the liver (Cui *et al.*, 2001), where it is conjugated by UGT1A1 to form mono- and di-glucuronides. Defects in clearance of bilirubin lead to accumulation of unsafe levels of unbound bilirubin ($\geq$ 20 mg/dL) and are associated with serious toxicities, including CNS toxicity, brain damage (kernicterus), and even death (Fujiwara *et al.*, 2010b; Ritter *et al.*, 1999). Therefore, control of bilirubin levels in the body is absolutely critical.

There are three forms of inheritable unconjugated hyperbilirubinemia diseases that occur in humans: Crigler-Najjar syndrome type I (CN-I), Crigler-Najjar syndrome type II (CN-II), and Gilbert's syndrome (Kadakol *et al.*, 2000). CN-I was first described by Crigler and Najjar in 1952 and is characterized by potentially lethal hyperbilirubinemia due to severely high serum bilirubin levels that range from 20–50 mg/dL (Crigler and Najjar, 1952a; Crigler and Najjar, 1952b). The severity of CN-I was later determined to result from the complete absence of UGT1A1 protein, and therefore activity (Seppen *et al.*, 1994). While phototherapy treatment has extended life expectancy, it eventually becomes less effective. Liver transplantation is currently the only restorative method available for patients with CN-I (Gourley, 1997). CN-II is characterized by intermediate hyperbilirubinemia (~7-20 mg/dL) due to decreased UGT1A1 activity (Arias, 1962; Seppen *et al.*, 1994). Induction therapy with PB to induce residual enzyme activity has been used to treat CN-II patients (Arias *et al.*, 1969). CN-I and CN-II syndromes both result from mutations in any of the five exons of the *UGT1A1* gene. Mutations that cause a premature stop codon or shift the reading frame ultimately alter or delete a large number of amino acid residues and always result in abolished UGT1A1 activity as seen in CN-I (Jansen *et al.*, 1995; Kadakol *et al.*, 2000; Bosma *et al.*, 1993; Ciotti *et al.*, 1998). In contrast, Gilbert's syndrome is a relatively harmless, commonly inherited condition characterized by mild hyperbilirubinemia (Kadakol *et al.*, 2000) and 3 to 10 percent of the general population is predicted to have it (Owens and Evans, 1975; Bailey *et al.*, 1977; Sieg *et al.*, 1987). The observed reduced UGT1A1 activity is due to an extra TA insertion in the TATA box of the *UGT1A1* promoter (A(TA)6TAA is normal), which results in decreased promoter activity. Bosma *et al.* documented a 70% decrease in UGT1A1 transcription in Gilbert's syndrome patients (Bosma *et al.*, 1995).

Characterization of *UGT1* polymorphisms has led to the identification of certain *UGT* gene variants as risk factors for cancer (Guillemette *et al.*, 2000a). Inherited variations in genes involved in the metabolism of estrogens have been implicated in the increased risk of breast cancer. Estrogens are essential for development of the reproductive system in women. However, estrogen exposure for lengthy periods of time may cause breast cancer since prolonged proliferation and genetic instability in estrogen target tissues has been thought to increase the likelihood of normal cells transforming into a malignant type (Guillemette *et al.*, 2004). UGT1A1 glucuronidation directly inactivates estrogens to facilitate their removal from estrogen-sensitive tissues. Investigation of the association between genetic variability in the UGT1A1 promoter region and estrogen-related cancer risk revealed that reduced levels of UGT1A1 increased breast cancer susceptibility (Guillemette *et al.*, 2000a). Many cancer-causing environmental contaminants, such as PAHs found in cigarette smoke are detoxified by glucuronidation (Bock *et al.*, 1999). Since UGT efficiency is critical for toxicity protection, polymorphic variation in the UGTs has the potential to alter sensitivity to PAHs present in diet and the environment (Hu and Wells, 1994). Enhanced DNA adduct formation has been observed in UGT1A deficient Gunn rats exposed to the PAH, benzo[a]pyrene (B[a]P) (Hu and Wells, 1992). UGT1A7 is an important extrahepatic UGT expressed in orolaryngeal tissues and lung. These tissues are in direct contact with cigarette smoke and therefore it is not surprising that UGT1A7 is responsible for the detoxification of several tobacco carcinogens

(Guillemette *et al.*, 2000b). When Zheng *et al*. examined the potential role of UGT1A7 genotype in orolaryngeal cancer risk, it was observed that *UGT1A7* allelic variants resulting in low enzyme activity were associated with increased risk of head and neck cancer (Zheng *et al.*, 2001). UGT1A7 has also been implicated as a cancer risk gene for liver and colon cancer (Vogel *et al.*, 2001; Strassburg *et al.*, 2002).

While UGT1 deficiencies have been studied with respect to disease susceptibility, a more immediate impact has recently emerged concerning treatment with drugs that have narrow therapeutic indices as well as more commonly used drugs with the potential for unwanted side effects. It is believed that polymorphisms within genes encoding for the UGTs can significantly affect drug response; therefore, patients with glucuronidation deficiencies are at a potentially higher risk for drug toxicities even when given normal therapeutic doses (Radu and Atsmon, 2001). The anticancer drug, irinotecan (CPT-11) is a classic example of pharmacogenetic influence on drug disposition and response. CPT-11 is hydrolyzed to its active metabolite SN-38, which is mainly eliminated through glucuronidation by UGT1A1. Individuals with Gilbert's syndrome that exhibit decreased enzyme activity have been determined to be at greater risk of irinotecan-induced toxicity (Wasserman *et al.*, 1997). Gilbert's syndrome patients are also susceptible to adverse drug reactions (ADRs) with the protease inhibitor atazanavir (Lankisch *et al.*, 2006) as well as a number of common therapeutic drugs, including acetaminophen (Douglas *et al.*, 1978) and ibuprofen (Radu and Atsmon, 2001). These findings support the effect of *UGT* genetic variation on individual sensitivities toward various therapeutic agents (Wells *et al.*, 2004). The future of pharmacy is geared toward understanding inherited differences in drug disposition or drug effects in hopes of improving drug safety and establishing personalized pharmacotherapy, which would involve optimized drug therapy based on a patient's unique genetic makeup (Strassburg, 2008).

Protein-Protein Interactions Between DMEs

Drug oxidation and conjugation by CYPs and UGTs, historically, have been considered to occur separately. However, recent studies have suggested that protein-protein interactions occur between DMEs and can even facilitate enzyme efficiency and function. Classic drug metabolism is typically understood as steps in which a polar moiety is introduced into a compound so that it will be more suitable for excretion. Although CYP metabolism generally leads to less active and more polar metabolites, bioactivation of pro-drugs and pro-carcinogens can result in pharmacologically or toxicologically active substances, respectively (Vandenbrink *et al.*, 2012; Ishii *et al.*, 2010). The UGTs and other Phase II enzymes, such as N-acetyltransferases (NATs), sulfotransferases (SULTs), and glutathione S-transferases (GSTs), play an important role in detoxifying these potent carcinogenic metabolites (Tukey and Strassburg, 2000; Operaña and Tukey, 2007; Ishii *et al.*, 2005). It would be reasonable to expect that a reactive metabolite produced by the CYPs could be directly transferred to other enzymes participating in its metabolism via protein-protein interactions, as physical interactions between DMEs would allow for the most efficient, concerted metabolism and aid in minimizing toxicity (Takeda *et al.*, 2005; Srivastava and Bernhard, 1987).

Increasing evidence of DME interactions has served to further validate that enzymes act in a cooperative manner to metabolize xenobiotics rapidly and efficiently (Taura *et al.*, 2000). In 2000, Taura *et al.* used affinity chromatography to demonstrate that CYP1A1 is associated with microsomal epoxide hydrolase (mEH), UGTs, and NADPH cytochrome P450-reductase (Taura *et al.*, 2000). Following in 2005, Fremont *et al.* investigated the immunoprecipitation of several human UGT isoforms and CYP3A4 in human liver microsomes by P450-immobilized affinity chromatography. These studies demonstrated successful co-immunoprecipitation of CYP3A4 with UGT2B7, UGT1A1, and UGT1A6 (Fremont *et al.*, 2005). With ample research suggesting CYP-UGT associations, it was important to address their potential functional relevance. Co-expression of CYP3A4 and UGT2B7 in COS cells was found to greatly increase UGT2B7-catalyzed glucuronidation of morphine, providing some of the earliest evidence of these postulated cooperative interactions in modulating enzyme function (Takeda *et al.*, 2005). However, another study found there to be no effect on rat UGT1A6 activity when it was simultaneously expressed with rat CYP1A1, suggesting that CYP-UGT interactions might actually be isoform specific (Ikushiro *et al.*, 2004; Takeda *et al.*, 2005; Ishii *et al.*, 2005). Characterization of isoform specificity of CYP-UGT interactions was tested via co-immunoprecipitation experiments, where CYP isoform-specific antibodies were used as probes to co-precipitate UGTs from solubilized rat liver microsomes. The data obtained indicated that CYP3A2, CYP2B2, CYP2C11/13, and CYP1A2 all co-precipitated with UGTs. However, there were large differences in the levels of UGTs that co-immunoprecipitated with each isoform (Ishii *et al.*, 2007). These overall findings support the hypothesis of UGT-CYP interactions facilitating multistep metabolism. Interestingly, the interactions of DMEs may be one of the major contributing factors responsible for inter-individual differences in drug sensitivity that cannot be explained by genetic variation (Ishii *et al.*, 2005).

UGT-UGT Interactions

Several studies have proposed that the UGTs themselves dimerize within the endoplasmic reticulum (ER). It was demonstrated at the beginning of the 1980s that rat liver UGTs tended to form aggregates and most likely existed as units larger than monomers (Matsui and Nagai, 1986; Matern *et al.*, 1982; Ikushiro *et al.*, 1997). Radiation inactivation analysis of the UGTs, a method by which to determine the molecular masses of membrane-bound enzymes *in situ*, later revealed significant molecular-weight differences among enzymes that were mathematical multiples of each other, suggesting that the UGTs were composed of one to four subunits of similar molecular weights (Peters *et al.*, 1984). Subsequent utilization of this method by Gschaidmeier and Bock indicated that UGTs are functional as dimers in monoglucuronide formation and as tetramers in diglucuronide formation (Gschaidmeier and Bock, 1994). Interactions between the UGT1s and UGT2B1 in rat microsomes were studied by immunopurification procedures with anti-peptide antibodies and chemical cross-linking experiments and revealed direct interactions between the UGT1A subfamily of enzymes and UGT2B1 (Ikushiro *et al.*, 1997). In 1997, Meech and Mackenzie performed mutation, co-expression, and SDS-PAGE analyses to propose that UGTs formed catalytically active dimers via their amino-terminal domains (Meech and Mackenzie, 1997). Knowing that the highly variable, substrate-binding N-terminus has been implicated in dimerization, it is not surprising that disrupting interactions within this region could alter K_m values and substrate binding specificity (Bock and Kohle, 2009). In 2007, Operaña *et al.* utilized FRET and co-immunoprecipitation experiments to investigate intermolecular interactions between UGT1A proteins in COS cells. Homo-dimerization was observed between all the UGT1As. These studies also revealed the promiscuous nature of UGT1A1 to heterodimerize with UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10. Additionally, co-expression of UGT1A1 with UGT1A7 increased 2-napthol glucuronidation in comparison to both single expression systems, implicating the potential role of oligomerization on activity (Operaña *et al.*, 2007). Studies by Fujiwara *et al.* in 2010 of protein-protein interactions between human UGT2B7 and UGT1As via double expression experiments in HEK293 cells revealed that UGT2B7 formed homo-oligomers, as well as hetero-oligomers, with UGT1A1, UGT1A4, UGT1A6, and UGT1A9. Kinetic analysis revealed co-expression of UGT1A enzymes altered UGT2B7-catalyzed zidovudine O-glucuronidation. Co-expression of UGT2B7 also modified the kinetics of estradiol 3-O-glucuruonidation by UGT1A1, imipramine N-glucuronidation by UGT1A4, serotonin O-glucuronidation by UGT1A6, and propofol O-glucuronidation by UGT1A9, clearly demonstrating the effect of interactions between human UGT2B7 and UGT1A on activity (Fujiwara *et al.*, 2010a). Overall, these findings support *in vitro* UGT-UGT interactions and their capacity to modulate activity. However, concrete evidence of interactions either *in vivo* or in more physiologically relevant *in vitro* systems has yet to be demonstrated. The ability to study UGT protein interactions in physiologically relevant, *in vitro* systems will help to further address their functional relevance as well as aid in identifying potential disconnects between UGT enzymology in single enzyme versus whole cell systems.

RNA Interference as a Tool to Study Glucuronidation

Research over the last decade has led to exciting new discoveries on the role of double-stranded RNA (dsRNA) in the cell. The diverse effects of dsRNA on gene expression are now known to include orchestrating epigenetic changes, repressing translation, and directing mRNA degradation in a sequence-specific manner. However, in the 1990s, injection of large amounts of antisense single-stranded RNA (ssRNA) had been the traditional approach by which to study gene function in nematodes (Rao and Sockanathan, 2005). Gene silencing, termed RNA interference (RNAi), was achieved through ssRNA injection, in the hope that it would pair with its complementary mRNA, block translation, and lead to an effective loss-of-function (Nellen and Lichtenstein, 1993). Experiments by Guo and Kemphues to assess *par-1*'s role in establishment of anterior-posterior polarity of *Caenorhabditis elegans (C. elegans)* embryo revealed that both sense and antisense RNA preparations were capable of causing interference (Guo and Kemphues, 1995). Additional work has also shown that, unlike cellular mRNAs, which tend to have relatively short half-lives, ssRNAs exhibit prolonged silencing effects and can even be inherited from one generation to the next (Seydoux and Fire, 1994). Despite the fact that the intrinsic differences between endogenous RNA and the interference-inducing substrate remained largely unexplained, antisense-mediated silencing still continued to be a widely used technique. Subsequently, in 1998 and at the time of the completion of the *C. elegans* genome project, Fire and Mello made an innovative discovery. In an attempt to address the observed discrepancies in previous silencing studies, they unfolded a new technology based on the silencing of specific genes by dsRNA. Fire *et al.* had previously noted that ssRNA samples prepared with bacteriophage RNA polymerases were often contaminated with ectopic transcripts. They hypothesized that the presence of dsRNAs could be the reason why both sense and antisense RNAs were capable of inducing silencing. To test their hypothesis, *C. elegans* were injected with either single- or double-stranded RNA targeting the *unc-22* gene. Over a 100-fold greater silencing effect was seen with dsRNA than with either strand individually. Furthermore, it was observed that the silencing effect could cross into the adult's gonads and be transferred to the worm's progeny after initial injection in its head, thus implicating an active transport mechanism necessary for achieving long-distance effects (Rao and Sockanathan, 2005). These results fueled further research, as RNAi became the standard means by which to investigate gene function in *C. elegans*. In 1999, studies of the interference process in *C. elegans* mutants resistant to dsRNA-mediate interference helped identify the genes required in RNAi (Tabara *et al.*, 1999). Earlier reports had observed gene silencing mediated by unknown substrates in several diverse organisms, including insects, plants, and fungi, but sequence comparison with the recently identified *C. elegans*' dsRNA-mediated silencing genes revealed similar machinery, confirming dsRNA-mediated silencing in other organisms (Hamilton and Baulcombe, 1999; Fagard *et al.*, 2000). RNAi has since been discovered in a wide variety of species, including fruit flies (Kennerdell and Carthew, 1998; Misquitta and

Paterson, 1999), trypanosomes (Ngo *et al.*, 1998), planaria (Sanchez Alvarado and Newmark, 1999), hydra (Lohmann *et al.*, 1999), zebrafish (Wargelius *et al.*, 1999), and mice (Wianny and Zernicka-Goetz, 2000) and appears to be related to the gene silencing phenomena observed in plants (Vaucheret *et al.*, 1998; Waterhouse *et al.*, 1998; Waterhouse *et al.*, 1999; Baulcombe, 1999) and fungus (Cogoni *et al.*, 1996; Cogoni and Macino, 1999a; Cogoni and Macino, 1999b; Zamore *et al.*, 2000). Early application of dsRNAs of varying sizes (38-1,662 bp) to commonly used mammalian cell culture systems, including HEK293, NIH/3T3 (mouse fibroblast), BHK-21 (Syrian baby hamster kidney), and CHO-K1 (Chinese hamster ovary) cells had surprisingly failed to result in potent and specific interference. This apparent lack of RNAi in mammalian cell culture was unexpected, as RNAi had been previously observed in mouse oocytes and early embryos (Wianny and Zernicka-Goetz, 2000; Svoboda *et al.*, 2000). However, researchers found that dsRNAs greater than 30 bp in the cytoplasm of mammalian cells provoked a potent interferon response (Stark *et al.*, 1998; Reynolds *et al.*, 2006). Today, it is well established that RNAi not only controls mRNA levels, but also serves as a protective mechanism against viral infections. To address the issues occurring with siRNA mammalian transfection, Elbashir *et al.* expanded on their previous experiments in which they had achieved successful RNAi with smaller, 21- and 22-bp RNA fragments in a *Drosophila in vitro* system (Elbashir *et al.*, 2001b). They demonstrated that transfection with 21-bp siRNA duplexes specifically suppressed expression of endogenous and heterologous genes in different mammalian cell lines, including HEK293 and HeLa cells (Elbashir *et al.*, 2001a). Therefore, 2001 marked the first description of siRNA use in mammalian cell culture and a surge of siRNA use in mammalian cells soon followed. Ultimately, these exciting discoveries implicate dsRNA-mediated silencing as more than just a new tool for loss-of-function studies, but rather an ancient phenomenon with an important, protective biological role (Rao and Sockanathan, 2005).

siRNA technology today provides a novel tool for systematically deciphering the functions and interactions of thousands of genes (McManus and Sharp, 2002). More modern uses of siRNA include targeting the expression of over expressed molecular targets in cancer therapy or for discerning the importance of an enzymatic pathway in an *in vitro* pharmacology assay (Rao *et al.*, 2009). However, to date, the use of siRNA to examine DME activity is fairly limited. Gene silencing via siRNA down regulation to study glucuronidation has been previously reported in both HeLa cells as well as in a Caco-2 cell system (Liu *et al.*, 2007; Jiang *et al.*, 2012). Identification of UGT1A6 as the primary UGT isoform involved in the glucuronidation of flavanoids in Caco-2 cells was determined through the use of RNAi, after a significant decrease in apigenin glucuronidation was observed with down regulation of UGT1A6 expression in tissues culture (Liu *et al.*, 2007). In the absence of selective UGT inhibitors, the use of siRNA technology provides a tool to selectively silence individual UGT isoforms that would allow for the assessment of changes in the enzyme activity both of the targeted UGT as well as other UGTs interacting on a protein level with the silenced UGT.

The Xenobiotic Receptors

Most animals, including humans, are exposed daily to a multitude of chemicals in the air, water, or food. While some of these chemicals are signaling molecules that carry valuable information about the animal's environment, such as the presence of food, predators, or members of the opposite sex, others are toxic and must be avoided or eliminated. Mammalian enzymatic defenses have evolved to facilitate the biotransformation and elimination of toxic compounds encountered in the environment (Hahn, 2002). Receptors that function as sensors of toxic byproducts derived from both endogenous and exogenous metabolism have been termed xenobiotic receptors. These include but are not limited to the farnesoid X receptor (FXR), liver X receptor (LXR), peroxisome proliferator activation receptors (PPARs), constitutive androstane/active receptor (CAR), pregnane X receptor (PXR), nuclear factor-erythroid 2-related factor 2 (Nrf2), and aryl hydrocarbon receptor (AhR) (Tolson and Wang, 2010). Among these, AhR, CAR, PXR and Nrf2 are the most extensively studied for their roles in the induction of cytoprotective genes, including the DMEs (Shen and Kong, 2009).

In order to understand how the DMEs are regulated, it is necessary to address the signaling mechanisms involving the xenobiotic receptors at the molecular level (Xu *et al.*, 2005). The xenobiotic receptors comprise a gene superfamily encoding for ligand-activated transcription factors, which transform endogenous and exogenous stimuli into cellular responses by regulating the expression of their target genes (Levine and Perdew, 2001; Wang and LeCluyse, 2003). Transcriptional regulation of gene expression by these receptors plays an essential role in the metabolism and clearance of many drugs and xenobiotics that are introduced into the body, for the purpose of protecting the body from the environmental insults (Li *et al.*, 1998;

Rushmore and Kong, 2002; Wang and LeCluyse, 2003).

The Aryl Hydrocarbon Receptor

The adaptive function of the aryl hydrocarbon receptor (AhR) has been studied for more than 30 years. Research initially sparked by the observed extraordinary toxic potency of chlorinated dibenzo-*p*-dioxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in animals (Higginbotham *et al.*, 1968; Schwetz *et al.*, 1973). Later focus on the potency of TCDD for eliciting biochemical effects (Poland and Glover, 1973a; Poland and Glover, 1974), such as induction of aryl hydrocarbon hydroxylase activity that is now known to be catalyzed primarily by CYP1A1 (Poland and Glover, 1973b; Poland and Glover, 1977; Goldstein *et al.*, 1977) paved the way for the discovery of AhR. The existence of AhR was first identified when PAH responsive strains of mice were found to express a receptor that bound TCDD with greater affinity when compared to PAH nonresponsive mice (Poland *et al.*, 1974). Poland *et al.* firmly established the presence of AhR via *in vitro* experiments on binding of [^{3}H]TCDD to hepatic cytosols from PAH responsive mice, which revealed a small pool of high-affinity binding sites that stereospecifically and reversibly bound TCDD (Poland *et al.*, 1976). Since the 1976 breakthrough, the mechanism by which AhR regulates the induction of adaptive enzymes has been under investigation (Whitlock, 1999).

In 1986, AhR was successfully purified upon the development of a photoaffinity ligand used to monitor the receptor (Poland *et al.*, 1986), thus enabling the subsequent cloning and characterization of the receptor (Ema *et al.*, 1992; Burbach *et al.*, 1992). AhR amino acid sequence analysis revealed a region similar to the basic region/helix-loop-helix (BR/HLH) motif found in many transcription factors that dimerize for function as well as that the N-terminal domain of AhR exhibited extensive sequence similarity to the human ARNT (aryl hydrocarbon receptor nuclear translocator) protein and two regulatory proteins of Drosophila, Sim and Per (Schmidt and Bradfield, 1996; Hankinson, 1995). In addition, it was determined that AhR binds an agonist at a domain that lies within this conserved N-terminal domain. These findings confirmed that AhR functions as a ligand-activated transcription factor with a helix-loop-helix motif similar to those found in a variety of DNA-binding proteins (Burbach *et al.*, 1992; Ema *et al.*, 1992). The AhR N-terminal region contains the basic residues that contribute to DNA binding, while the HLH domain facilitates protein-protein dimerization with other proteins and transcription factors (Murre *et al.*, 1989a; Murre *et al.*, 1989b; Davis *et al.*, 1990). The N-terminal region also contains the AhR nuclear localization signal and a nuclear export signal sequence (Ikuta *et al.*, 1998). The C-terminal portion contains the Per-Arnt-Sim (PAS) region and influences protein-protein interactions, DNA recognition, and ligand binding (Burbach *et al.*, 1992; Denis *et al.*, 1988; Perdew, 1988). It is now known that AhR is a member of the basic-helix-loop-helix (bHLH)-PAS gene superfamily of transcription factors (Gu *et al.*, 2000; Crews, 1998; Hahn, 1998). Proteins with PAS-related domains occur in organisms as diverse as animals, plants, fungi, bacteria, and archea, implicating the importance of adaptive inducibility in defense (Crews, 1998; Somers *et al.*, 2000; Ballario *et al.*, 1998; Pellequer *et al.*, 1998; Crosthwaite *et al.*, 1997; Taylor and Zhulin, 1999). It was originally believed that AhR had relatively narrow structural specificity (Poland and Knutson, 1982); however, it is now known that the receptor recognizes an impressive range of chemical structures, including non-aromatic and non-halogenated compounds (Denison and Nagy, 2003). In the context of the adaptive function of AhR, such substrate promiscuity is understandable and emphasizes its role as an environmental sensor.

The mechanism of AhR signaling has been extensively studied with respect to *CYP1A1* induction. Immunofluorescence microscopy using AhR-specific antibodies has revealed that the unbound receptor is localized in the cytosol (Pollenz *et al.*, 1994). Unbound AhR is retained in the cytosol through interactions with three heat shock proteins: Hsp90, which maintains AhR in a high affinity ligand binding state and prevents nuclear translocation and dimerization with ARNT, co-chaperone protein p23, and XAP2, an immunophilin-related protein involved in the regulation of AhR turnover (Perdew, 1988; Meyer *et al.*, 1998, Meyer and Perdew, 1999; Heid *et al.*, 2000; Dull *et al.*, 2002). Ligand binding induces a conformational change of the receptor that results in increased DNA binding affinity and decreased rate of ligand dissociation (Davis *et al.*, 1990), resulting in nuclear translocation of ligand-bound AhR and subsequent heterodimerization with its partner ARNT (Whitlock, 1999; Gonzalez *et al.*, 1996; Schmidt and Bradfield, 1996; Hankinson, 1995; Okey *et al.*, 1994). ARNT was originally thought to be necessary for nuclear translocation of AhR (Hoffman *et al.*, 1991), but it is now known that it is not required (Reyes *et al.*, 1992). The AhR/ARNT heterodimer complex recognizes and binds to distinct DNA sequences called xenobiotic response elements (XREs), leading to the transactivation and induction of target genes containing XREs in their promoter region. Electrophoretic mobility shift assays demonstrated that activated AhR recognized a specific DNA motif containing the XRE sequence: 5'-TGCGTG-3' (Denison *et al.*, 1989). Utilization of short segments of upstream regions of mouse and rat *CYP1A1* genes fused to the chloramphenicol acetyl transferase reporter gene that became activated upon TCDD or PAH treatment allowed for the initial identification of these XRE consensus sequences in both the mouse and human *CYP1A1* gene (Fujisawa-Sehara *et al.*, 1987; Denison *et al.*, 1988).

The XRE motif has since been identified in the regulatory regions of the UGTs, having first been observed in rat UGT1A6 (Emi *et al.*, 1996), human UGT1A6 (Munzel *et al.*, 1998; Munzel *et al.*, 2003), and human UGT1A1 (Yueh *et al.*, 2003). Treatment with the flavonoid, chrysin has been found to induce *UGT1A1* in both HepG2 and Caco-2 cells (Walle *et al.*, 2000; Galijatovic *et al.*, 2001; Galijatovic *et al.*, 2000). Since flavonoids are also capable of inducing *CYP1A1* (Allen *et al.*, 2001) in a *CYP1A1*-luciferase reporter HepG2 cell line (Chen and Tukey, 1996), it was hypothesized that induction of *UGT1A1* may occur through a similar mechanism. In 2003, Yueh *et al*. demonstrated induction of *UGT1A1* in HepG2 cells treated with specific AhR ligands, such as TCDD, β-naphthoflavone (BNF), and B[a]P metabolites, by monitoring increases in UGT1A1 mRNA, protein, and catalytic activity. Yueh and coworkers also performed nucleotide sequence analysis of the *UGT1A1* enhancer region, which revealed the presence of an XRE within that region. Mutation of this sequence eliminated binding of AhR and subsequent generation of enhancer constructs containing the same mutation resulted in a loss of TCDD and BNF induction of reporter gene activity (Yueh *et al.*, 2003). To date, human *UGT1A1*, *UGT1A3*, *UGT1A4*, *UGT1A6*, and *UGT1A9* have been shown to be regulated by AhR

(Mackenzie *et al.*, 2010; Yueh *et al.*, 2003; Bonzo *et al.*, 2007; Ou *et al.*, 2010; Chen *et al.*, 2005; Sugatani *et al.*, 2004; Lankisch *et al.*, 2008), thus implicating the importance of UGT-AhR interactions in protection against environmental insults.

Nuclear Receptors

The nuclear receptor superfamily is one largest group of transcription factors, with 49 distinct members presently identified in the human genome (Maglich *et al.*, 2001; Robinson-Rechavi *et al.*, 2001), and is responsible for regulating development and metabolism through control of gene expression. Many of these proteins directly bind to signaling molecules, which, because of their small, lipophilic character, can then easily enter target cells. Therefore, unlike membrane-bound receptors, the nuclear receptors are intracellular and function to directly control the activity of target genes (Mangelsdorf and Evans, 1995). While modern molecular biology studies of these receptors began around 15 years ago with the cloning of the estrogen and glucocorticoid receptors (Mangelsdorf *et al.*, 1995), the endogenous ligands for these receptors have been studied for more than 60 years, since hormone-regulated pathways were the target of interest for medicinal chemists even before the receptors were identified (Evans, 1988). Receptors characterized prior to the identification of their ligands were referred to as orphan receptors, and as their natural and synthetic ligands have become known, many of them have now been adopted (Chawla *et al.*, 2001). Over time, the synthesis and use of new ligands have facilitated in the discovery of new roles and relationships for the nuclear-receptor superfamily, ultimately emphasizing their importance as master regulators of genes (Weatherman *et al.*, 1999).

The nuclear receptors primarily act through the direct association with specific DNA sequences known as hormone response elements (HREs) (Evans, 1988; Beato, 1991). The combined achievements of the discovery of *puff* induction by ecdysone in giant chromosomes of insects (Clever and Karlson, 1960), the characterization and purification of hormone receptors, and the cloning of hormonally regulated genes culminated in the identification of hormone responsive sequences in the vicinity of genes regulated by steroid hormones (Beato, 1989). Since then, the speed of progress in the field has been extraordinarily fast, with dozens of regulatory elements for steroid hormones having been described and the cDNAs for virtually all known hormone receptors having been cloned (Evans, 1988). The powerful techniques of genetic engineering in combination with these clones provided insight into the molecular and functional architecture of the nuclear hormone receptors. In 1978, several protein chemical studies had already suggested that the steroid hormone receptors were structurally organized into different domains (Wrange and Gustafsson, 1978; Carlstedt-Duke *et al.*, 1982; Wrange *et al.*, 1984; Carlstedt-Duke *et al.*, 1987). This prediction was confirmed in 1986 and 1987, with the comparison of amino acid sequences of various hormone receptors (Kumar *et al.*, 1986; Hollenberg *et al.*, 1987; Rusconi and Yamamoto, 1987). All analyzed nuclear receptors to date have been found to be structured in a similar way, exhibiting a variable N-terminal region, a short and well-conserved cysteine-rich central domain, and a relatively well-conserved C-terminal half (Evans and Hollenberg, 1988), but can essentially be described in terms of their DNA-binding domain (DBD) and a ligand-binding domain (LBD). The DBD targets the receptor to specific HREs and contains several conserved cysteine residues. Eight of these cysteines are organized into two zinc fingers (Berg, 1989), a structural motif commonly identified in other genes for regulatory proteins, as well as enzymes (Klug and Schwabe, 1995). Identification of these repetitive zinc-binding domains within the receptor's central domain (Miller *et al.*, 1985), in addition to *in vitro* binding studies and functional evidence, verified that the domain was responsible for receptor DNA binding (Evans and Hollenberg, 1988). The C-terminal half of the receptor encompasses the LBD that possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiologic response. In its simplest terms, the LBD can be thought of as a molecular switch that when, bound by a ligand, shifts the receptor to a transcriptionally active state (Mangelsdorf *et al.*, 1995). Photo-crosslinking experiments (Carlstedt-Duke *et al.*, 1988; Simons *et al.*, 1987) as well as mapping of the LBD have helped to identify those residues responsible for direct ligand binding and those for facilitating interactions. Studies performed by Fawell *et al*. found that single amino acid substitutions of residues in the N-terminal half of the estrogen receptor, but not the C-terminal half, prevented dimerization (Fawell *et al.*, 1990). Ultimately, the sequence-specific DNA binding properties of the nuclear receptors are determined by these two conserved domains (DBD and LBD), which function in an interdependent manner to mediate protein-DNA and protein-protein interactions. The minimal target sequence recognized by the nuclear receptor DBD consists of a six base pair sequence. Several features of the core recognition motif are conserved for all members of the nuclear receptor superfamily. The detailed characterization of response elements within target genes that mediate their transcriptional activation has led to the identification of three distinct DNA-binding modes that are distinguished by whether the interactions occurring between response elements and nuclear receptors are monomers, homodimers, or heterodimers (Glass, 1994), the last two being the most common associations. Homodimeric recognition of response element sequences is typified by steroid hormone receptors and was first demonstrated in the cases of the estrogen receptor and glucocorticoid receptor (Kumar and Chambon, 1988; Tsai *et al.*, 1988). Steroid HREs are generally comprised of a pseudo-palindromic arrangement of two core recognition sequences. Crystallographic analysis revealed that each DBD of the dimer makes similar contacts with one of the core recognition motifs, forming in a symmetric structure (Luisi *et al.*, 1991; Schwabe *et al.*, 1993). Heterodimeric interactions between nuclear receptors and DNA response elements are classically demonstrated by the retinoic acid receptor (RAR), thyroid hormone receptor (TR), vitamin D receptor (VDR), and several of the orphan nuclear receptors, including the pregnane X receptor (PXR) and constitutive androstane receptor (CAR). These receptors form heterodimers with the retinoid X receptors (RXR) and bind to response elements consisting of direct, everted, or inverted repeat arrangements of the core consensus sequence (Forman and Evans, 1995; Mackenzie *et al.*, 2010; Yu *et al.*, 1991; Kliewer *et al.*, 1992; Leid *et al.*, 1992; Zhang *et al.*, 1992; Marks *et al.*, 1992). The identification of these distinct DNA-binding modes subsequently revealed three key response element features that regulate the specificity of DNA recognition by a particular set of nuclear receptors: the precise sequence of the core recognition motif, the orientation of core recognition motifs with respect to each other, and the spacing between those motifs (Glass, 1994). For example, synthetic DNA sites consisting of direct repeats of consensus hexamers separated by 3, 4, or 5 base pairs would be referred to as DR-3, DR-4, and DR-5. These distinct arrangements dictate the preferential responses to specific receptors (Baes *et al.*, 1994).

The Pregnane X Receptor and the Constitutive Androstane Receptor

The pregnane X receptor (PXR, NR1I2) belongs to the orphan nuclear receptor superfamily of ligand-activated transcription factors (Kliewer *et al.*, 1998) and has since been shown to play an essential role in both endobiotic (Zollner *et al.*, 2006; Iyer *et al.*, 2006; Cho *et al.*, 2009; Bhalla *et al.*, 2004; Bachmann *et al.*, 2004; Xie *et al.*, 2001; Sugatani *et al.*, 2005; Matic *et al.*, 2007) and xenobiotic (Francis *et al.*, 2003; Jones *et al.*, 2000; Tolson and Wang, 2010; Chirulli *et al.*, 2005) metabolism in humans, mice, and rats. Mouse PXR (mPXR) was first discovered and cloned in 1997 based on sequence homology with other nuclear receptors and was found to be activated by a variety of compounds, including natural and synthetic glucocorticoids, steroids, pregnane derivatives, anti-glucocorticoids, macrocyclic antibiotics, antifungals, and herbal extracts (Kliewer *et al.*, 1998; Jones *et al.*, 2000; Kliewer *et al.*, 1999; Lehmann *et al.*, 1998; Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Moore *et al.*, 2002). The human PXR (hPXR) ortholog was subsequently reported as the steroid and xenobiotic receptor (SXR) and pregnane activated receptor (PAR), which both exhibited structural features and activation patterns similar to mPXR (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998). Xie and colleagues finally confirmed SXR/PAR to be orthologous to mPXR with PXR knockout and transgenic mouse models. Targeted disruption of the mPXR abolished *CYP3A* induction by prototypic inducers, such as dexamethasone or pregnenolone-16α-carbonitrile (PCN), while an activated form of SXR caused constitutive upregulation of *CYP3A* gene expression and enhanced protection against toxic xenobiotic compounds in transgenic mice (Xie *et al.*, 2000a). PXR has since been cloned from a wide array of species, including rabbit, birds, amphibians, fish, pig, rhesus monkey, and dog (Jones *et al.*, 2000; Savas *et al.*, 2000; Handschin *et al.*, 2000; Moore *et al.*, 2002; Willson *et al.*, 2001). Ligand-activated PXR translocates from the cytoplasm to the nucleus of the cells (Squires *et al.*, 2004), where it then binds to DNA response elements as a heterodimer with RXR.

PXR was initially thought of as "conventional" nuclear receptor, as it appeared to exert its effects through a similar mechanism of action as the other steroid hormone receptors. However, cross-species comparisons have revealed surprising differences in the amino-acid sequence of their ligand-binding domains, indicating a relatively rapid and divergent evolution of these proteins (Willson and Kliewer, 2002). The ever-evolving library of structurally diverse PXR ligands has come to distinguish it as a unique, promiscuous, but integral mediator of inductive expression of many DMEs and transporters (Tolson and Wang, 2010). Microarray gene profiling analysis on liver samples derived from mice expressing a constitutively active variant, VP-hPXR, identified approximately 150 gene tags that were expressed in a PXR-dependent manner, including a spectrum of biologically important Phase I and II DMEs (Rosenfeld *et al.*, 2003). The extremely variable nature of PXR in ligand and target gene recognition verifies its ability to serve as a xenobiotic sensor (Tolson and Wang, 2010).

In the nuclear receptor superfamily tree, CAR (NR1I3) is the closest relative to PXR. Initially named MB67 in 1994, it was isolated by screening a cDNA library with a nuclear receptor DBD-based oligonucleotide as a probe (Baes *et al.*, 1994). This receptor was later re-designated as constitutive activated receptor (CAR), based on its ability to form a heterodimer with RXR that bound retinoic acid response elements to transactivate target genes in the absence of ligand in transfection assays (Baes *et al.*, 1994; Choi *et al.*, 1997). The first class of CAR ligands was then discovered in 1998, which included androstanol and androstenol. Interestingly, these compounds were characterized as inverse agonists because androstanol and androstenol were capable of inhibiting the constitutive activity of CAR by promoting co-activator release from the LBD (Forman *et al.*, 1998). Appropriately, this receptor is also referred to as the constitutive androstane receptor. Major progress in understanding the physiological roles of CAR came with the observation that CAR activation was linked to *CYP2B* gene induction by PB and PB-like inducers (Honkakoski *et al.*, 1998). Purification of CAR from hepatocytes by Negishi and colleagues identified it as a factor that is associated with the PB-responsive CYP2B regulatory element (Zelko and Negishi, 2000; Sueyoshi and Negishi, 2001), and it was subsequently shown to bind to the promoter as a heterodimer with RXR (Honkakoski *et al.*, 1998). Interestingly, endogenous CAR was found to reside in the cytoplasm of hepatocytes and therefore unable to affect gene transcription. Only upon exposure to PB did CAR translocate from the cytoplasm to the nucleus via a phosphorylation-dependent mechanism (Kawamoto *et al.*, 1999; Zelko *et al.*, 2001). Although there is no evidence that PB directly binds CAR, the PB-induced translocation of the receptor results in increased *CYP2B* gene transcription in the cell nucleus. Reconstitution of this phenomenon with cell-based reporter assays has proved difficult, as CAR expressed by cells that are either stably or transiently transfected with a CAR expression vector resides in the nucleus, regardless of its activation state (Sueyoshi *et al.*, 1999; Tzameli *et al.*, 2000). The generation of knockout mice has thus provided definitive evidence of the role of CAR in regulating *CYP2B* expression *in vivo*, as *Car* -/- mice exhibited no *Cyp2b* induction with PB or PB-like inducer TCPOBOP (1,4-bis(2-(3,5-dichloropyridyloxy))benzene) (Wei *et al.*, 2000). These findings triggered a wealth of subsequent studies that explored the role of CAR on xenobiotic metabolism (Honkakoski *et al.*, 2003; Qatanani and Moore, 2005). In addition to both CAR and PXR being highly expressed in the liver and small intestine, which are two key tissues greatly involved in drug metabolism (Lehmann *et al.*, 1998), these two receptors share significant cross-talk in both target gene recognition by binding to similar responsive elements in their target gene promoters and in accommodating a diverse array of xenobiotic activators (Xie *et al.*, 2000b; Wang and LeCluyse, 2003), further implicating their importance in xenobiotic defense mechanisms.

UGT1A1 is one of the most extensively characterized UGT isoforms, due to its important physiological role in the clearance of bilirubin as well as xenobiotics (Mackenzie *et al.*, 2003; Radominska-Pandya *et al.*, 2005). While PB treatment has been extensively used over the last 40 years to treat hyperbilirubinemia resulting from *UGT1A1* deficiencies (Yaffe *et al.*, 1966), the mechanism by which PB induces UGT1A1 activity has only been realized within the last decade. Sugatani *et al.*

(Sugatani *et al.*, 2001) first reported that PB-mediated induction of *UGT1A1* could be attributed to a 290-bp enhancer sequence upstream of the *UGT1A1* promoter. This sequence contained three putative nuclear receptor-binding motifs and was also responsive to CAR activation. In 2003, Xie and colleagues demonstrated that UGT1A1 expression was induced at both the mRNA and protein levels, in both transgenic VP-hPXR mice and in rifampicin-treated humanized PXR mice (Xie *et al.*, 2003). It was indepdently demonstrated that rodent-specific PXR agonist PCN increased UGT activity and expression only in wild-type, but not in PXR null mice (Chen *et al.*, 2003). In addition to *UGT1A1*, *UGT1A9* expression has been observed to increase with PXR activation (Chen *et al.*, 2003). *UGT1A3*, *UGT1A4*, *UGT1A6* and *UGT1A9* may also be PXR target genes, although exact DNA responsive elements required for these effects have yet to be found (Shelby and Klaassen, 2006; Vyhlidal *et al.*, 2004; Buckley and Klaassen, 2009).

UGT1A1 was the first UGT enzyme that was defined as a CAR target gene through the receptor's ability to recognize and bind to a distal phenobarbital-responsive enhancer module of *UGT1A1* (Sugatani *et al.*, 2001). Reduction in *UGT1A1* expression is associated with various clinical conditions, including Gilberts' syndrome characterized by mild, unconjugated hyperbilirubinemia. Polymorphism analysis of the *UGT1A1* promoter revealed a SNP of −3263 T > G located within the CAR enhancer sequence whose frequency was significantly higher in patients with Gilbert's syndrome (58%) in comparison with healthy volunteers (17%) (Sugatani *et al.*, 2002). In addition, this mutation significantly reduced CAR-mediated transcriptional activation of *UGT1A1* in cell-based luciferase assays, indicating that interplay occurs between gene polymorphism and nuclear receptor-mediated induction of *UGT1A1*. Following these findings, Qatanai and colleagues showed that CAR activation increased the major pathway of bilirubin clearance by inducing the expression of *UGT1A1*, *MRP2*, *SLC21A6*, *GSTA1*, and *GSTA2* (Qatanani *et al.*, 2005). Moreover, *Car -/-* mice pretreated with PB or TCPOBOP did not exhibit the marked increase in bilirubin clearance seen in wild-type mice. Bilirubin itself has also been documented as a CAR activator, suggesting a potential protective feedback mechanism when bilirubin accumulates in the body (Huang *et al.*, 2003). The list of UGTs as potential CAR target genes has recently been expanded to include *UGT1A3*, *UGT1A5*, *UGT1A6*, *UGT1A7*, *UGT1A8*, *UGT1A10*, *UGT2B1*, and *UGT2B5* (Shelby and Klaassen, 2006; Qatanani *et al.*, 2005). Overall, the role of PXR and CAR as mediators in the induction of UGTs expands the scope of these xenosensors. Mice lacking either PXR or CAR were hypersensitive to treatment with various xenobiotics, including the anesthetic tribromoethanol and the muscle relaxant zoxazolamine (Wei *et al.*, 2000; Xie *et al.*, 2000a), ultimately underscoring the importance of these two nuclear receptors in defending the body against a broad array of potentially harmful xenobiotics (Maglich *et al.*, 2002; Tolson and Wang, 2010).

The Oxidative Stress Sensor, NF-E2 related factor-2

NF-E2 related factor-2 (Nrf2) is a 66-kDa ubiquitous protein belonging to the small family of basic leucine zipper (bZip) transcription factors that binds an AP1/NF-E2 tandem repeat sequence (Gourdon *et al.*, 1992; Moi and Kan, 1990; Ikuta and Kan, 1991) and was originally implicated in regulating globin gene expression in hematopoietic cells (Andrews *et al.*, 1993; Moi and Kan, 1990; Ney *et al.*, 1990; Collis *et al.*, 1990; Chang *et al.*, 1992). Studies with both transfected cells and transgenic mice found a novel protein that was capable of activating this enhancer sequence within the locus control region of the globin gene, called nuclear factor-erythroid 2 (NF-E2) (Holtzclaw *et al.*, 2004). The tandem repeat NF-E2 recognition sequence was then used to screen a lambda gt11 cDNA expression library from K562 cells, which resulted in the identification of several other DNA binding proteins, one of which was called NF-E2-related factor 2 (Nrf2) (Moi *et al.*, 1994). While NF-E2 expression was restricted to erythroid and megakaryocytic cells, Nrf2 was ubiquitously expressed in a wide range of tissues, many of which are sites of expression for Phase II detoxification genes (Chan *et al.*, 1996; Chan and Kan, 1999; McMahon *et al.*, 2001; Moi *et al.*, 1994; Chan *et al.*, 1993). In addition, *Nrf2 -/-* mice generated by Chan *et al.* were not anemic, developed normally, and reproduced, indicating that Nrf2 was not required for the production of red blood cells (Chan *et al.*, 1996). However, additional challenge experiments in Nrf2 null mice did elucidate its importance in the oxidative stress response. In 1999, Chan and Kan observed that *Nrf2 -/-* mice died of acute respiratory distress syndrome when administered doses of the antioxidant butylated hydroxytoluene (BHT) that were normally tolerated by wild-type mice (Chan and Kan, 1999). Subsequent gene expression studies also showed that the expression of many detoxification enzymes becomes altered in the *Nrf2* null model. Several *in vivo* works have since shown that Nrf2 deficiency results in altered DME gene expression and subsequent detrimental conditions due to the inability to properly respond to stress. Mice deficient in Nrf2 have significantly lower and generally un-inducible levels of Phase II enzymes (McMahon *et al.*, 2001; Chanas *et al.*, 2002) and are much more sensitive than their wild-type counterparts to oxidative stress (Ishii *et al.*, 2000; Hirayama *et al.*, 2003), pulmonary toxicity (Cho *et al.*, 2002), and hepatotoxicity (Chan and Kan, 1999; Enomoto *et al.*, 2001). Nrf2 knockout mice were more susceptible to stomach tumor development in response to B[a]P administration due to a lack of protection against tumor formation, as protection was seen to result from induction of Phase II and antioxidant enzymes by oltipraz or sulforaphane (Ramos-Gomez *et al.*, 2001; Fahey *et al.*, 2002). The use of Nrf2 deficient mice highlights the crucial importance of elevated Phase II gene expression in cytoprotection.

The Nrf2 AP1-NFE2 recognition motif was later found to be a subset of the antioxidant response element (ARE) (Rushmore *et al.*, 1991; Xie *et al.*, 1995). Promoter analysis of genes encoding for antioxidant enzymes led to the identification of a DNA element that regulated their basal expression and coordinated induction in response to antioxidants and xenobiotics (Dhakshinamoorthy *et al.*, 2000; Jaiswal, 2000). Initially thought to be a unique XRE, this enhancer module was first identified in the promoters of genes encoding the two major detoxication enzymes, GSTA2 and NADPH:quinone oxidoreductase 1 (NQO-1) (Rushmore *et al.*, 1990; Friling *et al.*, 1990; Favreau and Pickett, 1993; Li and Jaiswal, 1992). These findings suggested that the regulation of gene expression by planar aromatic compounds, such as BNF and MC, was mediated by this unique DNA sequence that was distinct from the classical XRE sequence (Favreau and Pickett, 1991). Later work by Rushmore and Pickett found the sequence to also be responsive to hydrogen peroxide and phenolic antioxidants, such as t-butylhydroquinone (tBHQ), thus leading to its officially designation as the ARE (Rushmore and Pickett, 1990).

Elucidating the mechanism controlling Nrf2 activity was central to understanding how terrestrial organisms sensed destructive oxidative stress and subsequently activated an intrinsic cellular defense (Itoh *et al.*, 1999). Itoh *et al*. had observed that upon exposure to electrophilic agents, DNA-binding activity of Nrf2 was significantly induced, while Nrf2 steady-state mRNA levels remained constant. These results indicated that signals from oxidative stress agents might be transduced through an unidentified cellular receptor to the Nrf2 protein, which in turn mediates the protective response. In order to explore the molecular mechanisms that activate Nrf2 and thereby transduce oxidative stress signals, Itoh *et al*. started by examining the function of Nrf2's six domains (Neh1–Neh6) via transient co-transfection experiments in which each region was fused to the DBD of the yeast transcription factor Gal4 or a luciferase reporter gene (Itoh *et al.*, 1999). Neh4 and Neh5 were found to be independent activation domains, whereas the amino terminal Neh2 region was not. To determine Neh2's function, the authors prepared an Nrf2 mutant from which the Neh2 domain was deleted. Wild-type and mutant Nrf2 cDNAs were independently co-transfected into QT6 quail fibroblasts or HD3 chicken proerythroblasts with the luciferase reporter construct. The cells transfected with the mutant Nrf2 exhibited much higher luciferase activity than those transfected with the wild-type DNA, suggesting that a cellular repressor that interacts with the Neh2 domain of Nrf2 may exist. The Gal4–Neh2 construct was subsequently used as bait in a yeast two-hybrid system to isolate the repressor protein. Of the 300 clones recovered, 80 were analyzed and most contained the sequence of a single protein that was named Keap1 (Kelch-like ECH associated protein 1) because of its sequence homology with the drosophila actin-associated protein Kelch. Transfection of the Keap1-GFP construct into QT6 fibroblasts resulted in Keap1 localization within cytosol, while transfection of a Neh2-GFP into 293T cells revealed uniform fluorescence throughout the cells. However, co-transfection of Keap1 with Neh2-GFP displayed almost exclusive cytoplasmic fluorescence. Following these observations, treatment with the electrophilic agent diethyl maleate (DEM) was found to release the Nrf2 protein from the cytoplasm to the nucleus. Anti-Nrf2 immunoreactivity was detected predominately in the nucleus when Nrf2 expression plasmid alone was transfected into 293T cells, but when Keap1 was co-transfected with Nrf2, the cellular localization of Nrf2 was principally in the cytoplasm. Upon addition of DEM to the culture medium, Nrf2 was detected in the nucleus, even in the presence of co-transfected Keap1, thus showing DEM enables Nrf2 nuclear translocation from the cytoplasm (Itoh *et al.*, 1999). Complementary to these findings, Wakabayashi *et al.* subsequently developed a Keap1-deficient mouse and showed that the absence of Keap1 resulted in the constitutive accumulation of Nrf2 in the nucleus and therefore high expression levels of cytoprotective genes (Wakabayashi *et al.*, 2003). Additionally, examination of Keap1's sequence revealed it to be extremely cysteine rich and thus lead to the conclusion that Keap1 had the potential to serve as a perfect sensor for inducers (Dinkova-Kostova *et al.*, 2005). At its simplest, Keap1 retains the Nrf2 transcription factor in the cytoplasm until increases in cellular oxidative stress cause Keap1 to dissociate, allowing for Nrf2 nuclear translocation. Nrf2 then binds to the ARE present in the promoters of genes encoding several dozen cytoprotective proteins that enhance cell survival (Nioi *et al.*, 2003; Kensler *et al.*, 2007; Ishii *et al.*, 2000). Ultimately, these findings implicated the Keap1-Nrf2 complex as a cytoplasmic sensor system for oxidative stress.

Since Nrf2 was not only activated in response to H_2O_2, but specifically by chemical compounds with the capacity to either undergo redox cycling or be metabolically transformed to a reactive or electrophilic intermediate, this led researchers to believe that alterations in the cellular redox status due to elevated levels of reactive oxygen species (ROS) and electrophilic species and/or a reduced antioxidant capacity (e.g., glutathione) might be an important signal for triggering the transcriptional response mediated by this enhancer (Nguyen *et al.*, 2000). The involvement of Nrf2 was further supported by Nrf2 transactivation of reporter genes linked to the ARE sequence (Venugopal and Jaiswal, 1996; Venugopal and Jaiswal, 1998), *in vivo* studies in which expression of several ARE-dependent genes was found to be severely impaired in *Nrf2 -/-* mice (Itoh *et al.*, 1997; McMahon *et al.*, 2001), and by chromatin immunoprecipitation assays demonstrating direct interaction between endogenous Nrf2 and the ARE in H4IIE cells (Nguyen *et al.*, 2005).

Previous studies have shown that regulation of *UGT* expression is targeted by a number of xenobiotic receptors in response to xenobiotics, carcinogens, and stress signals. Induction of proteins following exposure to electrophiles and oxidants has been termed the antioxidant response and has been linked to Nrf2 activation (Holtzclaw *et al.*, 2004), as Nrf2 has been identified as the major regulator of cytoprotective genes encoding detoxification and antioxidant enzymes (Itoh *et al.*, 1995; Ishii *et al.*, 2000). The induction of UGT activity in mice treated with natural or synthetic chemopreventive agents represented an initial indication that UGT activity may be among the cytoprotective proteins induced by this signaling pathway (Lee *et al.*, 2003). This has also been supported in *Nrf2 -/-* mice, where treatment with the antioxidant tert-Butylhydroquinone (tBHQ) led to reduced glucuronidation activity in comparison with wild-type mice (Thimmulappa *et al.*, 2002). In 2001, Enomoto and coworkers showed that administration of acetaminophen induced more severe centrilobular hepatocellular necrosis in *Nrf2* null mice in comparison to wild-type mice. The authors suggested that the resulting sensitivity of *Nrf2 -/-* liver to electrophiles resulted from lack of cytoprotection due to decreased UGT and glutamate-cysteine ligase (GCL) expression (Enomoto *et al.*, 2001). In 2007, Yueh *et al*. performed a series of experiments that resulted in the identification of an ARE sequence within the *UGT1A1* promoter. HepG2 cells treated with the prooxidants tBHQ and BNF resulted in increased UGT1A1 glucuronidation. Loss of function analysis for Nrf2 conducted by siRNA targeted down regulation revealed that induction of *UGT1A1* was not seen in Nrf2 deficient cells. Transgenic mice bearing the human *UGT1* locus (*TgUGT1*) were treated with tBHQ to examine the contribution of oxidants toward the regulation of human *UGT1A1 in vivo*. *UGT1A1* was significantly increased in liver and small and large intestines. Gene mapping experiments including transfections of *UGT1A1* reporter gene constructs into HepG2 cells coupled with functional analysis of Nrf2 expression and binding to ARE ultimately confirmed the existence of an ARE in the phenobarbital-response enhancer module region of the *UGT1A1* gene (Yueh and Tukey, 2007). To date, the list of

ARE-driven genes includes rat *GSTA1*, mouse *GSTA1*, rat *GSTP1*, rat *NQO-1*, human *NQO-1*, human *GCL*, mouse *ferritin-L*, mouse *metallothionein-1*, multiple rat and mouse *UGTs*, and human *UGT1A1* (Lee and Johnson, 2004; Yueh and Tukey, 2007). The findings demonstrate a physiological role for Nrf2 in the *UGT* regulation.

The UGT1A1 Phenobarbital Response Enhancer Module

Increasing evidence has suggested that various xenobiotic receptors are capable of inducing a broad spectrum of hepatic and intestinal genes involved in xenobiotic metabolism (Qatanani and Moore, 2005; Ueda *et al.*, 2002). In 2001, Sugatani *et al*. reported that induction of *UGT1A1* expression by PB could be credited to a core module in the distal *UGT1A1* promoter region (Tolson and Wang, 2010). This region was found to contain three putative nuclear receptor-binding motifs and was activated by CAR in co-transfected HepG2 cells as well as in mouse primary hepatocytes treated with PB (Sugatani *et al.*, 2001). Following in 2003, Xie and colleagues demonstrated that UGT1A1 mRNA and protein were upregulated in both transgenic VP-hPXR mice and in rifampicin-treated, humanized PXR mice. Subsequent *in vitro* experiments and electrophoretic mobility shift assays (EMSAs) revealed that the PXR/RXR heterodimer bound a nuclear receptor enhancer element in *UGT1A1* promoter region (Xie *et al.*, 2003). Concurrently, independent studies also showed that the rodent-specific PXR agonist PCN increased UGT enzymatic activity and expression in wild-type, but not in *Pxr -/-* mice (Chen *et al.*, 2003). Yueh *et al.* later showed that significant induction of *UGT1A1* in HepG2 cells treated with prototypical AhR ligands, such as TCDD, BNF, and B[a]P metabolites was attributed to a xenobiotic response element that was discovered by nucleotide sequence analysis of this *UGT1A1* enhancer region (Yueh *et al.*, 2003). Moreover, in 2007, Yueh *et al.* also documented *UGT1A1* induction by antioxidants resulted directly from Nrf2 binding to an ARE sequence flanking the recently identified the AhR response element on the *UGT1A1* gene (Yueh and Tukey, 2007). It is of significant interest that the CAR and PXR responsive elements, the AhR XRE, and the Nrf2 ARE are all located within the same 290-bp enhancer region, as this implies that the major xenobiotic responsive sequences of *UGT1A1* tend to cluster together and may contribute to both induction efficacy and xenobiotic promiscuity (Tolson and Wang, 2010). This region of the *UGT1A1* promoter that contains the enhancer sequences is widely known as the phenobarbital response enhancer module (PBREM). Today, the PBREM has been determined to also include recognition sequences for the liver X receptor α (LXRα) (unpublished observations), PPARα (Senekeo-Effenberger *et al.*, 2007) and glucocorticoid receptor (Yueh *et al.*, 2003; Sugatani *et al.*, 2008; Sugatani *et al.*, 2005; Usui *et al.*, 2006). Mutations within this region significantly impact the enhancing effects of the classical UGT inducers, including chrysin, TCDD, B[a]P and MC (Sugatani *et al.*, 2004; Mackenzie *et al.*, 2010), further verifying the importance of the PBREM in facilitating the extensive breadth of glucuronidation in xenobiotic metabolism.

The NF-κB/IKK Pathway

Addressing the signaling mechanisms involving other relevant transcription factors, such as NF-κB, is necessary for fully understanding how the DMEs are regulated (Xu *et al.*, 2005). In 1986, NF-κB was originally identified as a B-cell nuclear protein and named after its ability to bind to an intronic enhancer, termed the κB motif, of the immunoglobulin κ-light chain gene (Sen and Baltimore, 2006; Sen and Baltimore, 1986). Activated NF-κB can bind these specific κB elements in target genes to regulate transcription of genes mediating inflammation, carcinogenesis and anti-apoptotic reactions (Chen *et al.*, 2001). Numerous studies since have identified NF-κB in several cell types and demonstrated activation by a wide range of inducers, including cytokines, mitogens, environmental and occupational particles or metals, intracellular stresses, viral and bacterial products, and UV light (Karin and Ben-Neriah, 2000; Chen *et al.*, 1999; Pahl, 1999; Gilmore, 1999; Sun and Ballard, 1999). However, it was initially observed that NF-κB activity could be induced in the absence of new protein synthesis. This led to examination of the state of NF-κB in unstimulated 7OZ/3 cells by Baeuerle and Baltimore. Surprisingly, they observed little NF-κB activity in either the nucleus or cytoplasm of unstimulated cells. Denaturation-renaturation of cytosolic fractions eventually revealed the presence of NF-κB in cytoplasm, implicating that NF-κB was a cytoplasmic protein that was inhibited in its DNA-binding activity. The authors then utilized mild detergents, such as deoxycholate (DOC), to gently separate the inhibitor-NF-κB complex and were able to release cytosolic NF-κB from an inhibitory protein, IκB (Baeuerle and Baltimore, 1988b). With the use of dissociating agents, they were also able to detect as much NF-κB in the cytosolic fraction from unstimulated 7OZ/3 cells as is found in the nuclear extract from phorbol ester-activated cells (Baeuerle and Baltimore, 1988a).

The discovery of this cytoplasmic inhibitor marked another milestone in NF-κB research and immediately increased interest in identifying the inhibitor and elucidating a physiologic mechanism for liberation of NF-κB from IκB (Hinz *et al.*, 2012; Kanarek and Ben-Neriah, 2012). It was later demonstrated that IκB exerted its inhibitory function by physically masking the NF-κB nuclear localization sequences (Huxford *et al.*, 1998; Beg *et al.*, 1992; Henkel *et al.*, 1992). Subsequent analysis of the IκB promoter and mRNA synthesis showed that transcription of IκB is regulated by NF-κB (Brown *et al.*, 1993; de Martin *et al.*, 1993; Sun *et al.*, 1993).

IκBα is the most abundant NF-κB inhibitory protein (Chen *et al.*, 1999). Successive experiments in cell lines implicated early on that stimulus-induced IκB phosphorylation caused the release of NF-κB, which could account for activation of the transcription factor (Ghosh and Baltimore, 1990). The IκB kinase (IKK) complex, which consists of two catalytic subunits, IKKα and IKKβ, was determined to phosphorylate IκB (Karin and Ben-Neriah, 2000). However, it was eventually established that IκB phosphorylation was insufficient for NF-κB activation (Alkalay *et al.*, 1995; DiDonato *et al.*, 1995; Chen *et al.*, 1995; Finco *et al.*, 1994) and that IκB degradation preceded NF-κB activation (Brown *et al.*, 1993; Sun *et al.*, 1993; Beg *et al.*, 1993; Mellits *et al.*, 1993). In 1993, Henkel *et al*. investigated the fate of IκB after treatment with various NF-κB inducers, including phorbol ester, interleukin-1, lipopolysaccharide (LPS), and tumor necrosis factor-α (TNFα). The authors observed IκB degradation after stimulation and that cell treatment with protease inhibitors or antioxidants completely inhibited IκB degradation and subsequent NF-κB activation, thus confirming degradation of IκB as necessary for NF-κB activation (Henkel *et al.*, 1993). Further elucidation of the IκB proteolysis mechanism followed, first through the studies of Palombella *et al.*, which demonstrated that proteasome inhibitor treatment abolished IκB degradation (Palombella *et al.*, 1994). Soon after, several laboratories ascertained that signal-induced ubiquitination and proteasomal degradation of IκB was required for NF-κB activation (Chen *et al.*, 1995; Palombella *et al.*, 1994; Alkalay *et al.*, 1995), implicating for the first time ubiquitin-dependent proteolysis as an integral step of signal-induced transcriptional activation (Kanarek and Ben-Neriah, 2012). Sequence homology comparisons and site-directed mutagenesis revealed a high degree of conservation in a short six amino acid N-terminal sequence of IκB. Among these, two conserved serine (Ser) residues were determined to be important in IκB phosphorylation. These residues were consistently phosphorylated post-phorbol ester simulation. In addition, signal-induced IκB ubiquitination, degradation, and NF-κB activation were abolished with their mutagenesis (Chen *et al.*, 1995; Brown *et al.*, 1995). In brief, inactive NF-κB is sequestered in the cytoplasm and bound to IκB, which masks NF-κB's nuclear localization signal to prevent nuclear uptake. Upon cellular activation by extracellular stimuli, IκB is phosphorylated and targeted for rapid degradation. This results in NF-κB release and translocation to the nucleus. Once within the nucleus, NF-κB can initiate or regulate early-response gene transcription by binding to κB motifs (GGGRNNYYCC) located in the promoter or enhancer regions of target genes.

While NF-κB was originally studied for its role in activation of innate immune responses and inflammation, in 1996 it became clear that another very critical function of NF-κB existed: inhibition of apoptosis (Liu *et al.*, 1996; Li *et al.*, 1999a; Wang *et al.*, 1998; Beg and Baltimore, 1996). The first clue indicating this important function was directly observed from *RelA* knockout mice that died at mid-gestation from uncontrolled liver apoptosis (Li *et al.*, 1999b). It was later determined that the extensive liver apoptosis in these mice was dependent on signaling via TNFR1 and that IKKβ- and IKKγ-deficient mice displayed similar yet more severe phenotypes (Li *et al.*, 1999a; Makris *et al.*, 2000). Over time, NF-κB has been documented as a transcriptional activator of many anti-apoptotic genes, including c-FLIP, c-IAP-1, c-IAP-2, X chromosome-linked IAP (XIAP), Bcl-x_L, Bcl-2 related protein *A1* (A1/Bfl1), Gadd45β, and SOD2 (Karin and Lin, 2002).

The NF-κB/IKK pathway has been shown to regulate transcription of important protective genes (Chen *et al.*, 2001). As such, changes in transcriptional activation can result in alterations in target gene expression and thus contribute to toxicity or disease development. In 1995, studies demonstrated that infection and inflammatory diseases generally depressed CYP activity in rodents and humans (Chang *et al.*, 1978; Renton and Knickle, 1990). Moreover, *in vivo* and *in vitro* treatments with inflammatory stimuli, including LPS and cytokines, significantly decrease CYP2B and CYP3A expression and activity (Abdel-Razzak *et al.*, 1995; Beigneux *et al.*, 2002; Li-Masters and Morgan, 2002; Muntane-Relat *et al.*, 1995; Pascussi *et al.*, 2000; Sewer and Morgan, 1997). Similarly, human hepatocytes treated with IL-1β and LPS showed a down-regulation of *UGT1A1*, *GSTA1*, and *GSTA2* expression (Assenat *et al.*, 2004). Apigenin treatment of CaCo-2 cells by Svehlikova *et al.,* found *UGT1A1* induction to be associated with NF-κB translocation, as co-treatment with the NF-κB nuclear translocation inhibitor, SN50, enhanced induction of *UGT1A1* by apigenin (Svehlikova *et al.*, 2004). In contrast, neonatal *hUGT1* mice that exhibit elevated serum bilirubin levels during development and have been treated orally with known activators of NF-κB, such as cadmium and LPS, (Hyun *et al.*, 2007; Luo *et al.*, 2004), experience marked decreases in serum bilirubin and significant increases in intestinal *UGT1A1* and *Cyp2b10*. An absence of induction of these genes was noted in the liver, implicating induction as intestinal specific. Fujiwara *et al.* consequently investigated the role of intestinal NF-κB in cadmium induction of *Cyp2b10* in a conditional knockout mouse model deficient in *Ikkα/β* specifically in intestinal epithelial cells (Fujiwara *et al.*, 2012). Although these mice do not carry the *UGT1* transgene, evaluation of *Cyp2b10* expression was still possible, since regulation occurs in a similar fashion to that of human *UGT1A1* in *hUGT1* mice. Induction of *Cyp2b10* expression was completely abolished in *Vil-Cre/Ikkα*$^{F/F}$*Ikkβ*$^{F/F}$ (GI knockout) mice treated with cadmium, compared to the cadmium-treated control *Ikkα*$^{F/F}$*/Ikkβ*$^{F/F}$ (WT mice). These results suggest the existence of a close relationship between NF-κB expression in intestinal cells and expression of *UGT1A1*.

The Mitogen Activated Protein Kinases

Addressing the signal transduction cascades, such as the Mitogen Activated Protein Kinases (MAPKs), in addition to the signaling mechanism involving the xenobiotic receptors and other relevant transcription factors, is required to fully understand DME regulation (Xu *et al.*, 2005). The discovery of the MAPKs occurred approximately 20 years ago, as evidence began to build that suggested insulin and mitogens acted through novel, possibly similar mechanisms to promote intracellular protein phosphorylation (Avruch *et al.*, 1982; Blackshear *et al.*, 1983) through the activation of protein Ser/threonine (Thr) kinases (Cobb and Rosen, 1983; Novak-Hofer and Thomas, 1984). Sturgill and Ray were the first to detect insulin-activated protein Ser/Thr kinase activity in extracts of 3T3-L1 adipocytes that were capable of phosphorylating a contaminating high molecular weight polypeptide identified as microtubule-associated protein-2 (MAP-2) (Sturgill and Ray, 1986). Insulin activation of this partially purified kinase was accompanied by an increase in ^{32}P incorporation onto its tyrosine (Tyr) and Thr residues (Ray and Sturgill, 1988a). Anti-phospho-Tyr antibodies also adsorbed kinase activity, confirming that Tyr phosphorylation of the kinase polypeptide occurred with its activation (Ray and Sturgill, 1988b). In addition, *in vitro* treatment with either a Tyr-specific or a Ser/Thr-specific protein phosphatase resulted in the deactivation of the kinase (Anderson *et al.*, 1990). At the time, few other regulatory Tyr phosphorylations had been identified apart from those on various receptor and nonreceptor Tyr kinases themselves (Hunter and Cooper, 1985). Furthermore, the absolute increase in Ser/Thr phosphorylation of cellular proteins exceeded the increase in Tyr phosphorylation observed in cells expressing constitutively active Tyr kinases by 100-1000 fold (Cooper and Hunter, 1981). The possibility that this MAP-2 kinase might be a ubiquitous effector of mitogenic stimuli was further supported by the finding that the MAP-2 kinase polypeptide was identical to previously characterized 41-43 kDa polypeptides whose Tyr phosphorylation was stimulated by several growth factors and active phorbol esters (Rossomando *et al.*, 1989; Nakamura *et al.*, 1983; Cooper *et al.*, 1984; Cooper and Hunter, 1985; Kohno, 1985; Gilmore and Martin, 1983; Bishop *et al.*, 1983). These findings ultimately fueled the renaming of the acronym "MAP" from "microtubule-associated protein" to "mitogen-activated protein", as we know it today. However, the MAP kinase (MAPK) was not the first insulin-mitogen activated protein (Ser/Thr) kinase described. Earlier work from several labs had shown that *in vivo* Ser/Thr phosphorylation of the ribosomal protein S6 in response to insulin or mitogen stimulation (Gressner and Wool, 1976; Haselbacher *et al.*, 1979; Smith *et al.*, 1979) was paralleled by the appearance of stably activated, 40S-S6 selective protein kinase activities in stimulated cell extracts (Cobb and Rosen, 1983; Novak-Hofer and Thomas, 1984). The first of these S6 kinases (now called Rsks) was purified from *Xenopus* oocytes (Erikson and Maller, 1985; Erikson and Maller, 1986). Direct microinjection into oocytes of Tyr kinase polypeptides, such as vSrc, vAbl, and the insulin receptor itself provided evidence that these S6 kinases were activated downstream of Tyr kinases (Spivack *et al.*, 1984; Maller *et al.*, 1985; Stefanovic *et al.*, 1986). Activation of the *Xenopus* S6 kinase was determined to occur exclusively through Ser/Thr phosphorylation, since the insulin-activated S6 kinase lacked incorporation of ^{32}P at its Tyr residue and its activity was eliminated by Ser/Thr-specific phosphatases (Maller, 1986). Amazingly, it was then found that the partially purified, insulin-activated MAPK also directly phosphorylated and activated the purified *Xenopus* S6 kinase (Sturgill *et al.*, 1988). Independent studies had concurrently identified a set of mitogen activated S6 peptide kinases in extracts of EGF-treated NIH3T3 cells that were activated by upstream, EGF-regulated kinases (Ahn *et al.*, 1990; Ahn and Krebs, 1990; Ahn *et al.*, 1991). The ability of the MAPK to activate an S6 kinase identified what proved to be the first physiologic MAPK substrate, marking an important milestone in growth factor signaling (Avruch, 2007). However, the concept of a protein kinase cascade was not particularly novel. Krebs and colleagues were responsible for defining the first example, in the activation of phosphorylase b kinase by the cyclic AMP-dependent protein kinase (Walsh *et al.*, 1968). It was also widely recognized that phosphorylation by an upstream kinase was required for AMP-activated protein kinase activity (Ingebritsen *et al.*, 1978). Then in 1990, a surprising aspect of the MAPK cascade was revealed via the molecular cloning of a cDNA encoding the MAPK polypeptide (Boulton *et al.*, 1990). It was found that the primary sequence of the p44 MAPK (designated as extracellular signal-related kinase 1(ERK1)) was nearly 50% identical to the sequences of two recently described *S. cerevisiae* protein kinases, KSS1 and FUS3, that had been identified as participants in the yeast mating pathway (Courchesne *et al.*, 1989; Elion *et al.*, 1990). The remarkably high conservation of structure across an enormous phyllogenetic distance indicates that the role of this family of protein kinases as mediators of receptor-regulated cellular differentiation and proliferation is both ancient and highly conserved (Avruch, 2007).

These findings were followed by an intense effort to define the order and biochemical actions of each of these yeast kinases, as well as the identity of the upstream activators of the MAPKs evident in various vertebrate systems (Avruch, 2007). In the 1990s, there were a series of reports of a partially purified cytoplasmic protein (50-60 kDa) that functioned as a MAPK activator. This activator was capable of promoting *in vitro* phosphorylation of MAPKs, ERK1 and ERK2, at both Thr and

Tyr residues, which resulted in increased MAPK activity (Ahn *et al.*, 1991; Gomez and Cohen, 1991; Ahn *et al.*, 1992). These findings eliminated the possibility that the MAPK was simply the direct substrate of a Tyr-specific kinase. The discovery that the MAPKs auto-activated slowly *in vitro* by auto-phosphorylation and of the inability of MAPK activators to catalyze significant phosphorylation of other polypeptides led to great uncertainty as to whether the MAPK activators were actually protein kinases (Avruch, 2007). However, this issue was eventually settled with the studies that demonstrated that MAPK activators could phosphorylate catalytically-inactive mutant MAPK polypeptides. These activators are now denoted as MAPK kinases (MAP2Ks) or MAP and ERK kinases (MEKs). Analysis of primary MAP2K sequences from various sources revealed that the vertebrate MAP2Ks were 30-40% identical in overall primary sequence to one of the essential protein kinases of the *S. cerevisiae* mating pathway, STE7 (Nakielny *et al.*, 1992; Crews and Erikson, 1992; Kosako *et al.*, 1992; Wu *et al.*, 1992; Crews *et al.*, 1992; Seger *et al.*, 1992), and consequently two kinases, MKK1 and MKK2, each capable of activating ERK1 and ERK2, were identified. Over time, subsequent studies led to the identification of MAP2K upstream kinase regulators (MAP3Ks and MAP4Ks) as well as the discovery of the two additional mammalian MAPK subfamilies, stress-activated protein kinase (SAPK)/ c-Jun NH2-terminal protein kinase (JNK) (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994) and p38 (Han *et al.*, 1994; Rouse *et al.*, 1994; Freshney *et al.*, 1994; Lee *et al.*, 1994). It was eventually determined that MAPKs were activated by dual phosphorylation of a tripeptide motif (Thr-Xaa-Tyr) located in the activation loop (T-loop). This phosphorylation is mediated by a MAP2K, which is activated by phosphorylation by a

MAP3K, thus demonstrating that MAPK activation occurs via a kinase signaling cascade. These exciting discoveries that unveiled the MAPK pathway in combination with identifying MAP3K/MAP2K/MAPK cassettes in *S. cerevisiae* and *S. pombe* that were distinct from their mating pathways (Levin and Errede, 1995), provided a forceful demonstration that evolution conserved not only critical housekeeping molecules, but useful regulatory modules and designs as well. Today, it is well established that MAPK cascades are evolutionarily conserved in all eukaryotes and play a key role in the regulation of gene expression (Schaeffer and Weber, 1999).

It has recently been shown through transient transfection studies in HepG2 cells as well as treatment with kinase inhibitors that induction of ARE-dependent Phase II detoxifying enzymes is mediated by a MAPK pathway (Yu *et al.*, 2000; Keum *et al.*, 2003; Shen *et al.*, 2004; Yu *et al.*, 1999). Specific to *UGT* gene regulation, investigation of the mechanism involved in *UGT1A1* induction by the important dietary flavonoid chrysin in cell culture identified a previously unknown mechanism of *UGT1A1* regulation through the MAPK pathway. siRNA down regulation of AhR, a typical mediator of *UGT1A1* expression, revealed that chrysin utilized AhR in conjunction with other factors through MAPK signaling pathways to maximally induce *UGT1A1*. Treatment with various MAPK inhibitors has also been observed to suppress chrysin induction of UGT1A1 luciferase activity (Bonzo *et al.*, 2007). Moreover, induction of UGT1A1 transcription by sulforaphane has also been linked to the MAPKs, as induction was completely abolished by PD98059, a selective inhibitor of MEK1, which is an upstream kinase regulating ERK1/2 (Svehlikova *et al.*, 2004). These findings demonstrate that the MAPKs can act as upstream activators of transcription factors that regulate important DMEs.

Objectives of the Dissertation

I have been very lucky to have such a well-rounded research experience throughout graduate school. My work has exposed me to two very important, yet separate areas of focus of the UGT superfamily of enzymes: protein structure and gene regulation. I investigated the functional relevance of UGT intermolecular interactions in tissue culture, utilizing human hepatocytes and siRNA technology, and I studied *UGT1A1* gene regulation by oral arsenic in a humanized mouse model system. Both projects emphasize the inherent complexities of the UGTs, as activity and subsequent drug metabolism and disposition can be affected by several factors, including dimerization, heredity, and environmental toxicant exposure.

When I officially joined the Tukey lab at the beginning of graduate school, one of the doctoral candidates (Dr. Theresa Operaña) was focusing the majority of her thesis work on intermolecular interactions of the UGTs through the use of FRET technology in combination with co-immunoprecipitation experiments. Her co-expression studies in COS cells revealed that all the UGT1A proteins homo-dimerized and that UGT1A1 hetero-dimerized with UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10. Additionally, co-expression of UGT1A1 and UGT1A7 increased 2-napthol glucuronidation in comparison to cells only expressing either UGT1A1 or UGT1A7, suggesting that protein interactions could impact enzyme function (Operaña and Tukey, 2007). This aspect of her work had always interested me and during the summer of 2011, I pursued an opportunity to further investigate UGT-UGT interactions as a graduate intern in the PKDM department at Amgen, Inc. With the abundance of *in vitro* data supporting the functional relevance of UGT dimerization, I became interested in addressing whether dimerization was capable of modulating enzyme functionality in biologically active systems, such as human hepatocytes. To test my hypothesis, I utilized selective siRNA down regulation experiments and activity studies to examine changes in metabolite formation due to disrupted protein interactions. Through my studies, I was able to demonstrate siRNA down regulation as an important process for evaluating UGT enzymology and that UGT-UGT interactions are a physiologically relevant phenomena whose effects can be observed in human hepatocytes. Therefore, human hepatocytes can potentially serve as a more reliable method for characterizing UGTs and phenotyping UGT substrates.

The advent of transgenic technologies in the 1980s opened many doors in medical and research fields (Dunn *et al.*, 2005; Gordon and Ruddle, 1981). This has been particularly the case for the study of *UGT1* gene regulation *in vivo* and understanding human *UGT1A1* deficiencies. In 2010, our laboratory successfully generated a fully humanized mouse model (*hUGT1*) by crossing *TgUGT1* mice expressing the entire human *UGT1* locus with *Ugt1*-null mice. While *Ugt1*-null mice died within seven days after birth due to a UGT1A1 glucuronidation deficiency, the humanized mice did not exhibit the same developmental lethality (Fujiwara *et al.*, 2010b). Further characterization of the *UGT1* humanized model revealed that during development, neonatal bilirubin levels rapidly increased, peaking at around 14 days after birth and then decreased to normal adult levels around 21 days. The observed rise and fall in bilirubin levels was determined to be directly due to developmental expression of small intestinal *UGT1A1* (Fujiwara *et al.*, 2010b).

Since numerous environmental toxicants induce *UGT1A1* through association with xenobiotic and environmental receptors, I speculated that induction of the *UGT1A1* gene by environmental contaminants might alter serum bilirubin levels. Earlier work in our lab had already demonstrated that treatment of the *hUGT1* mice with *UGT1A1*-inducing agents, such as PB and TCDD, led to a significant reduction in serum bilirubin. With *UGT1A1* gene expression being controlled and regulated *in vivo* by inducible and tissue specific factors, I decided to explore the association between exposure to prominent metal contaminants and gene expression through fluctuations in bilirubin levels.

Environmental arsenic contamination is a significant problem worldwide, with drinking water being the most common source of arsenic exposure. There is extensive epidemiological evidence linking arsenic exposure to various diseases (arteriosclerosis, cardiovascular disease, Blackfoot disease) (Navas-Acien *et al.*, 2005), numerous cancers (skin, bladder, lung, liver, prostate, kidney) (Kligerman and Tennant, 2007; Chen *et al.*, 1992), diabetes (Diaz-Villasenor *et al.*, 2007) and certain neurological ailments (Alzheimer's and Parkinson's) (Schmuck *et al.*, 2005; Vahidnia *et al.*, 2007), which suggests that arsenic has a very extensive effect and is capable of altering various signaling pathways. Therefore, understanding the development of toxicities associated with arsenic exposure is complicated.

With respect to the field of drug metabolism, there is very little information on how arsenic exposure modulates *UGT1* gene expression. This is of great concern, considering how prominent arsenic exposure has recently become in the news. The shocking and on-going prevalence of contamination led me to question whether arsenic was capable of affecting important biological processes, such as drug metabolism. More specifically, I wanted to investigate the effects of oral arsenic exposure on *UGT1A1* expression in our *hUGT1* mouse model. Treatment of neonatal *hUGT1* mice with arsenic caused decreases in serum bilirubin and significant induction of small intestinal *UGT1A1*. This then led me to further investigate the mechanism by which induction was occurring.

Many of the toxic effects of arsenic exposure can be attributed to an assortment of cellular responses, including altered nuclear transcription factor activities or signal transduction, inflammation, and oxidative stress, which led to the investigation of nuclear receptors (Kaltreider *et al.*, 2001; Bonzo *et al.*, 2005; Noreault *et al.*, 2005; Medina-Diaz *et al.*, 2009; Baldwin and Roling, 2009), NF-κB (Kapahi *et al.*, 2000; Roussel and Barchowsky, 2000), and Nrf2 (Pi *et al.*, 2003; Kumagai and Sumi, 2007; De Vizcaya-Ruiz *et al.*, 2009) in regulating intestinal *UGT1A1* induction. However, the contribution of arsenic to such a variety of different diseases also indicated to me that it might not function through one specific mechanism, but instead by eliciting a more global effect. Both abnormal cell cycle regulation (Bonzo *et al.*, 2005; Lau *et al.*, 2004; Eguchi *et al.*, 2011) and changes in cellular morphology due to arsenic-induced cytotoxicity (Yancy *et al.*, 2005; Li *et al.*, 2011) have been observed with exposure and can lead to changes in signaling that ultimately affect gene expression. This directed my attention towards immunohistochemical assessment of intestinal damage, changes in cellular morphology, and alterations in cellular proliferation.

These studies collectively led to the identification of a novel mechanism that consists of three potential pathways by which oral arsenic modulates small intestinal *UGT1A1* expression in *hUGT1* mice.

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CHAPTER 2

Evaluation of UGT Protein Interactions in Human Hepatocytes: Effect of siRNA Down Regulation of UGT1A9 and UGT2B7 on Propofol Glucuronidation in Human Hepatocytes

Introduction

The UDP-Glucuronosyltransferases (UGTs) are membrane bound proteins localized to the endoplasmic reticulum. This superfamily of enzymes catalyzes the formation of glucuronides by the transfer of glucuronic acid from the co-substrate uridine 5′-diphosphoglucuronic acid (UDPGA) to many endogenous and exogenous substrates, making them more suitable for excretion into the urine or bile (Dutton, 1980; Tukey and Strassburg, 2000). UGTs often work in concert with other key enzymes involved in drug metabolism, such as the cytochrome P450s (CYPs). The CYPs perform oxidation or reduction reactions to either activate or detoxify the parent compound. These metabolites can then be further detoxified through conjugation reactions, carried out by the UGTs or other Phase II DMEs, including the N-acetyltransferases (NATs), sulfotransferases (SULTs), and glutathione S-transferases (GSTs). There is accumulating evidence suggesting that protein-protein interactions occur between these enzymes and that these interactions play a significant role in modulating enzyme activity (Fremont *et al.*, 2005; Ikushiro *et al.*, 1997; Ishii *et al.*, 2010b; Iyanagi, 2007; Kurkela *et al.*, 2003; Lewis *et al.*, 2011; Nakajima *et al.*, 2007; Operaña and Tukey, 2007; Takeda *et al.*, 2005; Taura *et al.*, 2000). Co-localization and protein-protein interactions between DMEs allows concerted metabolism to occur more efficiently (Ishii *et al.*, 2005). Several studies also suggest that the UGTs themselves dimerize and are functional as dimers in monoglucuronide formation or as tetramers in diglucuronide formation (Gschaidmeier and Bock, 1994). It is the highly variable, substrate binding N-terminus that has generally been implicated in these protein-protein interactions, though evidence exists that the C-terminus may also have a role (Kurkela *et al.*, 2007; Meech and Mackenzie, 1997). Disrupting these interactions has been known to alter K_m values and substrate binding specificity (Bock and Kohle, 2009). However, while dimerization has been studied extensively utilizing recombinant systems, it has yet to be confirmed in a physiologically relevant *in vitro* system. The ability to study UGT protein-protein interactions in human hepatocytes may be valuable in identifying potential disconnects between UGT enzymology in single enzyme versus whole cell systems and in evaluating whether UGT dimerization is a physiologically relevant phenomena or simply an *in vitro* artifact.

Discovery of RNA interference (RNAi) has allowed for a more complete and systematic analysis of gene expression and function. RNAi is a natural cellular process that controls mRNA levels and serves as a protective mechanism against viral infections. In brief, the process begins with the cleavage of large double-stranded RNAs (dsRNAs) by Dicer, into small interfering RNAs (siRNAs). Each siRNA is then unwound into two single-stranded RNAs: the passenger strand, which is degraded, and the guide strand, which is incorporated into the RNA-Induced Silencing Complex (RISC). These siRNAs can then bind to other specific RNAs (mRNA), shutting down mRNA and protein synthesis nonspecifically (McManus and Sharp, 2002). Therefore, introduction of siRNA that targets a gene of interest results in its highly specific and selective down regulation (Rao *et al.*, 2009). In the absence of selective UGT inhibitors, the use of siRNA technology provides a tool to selectively silence individual UGT isoforms, which should allow for the assessment of changes in the enzyme activity both of the targeted UGT as well as other UGTs which may interact on a protein level with the silenced UGT.

The objective of this current work was to utilize selective siRNA down regulation to study the effects of UGT1A9-UGT2B7 protein interactions on glucuronidation activity in human hepatocytes. Co-expression of UGT1A9 and UGT2B7 in HEK cells has previously been shown to enhance the activity of both propofol and morphine glucuronidation when compared to singly expressed systems (Fujiwara *et al*., 2010) and as such, UGT1A9 and UGT2B7 represent a rational starting point for the evaluation of UGT protein interactions in human hepatocytes. Multiple siRNA primers were evaluated and quantitative PCR analysis was used to verify selective down regulation of two UGT isoforms previously shown to be involved in protein-protein interactions. Finally, changes in metabolite formation in hepatocytes treated with siRNA primers were measured by HPLC-MS/MS in order to assess the functional impact of silencing UGT expression on both the UGT isoform of interest as well as on isoforms that may interact with the silenced UGT.

Experimental

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Recombinant UGT Supersomes were purchased from BD Biosciences (San Jose, CA). Cryopreserved plateable human hepatocytes (Donor 4151, Donor 4199, Donor 4237) were purchased from CellzDirect Inc. (Tucson, AZ). BIOCOAT Cell Environmental Collagen I Cellware 96 well plates were obtained from BD Biosciences (San Jose, CA). In VitroGRO CP Plating Medium and Torpedo antibiotic mix was purchased from Celsis (Chicago, IL). Silencer Select Predesigned siRNA oligos were obtained from Ambion (Austin, TX). Lipofectamine, Dulbecco's

Modified Eagle Medium (DMEM), maintenance media supplements (100 U/mL penicillin and streptomycin, 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin, 5.35 µg/mL linoleic acid, 2mM GlutaMAX™, 15mM HEPES, pH 7.4), and RNAiMax Reagent were purchased from Invitrogen (Carlsbad, CA).

Assessment of Propofol Glucuronidation in Recombinant UGTs. Glucuronidation of propofol was evaluated against recombinantly expressed human hepatic UGT enzymes preparations (1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, 2B17). UGT enzymes (0.05 mg) were activated by pre-incubating with alamethicin (25 µg/mg) in 50 mM Tris buffer on ice for 30 min. At the end of the pre-incubation period, incubation mixtures were diluted with purified water and propofol was added to achieve a final concentration of 2.5, 10, or 20 µM. Following a second pre-incubation period (5 min) at 37 ºC, reactions were initiated by addition of UDPGA (1 mM, final concentration) and incubated for 30 min at 37 ºC (100 µL final incubation volume). Control incubations with inactive microsomes (prepared from membranes not expressing UGT enzyme) were treated identically as described above. Reactions were terminated by addition of 200 µL acetonitrile containing formic acid (0.1%, v/v) and 0.1 µM tolbutamide as an internal standard. Following centrifugation (10 min x 1460 g) the resulting supernatants were transferred to 96-well plates and analyzed for the presence of propofol glucuronide by mass spectrometry (LC-MS/MS). Data is plotted as the percent of total glucuronide formation (formation of glucuronide formation by a UGT as a fraction of the total glucuronide formation for all UGTs).

Plating and Transfection of Human Hepatocytes. Cryopreserved plateable human hepatocytes from three individual donors (Hu4199, Hu4237, and Hu4151) were placed in a 37°C water bath until thawed and then quickly transferred to fresh, pre-warmed In Vitro GRO CP media, supplemented with Torpedo antibiotics. Viable cell count was determined using the Trypan Blue exclusion method. Human hepatocytes were then plated on collagen coated, 96-well plates, at a density of 0.7 x 10^6 viable cells/mL and allowed to attach for 2-4 hours.

For transfection, 25 μM UGT2B7 siRNA (Ambion s14651; 5' → 3' sequence, CATTGAAGAGTAATTAA) and 50 μM UGT1A9 siRNA (Ambion s29248; 5' → 3' sequence, CGATCCTTTTGATAACTGT) stock solutions were prepared and each siRNA stock solution was separately added to conical tubes containing OPTIMEM, yielding final siRNA concentrations of 1.0 μM for UGT1A9 oligos and 0.5 μM for UGT2B7 oligos (siRNA + OPTI). Lipofectamine transfection reagent (TR) (50x) was diluted in a separate conical tube containing OPTIMEM to achieve a 1X solution (TR + OPTI). TR + OPTI was added to each siRNA + OPTI mixture (final siRNA concentrations of 0.5 μM for UGT1A9 and 0.25 μM for UGT2B7) and incubated at room temperature for 1 hour with occasional mixing (siRNA + TR + OPTI).

Plating media was removed from hepatocytes and cells were washed with maintenance media (DMEM plus maintenance media supplements), prior to the addition of siRNA + TR + OPTIMEM, giving a final siRNA concentration of 100 nM for UGT1A9 and 50 nM for UGT2B7. Cells were transfected with siRNA for a 72-hour period, which was previously determined to be optimal in regard to transfection efficiency and cellular integrity (data not shown). Cell media was refreshed every 24 hours with pre-warmed maintenance media.

Assessment of siRNA Down Regulation. RNA was isolated using the Ambion Total RNA Isolation Kit with Applied Biosystems MagMax Express 96 Magnetic Particle Processor. TaqMan® Probe-Based Gene Expression Analysis was used to quantify siRNA oligo efficiency and specificity. RNA quantity was assessed using the NanoDrop (Thermo Scientific, Wilmington, DE). All RNA samples were then normalized to 5 ng/µL with nuclease free water. cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit with a final volume of 20 µL and 66 ng total RNA according to manufacturer's protocol. After synthesis, the cDNA reactions were diluted to 160 µL total volume with nuclease free water. TaqMan reactions were run on a 7900HT Real-time PCR system in a 384-well optical reaction plate. Each reaction contained 10 µL 2x Gene Expression Master Mix, 5 µL nuclease free water, 1 µL 20x Primer and Probe mix, and 4 µL of cDNA. All reactions were run in duplicate using default cycling parameters. The endogenous control used was 18S1 (Taqman assay ID Hs03928985_g1). Assay ID numbers for the UGT TaqMan assays are as follows: UGT1A1, Hs02511055_s1; UGT1A3, Hs01592480_m1; UGT1A4, Hs01592480_m1; UGT1A6, Hs01592477_m1; UGT1A9, Hs02516855_sH; UGT2B4, Hs00607514_mH; UGT2B7, Hs00426592_m1. UGT expression was normalized to expression of the endogenous control 18S1 in each individual well prior to statistical analysis. Control data (100% of control expression) defines the expression of each respective mRNA in the absence of siRNA treatment and after normalization to the 18S1 control. Standard deviations were calculated from the standard deviations of the expression values for both the target UGT of interest and the 18S1 control as noted in equation 1:

$$(1) \qquad StDev_{mRNA} = \sqrt{StDev^2{}_{UGT} + StDev^2{}_{18S1}}$$

Activity Assays. Following siRNA transfection, hepatocytes were incubated with propofol (0 – 250 μM, final concentration) for 1 hour, conditions which had previously been shown to be linear with respect to incubation time and cell count (data not shown). Propofol was dissolved in DMSO and subsequently diluted into incubation media prior to addition to the hepatocyte cultures in order to maintain a DMSO concentration of less than 0.1% (v/v). The reaction was then quenched by transferring 100 μL of cell media to a deep well plate containing tolbutamide (0.1 μM) as an internal standard in acetonitrile. Samples were then centrifuged at 3000 rpm for 10 min. and transferred to a second 96-well plate for LC-MS/MS analysis.

Liquid Chromatography/Tandem Mass Spectral Analysis of Glucuronide Formation. Measurement of propofol glucuronide was performed using LC-MS/MS technology. The LC-MS/MS system consists of an Applied Biosystems 4000 Q-Trap spectrometer (operated in triple quadrupole mode) equipped with an electrospray ionization source (Applied Biosystems, Foster City, CA). The MS/MS system was coupled to two LC-20AD pumps with an in-line CBM-20A controller and DGU-20A5 solvent degasser (Shimadzu, Columbia, MD) and a LEAP CTC HTS PAL autosampler equipped with a dual-solvent self-washing system (CTC Analytics, Carrboro, NC). The injection volume was 20 µl for each analyte. HPLC separation was achieved using a Gemini C18 2.0 × 30 mm 5 µm column (Phenomenex, Torrance, CA). Gradient elution (flow rate = 500 µl/min) was performed using a mobile phase system consisting of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient conditions were 5% B for 1.0 min, increasing to 100% B from 1.0 to 3.0 min, holding at 100% B from 3.0 to 3.75 min, and returning to 5% B from 3.75 to 5.0 min. HPLC flow was diverted from the MS/MS system for the first 20 s to remove any nonvolatile salts. Generic mass spectrometry parameters included the curtain gas (12 arbitrary units), collision-assisted dissociation gas (medium), ion spray voltage (4500 V), source temperature (500°C), and ion source gas 1 and gas 2 (40 arbitrary units each). Multiple reaction monitoring mass transitions (Q1→Q3) were 352.9→176.9 for propofol glucuronide and 268.9→169.7 for tolbutamide, both utilizing negative ionization. Quantitation of propofol glucuronide was achieved by comparing peak areas in unknown samples to a standard curve of propofol glucuronide from 5 to 2000 ng/mL and weighted using 1/x scaling factor.

Results

Propofol Glucuronidation in Recombinant UGTs

Phenotyping experiments designed to characterize the enzymes responsible for the glucuronidation of propofol at therapeutically relevant concentrations were carried out using UGT Supersomes. At final substrate concentrations of 2.5, 10, or 20 µM, incubations with UGT1A9 accounted for the majority of propofol glucuronide formation *in vitro*. Incubations with UGT1A6 resulted in a minor amount of propofol glucuronide formation, while those with UGT1A1, UGT1A3, UGT1A4, UGT2B4, UGT2B7, UGT2B15 or UGT2B17 did not result in the detectable formation of propofol glucuronide (Figure 2-1). Data was normalized to total glucuronide formed for all UGT isoforms.

siRNA Characterization

UGT1A9 and UGT2B7 were chosen for selective siRNA down regulation because these isoforms have been previously implicated in several dimerization studies (Fujiwara *et al.*, 2010). Three oligo sets for each of the two isoforms were tested for down regulation efficiency and selectivity (data not shown). The exon regions and cDNA sequences targeted by the selected oligos are shown in Figure 2-2 and the experimental conditions for the chosen siRNA oligo sets are summarized in Table 2-1. Quantitative PCR analysis was used to assess relative expression of different UGT isoforms post-transfection and confirmed the successful, selective down regulation of only the desired target gene. In each case, expression of the targeted UGT isoform was reduced to less than 20% of control expression, with minimal changes to the expression of other UGT isoforms (Figure 2-3A, 2-3B). Individual donor variation in siRNA down regulation was minimal for the three individual donors examined (Figure 2-4A, 2-4B). Though increased variability in the data was observed due to incorporating the standard deviations of both the UGT of interest as well as the 18S1 housekeeping control to calculate the final standard deviation (equation 1), changes in UGT mRNA expression did not reach statistical significance except for the expected isoforms ($p < 0.0001$).

Table 2-1. **Summary of the experimental conditions used for siRNA down regulation of UGT1A9 and UGT2B7 in human hepatocytes.**

Target Gene	RefSeq	Exon Targeted	siRNA (µM)	Cell Density (cells/mL)	Well Volume (µL)	Incubation Time (hr)
UGT1A9	NM_021027.2	1	50	0.7×10^6	100	72
UGT2B7	NM_001074.2	5	25	0.7×10^6	100	72

Table 2-2. **Summary of kinetic parameters of propofol glucuronidation in human hepatocytes with and without siRNA treatment.**

	V_{max} (µmol/min)	K_m (µM)	K_i (µM)
OPTI Only	0.393 ± 0.009	7.99 ± 0.45	361.7 ± 31.3
TR Only	0.277 ± 0.005	7.62 ± 0.37	521.2 ± 46.3
UGT1A9 siRNA	0.073 ± 0.007	6.02 ± 1.6	448.4 ± 203.5
UGT2B7 siRNA	0.161 ± 0.027	10.5 ± 3.7	198.5 ± 88.9

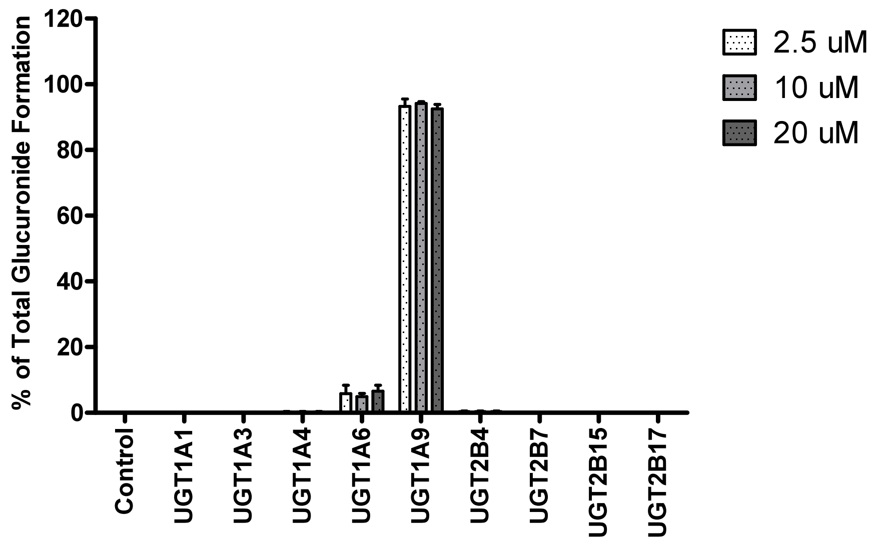

Figure 2-1. **Glucuronidation of propofol in recombinant UGTs at therapeutically relevant concentrations.** Propofol was primarily glucuronidated by UGT1A9 with a minor contribution from UGT1A6.

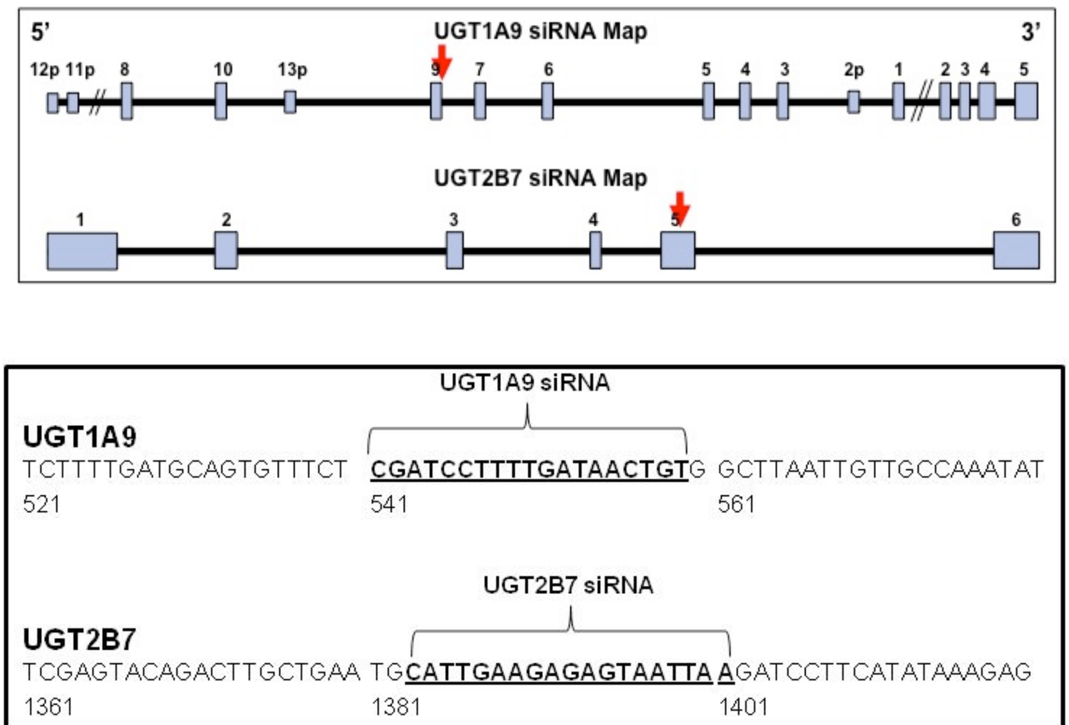

Figure 2-2. **siRNA maps for the selected UGT1A9 and UGT2B7 oligos illustrate the specific regions and consensus sequences (5' → 3') targeted for down regulation.**

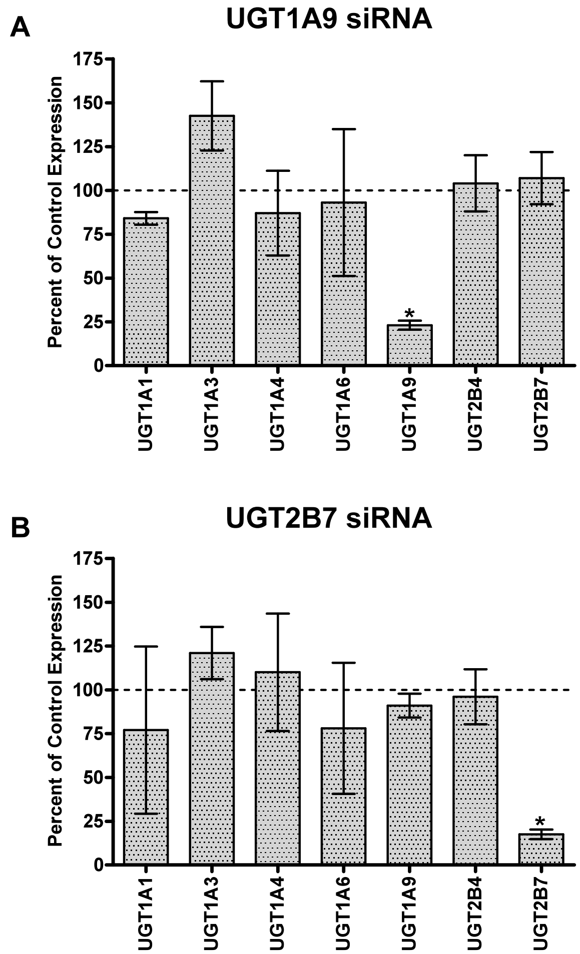

Figure 2-3. **siRNA down regulation of UGT1A9 and UGT2B7 in human hepatocytes.** Quantitative PCR analysis data for transfection with **(A)** UGT1A9 or **(B)** UGT2B7 siRNA indicates selective down regulation of the intended target gene. Control data (100% of control expression) defines the expression of each respective mRNA in the absence of siRNA treatment and after normalization to the 18S1 control. An asterisk (*) indicates a statistically significant difference, where $p < 0.0001$. Error bars indicate the standard deviation for replicate incubations (n=3).

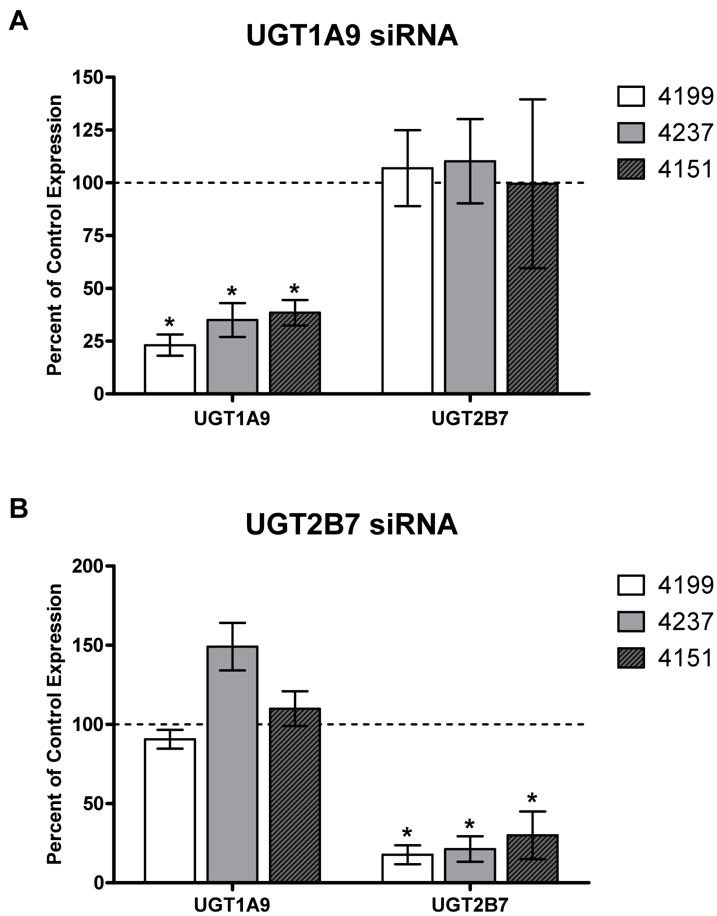

Figure 2-4. **Individual donor variation in siRNA down regulation was minimal for the three individual donors examined.** Quantitative PCR analysis data for transfection with **(A)** UGT1A9 or **(B)** UGT2B7 siRNA, indicates selective down regulation of the intended target gene, with minimal variation among hepatocyte donors (Hu4199, Hu4237, and Hu4151). An asterisk (*) indicates a statistically significant difference, where $p < 0.001$. Error bars indicate the standard deviation for replicate incubations (n=3).

Inhibition of Propofol Glucuronidation by siRNA Down Regulation in Human Hepatocytes

To examine the effect of protein-protein interactions on enzymatic activity, enzyme kinetic assays were performed and changes in metabolite formation were measured by LC-MS/MS. siRNA transfected hepatocytes were incubated with increasing concentrations of the UGT1A9 probe substrate propofol (0 – 250 µM, final concentration). Enzyme kinetic parameters are summarized in Table 2-2. Propofol glucuronidation in human hepatocytes was fit to a substrate inhibition model. As expected, propofol glucuronidation was significantly reduced relative to control in hepatocytes treated with UGT1A9 siRNA (Figure 2-5). The effects of silencing UGT1A9 expression is reflected by a 73.6% reduction in V_{max} values for cells transfected with UGT1A9 siRNA (V_{max} = 0.073 ± 0.007 µmol/min) compared to controls (OPTI Only V_{max} = 0.393 ± 0.009 µmol/min; TR Only V_{max} = 0.277 ± 0.005 µmol/min). UGT2B7 down regulation also resulted in a 42.2% reduction in V_{max} values for propofol glucuronidation (Figure 2-5; V_{max} = 0.160 ± 0.026 µmol/min). While the K_i values were generally similar for propofol glucuronidation in control hepatocytes and hepatocytes treated with UGT1A9 siRNA, a larger change (approximately 2.6-fold decrease) was observed in the propofol glucuronidation K_i when the hepatocytes were treated with UGT2B7 siRNA as compared to the transfection reagent control. K_m values for propofol glucuronidation were generally unchanged in the presence of either UGT1A9 or UGT2B7 siRNA.

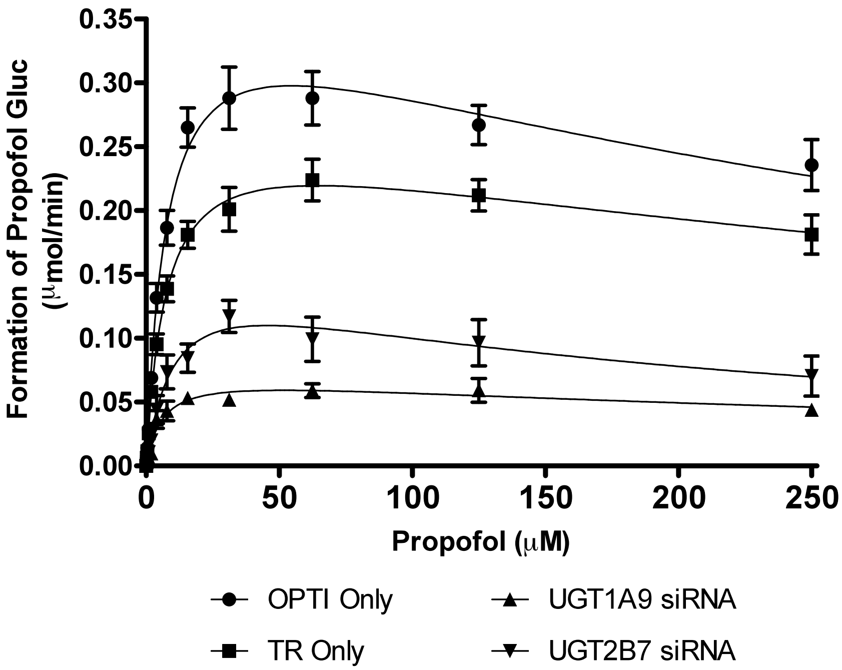

Figure 2-5. **Inhibition of propofol glucuronidation by siRNA in human hepatocytes.** Glucuronidation of the UGT1A9 probe propofol was significantly decreased with down regulation of UGT1A9 expression and to a lesser extent, with down regulation of UGT2B7 expression, compared to control incubations (OPTI Only, TR Only).

Discussion

Glucuronidation is one of the major pathways of metabolism for both endogenous compounds and xenobiotics, accounting for up to 35% of Phase II reactions (Ishii *et al.*, 2010a). With numerous pharmaceuticals such as propofol, irinotecan/SN-38 and opioids as well as many important endogenous compounds such as bilirubin, hormones, and bile acids known to undergo glucuronidation, *in vitro* systems capable of carrying out glucuronidation reactions have received a significant amount of attention in recent years (Foti and Fisher, 2012; Miners *et al.*, 2004).

The systems currently used for studying glucuronidation *in vitro* include tissue fractions such as human liver microsomes (HLMs) or S9 fractions, fresh or cryopreserved hepatocytes, and recombinant UGT enzymes. Human liver microsomes are generally considered the easiest to utilize, and contributions from Phase I and Phase II metabolic enzymes can easily be determined by the selective addition of the necessary cofactors for each pathway (Fisher *et al.*, 2002). Human hepatocytes are the most physiologically relevant *in vitro* system in which to study glucuronidation activity and generally result in the most accurate prediction of *in vivo* glucuronidation parameters from *in vitro* data (Engtrakul *et al.*, 2005; Soars *et al.*, 2002). Cellular systems that over-express one or multiple UGTs of interest have also been used to study glucuronidation phenomena (Coffman *et al.*, 1995; Fujiwara *et al.*, 2007; Nakajima *et al.*, 2007). Not only can the artificial environments of recombinant systems result in expression levels of the UGTs that may differ from native cells, but also the enzymatic contribution of each UGT isoform is very difficult to isolate, since the interactions vary depending on UGT isoform, substrate, and expression ratio. Additionally, post-translational modifications to the UGTs, such as phosphorylation and N-glycosylation, that have been shown to impact activity, may not occur in cell expression systems (Ishii *et al.*, 2010a; Miners *et al.*, 2006). Several studies have also demonstrated that variation in lipid composition between preparation and source of synthesis as well as general membrane circumstances contribute to *in vitro* intrinsic clearances that severely under-predict *in vivo* hepatic clearance (Fujiwara *et al.*, 2010; Miners *et al.*, 2004; Soars *et al.*, 2002). Finally, insect-expressed systems, such as Supersomes, while being a very commonly used phenotyping tool and perhaps the simplest system in which to study a single UGT isoform, lack the potential to exhibit the heterodimeric protein interactions that the more complex systems are capable of exhibiting.

With increasing evidence confirming discrepancies between recombinant systems and whole cell systems, propofol was chosen as a model compound with which to examine potential changes in glucuronidation activity due to protein interactions. While propofol has previously been shown to be a selective substrate for UGT1A9 (Court, 2005), recent data has indicated the importance of phenotyping compounds at therapeutically relevant concentrations (VandenBrink *et al.*, 2012). As such, a phenotyping assessment of propofol glucuronidation was carried out using concentrations that encompassed the peak plasma concentrations of propofol observed *in vivo* (Brunton *et al.*, 2006). At the concentrations tested, propofol was selectively glucuronidated by UGT1A9 with only a minor contribution from UGT1A6, indicating that it was an appropriate choice of model substrates with which to examine UGT activity in more complex *in vitro* systems.

To date, the use of siRNA to examine drug metabolizing enzyme activity is fairly limited. The technology is more commonly used to target the expression of over expressed molecular targets in cancer therapy or to discern the importance of an enzymatic pathway in an *in vitro* pharmacology assay (Rao *et al.*, 2009). The use of siRNA to study glucuronidation has been previously reported in both HeLa cells as well as in a Caco-2 cell system (Jiang *et al.*, 2012; Liu *et al.*, 2007). Upon down regulation of UGT1A6 expression in Caco-2 cells, a significant decrease in the glucuronidation of apigenin was observed, resulting in UGT1A6 being implicated as the primary UGT isoform involved in the glucuronidation of flavanoids in Caco-2 cells.

Previous investigations into the interactions between human UGT2B7 and UGT1A enzymes demonstrated that co-expression of UGT2B7 with UGT1A9 in HEK cells resulted in enhancement of propofol glucuronidation, in comparison with the UGT1A9 single expression system (Fujiwara *et al.*, 2010). Using siRNA inhibition in human hepatocytes, we have shown that down regulation of UGT2B7 expression also results in a decrease in the glucuronidation rate of propofol, presumably due to disruption of protein interactions between UGT1A9 and UGT2B7. While K_m values remained relatively unchanged when UGT2B7 was targeted, decreases in both the observed K_i and V_{max} values for propofol glucuronidation (fit to a substrate inhibition kinetic model) were observed as compared to the transfection reagent control. As K_i is the dissociation constant for an inhibitory enzyme-substrate complex, any modifications to protein structure or conformation could be expected to result in changes to the binding affinity for the inhibitory ligand in the UGT1A9 active site (Segel, 1975). As such, the more tightly bound inhibitor (as defined by the lower K_i value) may account for some of the observed decrease in UGT1A9 activity when UGT2B7 is silenced in the hepatocyte incubations. In addition, it has previously been shown that UGTs form catalytically active dimers through interactions of their amino-terminal domains, an interaction which serves to stabilize the resulting protein complex (Meech and Mackenzie, 1997). Conversely, the lack of such an interaction could conceivably serve to destabilize the UGT1A9 protein in hepatocytes pre-treated with UGT2B7 siRNA, resulting in the observed decrease in V_{max} values for propofol glucuronidation when UGT2B7 is down regulated. One final scenario that must be considered is that the incorporation of UGT2B7 siRNA may affect the translation or protein folding properties of UGT1A9, a phenomenon that we are currently investigating.

In summary, the data presented in this manuscript supports the utility of siRNA down regulation as an important process for evaluating UGT enzymology and suggests that UGT protein interactions are a physiologically relevant phenomena whose effects can be observed in human hepatocytes. The data also confirm previous interactions noted for UGT1A9 and UGT2B7 in over-expressed cellular systems. While single UGT expression systems will continue to be a useful tool both in characterizing UGTs and phenotyping drugs that undergo glucuronidation, the current data support the caution that should be taken in utilizing *in vitro* UGT systems in which heterodimeric protein interactions are unable to occur.

Chapter 2, in full, is a currently being prepared for submission in Archives of Biochemistry and Biophysics, 2012, Dickmann L., Tracy J., Tukey R.H., Wienkers L.C., and Foti R.S. I was the primary investigator and author of this paper.

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CHAPTER 3

The Regulatory Role of Oral Arsenic in *hUGT1* Mice

Introduction

Humanized Mice as a Sensor for Environmental Toxicant Exposure

The humanized mouse model that was utilized in this dissertation study was created by crossing heterozygous *TgUGT1* mice with heterozygous *Ugt1*-null mice. To generate the *TgUGT1* model, a BAC clone carrying the entire *hUGT1* locus was microinjected into the pronucleus of a fertilized mouse egg and subsequently transplanted into the oviduct of a pseudo-pregnant female. Breedings were then carried out to establish heterozygous *TgUGT1* founders. Characterization of the *TgUGT1* line confirmed that the human *UGT1* locus was successfully expressed, with all 9 *UGT1A* genes being regulated in a pattern concordant with the tissue specific expression profiles previously documented in humans (Chen *et al.*, 2005). The most dramatic expression of the *UGT1* locus and the *UGT1A1* gene was observed in the GI tract of the *TgUGT1* mice (Senekeo-Effenberger *et al.*, 2007; Chen *et al.*, 2005) and occurred in patterns similar to those seen in the human GI tract (Strassburg *et al.*, 2000; Strassburg *et al.*, 1999).

To be able to compare and contrast the role of human glucuronidation in disease and toxicity, the human *UGT1* locus needed to be expressed in an *Ugt1*-null background. The *Ugt1*-null model was created by inactivation of the entire murine *Ugt1* locus through targeted interruption of the *Ugt1* gene. This was accomplished by introducing a genetic lesion into conserved exon 4 (Nguyen *et al.*, 2008). Interruption of the conserved region of the locus leads to a complete absence of Ugt1a RNA and protein and the inability to metabolize bilirubin. Thus, deletion of the murine *Ugt1* gene leads to the accumulation of toxic levels of unconjugated bilirubin in the serum, evident early after birth by an orange tint of the skin, which eventually becomes lethal within 7 days after birth. Crossing the *TgUGT1* mice with the *Ugt1-null* mice results in the generation of the *Tg(UGT1)Ugt1*$^{-/-}$ mice, which represent the fully humanized *UGT1* (*hUGT1*) model (Fujiwara *et al.*, 2010b). *hUGT1* mice do not exhibit the developmental lethality observed in the *Ugt1*-null model that occurs in the absence of the *Ugt1a1* gene.

The regulatory region of the human *UGT1A1* gene is known as the PBREM and is where xenobiotic receptors, such as PXR, CAR, and PPARα, as well as the environmental sensors, such as AhR, and the antioxidant receptor Nrf2, can associate with DNA to induce transcription, thus playing a key role in the *in vivo* regulation of *UGT1A1*. Since numerous environmental toxicants can induce *UGT1A1* through association with these xenobiotic receptors and with UGT1A1 being the sole enzyme that can metabolize bilirubin, induction of *UGT1A1* directly leads to reduction of serum bilirubin levels. It has been shown that treatments of *hUGT1* mice with TCDD and PB, which are AhR and CAR agonists, dramatically induce *UGT1A1* expression, thus reducing their serum bilirubin levels (Fujiwara *et al.*, 2010b; Fujiwara *et al.*, 2012). Treatment of *hUGT1* mice with LPS can also induce *UGT1A1* expression and reduce serum bilirubin by generating oxidative stress (Fujiwara *et al.*, 2012). These findings indicate that *hUGT1* mice are responsive to toxic substances and have potential to serve as an *in vivo* biosensor for environmental toxicant exposure.

Environmental Arsenic Contamination

Environmental arsenic contamination is a significant problem worldwide, with drinking water being the most common source of exposure. This naturally occurring metalloid is ubiquitously distributed throughout Earth's crust, although typically complexed with pyrite. Under certain conditions it can dissociate and enter groundwater. Arsenic contamination of drinking water can also be attributed to certain anthropogenic sources, including agricultural and industrial practices (Kumagai and Sumi, 2007). Additionally, some crops and organisms tend to bioaccumulate arsenic and therefore regional and individual eating habits can greatly affect dietary arsenic intake (Bernstam and Nriagu, 2000). There is extensive epidemiological evidence linking arsenic exposure to various diseases (arteriosclerosis, cardiovascular disease, Blackfoot disease) (Navas-Acien *et al.*, 2005), numerous cancers (skin, bladder, lung, liver, prostate, kidney) (Kligerman and Tennant, 2007; Chen *et al.*, 1992), diabetes (Diaz-Villasenor *et al.*, 2007), and certain neurological ailments (Alzheimer's and Parkinson's) (Schmuck *et al.*, 2005; Vahidnia *et al.*, 2007). Arsenic absorption and toxicity greatly depends on the form in which it is ingested (Bernstam and Nriagu, 2000). Soluble inorganic species (sodium arsenite and sodium arsenate) have been determined as most readily absorbed from the GI tract, with typical absorption rates being 40–100% of the ingested amount (Pontius *et al.*, 1994). The predominant form of arsenic found in drinking water is arsenate (As^{5+}), but it is readily reduced *in vivo* to the more toxic species (As^{3+}) by either glutathione or arsenate reductase (Bode and Dong, 2002). Subsequent elimination of the trivalent species from the body occurs through reduction, methylation, and glutathione conjugation, to yield polar metabolites that are substrates for transporters (Kumagai and Sumi, 2007). However, very little is known about the effects of arsenic exposure on human UGT1A1 and the molecular mechanisms involved in UGT regulation by arsenic. This is of great concern, considering how prominent arsenic exposure has recently become in the news. Scientific articles and influential news websites have been bringing to light the presence of high levels of arsenic in many commonly consumed and everyday items, including apple and grape juice (Cohen, 2011), chicken-feed (Fairbrother, 2012) and many baby formulas, and rice cereals made from contaminated rice flour (Carroll, 2012). As of September 20, 2012, levels of arsenic in rice have skyrocketed, ultimately urging the FDA to define standards to protect consumers (Garber, 2012). Moreover, a FoxNews.com article from June 2012 has discussed the EPA's decision to decrease the arsenic standard for drinking water from 10 ppb to 2 -3 ppb, as 10 ppb has just been shown to stimulate adverse health effects in pregnant and lactating female mice as well as their offspring (Kozul-Horvath *et al.*, 2012; Grush, 2012). The shocking and on-going prevalence of contamination begs the question: what effect does arsenic have on important biological processes, such as xenobiotic metabolism?

Arsenic is known to alter multiple cellular pathways, including expression of growth factors, suppression of cell cycle checkpoint proteins, promotion of and resistance to apoptosis, inhibition of DNA repair, alterations in DNA methylation, decreased immunosurveillance, and increased oxidative stress, by disturbing the pro/antioxidant balance (Flora, 2011). This extensive list of potential targets explains the wide range of resulting disease manifestations due to exposure, such as carcinogenicity, genotoxicity, diabetes, cardiovascular, and nervous systems disorders. However, in 1998, Hu *et al.* analyzed the dose-response for arsenic inhibition of several purified human DNA repair enzymes, including DNA polymerase beta, DNA ligase I, and DNA ligase III. It was observed that most enzymes, even those with critical thiol groups, were surprisingly insensitive to arsenite and that only a few sensitive enzymes were responsible for arsenic-induced cellular toxicity (Hu *et al.*, 1998). In addition, arsenic concentrations required to deactivate certain enzymes have been determined to be much lower than what is required for direct binding to thiol groups. This was documented by Samikkannu *et al*. in 2003, who observed inactivation of pyruvate dehydrogenase activity by As_2O_3 to be about 38 times more potent in a human leukemia cell line (HL60) than in pure enzyme preparation, suggesting that while As_2O_3 inactivates activity in pure enzyme preparation by binding to the dithiols, this same mechanism may be different in HL60 cells. The IC50 values for As_2O_3 and phenylarsine oxide (PAO) to decrease the cellular vicinal thiol content of HL60 cells were determined to be 80.0 μM and 1.9 μM, respectively, confirming As_2O_3 as a weak thiol reacting agent in comparison to PAO. Dithiol compounds were also capable of suppressing PAO inhibition of pyruvate dehydrogenase activity but not for As_2O_3, and antioxidants suppressed As_2O_3 inhibition of pyruvate dehydrogenase activity but not for PAO. Lastly, As_2O_3 increased cellular H_2O_2 production in HL60 cells, while PAO did not, Fenton metal chelators decreased and Fenton metals increased As_2O_3 inhibition of pyruvate dehydrogenase activity, and HL60 treatment with H_2O_2 and Fenton metals decreased the pyruvate dehydrogenase activity, ultimately implicating inactivation of pyruvate dehydrogenase via ROS-mediated mechanisms (Samikkannu *et al.*, 2003). These findings suggest that arsenic-induced chromosomal damage and inhibition of DNA repair are not the result of direct enzyme inhibition, but instead an indirect effect caused by exposure-induced changes in cellular redox levels or alterations in signal transduction pathways with subsequent changes in gene expression (Hu *et al.*, 1998; Bernstam and Nriagu, 2000).

Arsenic-Induced Generation of Reactive Oxygen Species

Certain metals, including arsenic, have been reported to be potent carcinogenic or toxic agents in both humans and animals. While it is known that arsenic causes DNA damage and lipid peroxide formation *in vitro* and *in vivo*, the underlying molecular mechanisms of its toxicity have eluded scientists. However, what is well established is that reactive oxygen species (ROS), such as superoxide anion (O^{2-}), hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^{1}O_2$), and hydrogen peroxide (H_2O_2), generated by arsenic exposure have a wide potential for causing cellular injury and numerous toxic effects (Menghini, 1988; Ercal *et al.*, 2001), and thus may at least partly be contributing to the process of arsenic-induced toxicity (Sugiyama, 1994).

Aerobic organisms have evolved a sophisticated regulatory system by which to defend against oxidative damage. Overproduction or accumulation of oxygen free radicals in cells, termed oxidative stress, can damage DNA, proteins, lipids and other molecules (Cerutti, 1985; Lee and Ho, 1995). It therefore follows that these oxidative processes could potentially play important roles in arsenic-induced damage (Klein *et al.*, 1991; Sugiyama, 1994). The first oxidative stress theory, fueled by the documentation of arsenic-induced free radical formation, was presented in 1989 by

Yamanaka *et al*. *In vitro* experiments revealed diminished trivalent dimethylarsine induced DNA strand breaks upon addition of superoxide dismutase (SOD) and catalase (CAT), suggesting that reactive oxygen produced by exposure was involved in the induction of DNA damage (Yamanaka *et al.*, 1990; Yamanaka *et al.*, 1989; Kitchin, 2001). It was later confirmed that toxicity resulted from molecular oxygen reacting with dimethylarsine to form the dimethylarsinic radical and superoxide anion, and subsequent addition of another molecule of molecular oxygen formed the dimethylarsinic peroxyl radical that is detrimental to cells. Generation of O^{2-} and H_2O_2 post arsenic exposure has been demonstrated in various human cell lines, including human vascular smooth muscle cells (Lynn *et al.*, 2000), human–hamster hybrid cells (Liu *et al.*, 2001), and vascular endothelial cells (Barchowsky *et al.*, 1999), although HEL30 (Corsini *et al.*, 1999), NB4 (Jing *et al.*, 1999), and CHOK1 (Wang *et al.*, 1996) have only shown induction of H_2O_2. Arsenic-induced hydroxyl radical generation has also been reported in the rat brain (Garcia-Chavez *et al.*, 2003). While there is a plethora of direct evidence of arsenic induced ROS, reported indirect evidence also demonstrates arsenic's effect on the cellular antioxidant defense system. Low molecular weight donors of SH groups and enzymes, which can catalyze the reduction of SH groups in proteins and detoxify pro-oxidants by conjugation with glutathione, are part of the system that helps regulate the redox status of cellular thiols and protect SH-containing proteins from excessive oxidation (Samikkannu *et al.*, 2003). Arsenic affects many oxygen-radical-scavenging enzymes called antioxidant enzymes, such as SOD, CAT, glutathione peroxidase (GPx), GST, and glutathione reductase (Flora, 2011). Increases in protective enzymes, CAT and SOD, have been shown to suppress arsenic-induced sister chromatid exchanges in human lymphocytes (Nordenson and Beckman, 1991). Arsenic treatment of an X-ray sensitive Chinese hamster ovary cell line (XRS-5), which is sensitive to several free-radical generating agents, including H_2O_2, exhibited arsenic hypersensitivity, thus implicating that the genotoxicity of arsenite was mediated by ROS (Wang and Huang, 1994). In 1995, Lee *et al*. studied sodium arsenite induced oxidative stress in human fibroblasts. They observed increased formation of fluorescent dichlorofluorescein (DCF) by oxidation of the nonfluorescent form, which was also inhibited by a radical scavenger, confirming arsenic's ability to alter cellular redox levels. Treatment of human fibroblasts also revealed significantly increased heme oxygenase (HO-1) activity, ferritin level, glutathione levels and SOD activity, slightly decreased GPx activity, significantly decreased CAT activity, and no effect on glucose-6-phosphate dehydrogenase (G6PD) activity (Lee and Ho, 1995). These results demonstrate the effect of sodium arsenite on cellular antioxidant activities that can lead to enhanced oxidative stress in HFW. In 2000, Maiti and Chattetjee also reported tissue-specific protective mechanisms against arsenic exposure in male Wistar rats. The kidneys were observed to be more vulnerable to arsenic in rats exposed to 3.33mg/kg sodium arsenite per day for 14 days and displayed significant increases in lipid peroxidation and decreased SOD and CAT activities. Lipid peroxidation and SOD activity in liver remained unchanged post treatment due to significantly increased glutathione levels and protection of activities of glutathione reductase and GST from arsenite-induced oxidative damage by some antioxidant components, such as glutathione, GST, and G6PD (Maiti and Chatterjee, 2000). These findings clearly support that arsenic exposure results in ROS generation in various cellular systems. Since 1989, there has been increasing evidence supporting oxidative stress theory and greater scientific acceptance of this as a significant mode of action. As such, oxidative stress is currently the most widely accepted and studied mechanism of arsenic toxicity (Ercal *et al.*, 2001).

Arsenic Can Modulate Xenobiotic Transcriptional Activation

The *UGT1A1* gene contains a series of xenobiotic receptor enhancer sequences that recognize LXRα (unpublished observations), AhR (Yueh *et al.*, 2003; Bonzo *et al.*, 2007), CAR (Sugatani *et al.*, 2001; Sugatani *et al.*, 2005b), PXR (Xie *et al.*, 2003), GR (Sugatani *et al.*, 2005a), PPARα (Senekeo-Effenberger *et al.*, 2007), and Nrf2 (Yueh and Tukey, 2007) and is one of the few genes that can be independently regulated by activation of any one of these transcriptional factors. Therefore, there are numerous xenobiotics and environmental toxicants that can induce *UGT1A1* gene expression. However, very little is known with regards to how arsenic modulates *UGT* gene expression. Evidence of transcriptional regulation of other DMEs by arsenic can help provide guidance for elucidating mechanisms involved in arsenic-induced *UGT* expression.

There is emerging evidence that heavy metals regulate CYP1A1 activity by enhancing AhR in a metal and species-dependent manner (Wu *et al.*, 2009). Previous reports have confirmed arsenic's ability to alter CYP activities. Increases in B[a]P metabolites and BPDE adducts were observed in animals co-exposed to B[a]P and arsenic (Evans *et al.*, 2004). Since CYP1 enzymes, specifically CYP1A1, are known to be key in B[a]P metabolism (Shimada *et al.*, 2002), Wu *et al*. postulated in 2009 that these observed increases in B[a]P metabolism with arsenic exposure could be due to enhanced CYP1A1 expression and activity (Wu *et al.*, 2009). They were able to successfully demonstrate increased CYP1A1 mRNA expression in a human lung adenocarcinoma cell line treated with arsenic, as well as increased CYP1A1 expression and activity in lung tissues of arsenic-exposed mice. Elevated *CYP1A1* expression was also effectively blocked with an AhR antagonist, indicating that arsenic-induced CYP1A1 expression and activity occurred via AhR activation. In addition, arsenic-induced AhR activation and *CYP1A1* expression were both increased with pro-oxidant treatment and suppressed by antioxidants, such as *N*-acetylcysteine (NAC) and catalase. These findings illustrate that arsenic exposure enhances CYP1A1 expression and activity through AhR activation (Wu *et al.*, 2009). With *UGT1A1* being similarly regulated by AhR, it can therefore follow that arsenic may be capable of regulating *UGT1A1* gene expression.

The xenobiotic nuclear receptors PXR and CAR have been well established as xenobiotic sensors (Xie *et al.*, 2003; Blumberg *et al.*, 1998; Xie *et al.*, 2000). Arsenic-treated Tg-*CYP3A4* mice were shown to exhibit elevated hepatic CYP expression levels as well as increased PXR and RXR mRNA, which heterodimerize to regulate gene expression of several Phase I and II DMEs (Falkner *et al.*, 2001). However, when Noreault *et al.* investigated whether arsenite decreased CYP3A4 induction by PB or rifampicin, which is capable of inducing CYP3A4 either through CAR or PXR (Goodwin *et al.*, 2002a; Goodwin *et al.*, 2002b), the authors observed that treatment of human hepatocytes with arsenite in the presence of CYP3A4 inducers, PB or rifampicin caused major decreases in CYP3A4 mRNA, protein, and activity. CYP3A4 in untreated cells was also decreased following arsenite treatment. Since transcription of CYP3A4 is primarily regulated by heterodimers of RXR and PXR, the authors investigated the affect of arsenite on PXR and RXR expression. Arsenite failed to affect expression of PXR, yet caused marked decreases in PXR responsiveness to rifampicin. In addition, arsenite caused a large decrease in nuclear RXR protein and to a lesser extent in RXR mRNA. These findings suggest that arsenite inhibits both untreated and induced CYP3A4 transcription in primary human hepatocytes by decreasing the activity of PXR, as well as RXR expression (Noreault *et al.*, 2005). Since CAR and PXR are similar in that they form heterodimers with RXR, arsenic exposure that alters RXR expression can subsequently affect transcriptional activation of target genes. It can therefore be hypothesized that since the *UGT1A1* gene contains CAR and PXR enhancer sequences, *UGT1A1* gene expression can also be impacted by arsenic exposure.

Nrf2 is a redox-sensitive transcription factor that regulates the expression of genes encoding antioxidants and xenobiotic detoxification enzymes for cytoprotection against oxidative stress and xenobiotics (Singh *et al.*, 2006; Itoh *et al.*, 1995; Ishii *et al.*, 2000). In response to oxidative stress or direct xenobiotic stimulation, Nrf2 dissociates from Keap1 and enters the nucleus where it functions as a strong transcriptional activator. Arsenic has been shown to activate Nrf2, which in turn regulates the expression of genes encoding for many antioxidative response enzymes (Pi *et al.*, 2003). Upregulation of Nrf2 has been detected in an immortalized, non-tumorigenic human keratinocyte cell line (HaCaT) that was continuously exposed to environmentally relevant levels of inorganic arsenite for 28 weeks (Pi *et al.*, 2008). It has also been demonstrated that As^{3+} can activate Nrf2 by directly binding Keap1 (He *et al.*, 2006; He and Ma, 2010). Most importantly, our lab has previously documented that *UGT1A1* in *hUGT1* mice is regulated by the cellular antioxidant sensor Nrf2 following activation in response to ROS, and Nrf2 subsequently targets the antioxidant response element (ARE) in the promoter region of the *UGT1A1* gene (Yueh and Tukey, 2007). Since regulation of the *UGT1A1* gene by Nrf2 is sensitive to changes in ROS, Nrf2 may control those events that regulate expression of intestinal *UGT1A1* in response to arsenic exposure.

Arsenic Impacts the NF-κB/IKK Signaling Pathway

Arsenic causes oxidative stress, which in turn, activates signaling pathways involved in the regulation of early response genes, such as NF-κB, to respond to alterations in the intracellular redox status (Felix *et al.*, 2005; Kapahi *et al.*, 2000). Stress response transcription factors are particularly important in these early responses, as they regulate the expression of a variety of downstream target genes involved in cellular antioxidant defense mechanisms (Kapahi *et al.*, 2000). However, the molecular mechanisms by which arsenic affects the NF-κB pathway have yet to be identified, since controversial studies have shown that arsenic-induced changes in gene expression can occur by either suppression (Jeong *et al.*, 2004) or activation (Barchowsky *et al.*, 1996; Kitamura and Hiramatsu, 2010) of NF-κB, leading to the downregulation or upregulation of downstream genes, respectively. Arsenic-induced generation of ROS has been shown to activate the NF-κB (Baldwin, 2001) and NF-κB dependent gene transcription pathways, including NO, AP-1, p53, and p21 (Buzard and Kasprzak, 2000). Population studies of newborns whose mothers were exposed to varying levels of arsenic revealed differential expression of stress response and cell cycle regulatory genes (Fry *et al.*, 2007). In addition, it is widely known that NF-κB regulates genes encoding for cytokines, cytokine receptors, cell adhesion molecules, and growth regulators (Baldwin, 2001). As such, these findings implicate several avenues by which arsenic can modify cell cycle control, proliferation, and cellular morphology.

IκB phosphorylation and degradation has been identified as the most likely signaling step affected by oxidative stress (Li and Karin, 1999). Arsenic has been shown to exert its biological effects by reacting with IKK's free thiol to inhibit NF-κB signaling (Roussel and Barchowsky, 2000). Blocked IKK results in limited degradation of IκB and decreased NF-κB activation (Kapahi *et al.*, 2000). In contrast, very early research initially proposed ROS-induced oxidative stress as a universal mechanism for NF-κB activation by diverse agents, including arsenic (Schreck *et al.*, 1992b). Simply exposing various cell lines to H_2O_2 has provided direct evidence that ROS are capable of regulating NF-κB (Sen and Packer, 1996; Schreck *et al.*, 1991; Manna *et al.*, 1998; Li and Karin, 1999). However, other researchers were unable to detect NF-κB activation by H_2O_2 in Hela, HEK293, fibroblast, or Jurkat T cells, implicating H_2O_2-induced NF-κB activation as highly cell specific and that H_2O_2 is not a likely mediator of activation (Anderson *et al.*, 1994). Nonetheless, there still is extensive evidence that implicates reactive oxidative intermediates as NF-κB-activating signals, including the inhibition of NF-κB activation by various antioxidants and by overexpression of antioxidant enzymes (Schreck *et al.*, 1992a). Additionally, activation of NF-κB by arsenic trioxide (As_2O_3) at non-cytotoxic levels has been demonstrated in studies from several groups via gel shift assays by which to monitor activation and nuclear translocation of NF-κB and NF-κB-dependent reporter gene assays to indicate NF-κB activity (Kaltreider *et al.*, 1999; Chen and Shi, 2002a). It has also been recently demonstrated through Chen and Shi's research that arsenic is capable of activating NF-κB through the MAPKs. Their studies using wild-type and *stress-activated protein kinase (SAPK)/ERK kinase* (*sek1)* gene knockout mouse embryo stem cells suggested that As^{3+}-induced NF-κB occurred through a signaling pathway that involved SEK1 (MKK4)-JNK (Chen and Shi, 2002b) and that neither ERK nor p38 was required for As^{3+}-induced NF-κB activation. In contrast, blocked NF-κB activation was observed due to inhibition of ERK with either the specific inhibitor, PD98059, or in cells deficient of *Erk*, showing that ERK is required for NF-κB activation in mouse skin epidermal JB6 cells (Huang *et al.*, 2001). These findings implicate the potential for cross talk between the MAPKs and NF-κB/IKK signaling pathways in response to arsenic-induced oxidative stress, which adds to the complication of elucidating the mechanisms involved in arsenic exposure.

Previous work in our lab has confirmed activation of NF-κB/IKK signaling in *UGT1A1* induction with stress-inducing agents, LPS and cadmium (Fujiwara *et al.*, 2012). Since humanized neonatal *UGT1* mice treated with these NF-κB activators exhibited marked intestinal *UGT1A1* induction that resulted in significant decreases in bilirubin levels, it is reasonable to consider that other metal contaminants, such as arsenic, can regulate *UGT1A1* in a similar fashion (Fujiwara *et al.*, 2012). The role of arsenic in regulating *UGT1A1* expression, specifically in small intestine, via the NF-κB/IKK pathway has yet to be explained.

Arsenic Influences the MAPK Signaling Pathway

The MAPKs transmit extracellular signals to induce expression of various genes that mediate cell apoptosis, differentiation, proliferation, and transformation. Of the three major classes of MAPKs, ERKs are mainly involved in growth factor-induced cell differentiation, proliferation, and transformation signaling, while JNK and p38 mediate cytokine and numerous stress-induced cell response, cell growth arrest, and apoptosis (Qian *et al.*, 2003). Several environmental contaminants have been shown to induce apoptosis, or programmed cell death, in Hepa1c1c7 cells (Lei *et al.*, 1998), Daudi human B cells (Salas and Burchiel, 1998), human ectocervical cells (Rorke *et al.*, 1998), and A20.1 murine B-cells (Burchiel *et al.*, 1993). In a study assessing particulate matter (PM)-induced apoptosis in RAW 264.7 macrophage cells, carbon black particles containing B[a]P were shown to stimulate the release of TNF-α, a known MAPK initiator. Furthermore, cells treated with a MAPK kinase inhibitor did not undergo apoptosis, indicating that the MAPK pathway plays an important role in regulating PM- and TNF-α-induced apoptosis in this cell culture model (Chin *et al.*, 1998). In addition, B[a]P has been shown to activate JNK1 and induce caspase-3-mediated apoptosis in Hepa1c1c7 cells as well as upregulate α-PAK-exchange factor, which is upstream of JNK, in a manner concordant with activation of JNK1 in both HEK293 and HeLa cells (Yoshii *et al.*, 2001).

The cell deals with the onset of oxidative stress by programming the regulation of genes to protect against the potential hazards of ROS. As such, the JNK MAPKs have been identified as an important family of stress activated protein kinases that are regulated through complex signal transduction cascades and in turn phosphorylate and regulate the activity of various transcriptional factors. The JNK subfamily of MAPKs is activated in response to a variety of extracellular stimuli, including mitogens, pro-inflammatory stimuli, nutrients, and environmental stimuli or changes that induce oxidative stress (Davis, 2000; Ip and Davis, 1998). JNK was originally identified by its ability to bind (Kyriakis and Avruch, 1990; Adler *et al.*, 1992; Hibi *et al.*, 1993) and phosphorylate c-Jun (Pulverer *et al.*, 1991), an important component of transcription factor AP-1, thus enhancing its transcriptional activity. *In vitro* studies revealed that treatment of cells with cytokines, such as TNF and IL-1, as well as exposure to various environmental stresses, including osmotic, redox, and radiation, all resulted in JNK activation (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Ip and Davis, 1998). Phosphorylation of the AP-1 subunits is directly linked to apoptosis (Karin *et al.*, 1997; Colotta *et al.*, 1992). Apoptotic stimuli by DNA damaging agents ultimately lead to mitochondrial disruption and the release of death-promoting factors, such as cytochrome c. Previous work has demonstrated that apoptosis and mitochondrial damage are controlled in part by the Bcl-2 family of proteins, which can inhibit (i.e., Bcl-2 and Bcl-x_L) and or promote (i.e., Bax and Bak) cytochrome c release. Cytochrome c release initiates a self-amplifying cascade of proteolysis among cytosolic caspases that ends in cell death (Roth and Reed, 2002). Early apoptotic events in response to DNA damage lead to the promotion of DNA repair and the activation of poly(ADP-ribose) polymerase-1 (PARP-1) (Soldani and Scovassi, 2002), which transfers ADP-ribose to other nuclear proteins involved in DNA repair and transcription (D'Amours *et al.*, 1999). PARP-1 activation is also thought to be a cell death mediator, although the actual mechanism of PARP-1 induced cell death remains unknown. It has been speculated that utilization of nicotinamide adenine dinucleotide (NAD^+) in PARP-1-initiated ADP-ribosylation leads to depletion of NAD^+ stores. This eventually leads to disrupted mitochondrial function, which stimulates cytochrome c release and caspase activation (Chiarugi and Moskowitz, 2002). With PARP-1 being caspase substrate, it is targeted for cleavage and inactivated during apoptosis, possibly disrupting PARP-1 activity and subsequent DNA repair, which ultimately allows for the promotion of nuclear disintegration by endonucleases (Chen *et al.*, 2003c). Studies performed by Salas and Burchiel in which Daudi human B cells were treated with B[a]P and B[a]P-7,8-dihydrodiol revealed DNA fragmentation, decreased Bcl-2, and cleavage of PARP-1, further providing evidence that DNA damage may be the leading initiator of apoptosis (Salas and Burchiel, 1998).

Increasing evidence has revealed that arsenic differentially activates these MAPKs in a variety of human cell lines (Bode and Dong, 2002). Qu and colleagues generated a malignant transformed rat liver TRL1215 cell line by chronic arsenic treatment. Upon challenging the transformed cells with arsenic to monitor changes in apoptosis, they found that the transformed cells were resistant to arsenic-induced apoptosis in comparison to the control. Resistance could be removed by treating cells with a JNK specific activator, indicating arsenic suppression of JNK in transformed cells (Qu *et al.*, 2002). Subsequent studies demonstrated inhibition of JNK activity but increased ERK and p38 activities in these transformed cells. Substantial increases of two anti-apoptotic proteins, Bcl-x_L and Bcl-2, and significant decreases in expression of pro-apoptotic protein, Bax, were also observed. These findings show that arsenic treatment disrupts the JNK signaling pathway, which leads to inhibition of apoptosis. However, JNK has also been documented as one of several key stress response protein kinases that is activated in response arsenic (Cavigelli *et al.*, 1996; Chen *et al.*, 2003a) and is a central mediator of cellular apoptosis (Davis, 2000; Sabapathy *et al.*, 2001; Kamata *et al.*, 2005) following activation in response to pro-oxidants. Activation of ROS and JNK in response to toxic challenges can be identified by the subsequent induction of *c-jun*, *c-fos*, and *junB* gene expression (Cavigelli *et al.*, 1996). Arsenic is a potent inducer of oxidative stress, and has been shown to generate ROS, while inducing JNK1 and JNK2, as well as AP-1 activity. *In vitro* experiments by Cavigelli *et al*. demonstrated trivalent arsenic as a potent stimulator of AP-1 transcriptional activity and an efficient inducer of *c-fos* and *c-jun* gene expression. Induction of *c-jun* and *c-fos* transcription by trivalent arsenic additionally corresponded with increased activation of JNK and p38, which phosphorylate transcription factors that activate these immediate early genes (Cavigelli *et al.*, 1996). Interestingly, prolonged JNK activation, which promotes apoptosis stimulated by activation of mitochondrial pro-apoptotic proteins has been linked to tissue damage (Guma *et al.*, 2009), obesity (Hirosumi *et al.*, 2002), and cancer (Sakurai *et al.*, 2006), while JNK inhibition prevents mitochondrial apoptosis, oxidative stress, and lowers development of chemical-induced cancers (Shibata *et al.*, 2008). Therefore, the relationship between JNK and ROS may play an important regulatory role in control of intestinal *UGT1A1* gene expression following arsenic exposure. Regardless of the contrasting nature of the results, these studies reflect the ability of arsenic exposure to differentially affect the individual MAPKs to produce opposing effects on cell growth and differentiation (Qian *et al.*, 2003). Since these findings implicate the MAPK signaling pathways as important regulators of homeostasis during times of cellular stress, it is possible that DMEs may be regulated by the MAPKs to help mediate the response to various exposures. With previous evidence indicating that MAPK pathways impact *UGT1A1*, signaling alterations due to arsenic exposure can therefore potentially have significant effects on *UGT* gene expression.

Cell Cycle Dysregulation and Morphological Changes Occur with Arsenic Exposure

Exposure to the environmental toxicant arsenic is reported to produce a variety of effects, including disruption of signal transduction pathways, cellular proliferation, and apoptosis. It is therefore possible that arsenite may not have specific targets but instead extremely broad effects. Altered cell cycle regulation and morphological changes may contribute to the observed alterations in gene regulation and expression with arsenic exposure (Yancy *et al.*, 2005).

The events involved with the replication and partition of chromosomes are common to all cell cycles, as newly divided cells must receive a full genome complement to survive. In 1953, with the discovery of DNA's double helical structure by Watson and Crick, it was noted that the specific pairing within the double helix suggested a possible copying mechanism for genetic material (Watson and Crick, 1953). Around the same time, microspectrophotometric (Swift, 1950) and autoradiographic (Howard and Pelc, 1953) studies in eukaryotic cells revealed that DNA replication happened during a restricted portion of interphase termed the S phase. This work eventually led to the eukaryotic cell cycle being divided G1, S, G2, and M (mitosis) phase (Mitchison, 1971). Multiple signals exist to regulate the onset of cell cycle phases and ensure proper cell growth and tissue homeostasis (Nurse, 2000). In a variety of eukaryotic cells, the orderly progression of dividing cells through the cell cycle is controlled by a series of cell cycle regulatory proteins, mainly cyclins, that exert their function by binding to and activating a number of specific cyclin-dependent kinases (CDKs). CDK activity is further regulated by kinases and phosphatases that phosphorylate and dephosphorylate CDKs, respectively. In addition, several specific CDK inhibitory proteins and cell cycle checkpoint proteins, such as Gadd45, have been identified (Sheikh *et al.*, 2000).

Most cells within a normal tissue may be forced out of active cell cycling and into the quiescent (G_0) state, from which they may reenter cell cycling under some future circumstances. In mature tissues, cells may be induced to terminal differentiation by relinquishing their proliferative or cell cycling potential (Chen and Shi, 2002a). Emerging evidence has demonstrated that a variety of stress inducers, including DNA-damaging agents, can activate checkpoint function of cells, leading to cell cycle arrest. There are several checkpoints, existing in G1/S phase, G2 phase, and M phase of cell cycle, which have surveillance systems to detect specific DNA structures indicative of damage or ongoing repair and replication (Chen and Shi, 2002a). Microarray analysis, RT-PCR, and immunohistochemistry have shown that arsenic increases expression of cyclin D1 (Chen *et al.*, 2004), a key regulatory protein in cell proliferation and tumor formation (Vogt and Rossman, 2001). Increased expression of cell cycle regulatory genes (cyclin G1, PKC delta) is also observed in normal human epidermal keratinocytes treated with non-toxic doses of As^{3+} (Hamadeh *et al.*, 2002). Experiments carried out by Jia *et al.* to investigate the influence of arsenic on expression of cyclin related genes in HL60 cells revealed that 82 genes (including cyclin B1, PCNA, and insulin-like growth factor binding protein) exhibited changes in expression in response to As_2O_3 treatment (Jia *et al.*, 2003). It was subsequently hypothesized that cyclin B1, PCNA, and insulin like growth factor binding protein might play a significant role in the induction of apoptosis by As_2O_3. *In vitro* arsenic exposure also caused G2/M arrest in human fibroblast cells, which is evident from the delayed upregulation of cell cycle regulatory genes, such as CCNG1, CCNF, CDC2, CDC25A, and CDKN2A. Other cell cycle control genes, such as CCNB1, CLK1, and RAD9, were also enhanced by arsenite treatment. Ultimately, activation of both positive and negative regulators of cell cycle control by arsenite indicates that arsenite treatment causes cell cycle dysregulation (Yih *et al.*, 2002).

Toxic metals, such as arsenic, have also been identified as protein phosphatase inhibitors (Cavigelli *et al.*, 1996). Therefore, with phosphatases required for progression from G1/S phase and G2/M phase, metal-induced inhibition of phosphatase activity can delay cell cycle transitions. In addition, arsenic has been shown to alter cell cycle control, causing G1 and/or G2/M phase arrest with subsequent programmed cell death. The *in vitro* effect of As_2O_3 on proliferation, cell cycle regulation, and apoptosis was observed in human myeloma cell lines. As_2O_3

significantly inhibited proliferation in all of the myeloma cell lines via cell cycle arrest in association with induction of p21 and apoptosis (Park *et al.*, 2000). Since p53 plays a guarding role in maintaining genome integrity and accuracy of chromosome segregation, the mechanistic effects of arsenite on p53 activation were analyzed in human fibroblasts. Arsenite-induced DNA strand breaks were confirmed via comet assay and cell cycle retardation, and G2-M arrest was observed in 5-bromo-2'-deoxyuridine (BrdU) pulse-labeled cells by flow cytometry. Significant induction of p53 and its downstream protein p21 (Yih and Lee, 2000), as demonstrated by immunoblotting and immunofluorescence, associated with G1 and G2/M arrest and apoptosis suggests that modulations in cell cycle control by arsenite exposure may impact expression of other cellular components. However, studies by Bonzo *et al.* demonstrated that while arsenite interrupted cell cycle control by initiating G2/M arrest, arsenite-induced inhibition of AhR-mediated TCDD-inducible expression of *CYP1A1* occurred independent of cell cycle control (Bonzo *et al.*, 2005). Although cell cycle control was previously shown to influence *CYP1A1* expression through mechanisms involving AhR and other independent pathways, experiments using a range of arsenite concentrations from subcytotoxic to levels that cause cellular arrest and apoptosis demonstrated that inhibition of CYP1A1 induction occurred at concentrations of arsenite well below those that initiated cell cycle arrest and apoptosis. Investigation of arsenite effects on human *CYP1A1* gene expression in primary hepatocytes from transgenic mice in combination with polymerase II recruitment analysis has also provided additional evidence that arsenite inhibits *CYP1A1* expression by modifying transcription independent of cell cycle control

(Bonzo *et al.*, 2005).

Morphological alterations in arsenic-exposed cells have implicated underlying disruption of cytoskeletal structural elements responsible for cellular integrity, shape, and locomotion. However, specifics of these resulting structural changes are still not understood (Bernstam and Nriagu, 2000). *In vitro* studies with sodium arsenite have demonstrated similar cytogenetic alterations in a variety of cell systems (Lee *et al.*, 1985a; Yih and Lee, 1999; Wang and Huang, 1994; Oya-Ohta *et al.*, 1996). In addition, arsenite-treated Syrian hamster embryo cells exhibited induced cytogenetic changes that were closely associated with induced morphological transformation (Oshimura and Barrett, 1986; Lee *et al.*, 1985b). Perturbation of spindle dynamics was also observed with arsenite exposure in cultured human cells, which resulted in chromosome malsegregation during mitosis (Yih *et al.*, 1997; Huang and Lee, 1998). Furthermore, Yancy *et al.* reported on the effects of sodium arsenite on focal adhesion in H9C2 myoblasts. Sublethal arsenite concentrations decreased cell migration, cell attachment, single cell-spreading area, and distribution and number of focal adhesions. Phospho-protein detection revealed that arsenite decreased both Tyr phosphorylation of focal adhesion kinase (FAK) as well as its auto-phosphorylation at Tyr397, a typical indicator of FAK activation (Parsons, 2003). Decreased Tyr397 phosphorylation of FAK subsequently led to a reduced phosphorylation of the adhesion-related protein, paxillin. This mechanism is essential for focal adhesion formation and important for signaling events of arsenic-induced toxicity (Yancy *et al.*, 2005; Liu and Waalkes, 2005). These findings suggest that the genotoxicity of arsenic exposure may be due to its ability to induce cytogenetic alterations and/or genetic instability that can subsequently affect signaling cascades and therefore gene expression patterns (Yih and Lee, 2000).

Increased proliferation of the endoplasmic reticulum (ER) has also been directly linked to the increased expression of DMEs, including the UGTs. In 1976, Banjo and Nemeth measured the UGT activities and the concentrations of ER in 5- and 11-day chick embryo liver during culture, with and without phenobarbital treatment. It was found that UGT and ER always increased in a constant ratio of 2.2 x 10^{-9} units of transferase activity per square micrometer of membrane, thus demonstrating that synthesis and degradation of UGTs are coupled with ER synthesis and degradation (Banjo and Nemeth, 1976). It therefore follows that xenobiotics and environmental contaminants that cause proliferation can potentially affect DME content, including the UGTs.

Experimental

Materials. Primers for quantitative real-time polymerase chain reaction (Q-PCR) were commercially synthesized at Integrated DNA Technologies, Inc (IDT, San Diego, CA). The mouse anti-human UGT1A1 antibody was a gift of Dr. Joseph K. Ritter (Virginia Commonwealth University, Medical College of Virginia, Richmond, VA). The mouse anti-PCNA antibody was obtained from BD Biosciences (Franklin Lakes, New Jersey). The following antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA): rabbit anti-p44/p42 MAPK (Erk1/2), rabbit anti-phospho-p44/42 MAPK (Erk1/2) and rabbit anti-phospho- SAPK/JNK. Mouse anti-JNK1/2 was purchased from BD Biosciences (Franklin Lakes, New Jersey). The primary rabbit anti-Ki-67 antibody was obtained from Genetex, Inc. (San Antonio, Texas). The secondary biotin goat anti-rabbit Ig antibody was purchased from BD Biosciences (Franklin Lakes, New Jersey). The DAB Substrate Kit for peroxidase was purchased from Vector Laboratories (Burlingame, CA). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

Animals and Treatments. Humanized *UGT1* mice (*Tg(UGT1A1*^{*28}*)Ugt1*$^{-/-}$) were developed previously in a C57BL/6 background (Fujiwara *et al.*, 2010b). To generate *hUGT1/Car*$^{-/-}$ mice, *hUGT1* mice were crossed with *Car*-null mice provided to our laboratory by Dr. Masahiko Negishi at the National Institute of Environmental Health Sciences (Ueda *et al.*, 2002). *Car* null mice were then crossed into the C57BL/6 background before breeding with *hUGT1* mice. *IKK-α*$^{F/F}$*/IKK-β*$^{F/F}$ and *Vil-Cre/IKK-α*$^{F/F}$*/IKK-β*$^{F/F}$ mice were previously generated in a C57BL/6 background (Chen *et al.*, 2003b). All animals were housed in plastic cages with hardwood chips for bedding in a 12-hour light, 12-hour dark cycle with water and food (#7912, Harlan-Teklad, Indianapolis, IN) ad libitum. 10 mg/kg metal contaminant dosages were prepared by dissolving the metal salts in water. The control vehicle was water. 12-day-old mice were treated orally and tissues were collected at 14 days after birth. For tissue collection, mice were anesthetized by isoflurane inhalation and the liver was perfused with ice-cold 1.15% KCl. Then, the small intestine was either prepared for histological analysis or opened, rinsed in cold 1.15% KCl, and stored at -80°C for later use. All animal experiments were carried out following University of California San Diego Institutional Animal Care and Use guidelines.

Bilirubin Measurements. Blood was obtained from the submandibular vein and centrifuged at 2000 x g for 5 min. Serum samples (20 μL) were immediately measured for total serum bilirubin using a Unistat Bilirubinometer (Reichert, Inc., Depew, NY).

Q-PCR Analysis. Total RNA from whole tissues was isolated using TRIzol reagents according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). One microgram of RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Q-PCR was performed with qPCR MasterMix Plus for SYBR (Eurogentec, Seraing, Belgium), and the reactions were run in the Mx4000 Multiplex QPCR System (Stratagene, La Jolla, CA). The forward and reverse primers used were: UGT1A1-S, 5'-CCT TGC CTC AGA ATT CCT TC-3' and UGT1A1-AS, 5'-ATT GAT CCC AAA GAG AAA ACC AC-3'; mouse Cyp2b10-S, 5'-AAA GTC CCG TGG CAA CTT CC-3' and Cyp2b10-AS, 5'-CAT CCC AAA GTC TCT CAT GG-3'; mouse Cyp3a11-S, 5'-CTC AAT GGT GTG TAT ATC CCC-3', and Cyp3a11-AS, 5'-CCG ATG TTC TTA GAC ACT GCC-3'; mouse Cyp4a10-S, 5'-CAT GTT CGA GGG CCA TGA-3', and Cyp4a10-AS, 5'-TGT GGC CAG ATA GAA GAT-3'; mouse Gsta1-S, 5'-CAG CCT CCC CAA TGT GAA GAA-3', and Gsta1-AS, 5'-TGG CTC CAT CAA TGC AGC TT-3'; mouse Gsta2-S, 5'-AGC TTG ATG CCA GCC TTC TGA-3', and Gsta2-AS, 5'-TTT CTC TGG CTG CCA GGA TGT-3'; mouse SOD-1-S, 5'-CGA TGA AAG CGG TGT GCG TGC TG-3', and SOD-1-AS, 5'-TCT CCA ACA TGC CTC TCT TCA TC-3'; mouse SOD-2-S, 5'-

AGA GCA GCG GTC GTG TAA ACC T-3', and SOD-2-AS, 5'-CCA GAG CCT CGT GGT ACT TCT C-3'; mouse CAT-S, 5'-ACC AGG GCA TCA AAA ACT TG-3', and CAT-AS, 5'-GCC CTG AAG CTT TTT GTC AG-3'; mouse GPx-1-S, 5'-GGT TCG AGC CCA ATT TTA CA-3', and GPx-1-AS, 5'-TCG ATG TCG ATG GTA CGA AA-3'; mouse Nqo1-S, 5'-GGT GAT ATT TCA GTT CCC ATT GC-3', and Nqo1-AS, 5'-GCA GGA TGC CAC TCT GAA TC-3'; mouse HO-1-S, 5'-CAG GTG TCC AGA GAA GGC TTT-3', and HO-1-AS, 5'-TCT TCC AGG GCC GTG TAG AT -3'; mouse COX-2-S, 5'-GCA GGA TGC CAC TCT GAA TC-3', and COX-2-AS, 5'- GCT CGG CTT CCA GTA TTG AG -3'; mouse GSH-Re-S, 5'- GCG TGA ATG TTG GAT GTG TAC C -3', and GSH-Re-AS, 5'-TTC CCA TTG ACT TCC ACC GTG G-3'; mouse Tff1-S, 5'-AAA CAT GTA TCA TGG CCC-3', and Tff1-AS, 5'-GAA TTC GAG GAC TAA AAG TCT-3'; mouse Tff2-S, 5'-TGC TTT GAT CTT GGA TGC TG-3', and Tff2-AS, 5'-GGA AAA GCA GCA GTT TCG AC-3'; mouse Tff3-S, 5'-GCT GCC ATG CAG ACC AGA GCC-3', and Tff3-AS, 5'-TGG CCA CCA TCA GCA GCA GG-3'; mouse cyclophilin (CPH)-S, 5'-CAG ACG CCA CTG TCG CTT T-3' and mCPH-AS, 5'-TGT CTT TGG AAC TTT GTC TGC AA-3'. Each reaction contained 0.75 μL of cDNA and 0.25 μM of the primers in a total volume of 15 μL. PCR conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 20 sec, and 72°C for 40 sec.

Western Blot Analysis. Whole cell lysates from whole tissues were prepared with RIPA Buffer plus protease inhibitors. Briefly, 100 mg of tissue was homogenized on ice in 750 μl of the above buffer. Homogenates were incubated on ice for 20 min. and vortexed occasionally. Homogenates were then centrifuged at 14,000 rpm for 20 min. at 4°C and supernatants were collected for analysis. For phospho-protein detection, cytosolic fractions were prepared. Briefly, 100 mg of tissue was homogenized on ice in 1 mL of RIPA buffer plus protease and phosphatase inhibitors. Nuclei and unbroken cells were removed by low-speed centrifugation (1000 x g for 10 min. at 4°C) and the supernatant was centrifuged at 100,000 x g for 1 hour at 4°C. Supernatants were used for phospho-western analysis. All Western blots were performed using NuPAGE Bis-Tris polyacrylamide gels as outlined by the supplier (Invitrogen, Carlsbad, CA). Protein was heated at 70°C for 10 min in loading buffer and resolved in 4-12% Bis-Tris gels under denaturing conditions (50 mM MOPS, 50 mM Tris-base, pH 7.7, 0.1% SDS, 1mM EDTA). The resolved protein was transferred onto nitrocellulose membrane using a semidry transfer system. The membrane was blocked with 5% nonfat dry milk in 10 mM Tris74 HCl, pH 8, 0.15 M NaCl, and 0.05% Tween 20 (Tris-buffered saline) for 1 hour at room temperature. The membrane was washed in the Tris-buffered saline solution and incubated with primary antibodies in Tris-buffered saline, shaking at 4°C overnight. Membranes were then washed five times with Tris-buffered saline solution and incubated with horseradish peroxidase-conjugated secondary antibodies in Tris-buffered saline solution with 2% nonfat milk for 1 hour at room temperature. Membranes were washed five times with Tris-buffered saline solution and visualized using chemiluminescent reagents, on the BIORAD ChemiDoc XRS chemiluminescence detection system.

Histology. The small intestine was collected 48 hours after treatment, flushed with 10% formalin, and fixed overnight in 10% formalin. Tissues were then transferred to 70% ethanol, embedded in paraffin, and sectioned for slides. Slides were stained with hematoxylin and eosin for H&E or Schiff reagent for Periodic Acid Schiff (PAS) histological analysis. Ki-67 immunostaining was performed using the primary rabbit anti-Ki-67 antibody, secondary biotin goat anti-rabbit Ig antibody, and Strep-HRPO antibody.

Results

Intestinal UGT1A1 Induction Occurs with Oral Arsenic Exposure

The *UGT1A1* gene is responsive to a multitude of ligands because of the complex enhancer module distal to the promoter region. Since numerous environmental toxicants can induce *UGT1A1* through association with the xenobiotic receptors, it can be speculated that exposure of *hUGT1* mice with environmental toxicants can result in reduced serum bilirubin levels through induction of *UGT1A1* expression. To evaluate the *hUGT1* mice model as a sensor for environmental exposure, we treated mice orally with arsenic (As^{3+}), cadmium (Cd^{2+}), lead (Pb^{2+}), iron (Fe^{2+}), copper (Cu^{2+}), and Chromium (Cr^{6+}) with a dose of 10 mg/kg at 12 days after birth. Compared with the water-treated controls, As^{3+}- and Cd^{2+}-treated mice exhibited significantly lower serum bilirubin levels at 14 days after birth, while Pb^{2+}-, Fe^{2+}-, Cu^{2+}-, and Cr^{6+}-treated *hUGT1* mice still showed higher serum bilirubin levels, relative to the As^{3+}- and Cd^{2+}-treated mice (Figure 3-1A). To determine which tissue was responsible for increased bilirubin metabolism in the As^{3+}- and Cd^{2+}-treated *hUGT1* mice, RNA was isolated from liver and small intestine and Q-PCR analysis was performed for *UGT1A1*. In the liver, which is typically known as the major tissue for bilirubin metabolism, the expression level of *UGT1A1* was the same between the control and As^{3+}-treated mice (Figure 3-1B). In contrast, *UGT1A1* levels were markedly induced in the small intestine of As^{3+}-treated mice (Figure 3-1C).

CAR is not Involved in Regulating Arsenic-Induced UGT1A1 Expression

Arsenic can activate various transcription factors by triggering an oxidative stress response. It was therefore speculated that the induction of intestinal *UGT1A1* by As^{3+} could be modulated through the activation of certain transcription factors. The xenobiotic receptors, such as AhR, PPARα, CAR, and PXR are widely known for their involvement in regulating *UGT1A1* gene expression by binding specific enhancer sequences within the PBREM region (Sugatani *et al.*, 2005a; Senekeo-Effenberger *et al.*, 2007; Yueh *et al.*, 2003; Xie *et al.*, 2003). To determine if the xenobiotic receptors are responsible for *UGT1A1* induction in the small intestine with As^{3+} exposure, the expression of several xenobiotic receptor target genes was examined, including *Cyp1a1* (AhR), *Cyp2b10* (CAR), *Cyp3a11* (PXR), and *Cyp4a10* (PPARα) in control and As^{3+} treated mice. There was no induction of intestinal *Cyp1a1, Cyp3a11,* or *Cyp4a10*, eliminating involvement of AhR, PXR and PPARα in response to As^{3+} (Figure 3-2A,C, D). However, there was a statistically significant induction of *Cyp2b10* gene expression (Figure 3-2B), implicating a potential role for CAR in regulating *UGT1A1* in the GI tract following As^{3+} treatment. To directly examine the role of CAR in regulating *UGT1A1* gene expression, recently generated *hUGT1Car*$^{-/-}$

mice were treated with As^{3+}. CAR is non-functional in the *hUGT1Car*$^{-/-}$ mouse model, such that potent CAR agonist, PB, cannot induce *UGT1A1* and *Cyp2b10* in the mice. However, it was observed that *UGT1A1* expression was still induced and serum bilirubin levels decreased with As^{3+} treatment in neonatal *hUGT1Car*$^{-/-}$ mice (Figure 3-3A,B). In addition, while the CAR target gene, *Cyp2b10,* was not induced with PB treatment, its expression was still upregulated with As^{3+} treatment in neonatal *hUGT1Car*$^{-/-}$ mice. This indicates that induction of *UGT1A1* and *Cyp2b10* with As^{3+} exposure in *hUGT1* mice occurs independent of CAR.

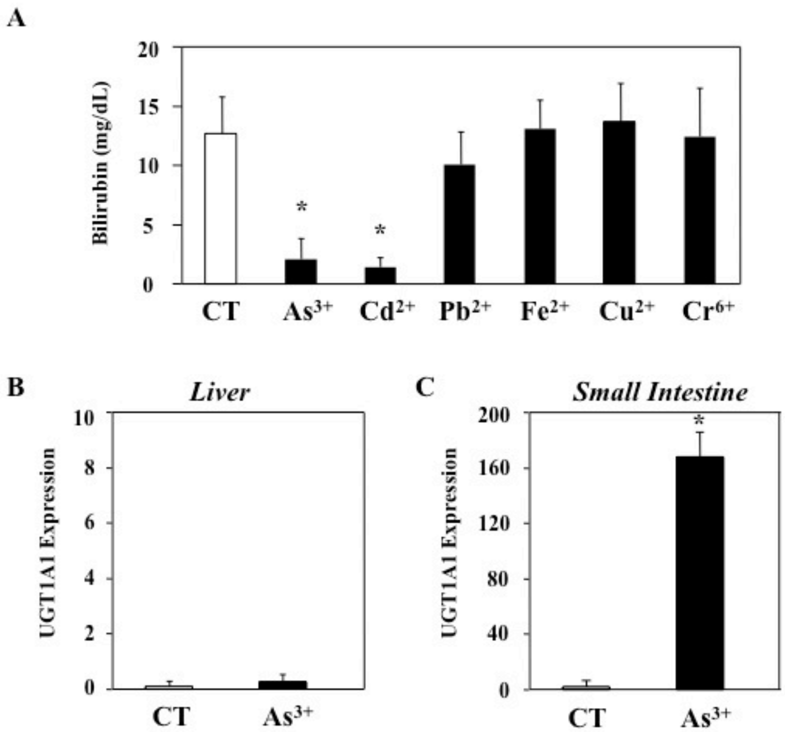

Figure 3-1. **Effects of metal contaminant exposure on serum bilirubin levels and *UGT1A1* expression in neonatal *hUGT1* mice.** At 12 days after birth, neonatal *hUGT1* mice were treated orally with 10 mg/kg of the indicated metal contaminants: sodium arsenite (As^{3+}), cadmium chloride (Cd^{2+}), lead (II) nitrate (Pb^{2+}), iron (II) sulfate (Fe^{2+}), cupric (II) chloride (Cu^{2+}), and potassium chromate (Cr^{6+}). Forty-eight hours post treatment, blood was collected and serum samples were prepared. (**A**) Serum bilirubin levels were measured using a Bilirubinometer. RNA was isolated from the liver and small intestine and Q-PCR was performed to determine *UGT1A1* expression **(B)** in the liver (**C**) and small intestine. Data is shown as fold induction of the value observed in mice treated with water alone (CT). Data is expressed as mean ± s.d., n = 6. * $P < 0.01$.

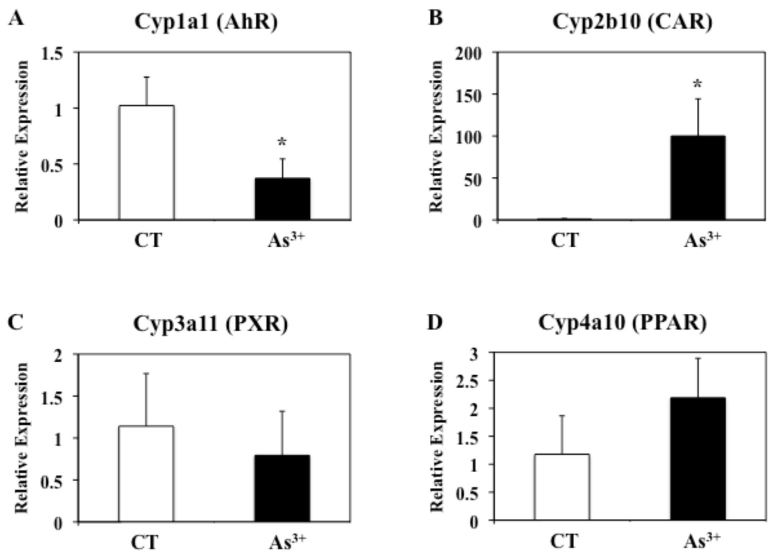

Figure 3-2. **Arsenic exposure induces *Cyp2b10* in the small intestine.** Forty-eight hours after oral As^{3+} exposure at 12 days, RNA was isolated from the small intestine at 14 days and Q-PCR was performed for *Cyp1a1, Cyp2b10, Cyp3a11, and Cyp4a10*, target genes of xenobiotic receptors AhR, PXR, CAR, and PPARα, respectively. Data is shown as fold induction of the value observed in mice treated with water alone (CT). Data is expressed as mean ± s.d., n = 3. * $P < 0.05$.

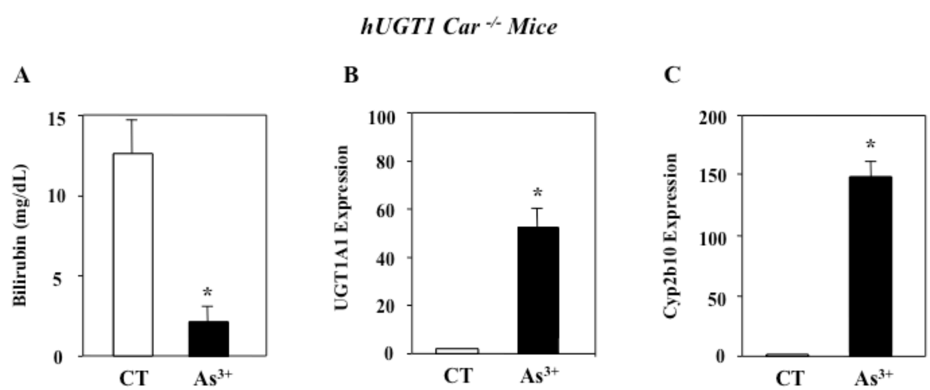

Figure 3-3. **Arsenic exposure induces *UGT1A1* and decreases serum bilirubin levels in *hUGT1Car*$^{-/-}$ mice.** At 12 days after birth, neonatal *hUGT1Car*$^{-/-}$ mice were treated orally with 10 mg/kg As^{3+}. At 14 days and 48 hours after treatment, **(A)** serum bilirubin levels were quantified and **(B)** intestinal *UGT1A1* levels determined by Q-PCR analysis. **(C)** *Cyp2b10* expression in the small intestine was also determined. Data is shown as fold induction of the value observed in mice treated with water alone (CT). Data is expressed as mean ± s.d., n = 6. * $P < 0.01$.

The NF-κB/IKK Pathway is not Involved in Regulating Intestinal UGT1A1

NF-κB prevents the generation of ROS in response to toxic challenges. In addition, previous work in our lab has demonstrated the existence of a close relationship between NF-κB expression in intestinal cells and regulating the expression of *UGT1A1* post cadmium exposure (Fujiwara *et al.*, 2012). These findings led to investigation of the involvement of the NF-κB pathway in modulating As^{3+}-induced *UGT1A1* expression. As^{3+} has been shown to exert its biological effects by reacting with IKK's free thiol to inhibit NF-κB signaling (Roussel and Barchowsky, 2000), as blocked IKK results in limited degradation of IκB and decreased NF-κB activation (Kapahi *et al.*, 2000). Western Blot analysis with small intestinal whole cell lysates revealed increased IκB protein in neonatal *hUGT1* mice exposed to As^{3+} (Figure 3-4A), initially suggesting *UGT1A1* induction may result from NF-κB repression. Further examination of the role of NF-κB/IKK signaling in the induction of gastrointestinal *UGT1A1* with As^{3+} exposure was done using mice in which NF-κB signaling through IκB-kinase (IKK)-α and IKK-β has been selectively ablated in the intestinal epithelium through the conditional knockout of the genes. To perform this experiment, transgenic mice expressing a *villin*-promoter driven Cre recombinase gene (*Vil-Cre*) only in intestinal epithelium were crossed with floxed $IKK\text{-}\alpha^{F/F}/IKK\text{-}\beta^{F/F}$ mice. Twelve-day-old $IKK\text{-}\alpha^{F/F}/IKK\text{-}\beta^{F/F}$ and $Vil\text{-}Cre/IKK\text{-}\alpha^{F/F}/IKK\text{-}\beta^{F/F}$ mice were then treated with 10 mg/kg As^{3+} and *Cyp2b10* expression in the intestinal epithelial cells was determined by Q-PCR. As^{3+} induced *Cyp2b10* in the intestinal epithelial cells of both the control $IKK\text{-}\alpha^{F/F}/IKK\text{-}\beta^{F/F}$ mice and $Vil\text{-}Cre/IKK\text{-}\alpha^{F/F}/IKK\text{-}\beta^{F/F}$ mice (Figure 3-4B). Although these mice do not carry the *UGT1* transgene, evaluation of *Cyp2b10* expression is still possible, since regulation occurs in a similar fashion to that of human *UGT1A1* in *hUGT1* mice. These findings suggest that induction of intestinal *Cyp2b10* gene expression by As^{3+} is independent of NF-κB/IKK signaling.

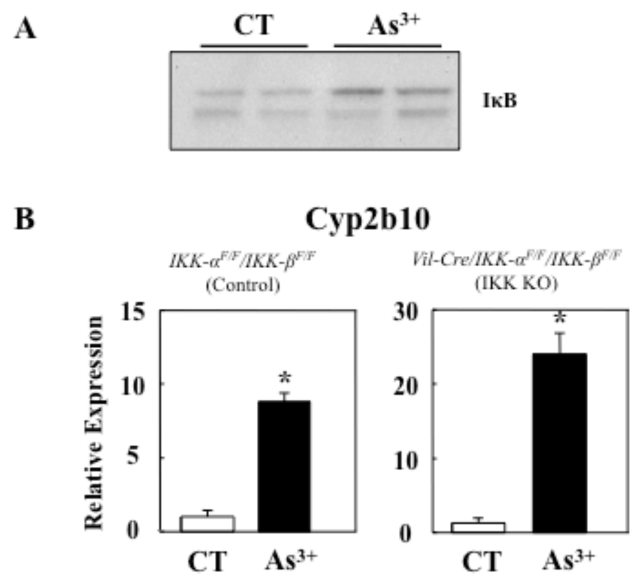

Figure 3-4. **Effects of Arsenic exposure in *IKK-α/IKK-β* conditional knockout mice. (A)** 12 days after birth, neonatal *hUGT1* mice were treated orally with 10 mg/kg As^{3+}. At 14 days and 48 hours after treatment, small intestine was collected and whole cell lysates were prepared for Western Blot analysis with the IκB antibody. **(B)** At 12 days after birth, neonatal $IKK\text{-}\alpha^{F/F}/IKK\text{-}\beta^{F/F}$ and $Vil\text{-}Cre/IKK\text{-}\alpha^{F/F}/IKK\text{-}\beta^{F/F}$ mice were treated orally with 10 mg/kg sodium arsenite. At 14 days after birth and 48 hours after the treatment, *Cyp2b10* levels in the intestinal epithelial cells were determined by Q-PCR. Data is shown as fold induction of the value observed in mice treated with water alone (CT). Data is expressed as mean ± s.d., n = 3. * $P < 0.01$.

Oxidative Stress Induced Nrf2 Activation Upregulates UGT1A1 Expression

As^{3+} has been identified as a potent inducer of oxidative stress and has been shown to generate ROS, which can lead to activation of signaling pathways involved in regulating genes involved in cellular defense mechanisms. Upregulation of cytoprotective genes, including DMEs, in response to environmental insults is known as the antioxidant response (Yueh and Tukey, 2007). To investigate whether induction of *UGT1A1* with As^{3+} exposure is upregulated as part of the antioxidant response, expression levels of several oxidative stress related genes were quantified. Two classic Nrf2 genes, *Gsta1* and *Gsta2*, were significantly upregulated in small intestine post exposure (Figure 3-5F,G). ROS can disrupt the Nrf2-Keap1 complex, leading to transcriptional activation of Nrf2 target genes, such as *UGT1A1* and the *GSTs* (Lee *et al.*, 2005). The involvement of the Nrf2-Keap1 signaling pathway was further investigated by performing co-treatment experiments with the potent antioxidant, NAC, to prevent ROS generation. Mice pre-treated with NAC 1 hour prior to As^{3+} exposure exhibit significant reductions in *UGT1A1*, *Cyp2b10*, and *Gsta1* expression (Figure 3-6). NAC pre-treatment caused *UGT1A1* induction to decrease by 98%, *Cyp2b10* by 77%, and *Gsta1* by 98%. These findings implicate that induction may occur through oxidative stress-induced Nrf2 activation.

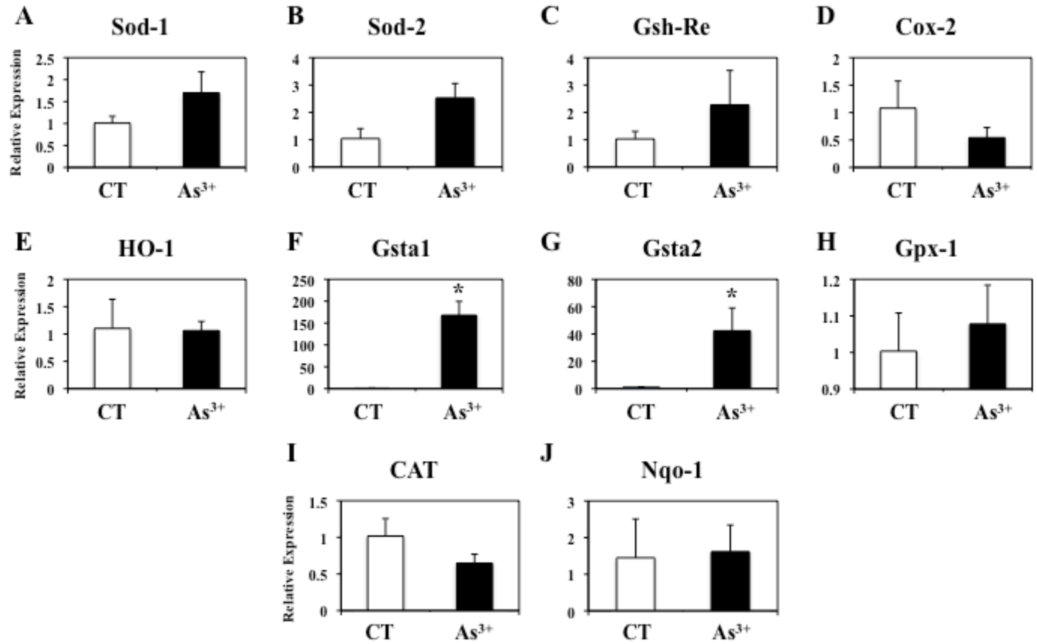

Figure 3-5. **Arsenic exposure also induces *Gsta1* and *Gsta2* in the small intestine.** Forty-eight hours after oral As^{3+} exposure, RNA was isolated from small intestine and Q-PCR was carried out for several oxidative stress related genes. Data is shown as fold induction of the value observed in mice treated with water alone (CT). Data is expressed as mean ± s.d., n = 3. * $P < 0.01$.

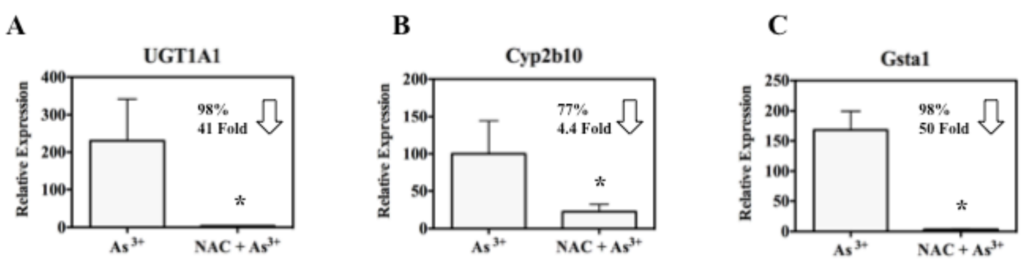

Figure 3-6. **Pre-treatment with NAC significantly reduces *UGT1A1*, *Cyp2b10*, and *Gsta1* expression in small intestine.** Mice were pre-treated with NAC 1 hour prior to As^{3+} exposure. Forty-eight hours after oral As^{3+} exposure, RNA was isolated from small intestine and Q-PCR was carried out for *UGT1A1*, *Cyp2b10* and *Gsta1*. Data is shown as fold induction of the value observed in mice treated with water alone (CT). Data is expressed as mean ± s.d., n = 3. * $P < 0.05$.

UGT1A1 Induction Occurs Independent of MAPK Activation

Recent studies have found that induction of ARE-dependent Phase II detoxifying enzymes is mediated by a MAPK pathway (Yu *et al.*, 2000; Keum *et al.*, 2003; Shen *et al.*, 2004; Yu *et al.*, 1999), and therefore *UGT1A1* induction may be occurring through MAPK activation of Nrf2. Changes in protein and phospho-protein expression of two MAPKs in small intestinal cytosolic fractions were used to assess whether oxidative stress induced in intestinal tissue of neonatal *hUGT1* mice affected MAPK activation that could lead to downstream changes in *UGT1A1* gene expression. As^{3+} exposure did not increase phosphorylation of either JNK or ERK MAPKs (Figure 3-7), indicting that these signaling pathways are not likely involved in transcriptional activation of *UGT1A1*.

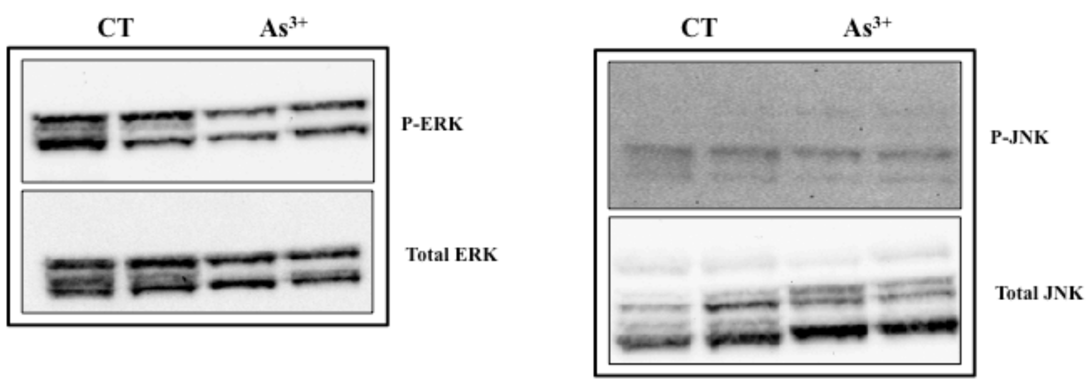

Figure 3-7. **Arsenic exposure does not activate JNK or ERK in intestine.** Forty-eight hours after oral As^{3+} exposure, cytosolic fractions were isolated from small intestine and prepared for Western Blot analysis with total JNK1/2, phospho-SAPK/JNK, total ERK1/2, and phospho-ERK1/2 antibodies. CT indicates mice treated with water alone.

Arsenic Exposure Causes Intestinal Damage, Changes in Cellular Morphology, and Increases Proliferation

The contribution of As^{3+} to such a variety of different diseases indicates that As^{3+} might not function through one specific mechanism, but instead by eliciting a more global effect. Both abnormal cell cycle regulation (Bonzo *et al.*, 2005; Lau *et al.*, 2004; Eguchi *et al.*, 2011) and changes in cellular morphology due to As^{3+}-induced cytotoxicity (Yancy *et al.*, 2005; Li *et al.*, 2011) have been observed with exposure and can lead to changes in signaling that ultimately affect gene expression. This directed initial experimental efforts toward the assessment of intestinal damage post exposure. The trefoil factor family (TFF) peptides are involved in maintaining the integrity of the gastrointestinal mucosa (Ribieras *et al.*, 1998), but can be ectopically expressed in cells of regenerating tissue surrounding compromised areas, therefore implicating their protective role in mucosal injury (Playford *et al.*, 1996). To determine if intestinal damage occurs with As^{3+} exposure, I measured changes in gene expression of *Tff1*, *Tff2*, and *Tff3* in control and As^{3+}-treated *hUGT1* intestinal tissue. *Tff1*, which is typically expressed in stomach, is significantly upregulated in the small intestine (Figure 3-8A), while *Tff2* and *Tff3* expression decreases or remains unchanged, respectively (Figure 3-8B,C). Abnormal induction of *Tff1* in the small intestine confirms intestinal injury.

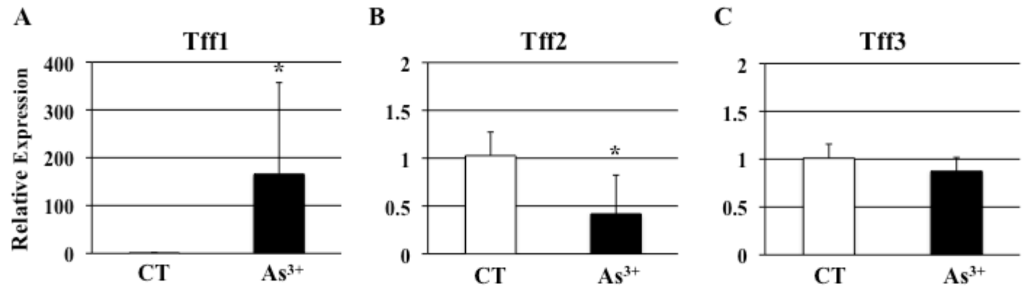

Figure 3-8. ***Tff1* is ectopically induced in small intestine with arsenic exposure.** Forty-eight hours after oral As^{3+} exposure, small intestine was collected and RNA isolated for Q-PCR analysis of *Tff1*, *Tff2*, and *Tff3*. Data is shown as fold induction of the value observed in mice treated with water alone (CT). Data is expressed as mean ± s.d., n = 3. * $P < 0.05$.

Significant induction of small intestinal *Tff1* led to immunohistochemical assessment of intestinal damage. H&E and PAS stainings of the small intestine were performed to assess any obvious morphological differences between control and As^{3+}-treated samples. H&E stained samples exhibit clear differences in goblet cell mucin secretion (Figure 3-9A,B), which is indicative of intestinal damage. However, abnormal expansion or vacuole formation at villi tips was also seen in As^{3+}-treated samples (indicated by black arrows in Figure 3-9B). PAS staining was subsequently used to confirm if the goblet cells and vacuoles were secreting glycoproteins, such as mucin, which is a sign of intestinal damage and part of the normal repair process. PAS staining revealed increased goblet cell mucin secretion in As^{3+}-treated samples, compared to the controls (mucin stains bright magenta in Figure 3-10A,B). However, an absence of PAS staining within the abnormal vacuoles formed at the villi tips suggests that this is not a normal maintenance response (indicated by black arrows in Figure 3-10B). Interestingly, PAS staining revealed that As^{3+}-treated samples exhibited stacked and enlarged nuclei (Figure 3-10B), which is indicative of increased cellular proliferation.

Arsenic is known to elicit profound and differential effects on cellular proliferation and apoptosis. In addition, increases in proliferation have been directly linked to increases in UGTs (Banjo and Nemeth, 1976). As such, Proliferating Cell Nuclear Antigen (PCNA) western blotting and Ki-67 immunostaining were used to investigate changes in cellular proliferation with As^{3+} exposure. PCNA is only expressed within the nuclei of cells during the DNA synthesis phase, while the Ki-67 antigen expression only occurs during late G1, S, G2, and M phases of the cell cycle and cannot be detected in cells in G_0 phase. Increases in both PCNA concentrations (Figure 3-11A) and Ki-67 immunostaining (Figure 3-11B) were observed in As^{3+}-treated samples. Active proliferation can be seen within the crypts, but there is significantly greater epithelial migration in As^{3+}-treated samples, confirming increased cellular proliferation (Figure 3-11B).

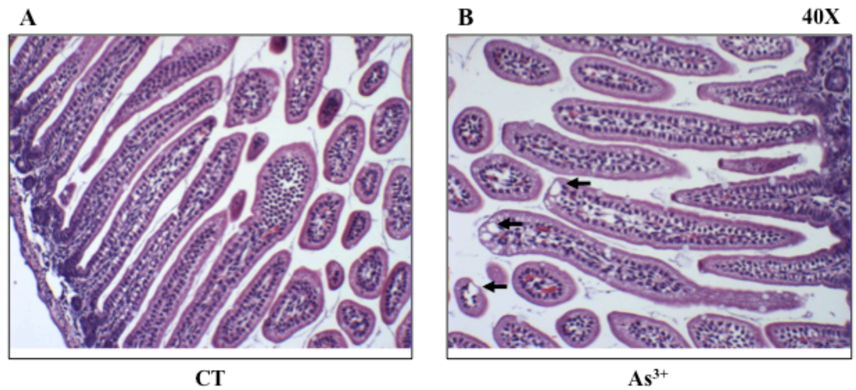

Figure 3-9. **H&E staining reveals abnormal vacuole formation.** The small intestine was collected 48 hours after As^{3+} treatment, flushed with 10% formalin, and fixed overnight in 10% formalin. Tissues were then transferred to 70% ethanol, embedded in paraffin, and sectioned for slides. Slides were stained with hematoxylin and eosin for H&E histological analysis.

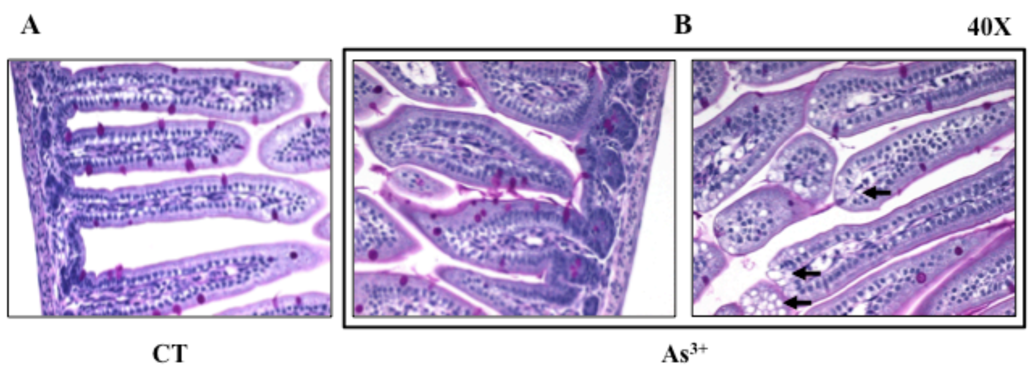

Figure 3-10. **PAS staining confirms abnormal vacuole formation.** The small intestine was collected 48 hours after As^{3+} treatment, flushed with 10% formalin, and fixed overnight in 10% formalin. Tissues were then transferred to 70% ethanol, embedded in paraffin, and sectioned for slides. Slides were stained with Schiff reagent for Periodic Acid Schiff (PAS) histological analysis.

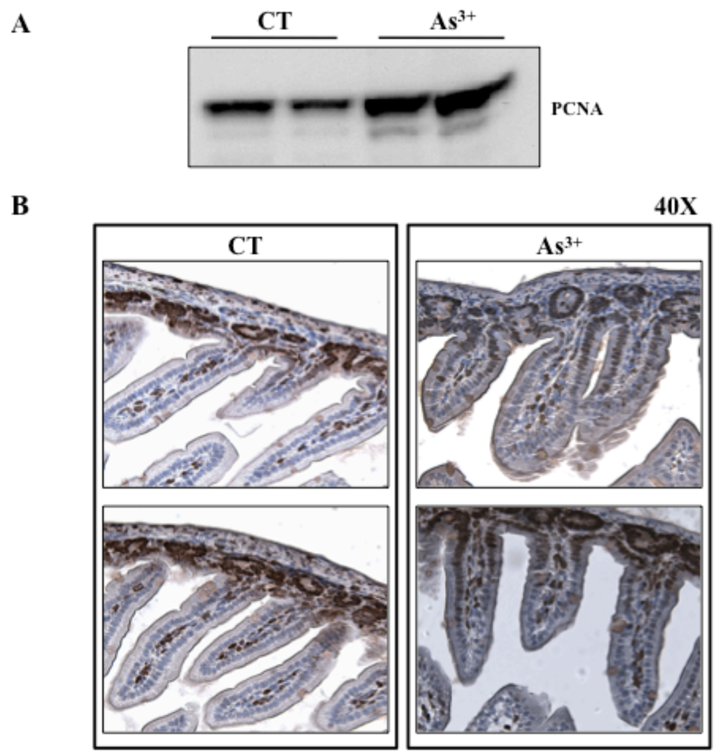

Figure 3-11. **Increased PCNA concentrations and Ki-67 immunostaining confirm increased proliferation.** **(A)** Forty-eight hours after oral As^{3+} exposure, whole cell lysates were isolated from small intestine and prepared for Western Blot analysis with the PCNA antibody. **(B)** The small intestine was collected 48 hours after treatment, flushed with 10% formalin, and fixed overnight in 10% formalin. Tissues were then transferred to 70% ethanol, embedded in paraffin, and sectioned for slides. Ki-67 immunostaining was performed using the primary rabbit anti-Ki-67 antibody, secondary biotin goat anti-rabbit Ig antibody and Strep-HRPO antibody.

Discussion

Much of our knowledge about the effects of As^{3+} has been inferred from indirect epidemiological and clinical observations, especially since As^{3+} has never been shown conclusively to be an initiating or a promoting agent of carcinogenesis in animals (Bode and Dong, 2002). Therefore, elucidation and understanding of the molecular mechanisms that determine the effects of As^{3+} exposure are critical for understanding its enigmatic nature. There is particularly little information about As^{3+}'s ability to modulate expression of DMEs, either directly or indirectly. The generation of a humanized *UGT1* mouse model, which expresses the entire human *UGT1* locus in a mouse *Ugt1* null background and exhibits elevated serum bilirubin levels during development (Fujiwara *et al.*, 2010b), has enabled studies that probe at the ability of prominent metal contaminants, including As^{3+}, to regulate the human *UGT* genes in a relevant *in vivo* model.

Since the *UGT1A1* gene contains a series of xenobiotic receptor enhancer sequences, it is one of the few genes that can be independently modulated by activation of numerous transcriptional factors. Therefore, it can be speculated that exposure of *hUGT1* mice to environmental toxicants can reduce serum bilirubin levels through induction of *UGT1A1* expression. Oral As^{3+} exposure in *hUGT1* mice caused marked decreases in serum bilirubin that corresponded with significant increases in intestinal *UGT1A1*. An absence of induction of *UGT1A1* was noted in liver, implicating induction as intestinal specific.

In addition to findings supporting the complex and extensive regulation of the *UGT1* locus by various nuclear receptors, studies demonstrating that As^{3+} is capable of affecting *CYP* gene expression by affecting AhR and PXR activation (Wu *et al.*, 2009; Noreault *et al.*, 2005) led to investigating nuclear receptor involvement in the regulation of As^{3+}-induced *UGT1A1* expression. Examination of the expression of several xenobiotic receptor target genes in control and As^{3+} treated mice eliminated involvement of AhR, PXR, and PPARα in response to As^{3+}. However, there was a statistically significant induction of *Cyp2b10* gene expression, which implicated a potential role for CAR in regulating *UGT1A1* in the GI tract following As^{3+} treatment. *hUGT1Car*$^{-/-}$ were utilized to directly examine the role of CAR in regulating *UGT1A1* gene expression. Serum bilirubin levels decreased and *UGT1A1* and *Cyp2b10* were still inducible with As^{3+} treatment in neonatal *hUGT1Car*$^{-/-}$ mice, indicating that induction of *UGT1A1* and *Cyp2b10* with As^{3+} exposure in *hUGT1* mice is not directly regulated by CAR.

Oxidative stress is currently the most widely accepted and studied mechanism of As^{3+} toxicity (Ercal *et al.*, 2001). As^{3+} causes oxidative stress and the generation of ROS, which in turn, activates signaling pathways involved in the regulation of genes encoding antioxidative response enzymes (Pi *et al.*, 2003). Upregulation of cytoprotective genes, including DMEs, in response to environmental insults is known as the antioxidant response. There are many pathways that are affected by ROS, including the NF-κB/IKK pathway, the Nrf2-Keap1 pathway, and the MAPK signaling pathways (Felix *et al.*, 2005; Yueh and Tukey, 2007; Bode and Dong, 2000).

The NF-κB/IKK pathway has been shown to regulate transcription of important protective genes (Chen *et al.*, 2001). As such, changes in its transcriptional activation can alter target gene expression. Arsenic is believed to exert its biological effects by reacting with IKK's free thiol to block NF-κB signaling. Blocked IKK results in decreased NF-κB translocation and limited degradation of IκB (Kapahi *et al.*, 2000). Increased IκB concentrations were observed in small intestinal whole cell lysates, suggesting NF-κB repression was involved in *UGT1A1* induction. However, previous work in our lab demonstrated that *hUGT1* mice treated orally with known NF-κB activators, such as Cd^{2+} and LPS, (Hyun *et al.*, 2007; Luo *et al.*, 2004), exhibited decreases in serum bilirubin and significant increases in intestinal *UGT1A1* and *Cyp2b10*, which are patterns identical to what is observed in As^{3+}-treated *hUGT1* mice. Fujiwara *et al*. further investigated the role of intestinal NF-κB in Cd^{2+}-induced expression of *Cyp2b10* in a conditional knockout mouse model deficient in *Ikkα/β* specifically in intestinal epithelial cells (Fujiwara *et al.*, 2012). Although these mice do not carry the *UGT1* transgene, evaluation of *Cyp2b10* expression was still possible, since regulation occurs in a similar fashion to that of human *UGT1A1* in *hUGT1* mice. *Cyp2b10* induction was completely abolished in *Vil-Cre/Ikkα*$^{F/F}$*Ikkβ*$^{F/F}$ (GI knockout) mice treated with Cd^{2+}, compared to the Cd^{2+}-treated control *Ikkα*$^{F/F}$*/Ikkβ*$^{F/F}$ (WT mice) (Fujiwara *et al.*, 2012). Subsequent investigation into the role of the NF-κB/IKK pathway in regulating intestinal *UGT1A1* expression post As^{3+} exposure in twelve-day-old *IKK-α*$^{F/F}$*/IKK-β*$^{F/F}$ and *Vil-Cre/IKK-α*$^{F/F}$*/IKK-β*$^{F/F}$ mice revealed sustained induction of *Cyp2b10* in the intestinal epithelial cells of both control *IKK-α*$^{F/F}$*/IKK-β*$^{F/F}$ mice and *Vil-Cre/IKK-α*$^{F/F}$*/IKK-β*$^{F/F}$ mice. While these results suggest that induction of intestinal *Cyp2b10* gene expression by As^{3+} occurs independent of NF-κB/IKK signaling, it is difficult to conclusively rule out NF-κB involvement in *UGT1A1* gene regulation without repeating As^{3+} treatments in a *hUGT1/IKK*-null model in which it is possible to directly measure UGT1A1 mRNA and protein expression.

UGT1A1 has been identified as an Nrf2 target gene that is induced in response to oxidative stress (Yueh and Tukey, 2007). To investigate whether induction of *UGT1A1* by As^{3+} exposure is upregulated as part of the Nrf2 antioxidant response, the expression levels of several oxidative stress related genes were quantified and it was established that two classic Nrf2 genes, *Gsta1* and *Gsta2*, were significantly upregulated in small intestine post exposure (Lee *et al.*, 2005). NAC pre-treatment of *hUGT1* mice to prevent ROS generation subsequently revealed that pre-treatment caused *UGT1A1* induction to decrease by 98%, *Cyp2b10* by 77%, and *Gsta1* by 98%. These findings implicate that induction may occur through oxidative stress-induced Nrf2 activation. As previous studies with Nrf2 deficient mouse have highlighted the crucial importance of elevated Phase II gene expression in cytoprotection, it is necessary to confirm Nrf2 involvement in regulating *UGT1A1* by treatment experiments in similar model. However, increasing evidence has revealed that As^{3+} differentially activates MAPKs (Bode and Dong, 2002), which can impact regulation of downstream target genes. Recent studies have found that induction of ARE-dependent Phase II detoxifying enzymes is mediated by a MAPK pathway (Yu *et al.*, 2000; Keum *et al.*, 2003; Shen *et al.*, 2004; Yu *et al.*, 1999), and therefore *UGT1A1* induction may be occurring through MAPK activation of Nrf2. Assessment of protein and phospho-protein of two MAPKs in small intestinal cytosolic fractions revealed that As^{3+} exposure did not increase phosphorylation of either JNK or ERK MAPKs, thus indicating that these signaling pathways are not likely involved in transcriptional activation of *UGT1A1*.

The contribution of As^{3+} to such a variety of different diseases indicates that it might not function through one, specific mechanism, but instead by eliciting a more global effect. Both abnormal cell cycle regulation (Bonzo *et al.*, 2005; Lau *et al.*, 2004; Eguchi *et al.*, 2011) and changes in cellular morphology due to As^{3+}-induced cytotoxicity (Yancy *et al.*, 2005; Li *et al.*, 2011) have been observed with exposure and can lead to changes in signaling that can affect gene expression. Initial assessment of intestinal damage by As^{3+} exposure was provided by quantifying changes in gene expression of the *Tffs*, which are involved in maintaining the integrity of the gastrointestinal mucosa (Ribieras *et al.*, 1998; Playford *et al.*, 1996). Abnormal induction of *Tff1* in small intestine, which is typically expressed in stomach suggested intestinal injury, which led to further investigation of damage by immunohistochemical analysis. H&E and PAS stainings of the small intestine were performed to assess any obvious morphological differences between control and As^{3+}-treated samples. While H&E stained samples showed clear differences in goblet cell mucin secretion, abnormal expansion or vacuole formation at villi tips was also seen in As^{3+}-treated samples. PAS staining was subsequently used to confirm if the goblet cells and vacuoles were secreting glycoproteins, such as mucin, which is a sign of intestinal damage and part of the normal repair process. PAS staining revealed increased goblet cell mucin secretion in As^{3+}-treated samples, compared to the controls. Interestingly, the absence of PAS staining within the abnormal vacuoles formed at the villi tips suggests that this is not a normal maintenance response. Furthermore, PAS staining revealed that As^{3+}-treated samples exhibited stacked and enlarged nuclei that are indicative of increased cellular proliferation. These findings were particularly significant, as both NF-κB and the MAPKs, which are classically involved in regulating cell growth and apoptosis, have been largely ruled out as potential regulators of As^{3+}-induced *UGT1A1* expression. Nonetheless, As^{3+} is known to elicit profound and differential affects on cellular proliferation and apoptosis (Qian *et al.*, 2003). Increases in both PCNA concentrations and Ki-67 immunostaining were observed in As^{3+}-treated samples. Active proliferation can be seen within the crypts, but there is significantly greater epithelial migration in As^{3+}-treated samples, ultimately confirming increased cellular proliferation.

Increases in proliferation have been directly linked to increases in UGT content (Banjo and Nemeth, 1976). It is very possible that proliferation could ultimately be underlying the induction that is observed with As^{3+} exposure. The overall contribution of proliferation can be further assessed in *hUGT1*/*growth arrest and DNA-damage-inducible gene 45β* (*Gadd45β*) deficient mice. The three *Gadd45* genes (*Gadd45α*, *Gadd45β*, and *Gadd45γ*) are all inducible by various environmental stresses, such as UV and γ-irradiation and oxidative stress. However, unlike the two other homologs, Gadd45β plays an anti-apoptotic role (Columbano *et al.*, 2005) and therefore Gadd45β null mice would exhibit decreased proliferation. Previous works showing the inducibility of the Gadd45 family by both partial hepatectomy and by treatment with TCPOBOP, a CAR activator that also produces a particularly strong and rapid proliferative response in mouse liver, have also linked hepatocyte proliferation by Gadd45β to CAR activation (Tian *et al.*, 2011; Columbano *et al.*, 2005; Costa *et al.*, 2005). Most recently, work from the Negishi lab has showed that CAR induction by PB results in the promotion of hepatocellular carcinoma (HCC) through Gadd45β and that the development decreased in *Car -/-* mice, thus confirming Gadd45β as a CAR target gene (Yamamoto *et al.*, 2010). The work in this dissertation has shown that *UGT1A1* remains inducible in *hUGT1/Car -/-* mice. Therefore, if histological examination reveals decreases in proliferation, it can follow that induction occurs through a different mechanism.

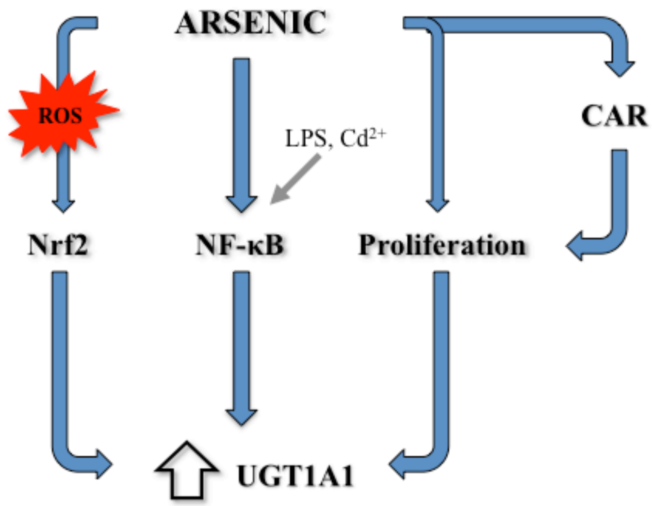

Figure 3-12. **The proposed mechanism for oral arsenic-induced *UGT1A1* gene expression.**

In the process of elucidating the mechanism by which As^{3+} modulates intestinal *UGT1A1*, I have narrowed my findings down to three possible mechanisms: the Nrf2-Keap1 pathway, the NF-κB/IKK pathway, and proliferation (Figure 3-12). Since *UGT1A1*, *Cyp2b10*, and *Gsta1* expression decreased significantly with NAC pre-treatment, it is reasonable to assume that ROS induced by As^{3+} activates Nrf2 and in turn upregulates *UGT1A1*. Maintained induction of Cyp2b10 in the *IKK-/-* mouse model indirectly suggested that *UGT1A1* induct is not mediated through NF-κB. However, previous experiments in our lab have demonstrated that typical NF-κB inducers, like Cd^{2+} upregulate *UGT1A1* and *Cyp2b10* via the NF-κB/IKK pathway in *hUGT1* mice. Lastly, proliferation could ultimately be underlying the induction we see. Although direct regulation of UGT1A1 by CAR has been negated, a proliferative response that results in increased UGT1A1 could be indirectly modulated by CAR. Figure 3-12 summarizes this proposed mechanism. These findings have provided advancements in understanding how As^{3+} regulates *UGT1A1* gene expression and highlight the enigmatic and complicated nature of its exposure. The shocking and on-going prevalence of contamination throughout the world is exactly why it is essential to understand how As^{3+} can affect important biological processes, such as drug metabolism.

Chapter 3, in part, is currently being prepared for submission for publication of the material. I was the primary investigator and author of this material.

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CHAPTER 4

General Conclusions

Numerous factors, including age (particularly the neonatal period), diet, disease states, drug-drug interactions (induction and inhibition), hormonal effects, ethnicity, genetic polymorphism, and protein-protein interactions can alter human UGT activity (Miners and Mackenzie, 1991; de Wildt *et al.*, 1999). This dissertation work has contributed to further identifying and understanding how these factors influence the metabolism and disposition of glucuronidated drugs. I have shown the functional relevance of UGT dimerization in human hepatocytes, as well as the ability of oral arsenic exposure to modulate *UGT1A1* gene expression during neonatal development. Determining how such factors affect UGT regulation, function, binding affinity, and substrate specificity will benefit the field of pharmacology and toxicology, in both drug design and risk assessment, respectively. In conclusion, my findings reflect the complicated nature of UGTs, thus emphasizing the importance of valid systems by which to study them and most importantly, recognizing the significant consequences of interindividual variability in pharmacotherapy.

Future Implications of this Work

UGT Interactions in Human Hepatocytes

Glucuronidation is one of the major metabolic pathways that serves as an essential clearance mechanism for a myriad of compounds, including drugs, dietary chemicals, environmental pollutants, and endogenous compounds. The significant number of substrates that are capable of undergoing glucuronidation has resulted in increased attention towards development of *in vitro* tools by which to help predict *in vivo* glucuronidation (Miners *et al.*, 2004; (Foti and Fisher, 2012). A simplified system that allows for the expression of a single gene product is inherently attractive for mechanistic studies of DMEs or to examine mechanisms of drug-drug interactions. However, it is important to understand the inherent difficulties and potential discrepancies that may arise in the use of these systems as predictors of *in vivo* xenobiotic metabolism (Remmel and Burchell, 1993). Tissues fractions (HLMs or S9), fresh or cryopreserved hepatocytes, and recombinant UGT enzymes are the current systems utilized for studying glucuronidation in vitro. HLMs are generally considered the easiest to use since contributions from Phase I and Phase II enzymes can easily be determined by the selective addition of the necessary cofactors for each pathway (Fisher *et al.*, 2002). Cryopreserved hepatocytes have been determined to provide the most accurate prediction of in vivo glucuronidation parameters from in vitro data, making them the most physiologically relevant system for studying in vitro activity (Soars *et al.*, 2002; Engtrakul *et al.*, 2005). Recombinant systems over-expressing one or multiple UGTs have also been utilized to study glucuronidation (Coffman *et al.*, 1995; Fujiwara *et al.*, 2007; Nakajima *et al.*, 2007). Unfortunately, UGT expression in these artificial environments may differ from native cells and the enzymatic contribution of each UGT isoform is very difficult to isolate due to the fact that interactions vary depending on isoform, substrate, and expression ratio. Additionally, post-translational modifications to the UGTs that have been shown to impact activity may not occur in these simplified expression systems (Miners *et al.*, 2006; Ishii *et al.*, 2010). Differences in lipid components between preparation and source of synthesis, as well as general membrane circumstances have been implicated in affecting UGT interactions and may account for *in vitro* intrinsic clearances that severely under-predict *in vivo* hepatic clearance (Soars *et al.*, 2002; Miners *et al.*, 2004; Fujiwara *et al.*, 2010). Insect-expressed systems, such as Supersomes, are another common method by which to study single UGT isoforms, but also lack the potential to exhibit the heterodimeric protein interactions exhibited in more complex systems. With accumulating evidence exemplifying discrepancies between recombinant systems and whole cell systems, it is necessary to address the use of siRNA knockdown as an alternative process for evaluating UGT enzymology. This work has provided confirmation of interactions previously noted for UGT1A9 and UGT2B7 in over-expressed cellular systems and suggests that UGT-UGT interactions are physiologically relevant phenomena whose effects can be observed in human hepatocytes. While further characterization with additional UGT isoforms as well as other cellular components (CYPs, transporters) is still necessary, the current data supports that caution should be taken in utilizing some of the more simplified, in vitro UGT systems in which heterodimeric protein interactions are unable to occur.

The clinical relevance of UGT interactions is not well studied, however, there is significant potential for impacting drug-induced toxicity. Previous observations have shown that heterodimerization of UGTs with mutant forms can lead to altered glucuronidation activity (Meech and Mackenzie, 1997; Koiwai *et al.*, 1996; Levesque *et al.*, 2007; Ito *et al.*, 2002; Nagar *et al.*, 2004). It is known that CN-I and CN-II are both autosomal recessively inherited conditions (Crigler and Najjar, 1952; Kadakol *et al.*, 2000). This is why heterozygous carriers are still capable of maintaining normal bilirubin levels. However, Koiwai *et al.* observed that heterozygous carriers of certain *UGT1A1* mutations displayed mild to moderate levels of hyperbilirubinemia. This suggests an autosomal dominant pattern of inheritance in which UGT1A1 mutants act as a dominant negative protein toward the functional UGT1A1 wild-type protein (Koiwai *et al.*, 1996). Dr. Operaña's dissertation work also tested this theory. To determine the effects of homo/heterodimerization on UGT function, she co-expressed several N-terminal truncated forms of UGT1A1 with the full-length form. The results all showed significant decreases in wild-type function, suggesting that the inactive, truncated forms function as dominant negative proteins. It can therefore follow that an individual expressing a mutant form of UGT1A1 may be at greater risk of ADRs due to lowered activity of the dimeric UGTs (Operaña, 2008). Investigation of the functional consequences of UGT dimerization has broadened the complexity of pharmacogenetics. These findings complement my dimerization work in human hepatocytes and further indicate dimerization among UGT proteins as an important factor in the pharmacokinetics and pharmacodynamics of a drug.

Intestinal Microflora and UGT1A1 Induction

Following ingestion, inorganic arsenic (iAs) is predominantly excreted as the methylated metabolite, dimethylarsinic acid (DMA^{V}) and to a lesser extent, monomethylarsonic acid (MMA^{V}). The identification of these pentavalent methylated metabolites in animal urine after exposure implicates biomethylation as a major detoxification mechanism. However, the formation of reactive trivalent intermediates, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) has forced researchers to reconsider methylation as an activation process. In addition, the discovery of new sulfur containing methylated As metabolites, monomethylmonothioarsonic acid ($MMMTA^{V}$) and dimethylmonothioarsinic acid ($DMMTA^{V}$) (Naranmandura *et al.*, 2007; Raml *et al.*, 2007) in human urine has provoked re-evaluation of arsenic biostransformation altogether (Hughes and Kenyon, 1998; Styblo *et al.*, 2000; Rehman and Naranmandura, 2012). These findings place particular emphasis on the importance of speciation analysis in evaluating risk from arsenic exposure (Van de Wiele *et al.*, 2010).

Although iAs may be the predominant form present in contaminated water and soils, the speciation changes that it undergoes during GI transit are not well characterized. The highly reducing environment as well as complex microbial community of the gut can greatly contribute to the pre-systemic (systemic being all metabolism carried out by human cells) biotransformation of ingested iAs (Kubachka *et al.*, 2009). The importance of pre-systemic metabolism by the microbe-rich environment of the GI tract has been demonstrated with *in vivo* animal models and *in vitro* experiments with animal microbiota (Hall *et al.*, 1997; Kubachka *et al.*, 2009). However, Van de Wiele *et al.* was the first to confirm these findings in humans, by exposing cultured bacteria from human intestine to iAs or four types of soils with arsenic naturally present. In the bacteria, MMA^{V} and the highly toxic MMA^{III} were formed from both pure iAs and iAs present in soils. These results validate that bacteria living in the human gut can increase the toxicity of arsenic ingested from contaminated food and water (Van de Wiele *et al.*, 2010).

Gut microbial communities represent one source of metabolic diversity, as shaping of the human microbial landscape is driven by a series of complex and dynamic interactions throughout life, including diet, life-style, disease, and antibiotic use. This developmental trajectory of the microbiome plays a key role in shaping the metabolic phenotype of the host and greatly influences host biochemistry and susceptibility to disease (Nicholson *et al.*, 2012; Yatsunenko *et al.*, 2012). Therefore, the unique environment of the gut, more specifically pre-systemic metabolism, could be a contributing factor in influencing *UGT1A1* induction patterns observed with oral arsenic exposure. Antibiotic treatment of *hUGT1* mice could aid in addressing the influence of intestinal microflora on activity and expression of DMEs in response to arsenic exposure.

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