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MOLECULAR INSIGHTS AND PHYSIOLOGICAL CONSEQUENCES OF ARYL HYDROCARBON RECEPTOR REGULATED PLASMINOGEN ACTIVATOR INHIBITOR-1 EXPRESSION

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MOLECULAR INSIGHTS AND PHYSIOLOGICAL CONSEQUENCES OF ARYL HYDROCARBON RECEPTOR REGULATED PLASMINOGEN ACTIVATOR INHIBITOR-1 EXPRESSION

by

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Dedication

Dedicated to my mother for always believing, my sister for her endless support in all aspects of life, and my nephew for inspiring me with the future!

Acknowledgements

I first want to acknowledgement to my mentor, Dr. Kees Elferink. His constant support, if only to tell me to relax, has been invaluable, and I would have been unable to accomplish this goal without him. I also must acknowledge past-post doctoral students in the lab, Kristen Mitchell, Gengming Huang, and Premnath Shetty, for their guidance, assistance, and friendship. I would like to thank current members of the lab – Tod Harper, Li Chen, Mehnaz Mustafa, and Hui Li – for their input and support throughout this process. I also must thank the members of the Lisa Elferink laboratory, especially Kristen Hill, for their support and for giving me space to work when Hurricane Ike made conditions in our laboratory impossible.

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MOLECULAR INSIGHTS AND PHYSIOLOGICAL CONSEQUENCES OF ARYL HYDROCARBON RECEPTOR REGULATED PLASMINOGEN ACTIVATOR INHIBITOR-1 EXPRESSION

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The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, attenuates liver regeneration *in vivo* when activated by its prototypical agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) following 70% partial hepatectomy (PH). One reported target of the AhR that may account for suppression of the regenerative response is plasminogen activator inhibitor-1 (PAI-1), which negatively regulates the cleavage and activation of hepatocyte growth factor (HGF) from its latent form in the extracellular matrix. Once activated, HGF signalling through its receptor cMet is a crucial component early in regeneration. Recent studies identified a sequence distinct from the canonical AhR binding site, the ncXRE, which confers TCDD-inducible expression to the PAI-1 promoter. Since the ncXRE shares partial sequence homology with the Kruppel-like factor 6 (KLF6) consensus binding site; **I hypothesize that the AhR interacts with KLF6 at the ncXRE, inducing transcription of PAI-1, suppressing HGF processing and its activation of cMet, inhibiting liver regeneration.** To test this hypothesis, co-immunoprecipitation on liver nuclear extracts and recombinant proteins confirmed that KLF6 and the AhR interact, likely dependent on the C-terminus transactivating domain of AhR and the DNA binding domain of KLF6. Both proteins bind the ncXRE *in vitro* and deletion analyses revealed that the N-terminal 27 amino acids of hKLF6 were required for complex formation. Chromatin immunoprecipitation studies deomonstrated that the AhR and KLF6 bind to the PAI-1 promoter *in vivo*. To assess the effects of AhR activation *in vivo*, C57BL/6 and PAI-$1^{-/-}$ mice were pretreated with TCDD, underwent PH, and liver samples and serum were collected at multiple time points post-PH to monitor PAI-1 expression, HGF processing, and cMet phosphorylation (activation) and DNA synthesis in the liver. I found that PAI-1 transcript and corresponding serum PAI-1 protein levels were markedly increased in TCDD-pretreated C57BL/6 mice, and this rise in PAI-1 levels inversely correlated to HGF processing and cMet phosphorylation. Hepatocytes in the periportal region of PAI-$1^{-/-}$ mice were able to overcome TCDD-mediated suppression of regeneration. The AhR-KLF6 interaction at the PAI-1 promoter, resulting in increased PAI-1 expression and decreased HGF processing and cMet activation, reveals a novel mechanism by which the AhR may contribute to liver homeostasis.

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LIST OF ABBREVIATIONS

AhR……..…..Aryl Hydrocarbon Receptor
TCDD……2,3,7,8-tetrachloro-dibenzo-*p*-dioxin
PH……..Partial Hepatectomy
PAS……Per-Arnt-Sim
Arnt…….....AhR nuclear translocator
XRE…...……Xenobiotic Response Element
AhRR…….AhR Repressor
G_1 ……..Gap 1
S…..……Synthesis
Cdk…...…….Cyclin dependent kinase
pRB……...hypophosphorylated Retinoblastoma protein
ppRB……..hyperphosphorylated RB
TNF-α……Tumor Necrosis Factor α
IL-6…….Interleukin-6
EGFR...……Epidermal Growth Factor Receptor
HGF……Hepatocyte Growth Factor
uPA ……..Urokinase Plasminogen Activator
uPAR……..Urokinase Plasminogen Activator Receptor
HCC……...Hepatocellular Carcinoma
PAI-1……..Plasminogen Activator Inhibitor-1
LRP……Low-density Lipoprotein Receptor-Related Protein
1-PP……1-Propynyl Pyrene
BrdU……...5-Bromo-2'-deoxyuridine
PCNA...…….....Proliferating Cell Nuclear Antigen
TGF-ß……Transforming Growth Factor ß
HIF...……...Hypoxia Inducible Factor
ncXRE……non-consensus XRE
EMSA…….Electrophoretic Mobility Shift Assay
ChIP……Chromatin Immunoprecipitation
KLF…….....Kruppel-like Factor
UTMB……University of Texas Medical Branch
qRT-PCR……quantitative Real Time Polymerase Chain Reaction
CKO……Conditional Knock Out

CHAPTER 1: INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a ubiquitous, ligand-activated transcription factor historically studied for its importance in mediating the response to toxicants, but the AhR has more recently garnered attention with an emerging role in physiological processes including development, apoptosis, and cell proliferation. While the AhR is activated by a variety of environmental contaminants, including polycyclic aromatic hydrocarbons and the poorly metabolized halogenated aromatic hydrocarbons (Rowlands and Gustafsson 1997; Safe 1986), it is both potently and persistently activated by 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD). Additionally, it has been demonstrated that the toxic effects of TCDD are likely mediated entirely by the AhR, as AhR null mice lack known responses and there is co-localization between areas of TCDD-induced hepatoxicity and AhR expression (Chang et al. 2005; Gonzalez and Fernandez-Salguero 1998; Mimura and Fujii-Kuriyama 2003; Poland and Knutson 1982). Thus TCDD serves as a useful tool for examining AhR activity in cellular processes as well as providing insight into the mechanisms involved in TCDD-mediated toxicity.

TCDD PRODUCITON AND TOXICITY

TCDD Production

TCDD is not a naturally occurring compound. It is created in a variety of processes, released into the environment as by-products or in industrial accidents, and humans become exposed mostly through ingestion with attendant bioaccumulation. Since most sources are actually a mixture of dioxin congeners, measurements of dioxin levels are reported as toxic equivalents, where TCDD, as most toxic, is assigned a value of 1 with the remaining congeners as fractions of 1 (McKay 2002). In a survey of 51 countries, both industrialized and developing, estimates of national dioxin emissions between 2000 and 2007 found that approximately 36kg toxic equivalents of dioxins are released into the environment each year by the surveyed countries. Of these, 45% of total emissions can be attributed to waste incineration, metal production, and power generation and heating while an additional 40% is released by uncontrolled combustion (Ren and Zheng 2009). Of waste incineration, the largest source of dioxins is from municipal and hospital waste incineration (Kulkarni et al. 2008). In addition to the aforementioned sources, pulp and paper mills have long been targeted as producers of dioxins due to the use of chlorine in the bleaching process. In waste water from one pulp mill in China during one week, 315 pg/L toxic equivalents of dioxins were released, 229.5 pg/L of TCDD specifically (Zheng et al. 2001). Although an increasing number of technologies are being

developed to reduce the release of dioxins into the environment and remediate contaminated sites (Kulkarni et al. 2008), these methods are being outpaced by the increased production of dioxins. As of 1996, the typical US body burden of dioxins in human adipose tissue was 22.83 toxic equivalents, 4.4 of which were attributable TCDD (Silkworth and Brown 1996). Thus, dioxin pollution and subsequent human exposure remains a significant health problem.

TCDD Toxicity in Humans

In humans, the toxic effects of TCDD are varied and with the exception of chloracne (hyperkeratosis of the skin), are largely based on epidemiological studies following large accidental exposures. In 1953, workers at a trichlorophenol production plant were exposed to TCDD and shown to have an increased likelihood of gastrointestinal system (liver, stomach, pancreas) cancers with increased TCDD exposure (Ott and Zober 1996). Perhaps one of the largest exposures occurred during an industrial explosion in a 2,4,5-trichlorophenol reactor in Seveso, Italy in 1976, exposing thousands to substantial quantities of TCDD. In the most recent health assessment of those exposed, the 20-year follow-up reported an increase incidence of lymphohemopoietic neoplasms and an overall increase in diabetes (Bertazzi et al. 2001), and women exposed in Seveso have a significant correlation between levels of dioxin in their serum and incidence of breast cancer (Warner et al. 2002). In addition to industrial accidents, significant accidental human exposure has occurred through the

intentional use of herbicides containing dioxins. An international cohort of workers exposed to dioxins in herbicides reported an increase in overall cancer rates and in soft-tissue sarcoma and non-Hodgkin's lymphoma in particular (Kogevinas et al. 1997). Vietnam Veterans exposed to large quantities of the dioxin-containing herbicide Agent Orange show correlations between TCDD levels and diabetes (Committee to Review the Health Effects in Vietnam Veterans of Exposure to Herbicides 2005). In 2004, Victor Yushchenko, presidential candidate for the Ukraine, was poisoned with a substantial quantity of TCDD (blood serum = 108,000 pg/g lipid weight; 50,000 fold above general population) and rapidly developed chloracne and was in critical condition. While the lasting results of this exposure beyond scarring have yet to surface, a three year study examining TCDD elimination determined a half-life of 15.4 months for high exposure, although 5 years is generally accepted for lower exposure (Sorg et al. 2009). There are several factors that confound this data including inadequate baseline measurements, potential exposure to toxic substances in addition to TCDD and related dioxins, and in some instances, poor follow-up. However, taken together, there is evidence supporting increase overall cancer risk and diabetes in humans exposed to TCDD. Due to these studies and those performed in animal models, the Environmental Protection Agency has classified TCDD as a Group B2, probable human carcinogen, and lists chloracne as the major effect seen in both acute and chronic exposure (U.S. Environmental Protection Agency 1997).

TCDD Toxicity in Animal Models

Given the complex nature of human exposure and reasonable lack of controlled clinical trials, researchers have utilized animal and cell models to provided the bulk of our knowledge regarding TCDD-induced toxicity. In a variety of animals, TCDD exposure induces wasting, thymic involution with resulting immunosuppression, hepatoxicity, chloracne and other epidermal abnormalities, and death (Poland and Knutson 1982). A thorough study by Vos and colleagues (Vos et al. 1974) examined the acute effects of high dose TCDD and subacute toxicity of low dose TCDD, all administered via gavage, in C57Bl/6 mice. Acute toxicity with a LD50 of 114 μg/kg resulted in wasting followed by severe edema before death, severe atrophy of the thymus and spleen, ocular hemorrhage, and degenerated and necrotic hepatocytes in the centrilobular areas with bile duct proliferation in the periportal areas of the liver. Subacute toxicity with 25 μg/kg TCDD administered weekly revealed similar, though less severe, manifestations at 2 weeks with increasing hepatic damage up to 6 weeks of administration and evidence of hepatic function impairment. Centrilobular necrosis, fatty change, and edema were also seen in ICR mice treated with high dose TCDD applied topically (Chang et al. 2005). The effects of TCDD toxicity specifically in the centrilobular regions of the liver is perhaps not surprising as the AhR and its well-known target gene *Cyp1A1* co-localize to this region in C57Bl/6J mice (Forkert 1997).

TCDD toxicity has also been implicated in carcinogenesis, though it remains unclear if it plays a role in tumor initiation or propagation, or both (Mandal 2005; Poland and Knutson 1982; Safe 2001). Rats subjected to a partial hepatectomy (PH), treated with one dose of diethylnitrosamine followed with low dose TCDD once every 2 weeks for 7 months indicate that TCDD is a promoter of hepatocarcinogenesis as only mice with both treatments developed tumors (Pitot et al. 1980), and this result is dependent on AhR (Viluksela et al. 2000). Transgenic animals with constitutively active AhR also display enhanced proliferative properties including induction of stomach tumors (Andersson et al. 2002) and promotion of hepatic tumors following *N*-nitrosodiethylamine treatment (Moennikes et al. 2004). These studies support further examination of the role of TCDD-induced AhR activity, especially in the liver.

ARYL HYDROCARBON RECEPTOR ACTIVITY

Classic Aryl Hydrocarbon Receptor Activity

The classic mechanism of AhR activity is depicted in **Figure 1.1**. The AhR is classified as a member of Per-Arnt-Sim (PAS) family of transcription factors and shares several features common to this family. The AhR is found in the cytoplasm of cells bound to HSP90 under steady state conditions (Perdew 1988; Probst et al. 1993). Upon ligand binding, and dependent on ligand binding, the AhR translocates to the nucleus, sheds its chaperonins, and heterodimerizes with another member of the PAS family, the aryl hydrocarbon nuclear translocator

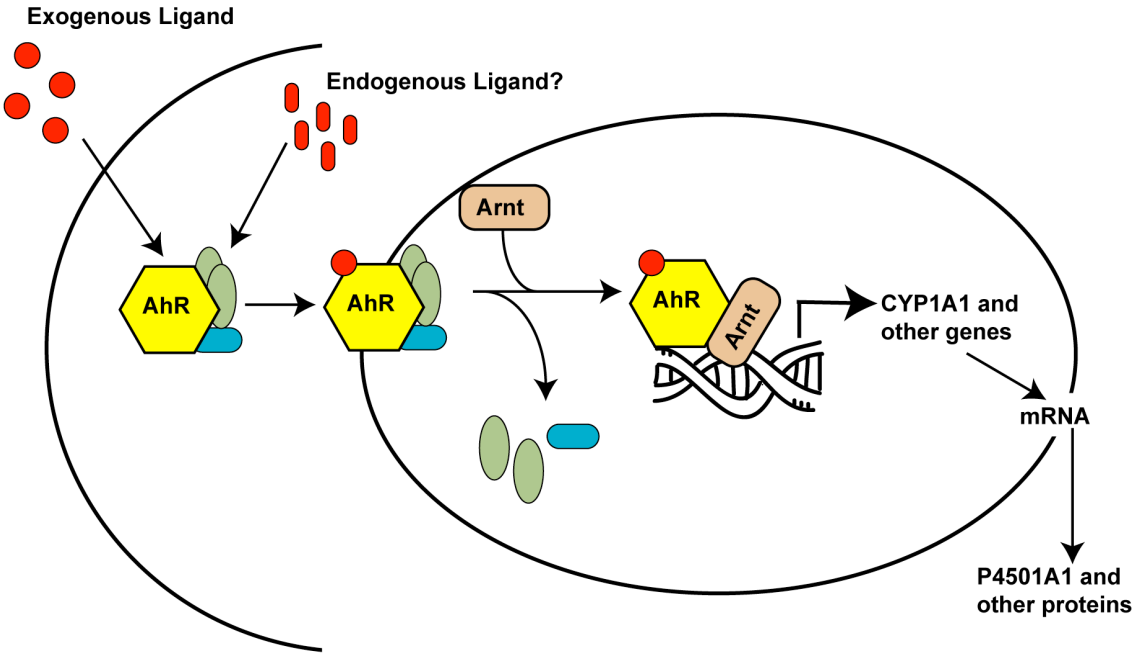

Figure 1.1. Classic pathway of AhR activation, translocation, heterodimerization with Arnt, binding to the canonical XRE, and inducing trancription of target genes.

(Arnt) (Hoffman et al. 1991; Ikuta et al. 1998; Lees and Whitelaw 1999; Probst et al. 1993; Prokipcak and Okey 1990). While nomenclature typically implies function, in actuality, Arnt is found almost exclusively in the nucleus (Holmes and Pollenz 1997), precluding its ability to facilitate the movement of AhR to the nucleus. Instead, Arnt functions to retain the receptor's nuclear localization and likely also plays a role in disruption of the AhR-HSP90 interaction (McGuire et al. 1996). Once formed, this transcriptionally active AhR-Arnt heterodimer binds the DNA consensus sequence (5'TnGCGTG-3') found in xenobiotic response elements (XRE), an enhancer element in the promoter region of target genes, notably the *Cyp1A* family of genes and the phase II enzymes UDP-glucuronosyltransferase and glutathione *S*-transferase implicated in biotransformation (Elferink and Whitlock 1994; Hankinson 1995; Probst et al.

1993; Wu and Whitlock 1993). It has been proposed that the AhR-Arnt heterodimer can enhance transcription at an alternate DNA site in the rat *Cyp1A2* gene when induced by 3-methylcholanthrene, but this study failed to show AhR binding to the site (Sogawa et al. 2004). While the classic mechanism of AhR activity has been well characterized, there is increasing growing evidence for alternate routes of activity.

Alternate Aryl Hydrocarbon Receptor Activity

In addition to Arnt, the AhR has been shown to interact with several other proteins affecting transcription. AhR binds GAC63, activating AhR-mediated transcription in reporter assays (Chen et al. 2006). Conversely, the AhR enhances the transcriptional activity of BRCA1 (Kang et al. 2008). The AhR independent of Arnt is capable of association with RelA, one member of the NF-κB transcription factor, and activation of NF-κB suppresses transcription at XREs (Ke et al. 2001; Tian et al. 1999), potentially by impairing chromatin remodeling involving p300/CBP (Ke et al. 2001), but the exact mechanism remains unclear. Interestingly, AhR also interacts with the NF-κB subunit RelB and induces TCDD-mediated transcription of IL-8 in macrophages at an as yet unidentified binding site in the IL-8 promoter (Vogel et al. 2007). This provides the first evidence of AhR interacting at a novel DNA binding site distinct from the XRE with a transcription partner other than Arnt, although the exact sequence remains ill

defined. These studies provide the first insights into AhR-mediated transcription that differs from the canonical pathway of strictly AhR and Arnt at an XRE.

Suppressing Aryl Hydrocarbon Receptor Activity

As with most biological systems, there are mechanisms to regulate AhR activity. In the absence of an exogenous ligand such as TCDD, it is speculated that the unknown endogenous ligand is metabolized by one of the target gene-encoded proteins in a feedback mechanism, thus preventing further activation of the receptor. Given its poor metabolism, TCDD-induced AhR activity is refractory to this process, but can be halted by two alternate mechanisms: 1) inhibition of its ability to induce transcription and 2) degradation of the protein.

In a proposed classic negative feedback loop, it has been demonstrated that one of the targets of AhR induction is the AhR repressor (AhRR) as its mRNA levels are decreased by two to three orders of magnitude in AhR knock-out mice and increased upon AhR activation as well as in mice with constitutively active AhR (Andersson et al. 2002; Bernshausen et al. 2006). As another member of PAS family, it was initially proposed that AhRR competes with the AhR for binding to Arnt, essentially sequestering Arnt and effectively halting AhR binding to XREs (Mimura et al. 1999). However, neither deletion of the C-terminus of the AhRR, which is typically is required for PAS family member interactions, nor overexpression of Arnt were able to abrogate suppression of the AhR (Evans et al. 2008). Furthermore, a point mutation inhibiting the ability of

AhRR to bind DNA still allowed AhR repression (Evans et al. 2008), indicating a mechanism other than interfering with AhR-Arnt heterodimerization and binding to XREs is responsible for AhRR mediated suppression of the AhR. It has been speculated that AhRR could interfere with AhR activation following nuclear translocation, specifically with its ability to dissociate from its chaperones in the nucleus (Mitchell and Elferink 2009), but this hypothesis has not been tested.

In addition to repression, actual degradation of AhR protein is another mechanism to suppress prolonged AhR activity. After activation with TCDD, AhR protein levels are decreased 5-fold in mouse hepatoma cells, 2-9 fold in rat liver, thymus, lung, spleen, and prostate, and high doses of TCDD decrease AhR protein in mouse centrilobular hepatocytes (Chang et al. 2005; Harper et al. 2006; Pollenz 2002). The AhR nuclear protein seems to be phosphorylated, ubiquitinated, and degraded without an appreciable amount being recycled to the cytoplasm (Prokipcak and Okey 1990; Roberts and Whitelaw 1999), and the transactivation domain of AhR appears to be important in this process (Pollenz et al. 2005), but the kinase and ubiquitin ligase involved and specific sites on AhR remain undefined. This pathway appears to be specific to the AhR fraction in the nucleus, implying that activation of the AhR is an important step in its eventual degradation. While there is ample evidence that AhR protein levels decrease following activation, AhR mRNA levels remain steady, and AhR protein degradation is likely via the 26S proteosome, little else is known about the targeting and actual mechanism of degradation (Pollenz 2002).

STRUCTURE OF THE ARYL HYDROCARBON RECEPTOR

Other than for its degradation, the structure of the AhR is has been well characterized and is depicted in **Figure 1.2** (Fukunaga et al. 1995; Gu et al. 2000). The N-terminus contains a nuclear localization sequence that is revealed upon ligand binding in the cytoplasm and a basic-Helix-Loop-Helix domain that is responsible for binding to XREs and contributes to both hsp90 and Arnt interactions. The AhR contains two PAS domains, both of which are necessary for binding to hsp90 and dimerization with Arnt. In addition, the ligand-binding region of AhR is located in the PAS B domain. The C-terminus has a glutamine-rich region, is responsible for transcriptional activation (Ge and Elferink 1998; Puga et al. 2000a).

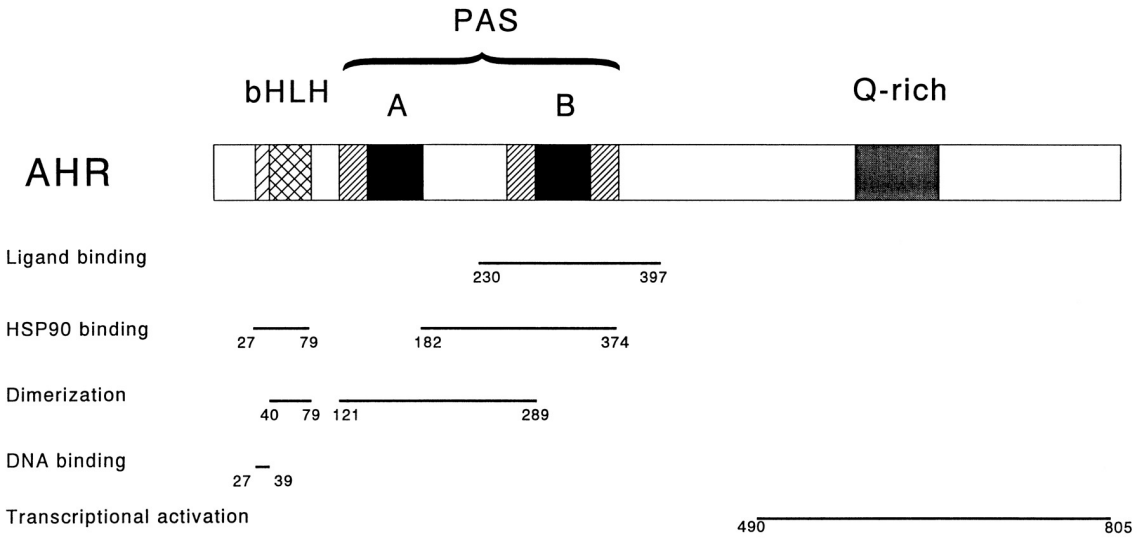

Figure 1.2. Structure of the AhR including its established functional domains (Fukunaga, Probst et al. 1995)

ARYL HYDROCARBON RECEPTOR NULL MICE

While the effects of TCDD-activation of the AhR have been studied for over four decades, investigators have begun to examine the role of the AhR in the absence of ligand by utilizing transgenic mice. AhR null mice were generated that lack AhR as early as *in utero* implantation. These mice do not exhibit pathology following TCDD administration, but do have hyperproliferation of the epidermis, hair follicles, liver blood vessels, and aged animals (11-13 months) develop sporadic adenocarcinomas of the liver and lung (Gonzalez and Fernandez-Salguero 1998). Given the seemingly complex nature of AhR activity *in vivo* with tumor formation in both animals that lack the AhR as well as animals with constitutively active AhR, it behooves us to examine the connection between AhR activation and cell proliferation.

PARTIAL HEPATECTOMY MODEL OF LIVER REGENERATION

The regenerative capacity of the liver has been appreciated since antiquity, and with the increasing interest in the physiological role of the AhR, scientists can exploit this property to study cell proliferation *in vivo*. As first described for the rat (Higgins and Anderson 1931), a partial hepatectomy (PH) is performed by ligating 2/3 of the liver, removing those lobes, closing the surgical site, and allowing the animal to recover. While the removed lobes do not actually “regenerate” per se, the remaining lobes undergo a compensatory hyperplasia until liver mass is restored in approximately 10 days, which is commonly referred

to as regeneration. This complex process requires a precise regulation with contributions from all hepatic cells: hepatocytes, biliary cells, endothelial cells, Kupffer cells, and stellate cells (Michalopoulos and DeFrances 1997). The majority of the liver, the hepatocytes, synchronously enters the cell cycle from a quiescent phase and begin the first round of DNA synthesis at approximately 12 hours post-PH with a peak at 24 hours in the rat (everything is time shifted 6-12 hours later for the mouse) (Michalopoulos and DeFrances 1997), making this a particularly attractive model to study cell cycle regulation, and potential perturbations, *in vivo*.

Cell Cycle Phases

Over two decades of work has revealed a complex process by which cells respond to external cues to grow, replicate their DNA, and divide. These stages can be separated into four main divisions: 1) G_1 (Gap 1), which is the initial growth phase, 2) S (Synthesis) phase during which DNA is actually replicated, 3) G_2 with additional growth before 4) Mitosis. The actual movement through and between these phases is a highly regulated and sequential activation of cyclin-dependent kinases (Cdks) by their corresponding cyclins (Sherr 1993) and their regulation by inhibitors (Sherr and Roberts 1999) as depicted in **Figure 1.3**. Influence from growth factors seems to be limited to the G_1 through S phase transition, as hepatocytes in the S-phase appear to be committed to division (Albrecht et al. 1993).

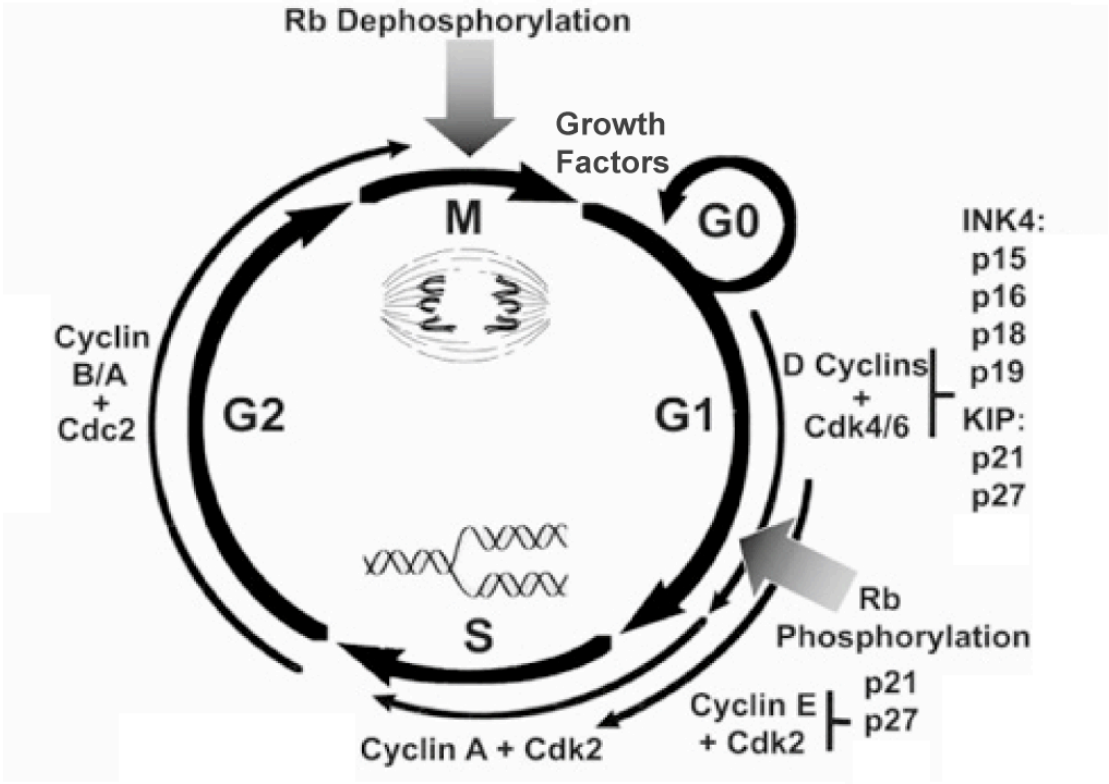

Figure 1.3. Following growth factor inducing cells to transition from G0 to G1, the cell cycle progresses through a highly regulated interaction between Cyclins and their associated Cdks and inhibiors as depicted. Modified from (Donovan and Slingerland 2000)

G_1 Phase of the Cell Cycle

The first regulator of the G_1 phase is Cyclin D and its associated kinases Cdk4 and Cdk6 (Albrecht et al. 1995; Sherr 1993; Sherr 1996). While the INK4 family of proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) inhibit the association of Cyclin D with Cdk4/6 (Hirai et al. 1995; Serrano et al. 1993), this interaction can be facilitated by $p21^{Cip1}$ and $p27^{Kip1}$ (LaBaer et al. 1997), and it is the balance of these various components that drive the initial steps in G_1 (Ortega et al. 2002). Cyclin D1 appears to be the dominant Cyclin D, rather than D2 and D3 in the mouse liver (Albrecht et al. 1993), and its induction has been detected

as early as 12 hours following PH in rats (Lu et al. 1992). When activated by Cyclin D1, Cdk4/6 phosphorylate pRB (Ortega et al. 2002), and essentially pass the torch to the pairing of Cyclin E and Cdk2.

G_1 to S Boundary of the Cell Cycle

Cyclin E expression seems to follow a similar time course to Cyclin D, but peaks at the boundary between G_1 and S phase (Albrecht et al. 1993; Lu et al. 1992; Sherr 1996). Cyclin E-Cdk2 also phosphorylates pRB (Ortega et al. 2002) to continue the progression through the G_1 phase, but data show that Cyclin E is also induced by E2F1 and suppressed by pRB (Geng et al. 1996). Cyclin E also associates with $p21^{Cip1}$ and $p27^{Kip1}$, but unlike with Cyclin D-Cdk4/6, this interaction prevents Cdk2 mediated phosphorylation of pRB (Harper et al. 1993; Polyak et al. 1994; Sherr 1996). $p21^{Cip1}$, first discovered as a target of p53 activation, is a potent growth inhibitor (El-Deiry et al. 1993; Wu et al. 1996) that also plays a role in promoting the formation of Cyclin D-Cdk4/6 complexes. During liver regeneration in mice, $p21^{Cip1}$ mRNA has a biphasic induction with levels rising between 1 to 24 hours post-PH in early G_1 and 48 to 72 hours, which is after S phase, independent of p53 (Albrecht et al. 1997). This time frame may correlate to the dichotomous function of $p21^{Cip1}$ promoting Cylin D-Cdk4/6 in G_1 and inhibiting Cdks thereafter, but mechanistic studies examining the precise regulation of these interactions in the context of a PH time course have not been examined. $p21^{Cip1}$ knockout mice develop normally, but are unable to G_1 arrest in

response to DNA damage (Deng et al. 1995), potentially indicating a role in response to stimuli whether PH or DNA damage, but not in normal development. Mice lacking $p27^{Kip1}$ seem to have increased growth, but are difficult to study due to infertility in female mice by disruption of the pituitary-ovarian axis (Kiyokawa et al. 1996). However, overexpression of $p27^{Kip1}$ does cause a G_1 arrest (Toyoshima and Hunter 1994), underscoring its importance in halting cell cycle progression.

pRB interacts with the E2F family of transcription factors, inhibiting their induction of target genes that are necessary for cell proliferation (Weinberg 1995). In order to remove the inhibition on E2F1 by pRB, it must be sequentially phosphorylated by Cyclin D-Cdk4/6 and then Cyclin E-Cdk2 (Lundberg and Weinberg 1998). The now hyperphosphorylated RB (ppRB) dissociates from E2F1, and E2F1 induces expression of genes important in cell cycle progression including itself and Cyclin E, to further stimulate RB phosphorylation (Geng et al. 1996; Sherr 1996). Once cells enter into the S-phase, there is rapid degradation of Cyclin E by the 26S-proteosome following phosphorylation by Cdk2 (Clurman et al. 1996), and the remainder of the proliferative process is growth factor independent. ppRB remains phosphorylated, likely through Cyclin A and B associated Cdks, and thus unable to inhibit E2F1 activity until anaphase of mitosis. Complete dephosphorylation of ppRB is evident by the following G_1 phase in synchronized cells (Ludlow et al. 1993).

PRIMING PHASE OF LIVER REGENERATION

While the cell cycle machinery has been well characterized, work continues to progress regarding the priming phase of the regenerative process. Many have speculated that hemodynamic changes, specifically the increase burden from the portal system that is seen in PH, could be one of the mechanistic triggers of regeneration, but there is no direct evidence that supports this hypothesis (Michalopoulos 2007). While it remains unknown what specifically triggers hepatocytes to enter the cell cycle, but it has been suggested that tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) are important components in the initiation (Cressman et al. 1996; Fausto 2000; Yamada et al. 1997), although they are not direct hepatomitogens (Michalopoulos 2007). There is evidence that epidermal growth factor receptor (EGFR) and its ligands are direct hepatomitogens in cell culture. In addition, EGFR is upregulated in the first 3 hours during regeneration following PH supporting additional evidence of EGFR's necessity in regeneration (Michalopoulos 2007).

Hepatocyte Growth Factor

In addition to the EGFR ligands epidermal growth factor (EGF) and TGFα, hepatocyte Growth Factor (HGF) is a direct hepatic mitogen capable of inducing clonal expansion of hepatocytes in culture (Block et al. 1996), and is considered one of the essential factors for the priming phase of liver regeneration following PH (Michalopoulos 2007). HGF is stored as a latent precursor in the extracellular

matrix (Masumoto and Yamamoto 1991; Masumoto and Yamamoto 1993) and is cleaved to become an active, potent mitogen by the serine protease urokinase plasminogen activator (uPA) when bound to its receptor uPAR (Kim et al. 1997; Mars et al. 1993; Naldini et al. 1992). Levels of single chain, inactive precursor HGF decrease in the liver within three hours following PH in the rat and are restored thereafter during the 72 hours post-PH, (Pediaditakis et al. 2001), correlating with the increase in hepatic HGF mRNA beginning at 3 hours and peaking at 12 hours post PH (Zarnegar et al. 1991), depicting the early use of stored HGF and subsequent replenishment.

cMet

Activation of the HGF receptor, cMet, is detected within 1 hour post-PH in the rat (Stolz et al. 1999) and has been shown to mediate all the effects of HGF (Naldini et al. 1991). cMet is critical for the liver response to injury as adult mice lacking cMet subjected to PH are unable to regenerate liver tissue with hepatocytes remaining in the quiescent phase (Borowiak et al. 2004). Likewise, mice lacking cMet in the adult liver are unable to adequately repair when challenged with FAS ligand or CCl_4 (Huh et al. 2004). Targeted suppression of cMet *in vivo* with RNAi before and after PH resulted in essentially no regeneration as long as cMet levels were suppressed, and microarray analysis showed a dysregulation of $p21^{Cip1}$ and suppressed Cyclin E levels (Paranjpe et al. 2007). Additionally, HGF has been shown to induce Cyclin D1 and $p21^{Cip1}$

expression (Albrecht et al. 1995; Albrecht et al. 1997). These studies reveal a critical role for cMet in the early stages of liver regeneration as hepatocytes in mice lacking cMet never progress to the G_1 phase of the cell cycle, but also indicate a potential interaction between cMet activation and regulators of cell cycle progression at the G_1-S transition once cells have reached this checkpoint.

Urokinase Plasminogen Activator and Its Receptor

Both uPA and uPAR display increased activity with HGF cleavage and extracellular matrix remodeling just minutes following PH (Kim et al. 1997; Mars et al. 1995a), and both have been reported as having increased transcript and protein levels in human hepatocellular carcinoma (HCC) samples compared to normal adjacent tissue, as well as exhibiting a positive correlation with tumor invasiveness (Zheng et al. 2000), implying a role for uPA activity in cell proliferation and possibly invasion. $uPA^{-/-}$ mice exhibit a decrease in processed HGF and subsequent delay in liver regeneration after PH and Fas-mediated hepatic apoptosis (Roselli et al. 1998; Shimizu et al. 2001a). These data portray HGF, following uPA-mediated cleavage and cMet activation, as a potent mitogen required early in the regenerative process for normal restoration of the adult liver following injury.

Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1) is a protease inhibitor often studied for its role in the fibrinolytic system, but is increasingly recognized as central in regulating uPA activity in the liver rather than the blood. PAI-1 not only binds to uPA and its receptor uPAR to inhibit protease activity, it also promotes the endocytosis of the uPA-UPAR-low-density lipoprotein receptor-related protein (LRP) complex and subsequent degradation of uPA in the lysosome (Cubellis et al. 1989; Cubellis et al. 1990; Degryse et al. 2001). Thus PAI-1 inhibits uPA activity as well as promoting its degradation, thereby suppressing HGF processing through two distinct mechanisms. In humans, PAI-1 protein levels are elevated in serum one day post-operative in recipients of adult living related partial liver transplantations (Sato et al. 2008), although it is unclear if this is due to signals within the liver for regeneration or response to the surgery. PAI-1 protein levels are also increased in human HCC samples compared to normal adjacent tissues (Zheng et al. 2000), mirroring what was previously seen with uPA expression. Likewise, PAI-1 induction is evident within two hours following PH in rodent models (Schneiderman et al. 1993; Thornton et al. 1994). The rapid induction of PAI-1 following PH would indicate a pivotal role in regulating proliferation. PAI-$1^{-/-}$ show accelerated regeneration in a Fas-induced hepatic apoptosis model, and this response is abrogated with anti-HGF antibody (Shimizu et al. 2001b), but no one has yet examined the regenerative response in a PH model. PAI-1 effects not only HGF processing, but also cleavage of the

extracellular matrix that appears to be necessary for tissue remodeling during the regenerative process and for providing additional growth factors and binding sites for the proliferating hepatocytes.

THE ARYL HYDROCARBON RECEPTOR AND CELL CYCLE REGULATION

Several studies support a role for the AhR in suppression of the basic cell cycle machinery, specifically at the G_1 to S transition. It has been previously established that TCDD-induced AhR activity slows proliferation of rat hepatoma cell lines by inhibiting progression into the S phase of the cell cycle (Weiss et al. 1996). Interestingly, the duration of AhR activation seems to be influential, because transient AhR activation observed following serum release of serum-starved 5L rat hepatoma cells promotes normal cell cycle progression through the S phase, but sustained AhR activity induced directly via TCDD treatment, or indirectly with the addition of the P4501A1 suicide substrate 1-Propynyl pyrene (1-PP) that facilitates prolonged endogenous AhR signaling, results in a G_1 arrest in these same cells (Levine-Fridman et al. 2004). Of note, both TCDD and 1-PP induce AhR-dependent expression of the Cdk inhibitor $p27^{Kip1}$, inhibiting cell proliferation in 5L rat hepatoma cells, which can be rescued with *Kip1* antisense RNA (Kolluri et al. 1999; Levine-Fridman et al. 2004; Marlowe et al. 2004). Several studies demonstrate a direct interaction between AhR and pRB, independent of Arnt, in yeast two-hybrid studies and in *in vitro* pull-down studies (Ge and Elferink 1998), and endogenously in MCF-7 cells once the AhR has

been activated and translocated into the nucleus (Puga et al. 2000a). In SAOS-2 cells, the direct interaction between the AhR and pRB synergizes to inhibit E2F-mediated transcription leading to cell arrest (Puga et al. 2000a). Using Hepa-1 mouse hepatoma cells, TCDD-activated AhR could bind pRB and displace the p300 coactivator from the E2F/DP quaternary complex driving E2F-regulated genes with resulting decreased expression of Cdk2, Cyclin E, and DNA polymerase α essential for DNA synthesis and S-phase progression, thereby triggering cell arrest at G_1 (Marlowe et al. 2004). Hence, the AhR was shown to inhibit cell cycle independent of direct DNA binding by functioning as a corepressor. Huang and Elferink (2005) demonstrated that TCDD-induced G1 arrest of 5L mouse hepatoma cells is at least partially dependent on Arnt protein function, consistent with transcriptional activation involving the canonical AhR/ Arnt dimer, and implies that AhR-mediated cell cycle arrest encompasses multiple mechanisms. However, examination of the AhR-Arnt-p300 interaction indicated that p300 interacted with Arnt, not AhR (Kobayashi et al. 1997), suggesting that the role for Arnt is likely more complex. While there is agreement that AhR activation leads to a G_1 arrest in proliferating cells, the mechanistic basis remains unclear.

Cell lines provide a useful model system for teasing out mechanisms of action in a highly controlled environment, but there are limitations to the wide-spread application of findings in cell lines. First, cell lines by their very nature

possess corrupted cell cycle checkpoint control and exhibit a highly proliferative capacity, and are thus far removed from their normal *in vivo* primary cell counterparts. Second, these cell lines are examined in isolation without contributions from the surrounding extracellular matrix and in the case of the liver, the non-parenchymal cells that populate the liver. Because of these considerations, I used a partial hepatectomy model to study the effects of TCDD-mediated AhR activity on cell proliferation *in vivo* with the intent of addressing some of the questions raised by the studies using cell lines.

THE ARYL HYDROCARBON RECEPTOR AND LIVER REGENERATION

The Aryl Hydrocarbon Receptor Suppresses Liver Regeneration

Using a 2/3 PH model, mice pretreated with TCDD prior to PH displayed suppressed proliferation in the regenerating liver as evaluated by BrdU incorporation, and this suppression was attributed to decreased Cdk2 activity (Mitchell et al. 2006). Rats treated with TCDD before surgery also displayed a significant decrease in hepatocyte proliferation determined by decreased proliferating cell nuclear antigen (PCNA) staining (Bauman et al. 1995). It was also observed that the cells that did proliferate seemed to be localized to the periportal region of the liver rather than the pan-lobular regeneration detected in control-PH animals using the rat model. However there was no discernable distinction in proliferation patterns of mice pretreated with TCDD prior to PH. This may reflect a species difference, or could possibly be due to differences in TCDD

dosing regimens: 14 days of pretreatment in the rats compared to 24 hours in the mice, but there is no evidence to directly support this hypothesis.

Activated Aryl Hydrocarbon Receptor During the Priming Phase

Although one explanation for the suppression of liver regeneration in TCDD treated mice may be attributable to a G_1 arrest, other considerations deserved inquiry including an exploration of TCDD effects on the priming phase. In silico analysis of the IL-6 promoter detected a putative XRE upstream of the transcriptional start site (Lai et al. 1996), however, IL-6 levels were not changed with TCDD administration during the regenerative process. Likewise, TNF-α levels remained unchanged (Mitchell et al. 2006). TCDD has been shown to decrease EGFR levels, ligand binding, and autophosphorylation in rats treated with diethylnitrosamine (Sewall et al. 1993), but primary hepatocytes exposed to mitogenic stimuli exhibit decreased DNA synthesis in response to TCDD without altering the EGFR receptor status or activity (Hushka and Greenlee 1995). While EGFR activity cannot be unequivocally ruled out as participating in TCDD-mediated suppression of regeneration, given the lack of response in mitogen-induced proliferation, I thought it was important to focus on alternate pathways involved in the priming phase, specifically HGF **(Figure 1.4)**.

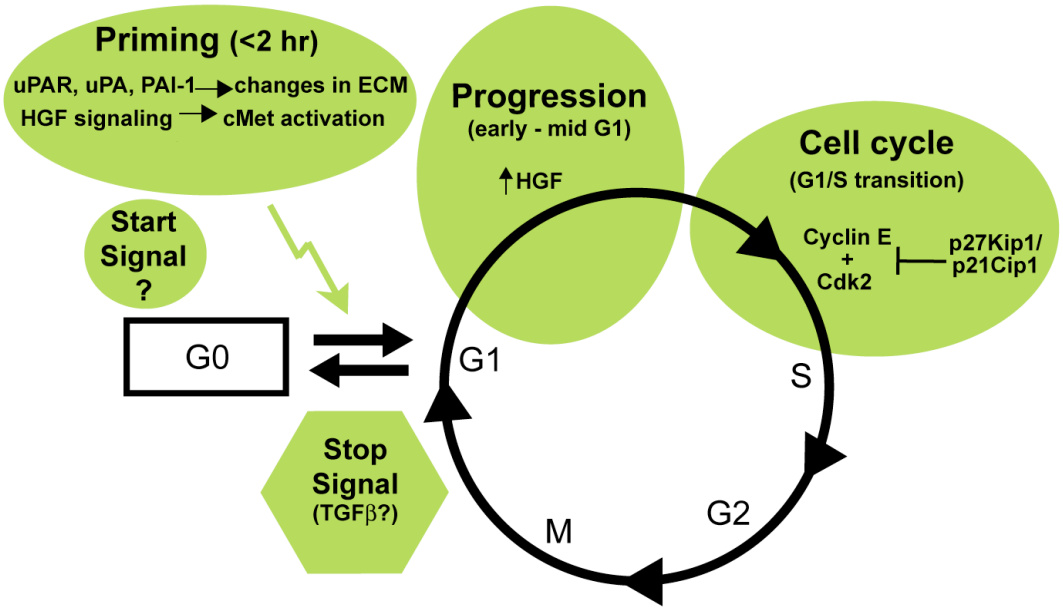

Figure 1.4. TCCD activation of AhR suppresses liver regeneration in rats and mice. Evidence to date suggests that AhR mediated disruption of Cyclin E-Cdk2 activity during the cell cycle is important in the suppression of regeneration. This work will focus on a potential role for the AhR during the priming phase of regeneration following PH.

The Aryl Hydrocarbon Receptor and the PAI-1 Promoter

Several recent studies provide compelling evidence that the AhR regulates expression of the PAI-1 gene. Two independent cDNA microarrays of human hepatoma HepG2 cells treated with TCDD identified PAI-1 to be a direct target of AhR activity as transcript levels were increased regardless of cyclohexamide pretreatment (Frueh et al. 2001; Puga et al. 2000b). Our laboratory confirmed PAI-1 induction by TCDD-activated AhR using primary mouse hepatocytes (unpublished data). In the mouse hepatoma cell line Hepa1c1c7 and its AhR deficient derivative, PAI-1 induction was observed with TCDD treatment, but only in cells with a functional AhR (Son and Rozman 2002). When examining the PAI-1 promoter (reviewed in Dimova and Kietzmann 2008), evidence supports PAI-1 gene regulation by a number of factors that have been implicated in AhR

cross-talk including transforming growth factor ß (TGF-ß) and hypoxia inducible factor-1 (HIF-1) or HIF-2 (Ahn et al. 2010; Dennler et al. 1998). Significantly, a luciferase reporter system demonstrated that the region between -161 to +73 of the PAI-1 promoter—a region lacking the TGF-ß, HIF-1 or HIF-2 binding sites—supported AhR-dependent TCDD inducibility and could be repressed using receptor antagonists (Son and Rozman 2002).

Using luciferase reporter constructs, TCDD responsiveness of the 161 base pairs upstream of the PAI-1 start site previously identified (Son and Rozman 2002) was explored in transiently transfected rat hepatoma cells by Dr. Huang in the Elferink laboratory. These studies confirmed that PAI-1 gene induction is both AhR dependent and TCDD inducible (**Figure 1.5**). Further characterization of the PAI-1 promoter using deletion constructs and point mutations pared this region down to a 36-nucleotide sequence, referred to as the non-consensus XRE (ncXRE), that the AhR bound in a TCDD-dependent fashion (**Table 1**). Not only is the sequence of the ncXRE completely different from the XRE, electrophoretic

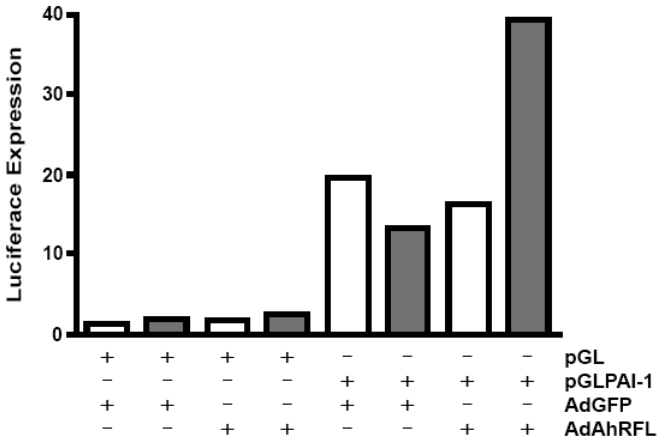

Figure 1.5. PAI-1 promoter-induced luciferase activity is both AhR and TCDD dependent. AhR deficient BP8 rat hepatoma cells were transfected with the pGL minimal reporter luciferase plasmid or pGLPAI-1 with the PAI-1 promoter driving luciferase activity. These cells were then infected with an adenovirus containing the reporter green fluorescent protein (AdGFP) or the full length AhR protein (AdAhRFL). Cells were grown to 70% confluence before treatment with TCDD or DMSO (vehicle) for 24 hours. Cell lysates were collected and measured for luciferase activity.

mobility shift assays (EMSAs) reveal a distinct protein-DNA complex formed at the ncXRE compared to that seen at an XRE. Specifically, a 100-fold excess of a 'cold' XRE competitor is unable to abolish formation of the ncXRE complex

TABLE 1

	Oligonucleotide sequence
XRE	5'-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3'
NC-XRE WT	5'-GTCCCAGCAAGTCACTGGGAGGGAGGGAGGGAGGGGGAG-3'

(**Figure 1.6**). Using *in vivo* chromatin immunoprecipitation (ChIP), evidence was obtained suggesting that the AhR binding to the PAI-1 promoter is Arnt-independent (**Figure 1.7**). Although this data does not categorically exclude a role for Arnt, failure to detect Arnt binding to the PAI-1 promoter, but not the Cyp1a1 promoter, implies that AhR binding to the ncXRE may involve an association with a new as yet unidentified factor.

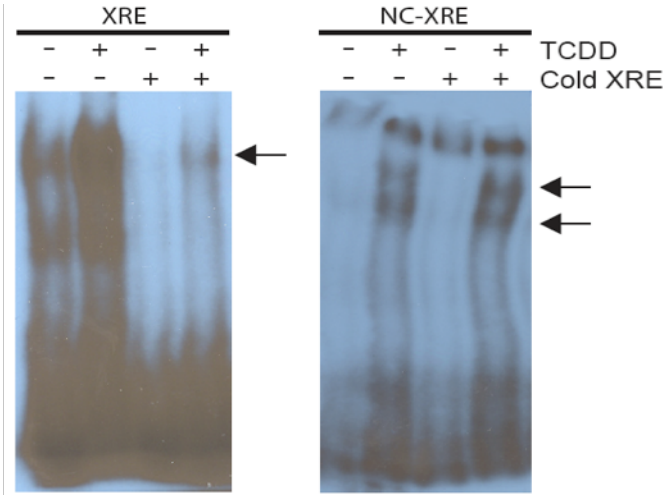

Figure 1.6. TCDD inducible EMSA product in the presence of NC-XRE shows a distinct binding to that seen with the XRE. Animals were treated with 20μg/kg TCDD (+) or vehicle (-), nuclear extracts were collected and analyzed via EMSA with either radioactively labeled XRE or NC-XRE. Competitive inhibition was performed with 100X cold XRE in both samples; no competitive inhibition is seen with the radioactively labeled NC-XRE.

Potential Role of Kruppel-Like Factor 6

Sequence comparisons reveals that the ncXRE shares homology with the classic binding site of the Kruppel-Like Factor Family (KLF) of proteins. This family is

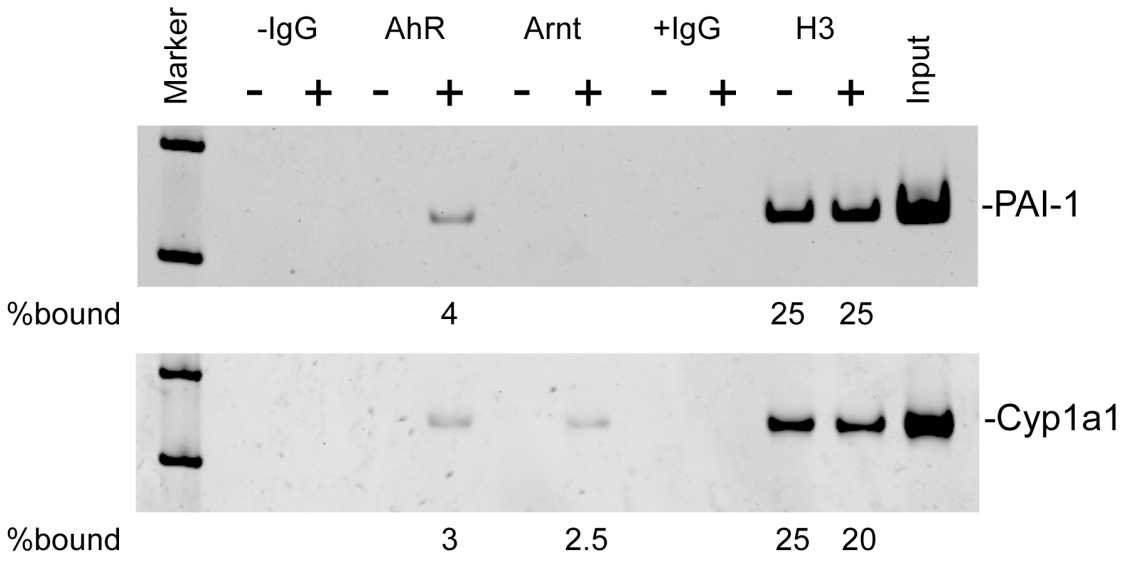

Figure 1.7. AhR associates with both the PAI-1 and P4501A1 promoters, but ARNT does not using a ChIP assay. Mice were treated 2 hours before liver removal with either 20μg/kg TCDD (+) or vehicle (-). Livers were homogenized, treated to crosslink DNA, probed with antibodies against AhR, ARNT, IgG (negative control), and the histone H3 (positive control). Samples then were un-cross-linked and PCR performed with primers for either the PAI-1 or P4501A1 (positive control) promoter regions. Shown are the PCR products with % of input calculated.

related to Sp1 and grouped based on three highly conserved zinc finger domains in the C-terminus, and thus have a similar binding site that include GC boxes and CACCC response elements (Bieker 2001; Philipsen and Suske 1999). There is a plethora of evidence that links the KLF family in cell growth and cancer (Black et al. 2001), making this an intriguing family when considering potential partners for AhR in suppressing proliferation. In addition, two separate KLF family members have been associated with the *Cyp1A1* gene: KLF9 can bind to regions in the rat *Cyp1A1* promoter and modulate its expression depending on the number of GC boxes (Imataka et al. 1992), and KLF4 inhibits *Cyp1A1* induction via an interaction with Sp1 in transfected Chinese hamster ovary cells (Zhang et al. 1998). However, neither KLF9 nor KLF4 are expressed in the liver. However, by exploring the expression profile of other KLF family members, I discovered that

not only is KLF6 found in the liver, it has been implicated in cell cycle regulation, and KLF6 binds the promoter region and induces expression of uPA in endothelial cells (Kojima et al. 2000). Hence, KLF6 was identified as a promising candidate for an AhR binding partner at the ncXRE. Evidence provided in the document supports this prediction.

KLF6 was first identified as an early gene response in liver stellate cells to liver injury by CCL_4 (Lalazar et al. 1997; Ratziu et al. 1998). Additional characterization confirmed KLF6 binding to GC box motifs (Suzuki et al. 1998), characteristic of KLF family members, almost exclusive peri-nuclear and nuclear localization, and expression and activity in human HepG2 cells (Ratziu et al. 1998), indicating that KLF6 can indeed function as a transcription factor in hepatocytes. Recent data has confirmed that wild-type KLF6 resides in the nucleus, and mutations in its nuclear localization sequence found within the zinc finger domains represses its ability to induce expression of target genes (Rodriguez et al. 2010). While much is not known about the exact mechanism of KLF6 transcriptional activation, there is evidence that KLF6 can interact with Sp1 to promote transcription (Botella et al. 2009).

KLF6 is commonly considered a tumor suppressor due to its conspicuous loss in several tumor types including prostate (Narla et al. 2005a; Narla et al. 2001), ovarian (DiFeo et al. 2006), colorectal (Reeves et al. 2004), and sporadic gastric tumors (Cho et al. 2005). Examining KLF6 mRNA levels in HCC samples

from patients with Hepatitis B revealed a statistically significant reduction of 57% and 73% compared to control livers and adjacent tissues respectively (Kremer-Tal et al. 2007). This finding was also seen in patients with Hepatitis C, and additionally, there was a significant decrease in KLF6 mRNA levels between cirrhosis and dysplastic tissues (Kremer-Tal et al. 2007), indicating the loss of KLF6 may be important in the early progression to hepatic neoplasms. In a separate study, KLF6 was down-regulated in 39.1% of HCC tissues (Pan et al. 2006), again supporting a connection between loss of KLF6 and tumor progression in the liver. There is also evidence of a KLF6 splice variant lacking a portion of the zinc-finger DNA binding domain that is up-regulated in HCC and prostate tumors which seems to antagonize the function of wild-type KLF6, although the mechanism remains unclear as this variant appears to be localized to the cytoplasm (Kremer-Tal et al. 2007; Narla et al. 2005a; Narla et al. 2005b).

KLF6 induces a number of genes whose corresponding protein inhibits cell cycle progression. KLF6 has been shown to increase levels of $p21^{Cip1}$ *in vitro* and *in vivo* in transgenic mice overexpressing KLF6 (Narla et al. 2001; Narla et al. 2007). In a set of 33 HCC samples compared to their surrounding tissue controls, there was a significant correlation between those with low $p21^{Cip1}$ and low wild-type KLF6 (Narla et al. 2007). Given that $p21^{Cip1}$ is an inhibitor of the S-Phase of the cell cycle, this provides one potential explanation as to the role of KLF6 in inhibiting cellular proliferation. In addition to increased levels of $p21^{Cip1}$, KLF6 binds Cyclin D, inhibiting its interaction with Cdk4/6, leading to a

redistribution of $p21^{Cip1}$ to Cdk2, decreased RB hyperphosphorylation, and a G_1 cell cycle arrest (Benzeno et al. 2004). Although the field of KLF6 is growing, much remains unknown about other KLF6 targets and the function of KLF6 outside of tumor suppression.

Hypothesis

Evidence supports the conclusion that TCDD treatment and subsequent activation of AhR can perturb cell cycle regulation, specifically at the G_1 to S transition, which in turn attenuates liver regeneration. Without detracting from the validity of that finding, I contend that additional AhR-regulated processes involving the uPA signaling cascade also contribute to the regenerative process, particularly during the priming phase, that are deserving of further investigation. While other components of the priming phase have been assessed, and found unchanged with TCDD treatment, the role of HGF, a known hepatomitogen, has been thus far overlooked. Specifically, evidence supports a connection between PAI-1 induction and AhR activation by TCDD, but this interaction has yet to be examined in the context of regeneration following PH.

Interestingly, early studies associating AhR with PAI-1 induction fail to include the common elements associated with AhR activity, specifically binding with Arnt at an XRE. While there is ample evidence of AhR interaction with proteins other than Arnt including RB, RelA and RelB, only RelB describes a novel AhR-DNA interaction, although the actual binding site remains

uncharacterized. Recent evidence from my laboratory identified a novel, well-defined DNA binding site, the ncXRE, for the AhR in the PAI-1 promoter that does not appear to require Arnt. Comparison of the EMSA products between the complex bound to the XRE and that to the ncXRE, imputes the involvement of additional protein species at the ncXRE given the two complexes with distinct binding properties from those observed with the XRE. With an established role as a tumor suppresser halting the cell cycle and the homology between its binding site and the ncXRE sequence, KLF6 provides a compelling candidate for binding with AhR at the ncXRE.

I hypothesize that AhR in conjunction with KLF6 binds to the ncXRE in the PAI-1 promoter, and that this protein-DNA complex is responsible for TCDD inducible PAI-1 expression affecting liver regeneration.

CHAPTER 2: TCDD MEDIATED ACTIVATION OF THE AHR INDUCES PAI-1, IMPACTING LIVER REGENERATION

INTRODUCTION

AhR activity has been associated with positively regulating cell proliferation in a number of model systems and in humans exposed to TCDD, the potent prototypical AhR agonist. In particular, humans exposed to TCDD have an increased general risk of cancer (Kogevinas et al. 1997; Ott and Zober 1996), transgenic animals expressing a constitutively active AhR develop tumors (Andersson et al. 2002), and mouse hepatoma cell lines lacking the AhR have a prolonged G_1 phase of the cell cycle that can be restored to normal with the reintroduction of full length AhR (Ma and James P. Whitlock 1996). This promotion of proliferation is also evident in the liver when AhR is activated by TCDD in the presence of known carcinogens (Moennikes et al. 2004; Pitot et al. 1980; Viluksela et al. 2000) or potent exogenous hepatomitogens (Mitchell et al. 2010), and both processes are dependent on the presence and TCDD-mediated activation of the AhR.

Conversely, a body of evidence is emerging supporting AhR-mediated suppression of cell proliferation, specifically at the G_1 to S transition of the cell cycle. Rat hepatoma cells exposed to TCDD exhibit cell cycle arrest at G_1

(Huang and Elferink 2005; Levine-Fridman et al. 2004; Weiss et al. 1996), potentially via AhR-mediated induction of the Cdk2 inhibitor $p27^{Kip1}$ (Kolluri et al. 1999) or direct interactions with pRB (Ge and Elferink 1998; Marlowe et al. 2004; Puga et al. 2000a), suppressing the activity of E2F1. In PH models of regeneration, TCDD pretreatment attenuates liver regeneration in rats (Bauman et al. 1995) and mice (Mitchell et al. 2006). Thus far, the TCDD-induced inhibition of proliferation has been attributed to suppressed Cdk2 activity, although the exact mechanism *in vivo* remains unclear as there is decreased Cyclin E association with Cdk2 despite comparable levels to vehicle pretreated animals of Cyclin E and no appreciable increased association between Cdk2 and its inhibitors (Mitchell et al. 2006). Collectively, the data indicate that the AhR can both promote and inhibit cell growth, although the precise circumstances leading to a particular cell fate defy a complete understanding to date.

Given the questions that still remain regarding TCDD-mediated suppression of liver regeneration *in vivo*, I was interested in examining a potential role for the AhR during the priming phase in liver regeneration. While EGFR and HGF activity through its receptor cMet have been identified as critical to induce hepatocyte proliferation in response to PH, additional factors including IL-6 and TNF-α are considered influential in mediating the hepatocyte transition from quiescence into the G_1 phase of the cell cycle (Michalopoulos 2007). It has been demonstrated that TCDD treatment prior to PH does not alter expression of IL-6

and TNF-α in mice compared to their vehicle pretreated controls (Mitchell et al. 2006), and EGFR status and activity remained unchanged in AhR-mediated suppression of DNA synthesis in primary hepatocytes (Hushka and Greenlee 1995). Interestingly, TCDD was shown to induce expression of PAI-1 (Son and Rozman 2002), a serine protease inhibitor that inhibits uPA activity thereby suppressing HGF activation.

I propose that TCDD treatment of animals prior to PH can induce expression of PAI-1, impair HGF processing and subsequent activation of its receptor cMet, to suppress regeneration. If so, liver regeneration should be refractory to TCDD treatment in PAI-1$^{-/-}$ mice. The studies described in this chapter provide evidence in support of the hypothesis that AhR regulation of PAI-1 expression contributes to receptor-mediated suppression of proliferation distinct from previous findings showing AhR-dependent control of G1 phase cell cycle progression.

MATERIALS AND METHODS

Animals. All care and procedure conditions were approved by the guidelines set by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (UTMB) at Galveston. C57Bl/6 and PAI-1 knock-out (PAI-1$^{-/-}$) mice (backcrossed onto a C57Bl/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME) and used at 8-10 weeks

of age. Animals were maintained in microisolator cages with unlimited access to drinking water and food while under a 12 hour light/dark cycle.

TCDD. TCDD (Cerilliant, Round Rock, TX) was dissolved in anisole and diluted to 2μg/ml in peanut oil, whereas the vehicle control consisted of peanut oil spiked with a corresponding amount of anisole. Animals were administered either vehicle or TCDD (20 ug/kg) by gavage 24 hours before surgery. This dose is sufficient to fully activate the AhR but not induce acute toxicity.

Surgical Procedures. All surgeries were performed under isoflurane-inhaled anesthesia and clean conditions. Both PH and sham surgeries were performed based on a procedure described previously (Higgins and Anderson 1931). Briefly, once anesthetized, an incision on the ventral side in both the skin and underlying fascia provided access to the liver where 70% of the lobes were ligated and removed. The fascia and skin were closed, and the mice were observed during recovery for signs of morbidity. Mice were sacrificed at the indicated times by cervical dislocation, and liver and blood were collected for the assays described below.

Quantitative Real Time RT-PCR (qRT-PCR) Analysis. RNA was isolated from frozen liver tissue at the indicated times after surgery with an RNAqueous kit (Ambion Inc, Austin, TX) according to the manufacturer's protocol. qRT-PCR was performed by the Sealy Center for Cancer Cell Biology and Real-Time PCR Core Facility at UTMB as described previously (Mitchell et al. 2006).

Serum PAI-1 Measurements. Plasma was collected from animal under anesthesia via cardiac puncture in heparin-coated syringes and tubes and centrifuged to pellet the red blood cells. Plasma was flash frozen in liquid nitrogen and stored at -80°C until used, avoiding repeat freeze-thaw. Plasma samples were assayed in triplicate by ELISA for active PAI-1 according to the manufacturer's instructions (Molecular Innovations, St. Louis, MO). The sensitivity of these assays was 0.05 ng protein per ml of plasma.

5'-Bromo-2'-deoxyuridine Labeling and Histopathology. Evaluation of liver proliferation was performed as described previously (Mitchell et al. 2006) with 5'-Bromo-2'-deoxyUridine (BrdU; Sigma Aldrich, St. Louis, MO). Mice were injected intraperitoneally with 50 mg/kg BrdU 2 hours before sacrifice or given continuously in their drinking water (0.8 mg/ml BrdU) that was protected from light and replenished daily for 72 hours post-PH. Formalin-fixed liver tissues were prepared for immunohistochemical analysis by standard procedures in the UTMB Research Histopathology Core Facility. Paraffin-embedded sections stained with an anti-BrdU antibody (Molecular Probes, Carlsbad, CA), anti-AhR antibody (Enzo Life Sciences, Farmingdale, NY) or with hematoxylin and eosin (H&E). The number of BrdU-positive hepatocyte nuclei in four randomly chosen fields were counted blind, and reported as a percentage of total nuclei in the field.

Immunoprecipitation and Western Blot Analysis. Frozen liver tissue was homogenized in ice cold TGH buffer (50 mM Hepes (pH 7.4), 150 mM NaCl,

10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA (pH 8.0), 1% Triton X-100) with 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitor cocktail, and 1 µg/ml BSA added just prior to use. Cell lysate was cleared by centrifugation at 12,000 x g for 20 minutes. Protein concentrations of the liver homogenates were determined with a protein assay kit (BioRad Laboratories, Inc. Hercules, CA). 1 mg of homogenate was incubated with 5 µg anti-HGFR antibody (R&D Systems, Minneapolis, MN) overnight, rotating, at 4°C. The complex was precipitated with 50 µl prewashed Protein A/G Agarose beads (Thermo Scientific, Rockford, IL) for 4 hours, rotating, at 4°C before centrifugation at 1000 x g for 5 minutes to collect the beads. The immunoprecipitated material was washed 5 times in TGH buffer with inhibitors, resuspended in 30 µl 2X SDS loading buffer, boiled for 10 minutes, and the protein fractionated by 8% SDS-PAGE. After transfer to polyvinylidene difluoride membranes (BioRad Laboratories, Inc., Hercules, CA), membranes were blocked in a Tris Buffered Saline-Tween 20 (TBST) solution of 5% BSA and probed with anti-phosphotyrosine, clone 4G10 (Millipore, Billerica, MA). Filters were visualized with a chemiluminescent oxidizing detection system (Perkin Elmer, Waltham, MA). The membrane was stripped and re-probed for total cMet using the same antibody for immunoprecipitation. For HGF, 10 µg total liver homogenate was separated by 10% SDS-PAGE, transferred to membranes, blocked with 5% fish gelatin in TBST and probed using an anti-HGF antibody (R&D Systems, Minneapolis, MN).

Statistical Analysis. Data were analyzed using Graph Pad Prism Software (San Diego, CA) by a two-way ANOVA (with a Bonferroni Post-Hoc Test) and by a Mann-Whitney U Test. The data represent the mean ± Standard Deviation and were considered statistically significant with a confidence interval set at 95% or $p \leq 0.05$.

RESULTS

Activated AhR induces PAI-1 expression in the liver and subsequent elevated protein levels in the serum during liver regeneration in C57Bl/6 mice compared to vehicle-pretreated controls. Consistent with the hypothesis that the AhR can regulate PAI-1 expression, PAI-1 mRNA levels are elevated in TCDD pretreated mice subjected to sham surgery compared to their vehicle pretreated counterparts (**Figure 2.1A**). However, TCDD-induced PAI-1 mRNA levels are increased a further 7-fold as a result of PH triggering a regenerative response. PAI-1 mRNA levels peak at 8 hours post-PH and gradually decline until reaching baseline levels by 36 hours post-PH (**Figure 2.1A**). A slight increase in PAI-1 mRNA levels is detected at 72 hours post-PH in the TCDD treated group.

The induction of PAI-1 by TCDD in the absence of PH is mirrored in PAI-1 serum protein levels, peaking at 12 hours following sham surgery. In keeping with the mRNA data, PAI-1 serum protein levels are significantly higher in animals pretreated with TCDD and subjected to PH (**Figure 2.1B**). Interestingly, there

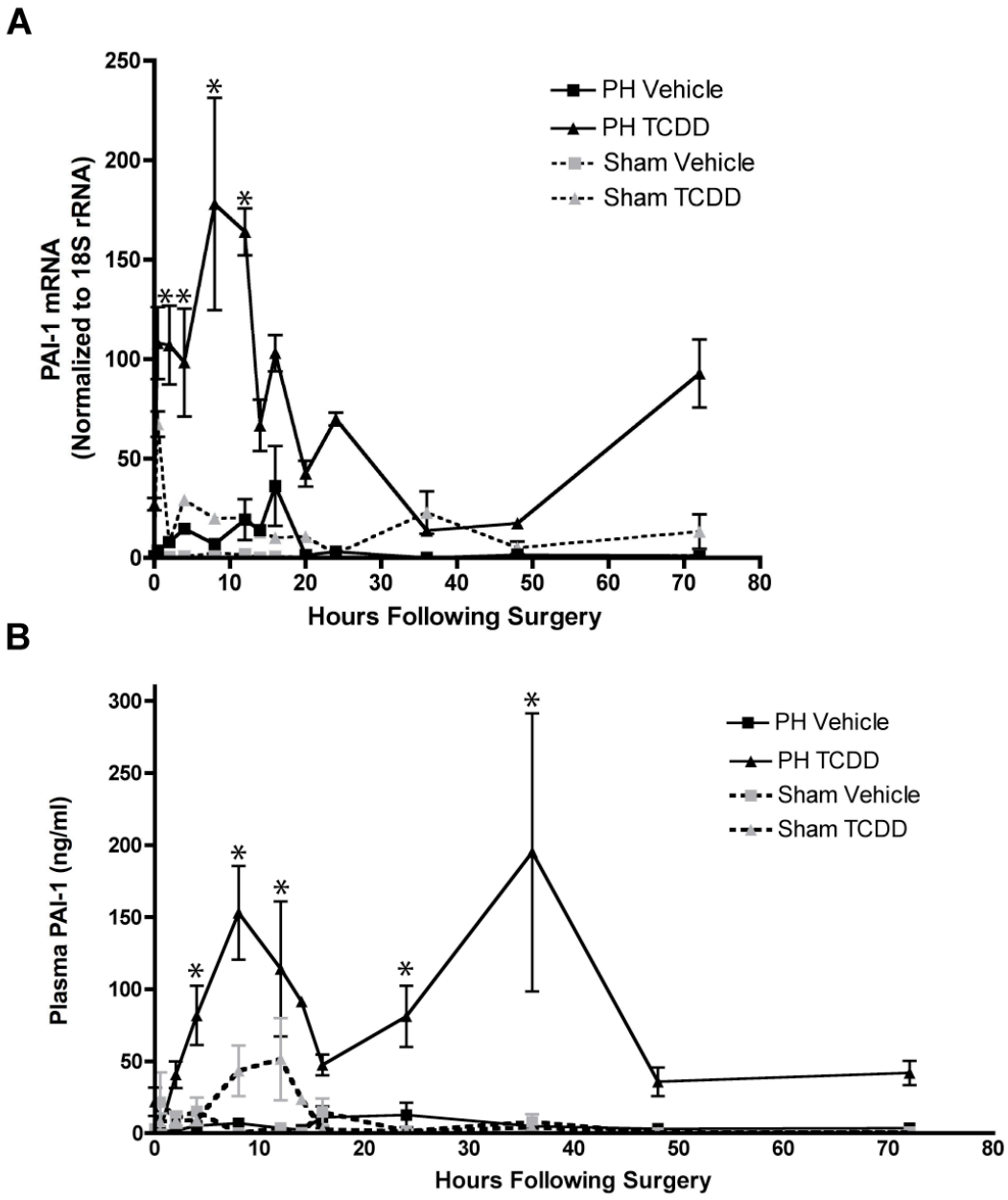

Figure 2.1. TCDD-mediated activation of the AhR induces PAI-1 transcription (A) and corresponding increase in plasma PAI-1 protein levels (B) during liver regeneration. C57Bl/6 mice were treated with vehicle (peanut oil) or TCDD 24 hours prior to partial hepatectomy (PH) or sham surgery. Animals were sacrificed at 0, 0.5, 2, 4, 8, 12, 14, 16, 24, 36, 48, and 72 hours post surgery. A) RNA was isolated from remaining liver tissue, quantified by qRT-PCR for PAI-1 transcript, and normalized to 18S rRNA. Graph depicts data ran in triplicate from two different experiments. B) Plasma PAI-1 protein levels were assessed using ELISA. Graph represents mean PAI-1 plasma concentration at each time point (n=4-9). *$p<0.001$, **$p<0.05$ compared to PH Vehicle.

appears to be a biphasic increase in serum protein levels, with peaks at 8 and 36 hours following PH in TCDD pretreated animals, a finding not reflected at the transcript level in the liver. Although the basis for the secondary increase in serum PAI-1 is unclear, the data demonstrate that TCDD induces both PAI-1 gene expression and the serum protein level, a response dramatically augmented by the regenerative process.

AhR activation by TCDD suppresses HGF processing and subsequent cMet activation in C57Bl/6 mice, but not PAI-1$^{-/-}$ mice. The enhanced PAI-1 expression is predicted to interfere with HGF processing and subsequent cMet signaling, a critical event in liver regeneration. In order to examine this directly, HGF processing and cMet activation were evaluated immunologically by probing for cleaved (activated) HGF and monitoring cMet phosphorylation, respectively, at multiple time points following PH in C57Bl/6 and

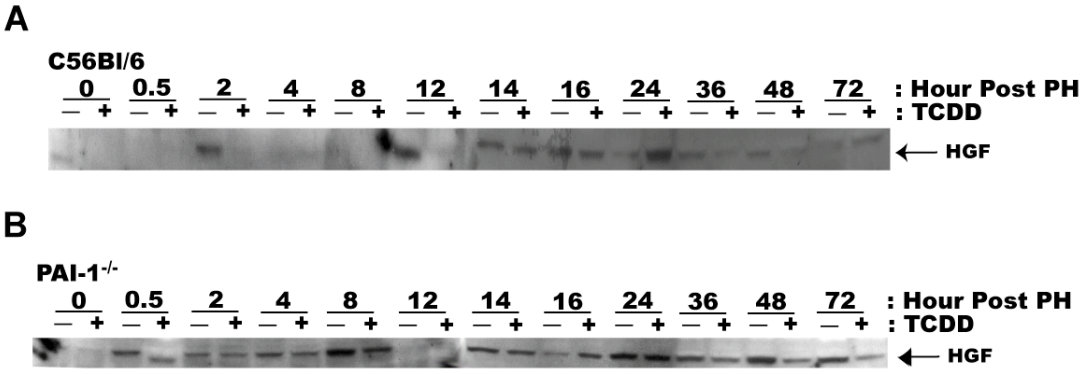

Figure 2.2. TCDD-mediated activation of AhR results in a suppression of processed (active) hepatocyte growth factor (HGF) in PAI-1$^{-/-}$ mice at 2 and 12 hours post-PH. C56Bl/6 (A) and PAI-1$^{-/-}$ (B) mice were treated with vehicle (-) or TCDD (+) 24 hours prior to PH. Mice were sacrificed at the indicated time points post-PH, and liver remnants were collected. Total liver homogenates were immunoblotted for active, heavy chain HGF (hcHGF).

PAI-1$^{-/-}$ mice. Cleaved HGF is readily detected at 2 and 12 hours following PH in vehicle pretreated mice, but suppressed in PH mice exposed to TCDD (**Figure 2.2A**). These are critical times in the regenerative program covering both the priming phase and early G1 phase of the cell cycle. Hepatic HGF processing thereafter is comparable between vehicle and TCDD pretreated mice, with a possible enhancement seen in TCDD treated animals at 24 hours post-PH (**Figure 2.2A**). In contrast, there is no appreciable difference in hepatic HGF processing in PAI-1$^{-/-}$ mice during the early stages of regeneration, although there does appear to be an increase in HGF at 48 and 72 hours post-PH in vehicle pretreated animals (**Figure 2.2B**).

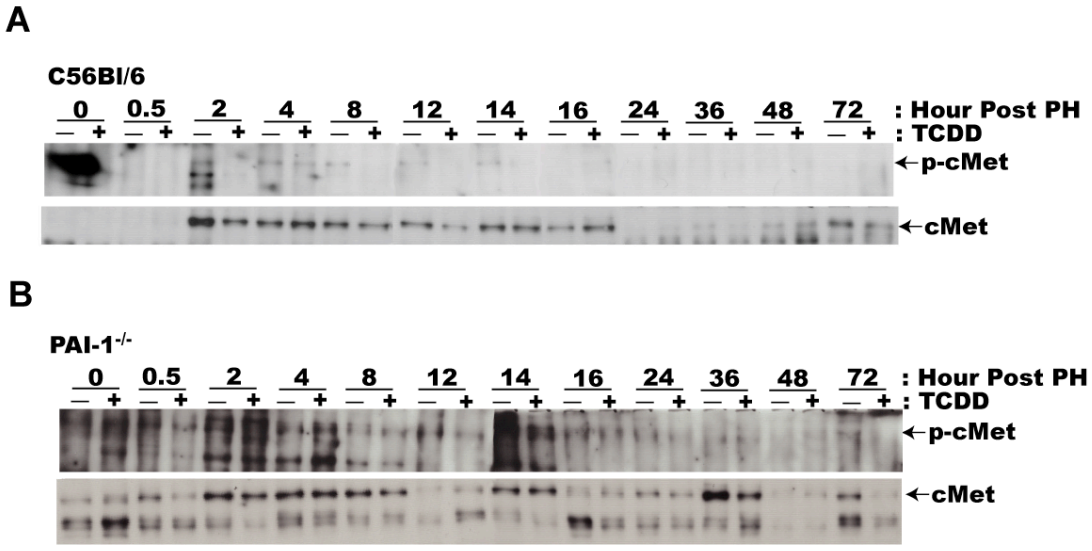

Figure 2.3. cMet activation, indicated by an increase in cMet phosphorylation (p-cMet), is suppressed by TCDD-mediated AhR activation in C56Bl/6 (A), but not PAI-1$^{-/-}$ (B), mice. Mice were pretreated with vehicle (-) or TCDD (+) 24 hours prior to partial hepatectomy (PH). Following sacrifice at the indicated time points, remnant livers were collected, homogenized, and immunoprecipitated for cMet. Samples were immunoblotted using a non-specific anti-phosphotyrosine antibody (4G10) to measure phosphorylation and immunoblotted for total cMet levels. A) C57Bl/6 mice have suppressed cMet activation until 16 hours post-PH. B) In PAI-1$^{-/-}$ mice, AhR activity is unable to effectively prevent cMet activation following PH.

Corresponding to HGF processing, cMet activation is suppressed in TCDD pretreated C56Bl/6, but not PAI-1$^{-/-}$ mice. Due to a lack of specific antibodies suitable for monitoring cMet phosphorylation sites in the mouse, cMet activation was measured by immunoprecipitating cMet from the liver following PH and measuring tyrosine phosphorylation using the non-specific phosphotyrosine-antibody 4G10. cMet activation was detected in vehicle pretreated, but not TCDD pretreated, C56Bl/6 mice, beginning at 2 hours following PH and continuing through 14 hours post-PH (**Figure 2.3A**). In the PAI-1$^{-/-}$ mice, the TCDD effect is absent, and cMet activation is equivalent between vehicle and TCDD pretreated mice (**Figure 2.3B**). As seen with HGF processing, increased cMet activation is detected in vehicle pretreated animals at 72 hours post PH in the PAI-1$^{-/-}$ mice compared to TCDD, although this is well beyond the priming phase of the regenerative process.

TCDD suppresses regeneration in centrilobular, but not periportal, zones of the liver of PAI-1$^{-/-}$ mice. Mice were administered BrdU via intraperitoneal injection 2 hours prior to sacrifice at 24, 36, 48, and 72 hours post-PH. BrdU is incorporated into DNA during active DNA synthesis and is a marker for S phase cell cycle progression. Subsequent immunohistochemical staining identifies BrdU-positive nuclei that can be counted as an indirect measure of DNA synthesis during the two hour pulse, and is a reliable indicator of ongoing liver regeneration. BrdU positive nuclei were counted in liver sections and reported as a percentage of the total number of nuclei. Representative slides are

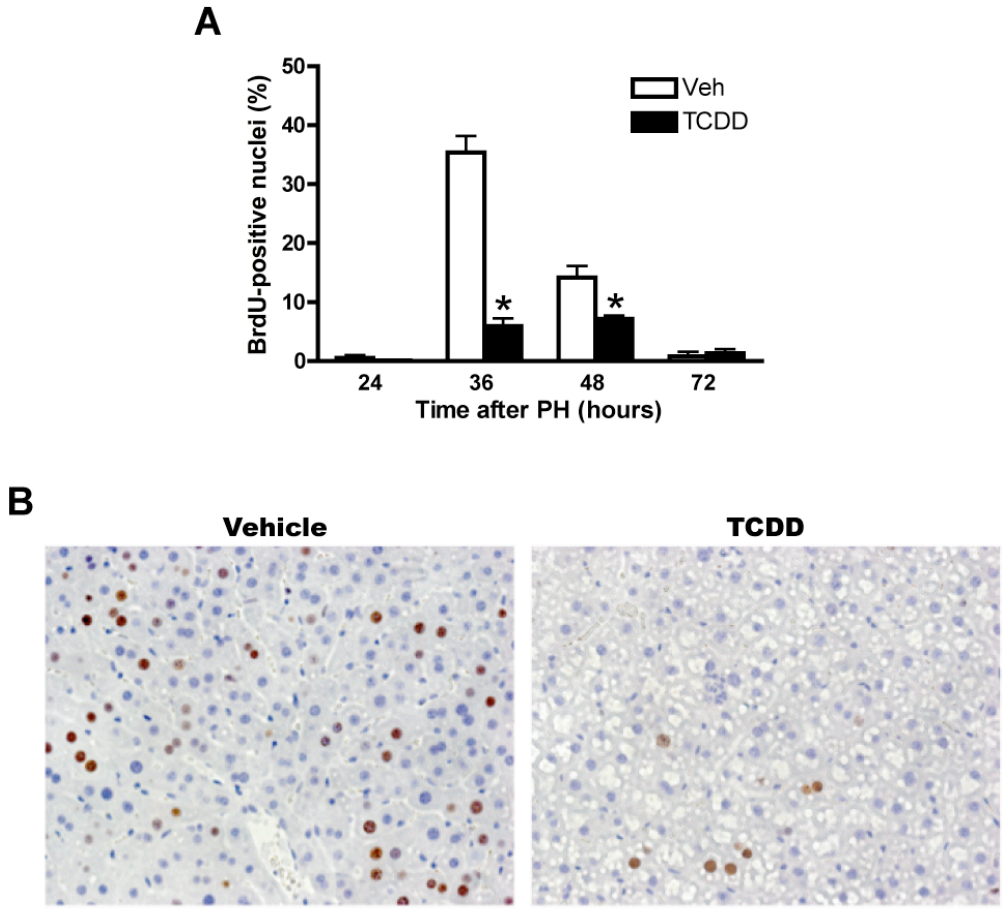

Figure 2.4. AhR-mediated suppression of liver regeneration is independent of PAI-1. PAI-1$^{-/-}$ mice were treated with vehicle or TCDD 24 hours before partial hepatectomy (PH). Two hours prior to sacrifice at the indicated times post-PH, mice were pulsed with BrdU to follow DNA synthesis. Remnant liver tissue was harvested and prepared for immunohistochemistry. A) BrdU-positive nuclei were counted in four separate, random fields per liver section (n=3 per treatment group). The graph depicts the mean for each group. B) Representative photomicrographs (40X) of liver sections from 36 hours post-PH demonstrating fewer BrdU positive nuclei between vehicle and TCDD-treated animals.

depicted (**Figure 2.4B**). In C57Bl/6 mice, peak DNA synthesis occurs at 36 hours post-PH, which is confirmed in the PAI-1$^{-/-}$ mice by the peak of BrdU-positive nuclei in vehicle pretreated animals at this time point (**Figure 2.4A**). TCDD pretreatment suppressed regeneration as indicated by decreased BrdU

incorporation, reflecting decreased DNA synthesis, at both 36 and 48 hours post-PH (**Figure 2.4A**).

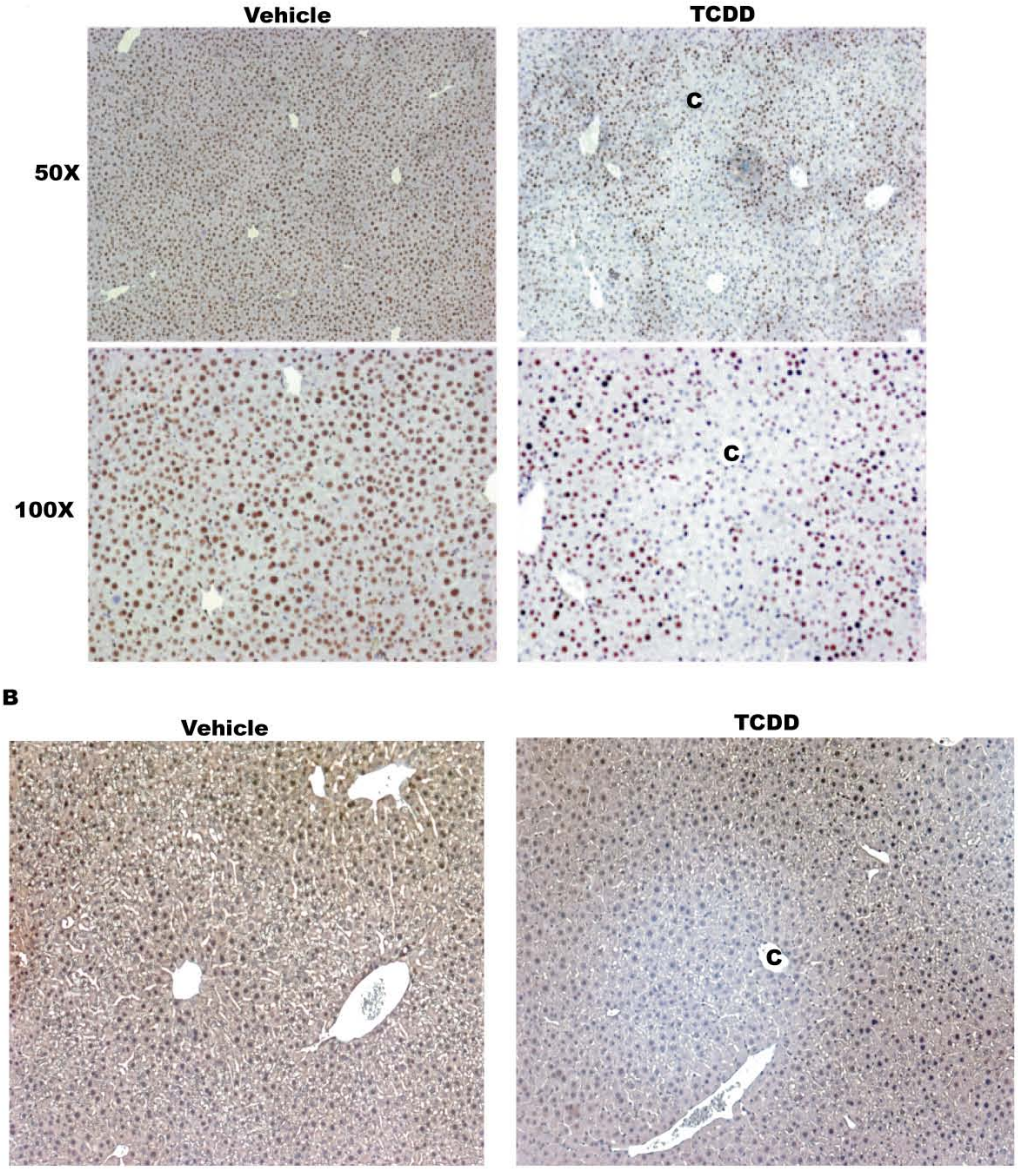

Figure 2.5. AhR-mediated suppression of liver regeneration (A) and AhR degradation (B) are localized in the centrilobular region (labeled with a C) in PAI-$1^{-/-}$ mice. Animals were treated with vehicle or TCDD 24 hours before partial hepatectomy (PH). Continuously throughout regeneration, BrdU was available in the drinking water to follow DNA synthesis until 72 hours post-PH. Remnant liver tissue was harvested and prepared for immunohistochemistry for BrdU and AhR. A) BrdU-positive nuclei were primarily periportal in TCDD treated mice, and pan-lobular in vehicle. B) AhR can be found in periportal hepatocyte nuclei, but is absent in the centrilobular regions at 72 hours post-PH in TCDD treated mice compared to vehicle.

In order to examine total DNA synthesis over the entire regenerative process rather than in 2-hour pulses at selected time points, PAI-1$^{-/-}$ mice were continuously administered BrdU in the drinking water for 72 hours following PH. Following sacrifice, liver sections were stained for BrdU incorporation. As depicted in **Figure 2.5A**, pan-lobular DNA synthesis is evident in vehicle-pretreated mice. In mice pretreated with TCDD however, DNA synthesis was primarily localized primarily to the periportal zone in liver acini, with the centrilobular region yielding few BrdU positive nuclei. Interestingly, AhR protein expression is decreased in the centrilobular zone of the liver at 72 hours post-PH in PAI-1$^{-/-}$ mice, but is readily detected in the zones more proximal to the periportal region (**Figure 2.5B**). The potential implication of this finding is discussed below.

DISCUSSION

While the regenerative capacity of the liver has been appreciated since antiquity as evidenced by the fate of Prometheus in Greek mythology, our mechanistic knowledge of the process responsible for initiating the transition of hepatocytes from a quiescent phase into G_1 of the cell cycle remains incomplete. This early phase of the regenerative process is commonly referred to as the priming phase of liver, and with the exception of EGFR agonists and HGF, several other factors identified contribute to this process but are neither necessary nor sufficient to promote hepatocyte proliferation (Michalopoulos

2007). In order to become active and signal through its receptor cMet, latent HGF located within the extracellular matrix must first be cleaved by the serine protease uPA (Kim et al. 1997; Mars et al. 1995b), a process negatively regulated by PAI-1. In this context PAI-1 was proposed to play a role in the TCDD-mediated AhR-dependent inhibition of liver regeneration following PH. This involves a process distinct from that identified previously by the laboratory (Mitchell et al. 2006), involving inhibitory events affecting Cdk2 activity during G1 phase of the cell cycle. Moreover, TCDD treatment has recently been linked to PAI-1 induction in microarray studies and *in vitro* assays (Frueh et al. 2001; Puga et al. 2000b; Son and Rozman 2002), Thus, I examined the connection between TCDD and PAI-1 during liver regeneration *in vivo*.

I confirmed that TCDD is indeed able to induce PAI-1 transcription *in vivo*, and this induction translates into increased PAI-1 protein in the serum (**Figure 2.1A** and **2.1B** respectively). Moreover, this induction is augmented during regeneration as PAI-1 levels are maximally induced in PH animals exposed to TCDD (**Figure 2.1**). Since PH alone has little effect on PAI-1 expression, the evidence implicates the involvement of other factors that synergize with the AhR to enhance PAI-1 expression during the regenerative process. The nature of this cross-talk is unknown, but suggests that impromptu AhR activation at critical stages during the regeneration can undermine normal liver repair processes.

Evaluation of PAI-1 expression at the mRNA and protein level as a function of the PH-induced regenerative response implicates additional regulatory events affecting PAI-1 protein levels. Specifically, the biphasic pattern of PAI-1 protein in the serum is not obviously reflected at the transcript level (**Figure 2.1B**). Since the animals are treated with TCDD 24 hours prior to surgery, the PAI-1 transcript levels are already elevated in these animals compared to vehicle control at the time of surgery (**Figure 2.1A**), hence it is probable that the initial peak in PAI-1 protein reflects enhanced PAI-1 gene expression prior to PH, a response also detected in the TCDD-treated sham surgery (non-resected) animals. It is noteworthy that this increase in PAI-1 mRNA precedes the increase in serum protein detected at 8 hours post-PH. In contrast, the second transient increase in serum PAI-1 in TCDD-treated PH mice occurs during a period of declining mRNA expression. Therefore, the increased serum PAI-1 level seen peaking at 36 hours post-PH is likely due to a post-transcriptional event reflecting both TCDD exposure and the regenerative process, since this increase was not evident in the TCDD-treated sham surgeries, nor the non-TCDD treated PH animals. The second wave of PAI-1 protein could reflect a second round of proliferation that many hepatocytes undergo to restore full liver mass although the timing—at 36 hours post-PH—is inconsistent with the rigid temporal program during normal regeneration. Alternatively, while hepatocytes represent the bulk of the liver, it is possible that the second increase in PAI-1 levels can be attributed to the non-parenchymal

cells (endothelial cells, Kupffer cells, and stellate cells) that also proliferate during regeneration, but this tends to occur 2-3 days after the initial tissue injury (Michalopoulos and DeFrances 1997), challenging the validity of this explanation. Instead, the transient increase in serum PAI-1 detected at 36 hours post-PH may reflect a post-translational event, such as diminished PAI-1 endocytosis. Reduced PAI-1 endocytosis may be due to a reduction in the level of the uPA/uPAR expressed at the cell surface to which PAI-1 binds prior to being internalized. Judging by the amount of cleaved HGF and cMet activation between 36-72 hours in both wild-type and PAI-$1^{-/-}$ mice (**Figures 2.2** and **2.3**), the second increase in serum PAI-1 appears to have minimal effect on cMet signaling. Therefore, the physiological impact of the secondary PAI-1 increase on regeneration is likely to be muted.

While the induction of PAI-1 by AhR *in vivo* is noteworthy as being a novel mechanism of PAI-1 regulation, importantly, it appears this induction has functional consequences. Following PH in TCDD pretreated mice, there is decreased HGF processing (**Figure 2.2A**) and cMet phosphorylation (**Figure 2.3A**) in the C57Bl/6 mice during the initial stages of liver regeneration, which is completely restored in the mice lacking PAI-1 (**Figures 2.2B** and **2.3B**). This suggests that the increased PAI-1 levels detected in the C57Bl/6 mice exposed to TCDD are sufficient to inhibit uPA/uPAR activity during this critical phase of liver regeneration.

Given the TCDD induction of PAI-1 and its suppressive effects on HGF cleavage and cMet activation, which were not seen in the PAI-1$^{-/-}$ mice, I was at first surprised that regeneration continued to be suppressed by TCDD in the PAI-1$^{-/-}$ mice as indicated by the BrdU pulse incorporation (**Figure 2.4A**). However, further analysis of cumulative BrdU throughout regeneration in these mice revealed that TCDD continued to suppress regeneration, but specifically in the centrilobular zones of the liver (**Figure 2.5A**). Interestingly, cells in the periportal region of rats were also able to escape TCDD mediated suppression of liver regeneration following PH (Bauman et al. 1995), but both of these findings contrast to the panlobular suppression seen in TCDD pretreated C57Bl/6 mice following PH (Mitchell et al. 2006).

In examining AhR protein expression at the same time point in these mice, the seeming co-localization between the lack of proliferation and lack of AhR (**Figure 2.5**) can be most easily explained by this region actually having high AhR activity leading up to 72 hours post-PH. Given the nuclear localization of the surrounding cells with AhR (**Figure 2.5B, TCDD**), the AhR is being activated in these animals. Others have reported AhR localization in the liver to be primarily centrilobular and to correspond with centrilobular patterns TCDD-mediated toxicity (Chang et al. 2005; Forkert 1997). With the ample evidence that supports AhR protein degradation following activation (Harper et al. 2006; Pollenz 2002), specifically in mouse centrilobular hepatocytes (Chang et al. 2005), it is formally possible that the PAI-1$^{-/-}$ mice actually have increased AhR activity in this region,

resulting in the apparent loss of AhR in the same cells that are BrdU negative, i.e. not proliferating. This high activity of AhR is likely regulating other known targets of the AhR involved in the cell cycle such that the suppressive effects from other proteins, such as $p27^{Kip1}$ (Kolluri et al. 1999), outweigh the proliferative drive of restored HGF levels in the PAI-$1^{-/-}$ mice.

This work demonstrates a novel regulation of PAI-1 expression by the AhR, negatively impacting HGF processing and cMet activation during the critical priming phase of regeneration following PH. Given the low dose of TCDD used in these studies, these findings may be applicable to risk assessment in humans with comparable levels of dioxin exposure. Whether or not the AhR-mediated induction of PAI-1 plays a role in other modes of liver injury could provide insight into individual responses to hepatic insults and provide potential targets in treating disorders of hepatic regulation.

CHAPTER 3: THE AHR AND KLF6 INTERACT AT THE NC-XRE OF THE PAI-1 PROMOTER *IN VITRO* AND *IN VIVO*

INTRODUCTION

The AhR, a member of the Per/Arnt/Sim (PAS) family of transcription factors, is typically found in the cytoplasm bound to HSP90 and another chaperonin, known as ARA9/AIP/XAP2. Upon ligand activation, such as with the potent, persistent toxicant TCDD, the AhR translocates to the nucleus. In the classic pathway of AhR activity (**Figure 1.1**), the AhR heterodimerizes with another member of the PAS family, Arnt, binds to XREs in the promoter region of target genes and induces their transcription.

Emerging evidence indicates that AhR activity may be more complex. In addition to Arnt, AhR has been shown to associate with other transcription factors. The Ahr-Arnt heterodimer interacts with the transcription factor Sp1 and enhances Sp1 binding to its target DNA when the binding site is in close proximity to an XRE (Wang et al. 1999). There is also evidence of AhR interacting with proteins independent of Arnt, specifically pRB, and the RelA and RelB, subunits of NF-κB (Ge and Elferink 1998; Ke et al. 2001; Puga et al. 2000a; Tian et al. 1999; Vogel et al. 2007). Further examination of the AhR-RelB interaction identified a novel DNA binding site, distinct from the XRE, in the IL-8

promoter that mediated TCDD-induced transcription, although the exact sequence remains ill-defined.

With the evidence that TCDD-activated AhR can induce PAI-1 transcription in cell lines (Son and Rozman 2002) and *in vivo* (**Figure 2.1**), our laboratory began work examining the molecular mechanisms of AhR activation at the PAI-1 promoter. Dr. Huang identified a DNA sequence referred to as the ncXRE (**Table 1**) in the PAI-1 promoter that forms a complex when AhR is activated in EMSAs (**Figure 1.6**) and that TCDD-activated AhR, but not Arnt, can bind the PAI-1 promoter *in vivo* (**Figure 1.7**). The lack of sequence homology between the XRE and ncXRE, the distinct complex formed at the ncXRE in EMSA, and the apparent lack of Arnt involvement indicate a potentially novel AhR interaction with new binding partner(s) at the ncXRE.

KLF6 is a hepatic member of the recently identified and growing KLF superfamily of transcription factors (Ratziu et al. 1998) and is considered a tumor suppressor due to its loss in multiple carcinomas. The KLF family is characterized by three zinc finger DNA binding domains in their C-termini, thus allowing them to bind and induce transcription at GC boxes and CACCC response elements in target genes (Bieker 2001; Philipsen and Suske 1999), elements that share sequence homology with the ncXRE. Found in the nucleus, KLF6 has been shown to induce transcription of uPA and $p21^{Cip1}$ (Kojima et al.

2000; Narla et al. 2001; Narla et al. 2007), but other target genes related to cell proliferation have yet to be identified.

Given the sequence homology between the KLF6 binding site and the ncXRE, KLF6 expression in hepatocytes, and a proposed role for KLF6 in proliferation, I hypothesized that the AhR interacts with KLF6 at the ncXRE in the PAI-1 promoter. In order to study this interaction, I performed immunoprecipitation studies on *in vitro* coupled transcription and translation with human AhR, Arnt, and KLF6 constructs. Using the human AhR and KLF6 proteins, I performed EMSAs to provide a preliminary characterization of the complex bound to the ncXRE. In order to examine this interaction *in vivo*, I used nuclear extracts from mice in the presence and absence of TCDD in co-immunoprecipitation studies to assess protein-protein interactions as well as chromatin immunoprecipitation (ChIP) assays to determine if the AhR and KLF6 bind to the ncXRE in the PAI-1 promoter. In order to characterize which domains of AhR and KLF6 are important for these interactions, I synthesized deletion constructs encoding the murine proteins. Although the initial AhR-KLF6 studies focused on the human proteins, the murine proteins were chosen in the follow up studies to complement the *in vivo* work, co-immunoprecipitations and EMSAs with nuclear extracts.

MATERIALS AND METHODS

Animals. All care and procedure conditions were approved by the guidelines set by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (UTMB) at Galveston. 8-10 week old female C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in microisolator cages with unlimited access to drinking water and food while under a 12 hour light/dark cycle.

TCDD. TCDD (Cerilliant, Round Rock, TX) was dissolved in anisole and diluted to 2 μg/ml in peanut oil or to 200 nM in DMSO, whereas the vehicle control consisted of peanut oil spiked with a corresponding amount of anisole or DMSO alone for *in vivo* and *in vitro* experiments respectively. Animals were administered either vehicle or TCDD (20 μg/kg) by gavage 2 hours before sacrifice via cervical dislocation. *In vitro* transcribed and translated proteins were activated with 20 nM TCDD for 1 hour at 30°C or 20 minutes at 35°C for immunoprecipitation and EMSA studies respectively.

AhR, Arnt, and KLF6 Constructs. Full length human AhR and Arnt were cloned into the pSport vector using KpnI and SalI sites, are Sp1 promoter driven, and were a kind gift from Dr. William Chan. Human KLF6 full length and mutant constructs were graciously provided by Dr. Scott Friedman in the pCS2+MT vector under the T7 promoter. Due to poor expression levels, the human KLF6 constructs were subcloned into the pSport vector via KpnI and SalI sites. Using

the full-length human KLF6/pCS2+MT construct as a template, PCR was performed with the following primers: WT hKLF6 (forward 5'-GGTACCATGGATTACAAGGATGACGACGATAAGATGGACGTGCTCCCCATGTGCAG-3', reverse 5'-GTCGACTCAGAGGTGCCTCTTCATGTGCAG-3'); Δ27 hKLF6 (forward 5'-GGTACCATGGATTACAAGGATGACGACGATAAGCTGGAGGAGTACTGGCAACAGACC-3', reverse is same as WT); Δ128 hKLF6 (forward 5'-GGTACCATGGATTACAAGGATGACGACGATAAGCCCATTGGCGAAGTTTTGGTCAGC-3', reverse is the same as WT); Δ178 hKLF6 (forward 5'-GGTACCATGGATTACAAGGATGACGACGATAAGACTTCGGGGAAGCCAGGTGACAAG-3', reverse is the same as WT). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and cloned into the TOPO TA vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. Colonies selected on kanamycin-resistant plates were picked, grown in luria broth with kanamycin, and the plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and sent to the UTMB Protein Chemistry Core for sequence verification. Following verification, clones were digested with KpnI and SalI (New England BioLabs, Ipswich, MA), ligated into the pSport vector using T4 DNA ligase (New England BioLabs, Ipswich, MA), and transformed into TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). Plasmids were purified and sequence verified as described for the TOPO cloning.

Mouse AhR constructs are in the pcDNA1/neo vector under the T7 promoter and were a kind gift from Dr. Oliver Hankinson. Mouse KLF6 constructs were generated using the same basic strategy as for the human KLF6 constructs. Primers designed were based on the mouse KLF6 sequence (Inuzuka et al. 1999) and are as follows: WT mKLF6 (forward 5'-GGTACCATGGATTACAAGGATGACGACGATAAGATGAAACTTTCACCTGCGC TCCCGGGAACA-3, reverse 5'-GTCGACTCAGAGGTGCCTCTTCATGTGCAG-3'); Δ34 mKLF6 (forwards 5'-GGTACCATGGATTACAAGGATGACGACGATAAGATGGATGTGCTCCCAATGT GTAGCATCTTC-3', reverse is the same as WT); Δ61 mKLF6 (forward 5'-GGTACCATGGATTACAAGGATGACGACGATAAGCTGGAGGAATATTGGCAAC AGACC-3', reverse is the same as WT); Δ128 mKLF6 (forward 5'-GGTACCATGGATTACAAGGATGACGACGATAAGTTTAATTATAACTTAGAGAC CAATAGCCTG-3', reverse is the same as WT); Δ212-318 mKLF6 (forward is the same as WT, reverse 5'-GTCGACTCAACTTCGAACCTTCCCAGGTGAGGGCAGGTC-3'). The template was cDNA reverse transcribed from liver RNA using the primer 5' CTCTTTTAGCCTACAGGATTCGTC-3'.

Coupled *in vitro* Transcription and Translation. Constructs were expressed using the TnT Coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's protocol. For each 50 µl reaction, 2

μg of each construct was added and expression was driven by T7 for the AhR constructs or Sp6 for KLF6. In order to radioactively label proteins, ^{35}S-methionine was purchased from Pierce (Rockford, IL), and 3 μl was used per 50 μl reaction.

Nuclear Extract Preparation. Nuclear extracts were obtained using sucrose density ultracentrifugation and nuclear lysis. Following sacrifice, livers were blanched with ice-cold phosphate-buffered saline, removed, and placed on a petri dish on ice. The liver was finely minced with a razor blade. The liver was transferred to a dounce homongenizer and suspended in 1.6 ml sterile PBS, 4 mL 2.2 M sucrose solution (2.2 M sucrose, 10 mM Hepes, 15 mM KCl, 2 mM EDTA (pH 8.0), and added just prior to use 0.15 mM Spermine, 0.5 mM Spermidine, 1 mM DTT, 1 mM PMSF, and 25 ng/mL protease inhibitor cocktail), and an additional 50 ng of protease inhibitor cocktail. Samples were homogenized on ice 5-6 times with the loose pestle and 7 times with the tight pestle before being filtered through cheesecloth to remove any remaining solid tissue. An additional 3 mL of the 2.2 M sucrose solution was added to each sample and mixed gently. This was layered onto 4 mL of a 2.05 M sucrose cushion with the same composition as the 2.2 M sucrose solution, but with a lower concentration of sucrose, being careful not to disturb the lower layer. Tubes were balanced and the samples were subjected to ultracentrifugation at 30,000 rpm in a Beckman Instruments (Fullerton, CA) MLS-50 rotor for 1 hour at

4°C. The white, pelleted nuclei were resuspended in 1 mL Nuclear Lysis Buffer (NLB: 10 mM Hepes, 102 mM KCl, 0.1 mM EDTA (pH 8.0), 11.4% glycerol, 0.15 mM Spermine, 0.5 mM Spermidine, 1 mM NaF, 1 mM Na_2VO_4, 1 mM $ZnSO_4$, and 1 mM protease inhibitor cocktail) on ice or in the cold room. Collect the nuclei by centrifugation at 1500 x g and remove the supernatant. Repeat the wash. The nuclei were resuspended in 200 µl NLB and divided between two tubes for ultracentrifugation. An equal volume of 2X NUN buffer (2 M urea, 2% NP40, 650 mM NaCl, 50 mM Hepes, and 2 mM DTT added freshly) was added, drop by drop. Samples were incubated on ice for 20 minutes with occasional mixing. Following centrifugation at 55,000 rpm for 20 minutes at 4°C. The supernatant was collected, protein concentrations of the nuclear proteins were measured with a protein assay kit according to manufacturers instructions (BioRad Laboratories, Inc. Hercules, CA), aliquoted, and stored at -80°C.

Immunoprecipitation and Analysis. Nuclear extracts or *in vitro* transcribed and translated proteins were suspended in ice cold TGH buffer (50 mM Hepes (pH 7.4), 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA (pH 8.0), 1% Triton X-100) with 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitor cocktail, and 1 µg/ml BSA added just prior to use. Nuclear extracts were incubated with an antibody against KLF6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight, rotating, at 4°C. The remaining immunoprecipitation protocol, SDS-PAGE and transfer protocols are as

described in Chapter 2. Membranes were blocked in 5% milk in TBST and probed with anti-AhR (Enzo Life Sciences, Farmingdale, NY) and anti-KLF6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies. Proteins expressed *in vitro* were incubated overnight with antibodies against AhR (Abcam, Cambridge, MA) or KLF6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C with rotation. Immunoprecipitation was performed as described in Chapter 2. The immunoprecipitated proteins were separated on 4-20% polyacrylamide gels, fixed in a solution containing 50% methanol and 50% acetic acid for 20 minutes, rinsed with water, and dried for 1 hour under vacuum. Dried gels were exposed to film or phosphoscreens and imaged on the Typhoon Trio Variable Mode Imager (GE Healthcare, Piscataway, NJ).

Electrophoretic Mobility Shift Assay (EMSA). For EMSA, either 10 µg of nuclear extract or 7 µl of each protein component from *in vitro* transcription and translation were used in each reaction. Each reaction was incubated in HEDG buffer (25 mM Hepes, 1 mM EDTA, 1 mM DTT, and 10% glycerol) with 100-150 ng polydIdC, 100 mM KCl, and 5 mM DTT for 10 minutes at room temperature. 300 ng of ncXRE was added and incubated at room temperature for an additional 15 minutes. Loading buffer was added, the samples were loaded onto 6% non-denaturing polyacrylamide gels, and ran for 30 minutes at 70V, 30 minutes at 90V, and 20 minutes at 120V in TAE buffer that was recycled between each step-up in voltage. The gels were dried onto paper for 1 hour under heat

and vacuum. The dried gels were exposed to film or phosphoscreens and imaged on the Typhoon Trio.

Chromatin Immunoprecipitation (ChIP). Following vehicle or TCDD treatment for 2 hours, C56Bl/6 mice were sacrifice via cervical dislocation. The liver was quickly removed and placed on ice. The gall bladder was discarded, and the liver was finely minced with a razor blade. The chromatin was cross-linked by a 10 minute incubation at room temperature, rotating, in ~2.5% formaldehyde and stopped by adding glycine to a final concentration of 200 mM. The sample was centrifuged at 3200 x g for 5 minutes at 4°C, the supernatant was removed, and the sample was re-suspended in ~6 ml cold phosphate buffered saline and transferred to a Dounce homogenizer. After 7 strokes with the loose pestle, the homogenate was centrifuged at 3200 x g again. The supernatant was discarded, and the sample was re-suspended in ~6 ml Cell Lysis Buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP40) plus 1:100 protease inhibitor cocktail added just before use. The samples were homogenized again with 4 strokes of the loose pestle, divided into 1.5 ml tubes (~1 ml/tube), and incubated on ice for 15 minutes with occasional mixing. Following centrifugation at 3,000 x g for 5 minutes at 4°C, the supernatant was removed and the samples were sufficiently processed for use in the ChIP-IT Express Enzymatic Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Briefly, the pellet was re-suspended in Lysis Buffer plus protease inhibitor cocktail and PMSF,

incubated on ice for 30 minutes, and homogenized with 30 strokes to release the nuclei. The nuclei were pelleted by centrifugation at 3,000 x g for 10 minutes at 4°C. The nuclei were re-suspended in Digestion Buffer with protease inhibitor cocktail and PMSF and "sheared" with Enzymatic Shearing Cocktail for 15 minutes at 37°C. The reaction was stopped with EDTA, and the sheared samples incubated on ice for 10 minutes. Following an 18,000 x g centrifugation, the nuclear debris was pelleted, and the sheared chromatin was collected in the supernatant. An aliquot was removed to serve as the Input DNA after being phenol/chloroform extracted and ethanol precipitated. The remaining chromatin was incubated overnight with rotating at 4°C with Protein G Magnetic Beads and buffers supplied with the kit in addition to antibodies against either IgG (R&D Systems, Minneapolis, MN), H3 (Abcam, Cambridge, MA), AhR (Abcam, Cambridge, MA), or KLF6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Following the protocol in the kit, the samples immunoprecipitated samples were collected, washed with the supplied buffers, eluted, reverse cross-linked, incubated at 65°C for 2.5 hours, treated with Proteinase K, and incubated at 37°C for 1 hour. Immunoprecipitated and Input DNA were PCR amplified using primers specific to the ncXRE in the PAI-1 promoter (forward 5'-GTCCCAGCAAGTCACTGGGAGG-3', reverse 5'-CTGGAGGCGGGTGTGCGGCG-3'), separated on a 5% polyacrylamide gel, stained with SYBR Green I (Invitrogen, Carlsbad, CA) for 20 minutes, and

imaged on the Typhoon Trio. Band intensity was quantified using the ImageQuant software and reported as a percentage of Input DNA for each sample.

Statistical Analysis. Data were analyzed using Graph Pad Prism Software (San Diego, CA) by a two-way ANOVA (with a Bonferroni Post-Hoc Test). The data represent the mean ± Standard Error of the Mean and were considered statistically significant with a confidence interval set at 95% or $p \leq 0.05$.

RESULTS

The human AhR and KLF6 interact *in vitro* and are necessary and sufficient for TCDD-dependent complex formation at the ncXRE. Human AhR, Arnt, and KLF6 constructs were expressed *in vitro*, and KLF6 was radioactively labeled with ^{35}S-methionine incorporation during translation. Equal volumes of expressed proteins were incubated with vehicle or TCDD and immunoprecipitated with both AhR and Arnt. **Figure 3.1A** demonstrates that KLF6 interacts with the AhR but not Arnt *in vitro,* in a manner dependent on activation of AhR by TCDD. Using these same KLF6 and AhR proteins for EMSA, it could be demonstrated that a complex can be formed with unlabeled ncXRE oligonucleotide that required the presence of the AhR, KLF6, and TCDD activation (**Figure 3.1B**). Lack of the AhR (**Figure 3.1B, lanes 5 and 6**), or KLF6 (**Figure 3.1B, lanes 4 and 6**), or TCDD (**Figure 3.1B, lanes 1 and 2**) prevented

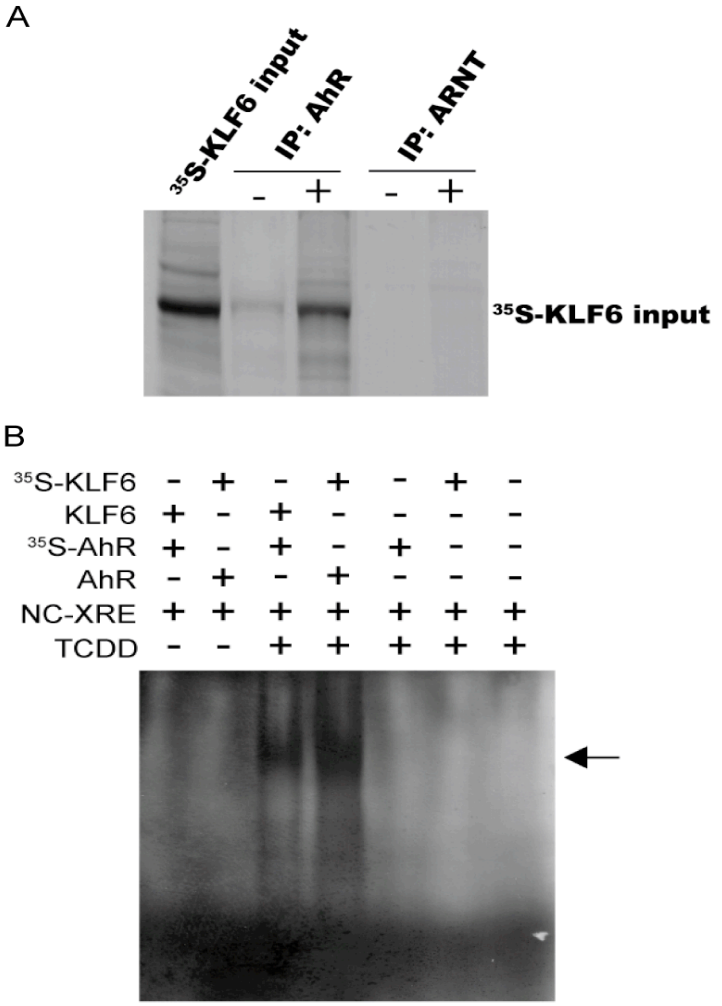

Figure 3.1. Human AhR and KLF6 coimmunoprecipitate (A) and bind to the ncXRE (B) *in vitro* in a manner dependent on TCDD activation of AhR and the presence of both proteins. A) Using human AhR, Arnt, and KLF6, labeled with ^{35}S-methionine, constructs expressed in *in vitro* coupled transcription and translation, equal volumes of resulting protein were incubated with each other in the presence (+) or absence (-) of 20nM TCDD. Samples were immunoprecipitated with antibodies against AhR or Arnt, and the resulting pull-down was separated on a gel and analyzed for the presence of the ^{35}S-labeled KLF6. B) Various combinations, as listed, of ^{35}S-labeled and "cold" hAhR and hKLF6 proteins were incubated with TCDD and the ncXRE and complex formation was detected using EMSA. This reveals that both AhR and KLF6 must be present for complex formation in a TCDD-dependent manner.

complex formation. By variously using ^{35}S-AhR (**Figure 3.1B, lane 3**) and ^{35}S-KLF6 (**Figure 3.1B, lane 4**), the evidence demonstrates that both proteins are required for the interaction with the ncXRE.

The N-terminal 27 amino acids of human KLF6 are necessary for complex formation at the ncXRE. In an initial attempt to characterize the specific region of KLF6 necessary for ncXRE complex formation *in vitro*, Dr. Scott Friedman kindly provided constructs comprising sequential deletions of the human KLF6 protein. These constructs yielded poor expression *in vitro*, and the cDNA inserts were subcloned into the pSport vector under control of the Sp6

promoter. The subsequent constructs expressed recombinant proteins adequately *in vitro* and could be readily radiolabeled with ^{35}S-methionine (**Figure 3.2A)**. EMSA was performed using the radiolabeled KLF6 proteins, and

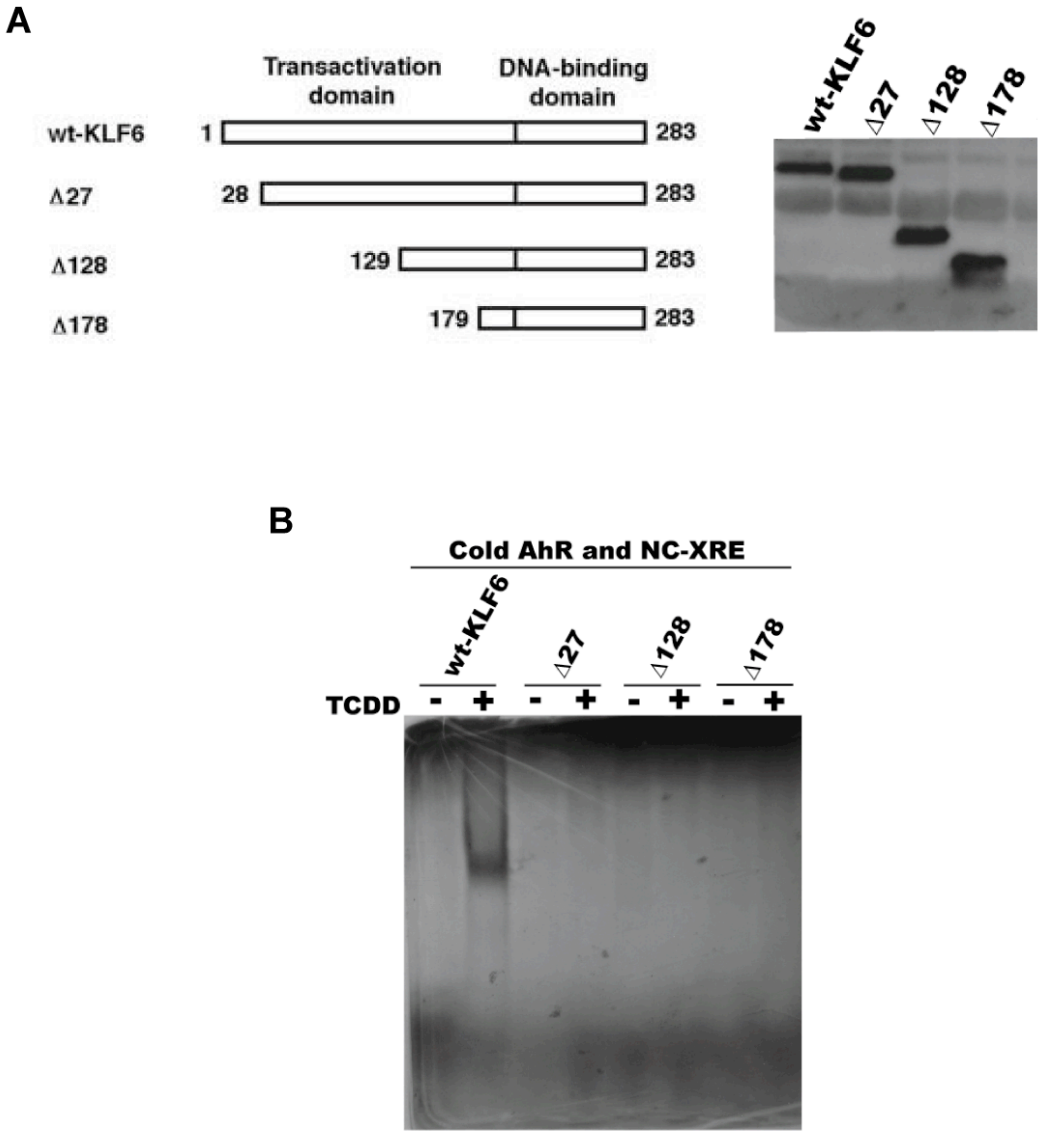

Figure 3.2. The N-terminal 27 amino acids of hKLF6 are necessary for binding to the ncXRE *in vitro*. A) Human mutant KLF6 proteins were express *in vitro* and labeled with ^{35}S-methionine. B) EMSA was performed using "cold" hAhR and ^{35}S-labeled hKLF6 constructs with sequential deletions beginning at the N-terminus in the presence (+) or absence (-) of TCDD. A shift product is readily detected in the wt-KLF6 construct, but none others, indicating that the first 27 amino acids of the hKLF6 are important in complex formation at the ncXRE.

unlabeled AhR and the ncXRE oligonucleotide in the presence and absence of TCDD. Full length KLF6 (wt-KLF6) forms a complex following activation with TCDD (**Figure 3.2B**) consistent with earlier findings (**Figure 3.1B**). However, no complex formation is detected with any of the mutant constructs (**Figure 3.2B**), suggesting that the N-terminal 27 amino acids in KLF6 are critical for complex formation.

KLF6 binds the AhR and is part of the ncXRE-associated complex *in vivo* in mice. In order to study the interaction between AhR and KLF6 *in vivo*, nuclear extracts were prepared from mice pretreated with vehicle or 20μg/kg TCDD 2 hours prior to sacrifice, and KLF6 and its associated proteins were immunoprecipiated. Subsequent immunoblotting revealed that AhR was associated with KLF6 in a TCDD dependent manner (**Figure 3.3A**). I performed an EMSA on these nuclear extracts pre-incubated with an IgG control antibody and an anti-KLF6 antibody (**Figure 3.3B**). Although the EMSA did not produce a supershift complex, the KLF6 antibody specifically abolished formation of the protein-DNA complex suggesting that KLF6 is indeed a component of the ncXRE binding complex in the mouse liver.

The AhR and KLF6 bind the ncXRE *in vivo* in response to TCDD. To independently verify AhR and KLF6 association with the ncXRE *in vivo*, I performed ChIP assays using a control IgG antibody (negative control), or antibodies against H3 (positive control), the AhR and KLF6 to immunoprecipitate

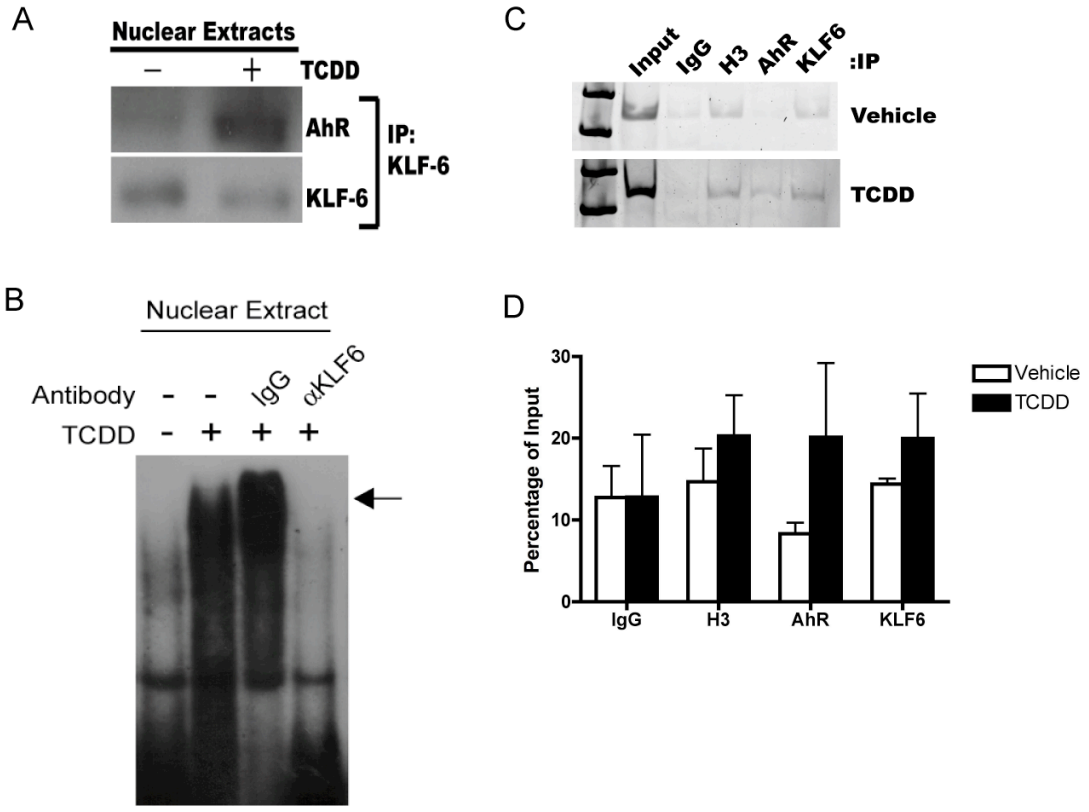

Figure 3.3. AhR and KLF6 interact and bind the ncXRE of the PAI-1 promoter *in vivo*. C57Bl/6 mice induced with vehicle (-) or 20μg/kg TCDD (+) via gavage 2 hours before sacrifice and either nuclear extracts were prepared (A and B) or the liver was utilized for ChIP (C and D). A) Nuclear proteins were immunoprecipitated with an antibody against KLF6 and immunoblotted for the presence of AhR and KLF6, revealing that AhR and KLF6 interact *in vivo* when the AhR is activated by TCDD. B) 10μg of nuclear extract was pre-incubated with antibodies against IgG and KLF6 before the addition of the ncXRE in an EMSA. Abolishment of complex formation with the antibody against KLF6, but not IgG, indicates a role for KLF6 in binding to the ncXRE. ChIP was performed with antibodies against IgG (negative control), H3 (positive control), AhR or KLF6. DNA bound to the respective proteins was recovered and PCR performed for the ncXRE of the PAI-1 promoter. C) The PCR products were separated on a 5% polyacrylamide gel and stained with SYBR Green 1. D) Bands from 3 separate experiments were quantified, averaged, and reported as a percentage of input DNA. AhR binding to the ncXRE is TCDD dependent, while KLF6 appears to be bound in steady states.

the target proteins from liver nuclei in vehicle or TCDD treated mice. PCR was subsequently performed in the cross-linked DNA using primers specific to the PAI-1 promoter encompassing the region harboring the ncXRE, and the anticipated 240bp PCR product resolved on a polyacrylamide gel, stained with SYBR green, and quantified as a percentage of input DNA (**Figure 3.3D**). A

representative gel is depicted in **Figure 3.3C**. The result reveals that the murine AhR binds to the PAI-1 promoter *in vivo* in a TCDD-dependent manner, whereas KLF6 binds the PAI-1 promoter constitutively (**Figure 3.3D**).

Characterization of the murine AhR and KLF6 interaction *in vitro*. In order to study the murine AhR and KLF6 protein-protein and ncXRE protein-DNA interactions, I used AhR expression clones kindly provided by Dr. Oliver Hankinson and generated KLF6 deletion constructs from mouse liver mRNA reverse-transcribed into cDNA and cloned into the pSport expression vector provided by Dr. William Chan. AhR constructs encoded mutants harboring deletions spanning the basic Helix-Loop-Helix domain, the basic (DNA binding) and Helix-Loop-Helix domains separately, each Helix, the PAS B domain, and the C terminus (containing the transactivation domain). I generated a series of mouse KLF6 deletion mutants lacking the N-terminal 34 amino acids (Δ34), 61 amino acids (Δ61), and 128 amino acids (Δ128). It is noteworthy that the mouse KLF6 protein contains an extra 34 N-terminal residues compared with the human protein, hence the Δ34 and Δ61 murine proteins are akin to the human full-length and Δ27 KLF6 proteins, respectively as depicted (**Figure 3.4**). In addition, a construct encoding the mouse KLF6 lacking the DNA binding domain (Δ212-318) was generated. Using *in vitro* coupled transcription and translation, each protein species was expressed and radioactively labeled (**Figure 3.4**).

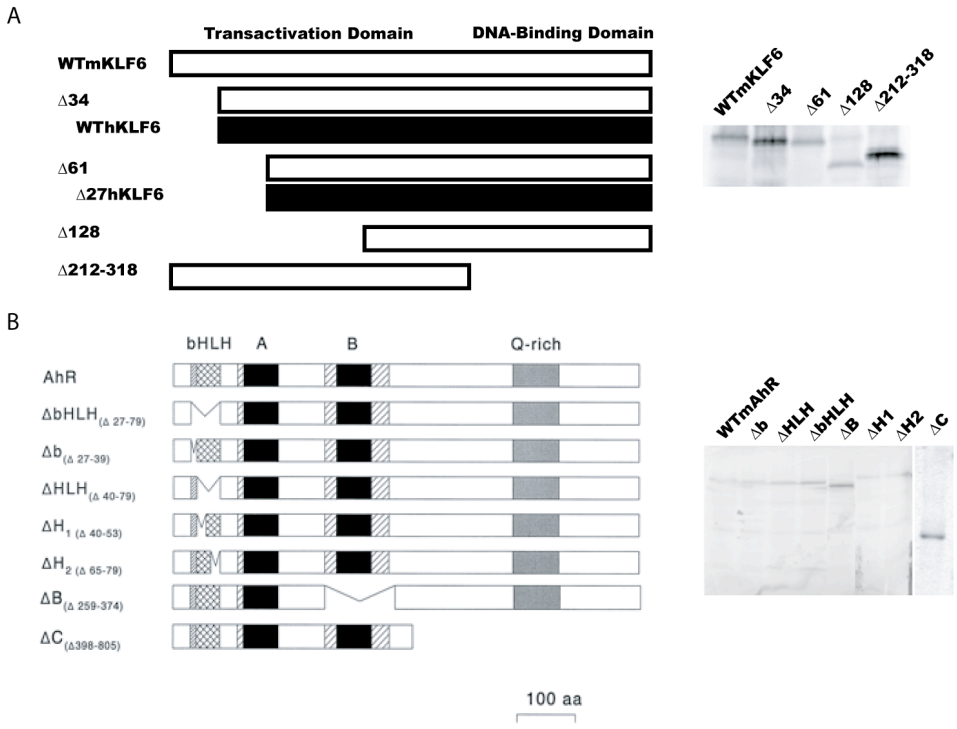

Figure 3.4. Structure and expression of murine KLF6 (A) and AhR (B) deletion constructs *in vitro*. Mutant mKLF6 constructs were created as described in the materials and methods and are correlated to their human counterparts. The mAhR constructs were a kind gift from Dr. Oliver Hankinson (Fukunaga et al. 1995).

Reciprocal co-immunoprecipitation using antibodies against the AhR and KLF6 evaluated protein-protein interactions. The results reveal that murine KLF6 binding to the AhR requires the receptor's C-terminal region (**Figure 3.5B**), whilst AhR binding to KLF6 occurs within the N-terminal 61 amino acids (**Figure 3.5A**), as depicted (**Figure 3.5D)** and in keeping with the human protein studies. In contrast to the findings with the human proteins however, the interaction between the mouse proteins appears to be TCDD independent *in vitro*.

EMSA was subsequently performed using the recombinant mouse proteins expressed *in vitr*o using the full-length mouse AhR and mouse KLF6 deletion mutants (**Figure 3.5C**). The results reveal an EMSA complex with the

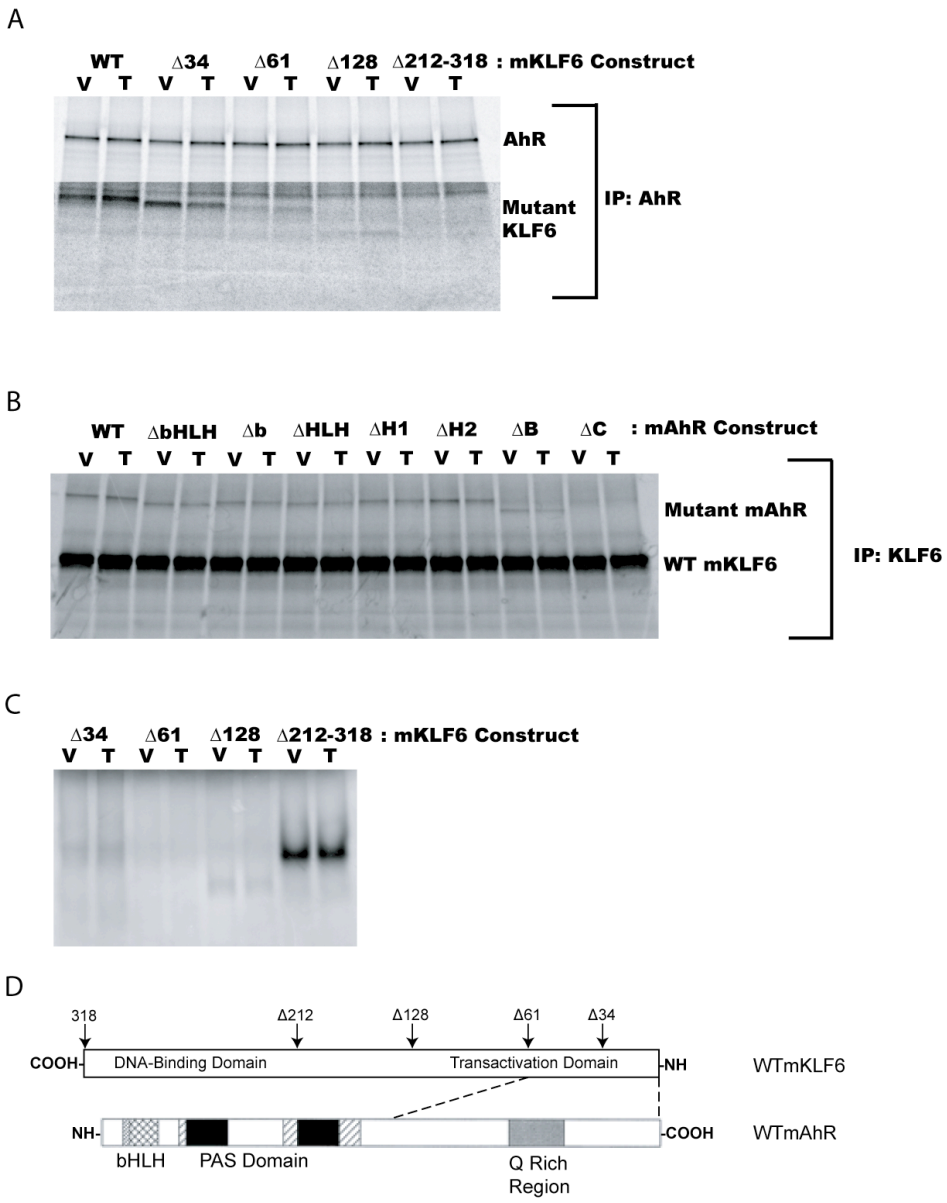

Figure 3.5. mAhR and mKLF6 interact *in vitro* through the C-terminus of mAhR and the DNA binding domain of mKLF6 independent of TCDD, but do reconstitute a complex at the ncXRE. 2µg of mAhR and mKLF6 mutant constructs were expressed using an *in vitro* coupled transcription and translation and labeled with ^{35}S-methionine. A) Mutant mKLF6 constructs were combined with an equal volume of wt mAhR and incubated with 20nM TCDD (T) or DMSO (V). Samples were immunoprecipitated with antibody against AhR and the resulting proteins were separated on a 4-20% polyacrylamide gel. B) The same process as for (A) was utilized but with mutant mAhR and wt mKLF6. C) Radioactively labeled wt mAhR and the mKLF6 mutant constructs were incubated with 20nM TCDD (T) or DMSO (V) and subsequently with 300ng ncXRE. D) Pictoral represenation of the interaction between mAhR and mKLF6.

Δ34 KLF6 mutant, but not the Δ61 mutant. As with the protein-protein interaction, DNA binding is TCDD independent. A smaller protein-DNA complex is also observed with the Δ128 KLF6 mutant, although the composition of this complex needs to be verified. Interestingly, the KLF6 protein lacking the DNA binding domain produced a robust EMSA product suggesting that the DNA binding domain may be expendable for complex formation. However, additional studies will need to be performed to validate these results.

DISCUSSION

The aim of this work was to begin to characterize the complex formed at the ncXRE. Previous studies in the laboratory of Cornelis Elferink indicated that the interaction at the ncXRE is distinct from that seen with the traditional XRE. This was based on the difference in the sequences between these two response elements, the inability for excess XRE to compete for protein binding with the ncXRE, and the apparent lack of Arnt binding at this site. Based on sequence homology between the ncXRE and the consensus binding site associated with the Kruppel-like factor family of transcription factors, and its expression in the liver, I hypothesized that KLF6 could be one of the components of the complex at the ncXRE. I used a combination of *in vivo* studies in the mouse liver as well as *in vitro* experiments using both human and mouse recombinant proteins to characterize this novel interaction involving the AhR and KLF6.

I successfully demonstrated that the AhR and KLF6 interact *in vivo* (**Figure 3.3A**) as evidenced by the AhR co-immunoprecipitating with KLF6 in nuclear extracts, specifically when activated by TCDD. These data do not distinguish whether the AhR-KLF6 interaction is direct or indirect, nor reveal the identity of other possible components, but does illuminate the existence of a novel transcription factor interaction for both proteins. Also using nuclear extracts, the addition of KLF6 antibody to proteins prior to EMSA abolished the formation of a complex at the ncXRE (**Figure 3.3B**), implying a role for KLF6 in binding to the ncXRE.

Evidence supporting an AhR and KLF6 interaction with the ncXRE *in vivo* came from the ChIP assay, which pulled-down the PAI-1 promoter region encompassing the ncXRE with antibodies for both proteins. The high GC content within this region of the PAI-1 promoter constrained primer design and prevented qPCR (TaqMan). Instead, PCR products were detected by staining with the highly sensitive SYBR Green-1 quantified using the Typhoon Trio. Since the ChIP assay PCR product closely overlaps with the -161 to +73 bp PAI-1 promoter region shown to contain the ncXRE previously (see Chapter 1), I surmise that AhR and KLF6 DNA-binding observed is due to an interaction with the ncXRE specifically. Using this technique, I found that the AhR was associated with the ncXRE in a TCDD-dependent manner, while KLF6 DNA binding was constitutive *in vivo* (**Figures 3.3C and 3.3D**). This suggests that the AhR is not essential for

initial binding of KLF6 to the ncXRE, but that TCDD inducibility of the PAI-1 gene depends on receptor activation and nuclear translocation in cells.

Studies using both human and mouse AhR and KLF6 recombinant proteins expressed *in vitro* confirm an interaction between the two proteins. Moreover, the data suggest that the mAhR C-terminus and the DNA binding domain of mKLF6 are important in conferring this interaction (**Figures 3.1A, 3.5A, and 3.5B**). However, the *in vitro* interaction between the human proteins appears to be TCDD dependent (**Figure 3.1A**), a property not retained by the mouse counterparts (**Figures 3.5A and 3.5B**). Interestingly, the interaction between mAhR and mKLF6 in nuclear extracts from the mouse indicate that the interaction, at least *in vivo*, does indeed depend on TCDD activation (**Figure 3.3A**). It could be that TCDD is required *in vivo* to initiate translocation of the AhR to the nucleus, a step in AhR activation that is bypassed by the *in vitro* studies presented here under cell free conditions. Accordingly, DNA binding by the human proteins to the ncXRE *in vitro* required TCDD activation of the AhR (**Figure 3.1B**), whilst ncXRE binding by the murine proteins occurred in the absence of TCDD activation. The simplest explanation is that the difference is attributable to species variation, a property that could be explored further in future domain swap experiments.

With the exception of the identification of the mKLF6 sequence (Inuzuka et al. 1999), the vast majority of data regarding KLF6 activity is centered on the human protein: either by analyzing human samples and cells, studies with human

cDNA constructs transfected into heterologous mammalian cells, or *in vitro* coupled transcription and translation experiments using the human protein. On the other hand, many studies have indicated a difference between human and mouse AhR proteins. Yeast reporter systems with hAhR and mAhR revealed a difference in ligand binding affinities (Kawanishi et al. 2003), a property also observed using *in vitro* transcribed and translated proteins, where the hAhR had a lower affinity for a dioxin compared to mAhR (Ramadoss and Perdew 2004). Some of the apparent species variability may be related to the experimental conditions however, for instance, Ramadoss and Perdew (2004) obtained more consistent results with transient transfection studies involving the two constructs, versus *in vitro* transcription and translation studies plagued by comparatively low protein expression. Thus it is possible that some of the binding characteristics observed may be a consequence technical limitations associated with the *in vitro* assays.

In addition to binding ligand, recent studies have demonstrated a functional difference between hAhR and mAhR in response to the same ligand. While the N-terminus is highly conserved between species, the transactivating domain in the C-terminus only shares 53% homology, and has been shown to differentially affect hAhR versus mAhR ability to activate transcription using a XRE-driven reporter assay (Flaveny et al. 2008). Studies in neurosphere cultures indicate that a hAhR is less responsive to TCDD than mAhR (Gassmann et al. 2010), but hAhR is more responsive to the proposed endogenous ligand

indoxyl-3-sulfate than mAhR in hepatoma cells lines (Schroeder et al. 2010). Thus in addition to the restrictions encountered with relatively low expression levels of the proteins using *in vitro* transcription and translation, there seems to be a significant difference in the hAhR and mAhR in functional studies, providing another explanation to the differences between the *in vitro* studies presented in this dissertation.

It is somewhat surprising to see a robust complex formation at the ncXRE in the murine KLF6 protein lacking the DNA binding domain (**Figure 3.5C, lanes 7 and 8**) considering the binding site for KLF6 provided one of the initial rationales for suspecting its involvement at the ncXRE. However, the KLF6 splice variant found to be increased in HCC and prostate tumors lacks a portion of the zinc-finger DNA binding domain, and is antagonistic to wild-type KLF6 transcriptional induction (Kremer-Tal et al. 2007; Narla et al. 2005a; Narla et al. 2005b). Perhaps the apparent binding at the ncXRE by my Δ212-318 mutant mKLF6 would lead to repression of transcription rather than induction. This could be examined using the PAI-1 reporter constructs generated in the laboratory previously in conjunction with a construct that expressed the actual protein sequence found in the KLF6 splice variant.

In conclusion, this work presents the first evidence of a novel interaction between the AhR and KLF6 at the ncXRE in the PAI-1 promoter in the liver. Despite evidence for species differences between the human and mouse

proteins, both support an interaction between the two proteins, although the precise mechanism of binding to the ncXRE remains unclear. The ChIP data suggest that the AhR and KLF6 are both found at the ncXRE *in vivo*, supporting a functional role for the two proteins in regulation PAI-1 transcription. Not only is this a novel protein-protein interaction and new target gene for both proteins, it is the first evidence of the AhR binding to a novel, defined DNA binding site *in vivo* distinct from the canonical XRE. Identifying additional genes that contain ncXREs will open a new avenue of AhR research and potentially shed light on the physiological role of the AhR.

CHAPTER 4: CONCLUSION AND FUTURE AIMS

CONCLUSIONS

The ligand activated transcription factor AhR has long been studied for its ability to mediate the toxic effects of multiple environmental contaminants including the potent, persistent, prototypical AhR agonist TCDD. A growing body of evidence, largely initiated by work using AhR null mice, supports a role for the AhR in physiological processes including development, apoptosis, and cell proliferation. Administration of sub-toxic TCDD doses fully activates the AhR, and has been used to examine the receptor's role in physiological processes, specifically hepatocyte proliferation induced by PH. Although the risks to humans of high-dose dioxin exposures are known, including increased incidence of tumors and diabetes, using these levels of TCDD in acute dosing paradigms will also help identify potential risks associated with body burdens found in the general population due to chronic low-dose TCDD exposures .

The well-characterized and accepted mode of AhR activity involves its translocation to the nucleus where the AhR heterodimerizes with Arnt to bind XREs in the promoter region of target genes and induces transcription. However, a growing body of evidence indicates that once in the nucleus, the AhR binds a number of proteins in addition to and independent of Arnt, indicating a more complex network of protein-protein interactions than previously thought. Studies using microarrays reveal many apparent targets of AhR mediated induction with

TCDD activation (Frueh et al. 2001; Puga et al. 2000b), yet in silico analysis of the promoter region of many of these genes, including PAI-1, fail to detect the canonical XRE. Thus, recognizing that the AhR can function in roles separate from those involving toxicological endpoints, coincides with several studies supporting new modes of receptor action including new nuclear binding partners and DNA binding sites such as KLF6 and the ncXRE, respectively.

Extensive work examining the role of the AhR in cell proliferation has focused on the G_1 phase and G_1-S transition in a multitude of cell lines and initial forays into *in vivo* models. However, cell proliferation *in vivo* following PH involves a precisely orchestrated series of events, referred to as the priming phase, to induce quiescent cells to enter the cell cycle, driven largely by EGFR and its ligands and HGF via its receptor cMet. An important, early component of this phase includes latent HGF in the extracellular matrix being cleaved by the protease uPA, a process inhibited by PAI-1. Prior to the work presented in this dissertation, little was know about the effect of the AhR on the priming phase of the cell proliferation, and none on the impact of receptor activity on the critical signaling molecule HGF. In this context, the previous studies from the Elferink laboratory showing TCDD-mediated AhR induction of PAI-1 involving the ncXRE appear to be germane to HGF signaling, but the effects of this induction *in vivo* had yet to be explored.

The overall goal of my dissertation was to further refine our understanding of the role of the AhR in the physiological process of cell proliferation *in vivo* and provide initial insights into the molecular mechanisms governing an alternate mode of AhR activity.

In Chapter 2, I examined the effect of AhR activation by TCDD in cell proliferation, including the initial priming phase following PH, specifically its ability to induce PAI-1, suppressing HGF activation and cMet phosphorylation, and subsequent impact on hepatocyte proliferation. In agreement with preliminary work from our laboratory using reporter constructs with the PAI-1 promoter, TCDD activation of the AhR induced PAI-1 mRNA expression *in vivo*, and this expression was dramatically enhanced in animals subjected to PH during the priming phase of regeneration. Measurement of serum PAI-1 levels demonstrated a concomitant increase of PAI-1 protein. Furthermore, HGF processing and cMet phosphorylation was suppressed by TCDD exposure in the early stages of regeneration in a manner dependent on PAI-1. These results suggest that TCDD-induced PAI-1 expression can inhibit the regenerative process following PH. Accordingly, the finding in PAI-$1^{-/-}$ mouse livers that TCDD treatment failed to suppress cMet activation or regeneration in the periportal regions indicated by nascent DNA synthesis, supports the contention that PAI-1 plays an important role. However, the lack of regeneration in the centrilobular regions in TCDD treated PAI-$1^{-/-}$ mice where AhR activity is the highest, suggests the existence of additional receptor-mediated growth inhibitory processes. Given

the extensive evidence that AhR activation can suppress cell proliferation at the G_1-S transition by disrupting Cdk2 activity, it stands to reason that TCDD effects targeting downstream signaling events could retard regenerative processes initiated by normal levels of cMet activation. This implies however, that the periportal growth arrest seen in TCDD treated hepatectomized wild-type mice attributed to suppressed Cdk activity is insufficient to stifle liver regeneration in this zone in PAI-$1^{-/-}$ mice. In summary, TCDD activation of AhR induces PAI-1 mRNA and protein expression in the priming phase of liver regeneration following PH, suppressing HGF cleavage and subsequent cMet activation, and providing an additional mechanism responsible for TCDD-mediated attenuation of liver regeneration.

With the demonstrated TCDD mediated, AhR dependent induction of PAI-1 in cells and *in vivo*, and the identification of a novel DNA binding site distinct from the canonical XRE in the PAI-1 promoter that confers these characteristics, referred to as the ncXRE, I began to characterize the molecular mechanism of AhR activity at the ncXRE in Chapter 3. Immunoprecipitation of human proteins *in vitro* demonstrated that hKLF6 binds to the hAhR, but not hArnt, in a manner dependent on TCDD activation of the AhR. Nuclear extracts from mice pretreated with vehicle or TCDD confirmed that mAhR and mKLF6 interacted *in vivo* by immunoprecipitating with KLF6 antibodies. A complementary analysis of murine proteins expressed *in vitro* also demonstrated an interaction between mAhR and mKLF6. Specifically, an analysis using mutation constructs

for both of these proteins revealed that the C-terminus of mAhR is required for KLF6 binding. Removal of the N-terminal 61 amino acids in KLF6 also severely disrupted the AhR interaction. However, unlike the human proteins, this interaction did not appear to be dependent on TCDD activation of the AhR in mice, indicating species variance.

ChIP assays revealed constitutive binding of mKLF6 to the PAI-1 promoter *in vivo*, and a TCDD-dependent binding of the mAhR to the same promoter region, suggesting a potential role for KLF6 in PAI-1 induction and evidence of AhR binding to the promoter *in vivo.* By performing EMSAs with hAhR, full-length hKLF6, and a series of progressive hKLF6 deletions beginning at the N-terminus, I found that ncXRE DNA binding by the hAhR-hKLF6 dimer requires the N-terminal 27 amino acids of hKLF6 and is TCDD dependent *in vitro*. This N-terminal 27 residue region in hKLF6 is homologous to the sequence located between residue 34 and 61 in the mKLF6 protein, which when deleted in the Δ61 mKLF6 deletion construct largely attenuated binding to the mAhR. It is noteworthy that this same region in the mKLF6 protein also appeared to confer DNA binding in the EMSA because its removal abolished formation of a gel shift complex. Interestingly, the DNA binding domain of mKLF6 does not appear necessary for this complex formation at the ncXRE, and in fact seems to enhance formation of a protein-DNA complex at the ncXRE. Although an unexpected finding, it may provide insight into the mechanism behind a splice

variant of KLF6 that lacks a portion of this region and is capable of antagonizing full-length KLF6 activity. Further studies are required to follow up on this observation. Much like the *in vitro* immunoprecipitation, using the recombinant mouse proteins for EMSA confirmed complex formation at the ncXRE that was also independent of TCDD activation, again highlighting a potential species variation. In summary, the evidence shows that the AhR and KLF6 physically interact and bind to the newly identified ncXRE located in the PAI-1 promoter both *in vivo* and *in vitro.* These features are dependent on TCDD activation of the AhR in human proteins *in vitro* and *in vivo*, although there is evidence of species variation of this dependence when using murine proteins *in vitro*.

In conclusion, this dissertation provides the first evidence that AhR activation by TCDD negatively impacts the priming phase of regeneration following PH by inducing expression of PAI-1, thus revealing additional complexity to the established mechanism of the AhR in suppressing cell proliferation at the G_1-S transition during the cell cycle *in vivo* in response to physiological stimuli. In addition to the supplementary role for the AhR in liver regeneration, I identified a hitherto unknown interaction between the AhR and KLF6 that is capable of functioning through the novel ncXRE to regulate PAI-1 gene expression. Thus, the evidence provides the first molecular insights into a role for the AhR distinct from the well-defined Arnt-dependent and XRE-mediated mechanism depicted in **Figure 4.1**. Moreover, these findings invoke broad

implications for AhR biology in gene expression germane to normal physiological processes.

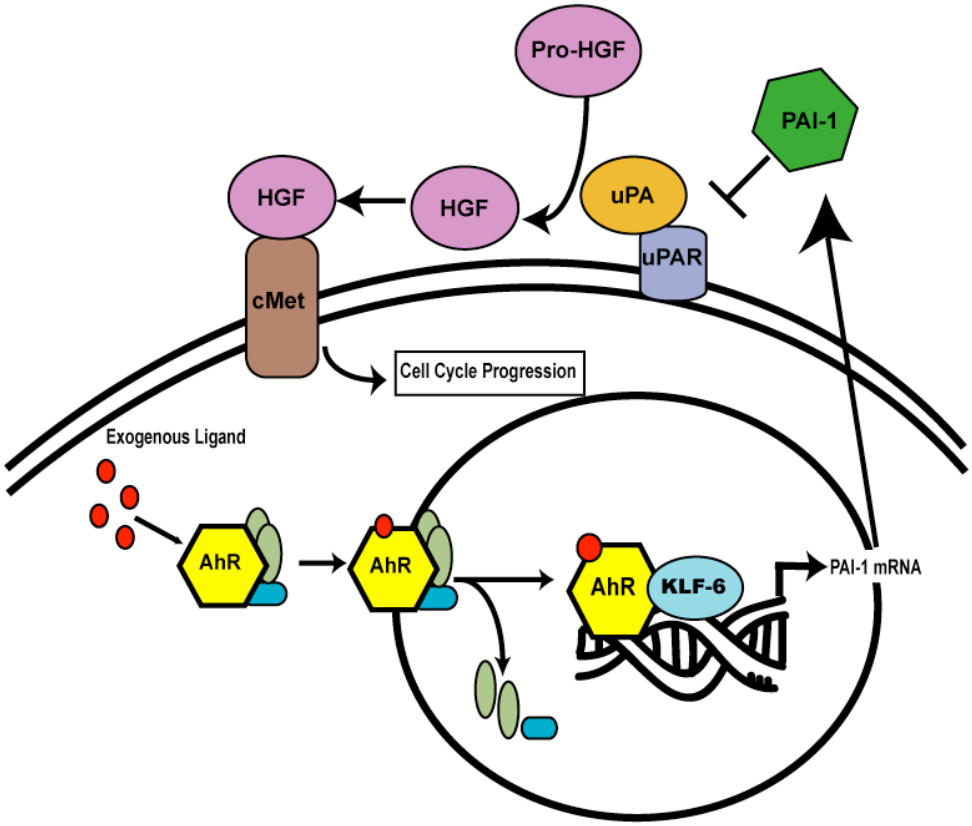

Figure 4.1. Schematic of the novel AhR interaction with KLF6 at the PAI-1 promoter in response to TCDD, inducing PAI-1 expression leading to suppression of HGF processing and subsequent activation of its receptor cMet.

FUTURE DIRECTIONS

Further characterize the role of KLF6 and AhR at the ncXRE *in vitro*.

I have presented the first evidence demonstrating a novel interaction between AhR and KLF6 as well as their ability to bind the ncXRE-containing region of the PAI-1 promoter *in vivo*, but additional experiments are indicated to fully characterize the contributions of each protein to complex formation at the

ncXRE. First, complete sets of deletion constructs of both the human and murine proteins will need to be generated and evaluated in additional expression systems to confirm the *in vitro* transcription and translation studies. This will serve to further characterize the work I have already embarked upon and begin to address the species variability seen by myself and others when comparing the activity of hAhR and mAhR proteins. The use of cell-based over-expression systems should provide conditions necessary to effectively examine the AhR-KLF6 interaction, including the role for any additional factors that might facilitate complex formation that are found in the nuclear extracts but not in the reticulocyte lysate used for *in vitro* transcription and translation.

Identify additional protein components at the ncXRE.

While the studies of KLF6 and AhR interaction and involvement at the ncXRE *in vitro* and *in vivo* represents the first glimpse into a novel transcriptional activity for the AhR, but much work remains to fully characterize the complex found at this site. To this end, I developed a protocol to identify additional protein components associated with the ncXRE using nuclear extracts from TCDD activated mice that is currently being utilized by a colleague in the lab. Briefly, nuclear extracts prepared from animals pretreated with vehicle or TCDD 2 hours prior to sacrifice, as described in Chapter 3, were blocked with polydIdC to prevent non-specific binding and then incubated with biotinylated ncXRE under conditions similar to that used in EMSA. Difference Gel Electrophoresis (2D-

DIGE) is being used to interrogate mouse liver nuclear extract for TCDD-dependent ncXRE-associated proteins by differentially labeling with Cy-dyes and separating extracts using 2D gel electrophoresis. Using the DyCyder 2D software analysis suite, protein species that bind the ncXRE and are enriched in the TCDD treated nuclear extracts can be identified and picked for mass

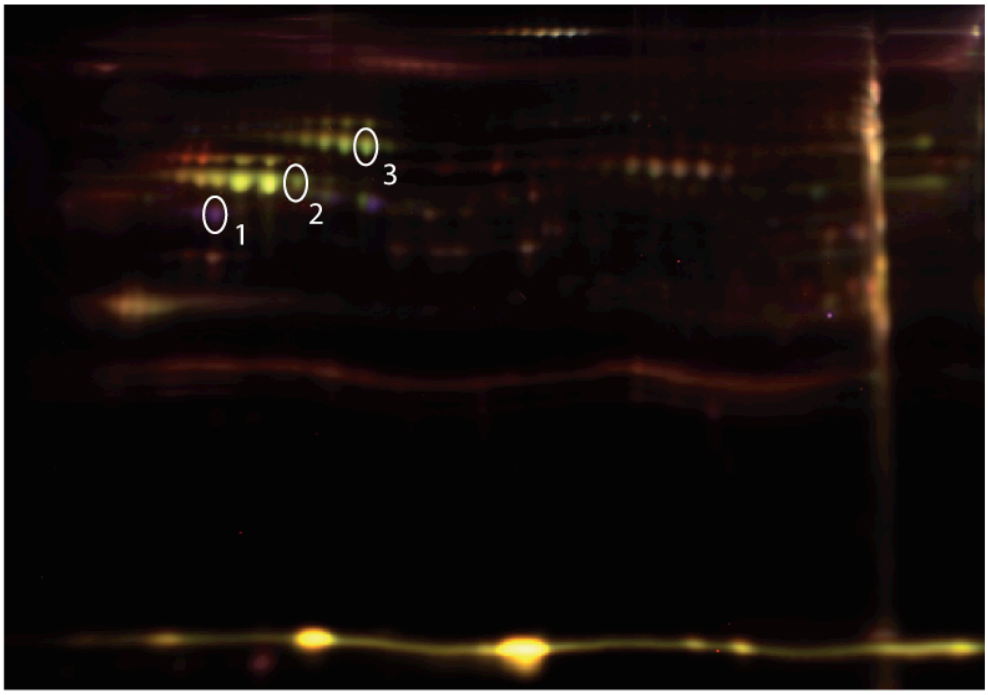

Figure 4.2. 2D gel electrophoresis of proteins bound to the ncXRE. Proteins from nuclear extracts of mice pretreated with TCDD are depicted in green, vehicle in blue, control in red. Selection 1 indicates a protein species found in vehicle only whereas selections 2 and 3 are green, and thus found only in TCDD treated animals.

spectrometry sequence identification. As depicted in **Figure 4.2**, I have demonstrated the feasibility of this approach. Proteins from vehicle treated animals were labeled with Cy3, from TCDD treated animals were labeled with Cy5, and from a control pool containing representative fractions from all animal groups were labeled with Cy2, and all were separated on 2 dimensions. This

experiment requires several rounds of repetition to identify proteins worthy of further analysis, but will reveal additional protein species capable of interacting at with the AhR at the ncXRE.

Identify the role of the AhR and KLF6 at the PAI-1 promoter *in vivo* and the potential effects on regeneration in the absence of an exogenous ligand.

I have demonstrated that the AhR and KLF6 associate with the PAI-1 promoter containing the ncXRE and that TCDD activation of the AhR induces PAI-1 expression *in vivo*, but future work is needed to assess whether or not this interaction has a functional consequence. In order to perform experiments to address this, it would be ideal to use animals that lack the AhR and KLF6 and compare the results to wild type animals. Complete $AhR^{-/-}$ (KO) mice are viable, but they display several developmental abnormalities, especially regarding the vasculature in the liver (Harstad et al. 2006). While these mice could be used, I think a more elegant study could be conducted using AhR liver-specific conditional knockout (CKO) mice. AhR floxed mice have been kindly provided by Dr. Christopher Bradfield (Walisser et al. 2005) and can be bred to generate the desired CKO mice. The floxed mice have loxP sites flanking exon 2 of the AhR gene and are crossed with mice harboring a gene for Cre-recombinase driven by the hepatocyte-specific albumin promoter. The resulting animals lose the AhR specifically in hepatocytes just prior to birth, allowing for normal development of the mouse during gestation. To date, every generated $KLF6^{-/-}$ mouse is

embryonic lethal, largely due to impaired vasculature development early in the embryo. However, thanks to a kind gift from Dr. Scott Friedman, I recently obtained floxed KLF6 mice with loxP sites flaking exons 2 and 3 (Leow et al. 2009), and following cross breeding with the Cre^{Alb} mice, these mice should lose KLF6 specifically in the hepatocytes as well. A KLF6 CKO mouse where KLF6 expression was eliminated in the prostate is viable and fecund, hence we are confident that a liver specific CKO mouse will also be viable. After confirmation studies demonstrate the loss of KLF6, these mice could be crossed with the AhR conditional knock out mice providing 3 different strains: mice lacking AhR, KLF6, or both proteins in hepatocytes. Using these mice would allow me to monitor the contributions of each protein component to PAI-1 induction *in vivo* and the potential effects on regeneration utilizing the methods described in Chapter 2.

Evidence supports a complex role of TCDD-activated AhR *in vivo* as the AhR can promote proliferation in response to carcinogenic or hyperplastic stimuli, but suppress regeneration activated by endogenous, homeostatic signals. Given this complex nature of AhR activity, studies are warranted examining the role of the AhR in the absence of an exogenous agonist. By examining regeneration that is solely due to the presence or absence of AhR using the conditional knockout mice, I will shed light on the physiological role of the AhR in this process without any potential complications attributable to introducing a toxicant to the system.

Use "humanized" mice to study species variations of the AhR and KLF6 at the ncXRE of the PAI-1 promoter *in vivo*.

If further characterization of the AhR and KLF6 mechanism of action at the ncXRE *in vitro* maintains the species variation between human and murine proteins as described in Chapter 3, one attractive way to examine this potential difference, and thus increase applicability to human risk assessment and identification of potential therapeutic targets, is the use of "humanized" mice. Transgenic mice containing full-length hAhR were crossed with AhR conditional knockout mice lacking the mAhR specifically in hepatocytes and have already been used to demonstrate differences in ligand binding and target gene induction compared to wild-type mice (Flaveny et al. 2009). Transgenic mice containing hKLF6 driven by the transthyretin promoter express hKLF6 specifically in the liver (Narla et al. 2007), but these mice still contain mKLF6. In order to create a truly humanized KLF6 mouse, it would be preferable to cross these mice with KLF6 conditional knockout mice. Using these two strains that contain the human AhR and KLF6 proteins will elucidate the interaction of these two proteins *in vivo*.

SUMMARY

Both the AhR and KLF6 have been implicated in carcinogenesis, albeit from opposing sides. However, recent studies regarding the role of the AhR in tumor promotion have revealed a complex regulation in which, depending on the stimulus, AhR can either promote or attenuate regeneration. I hypothesized that activated AhR, through a novel interaction with KLF6 at the ncXRE in the PAI-1 promoter, could attenuate liver regeneration in the presence of physiological stimuli following PH. A detailed study unequivocally linking KLF6 activity to PAI-1 induction *in vivo* will confirm an additional target gene for KLF6 and contribute to the knowledge of its ability to suppress proliferation. The discovery of a binding site distinct from the canonical XRE known to mediate the toxic effects of TCDD and the AhR's association with a transcription factor classified as a tumor suppressor at this site begins to explain, in part, how the receptor can function to suppress proliferation *in vivo*. This data provides additional considerations when assessing human risk following exposure to TCDD.

REFERENCES

Ahn, Y.-T., Chua, M.-S., James P. Whitlock, J., Shin, Y.-C., Song, W.-H., Kim, Y., Eom, C.-Y., and An, W. G. (2010). "Rodent-specific hypoxia response elements enhance PAI-1 expression through HIF-1 or HIF-2 in mouse hepatoma cells." *International Journal of Oncology*, 37, 1627-1638.

Albrecht, J. H., Hoffman, J. S., Kren, B. T., and Steer, C. J. (1993). "Cyclin and cyclin-dependent kinase 1 mRNA expression in models of regenerating liver and human liver diseases." *American Journal of Physiology: Gastrointestinal and Liver Physiology*, 265, G857-G864.

Albrecht, J. H., Hu, M. Y., and Cerra, F. B. (1995). "Distinct patterns of cyclin D1 regulation in models of liver regeneration and human liver." *Biochemical and Biophysical Research Communications*, 209(2), 648-655.

Albrecht, J. H., Meyer, A. H., and Hu, M. Y. (1997). "Regulation of cyclin-dependent kinase inhibitor p21WAF1/Cip1/Sdi1 gene expression in hepatic regeneration." *Hepatology*, 25, 557-563.

Andersson, P., McGuire, J., Rubio, C., Gradin, K., Whitelaw, M., Pettersson, S., Hanberg, A., and Poellinger, L. (2002). "A constitutively active dioxin/aryl hydrocarbon receptor induces stomach tumors " *Proceedings of the National Academies of Science USA*, 99, 9990-9995.

Bauman, J. W., Goldworthy, T. L., Dunn, C. S., and Fox, T. R. (1995). "Inhibitory effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on rat hepatocyte proliferation induced by 2/3 partial hepatectomy." *Cell Proliferation*, 28, 437-451.

Benzeno, S., Narla, G., Allina, J., Cheng, G. Z., Reeves, H. L., Banck, M. S., Odin, J. A., Diehl, J. A., Germain, D., and Friedman, S. L. (2004). "Cyclin-dependent kinase inhibition by the KLF6 tumor suppressor protein through interaction with cyclin D1." *Cancer Research*, 64, 3885-3891.

Bernshausen, T., Jux, B., Esser, C., Abel, J., and Fritsche, E. (2006). "Tissue distribution and function of the Aryl hydrocarbon receptor repressor (AhRR) in C57BL/6 and Aryl hydrocarbon receptor deficient mice." *Archives of Toxicology*, 80, 206-211.

Bertazzi, P. A., Consonni, D., Bachetti, S., Rubagotti, M., Baccarelli, A., Zocchetti, C., and Pesatori, A. (2001). "Health effects of dioxin exposure: a 20-year mortality study." *American Journal of Epidemiology*, 153(11), 1031-1044.

Bieker, J. J. (2001). "Kruppel-like factors: three fingers in many pies." *The Journal of Biological Chemistry*, 276(37), 34355-34358.

Black, A. R., Black, J. D., and Azizkhan-Clifford, J. (2001). "Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer." *Journal of Cellular Physiology*, 188, 143-160.

Block, G. D., Locker, J., Bowen, W. C., Petersen, B. E., Katyal, S., Strom, S. C., Riley, T., Howard, T. A., and Michalopoulos, G. K. (1996). "Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF-alpha in a chemically defined (HGM) medium." *The Journal of Cell Biology*, 132(6), 1133-1149.

Borowiak, M., Garratt, A. N., Wüstefeld, T., Strehle, M., Trautwein, C., and Birchmeier, C. (2004). "Met provides essential signals for liver regeneration." *Proceedings of the National Academies of Science USA*, 101(29), 10608-10613.

Botella, L., Sanz-Rodriquez, F., Komi, Y., Fernandez, A., Varela, E., Garrido-Martin, E., Narla, G., Friedman, S. L., and Kojima, S. (2009). "TGF-ß regulates the expression of transcription factor KLF6 and its splice cariants and promotes co-operative transactivation of common target genes through a Smad3-Sp1-KLF6 interaction." *Biochemical Journal*, 419(2), 485-495.

Chang, H., Wang, Y.-J., Chang, L. W., and Lin, P. (2005). "A histochemical and pathological study on the interrelationship between TCDD-induced AhR expression, AhR activation, and hepatotoxicity in mice." *Journal of Toxicology and Environmental Health, Part A*, 68, 1567-1579.

Chen, Y.-H., Beischlag, T. V., Kim, J. H., Perdew, G. H., and Stallcup, M. R. (2006). "Role of GAC63 in transcriptional activation mediated by the aryl hydrocarbon receptor." *The Journal of Biological Chemistry*, 281(18), 12242-12247.

Cho, Y. G., Kim, C. J., Park, C. H., Yang, Y. M., Kim, S. Y., Nam, S. W., Lee, S. H., Yoo, N. J., Lee, J. Y., and Park, W. S. (2005). "Genetic alterations of the KLF6 gene in gastric cancer." *Oncogene*, 24(28), 4588-4590.

Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M., and Roberts, J. M. (1996). "Turnover of cyclin E by the ubiquitin-proteosome pathway is regulated by cdk2 binding and cyclin phosphorylation." *Genes & Development*, 10, 1979-1990.

Committee to Review the Health Effects in Vietnam Veterans of Exposure to Herbicides. (2005). "Veterans and Agent Orange: Update 2004". The National Academies Press: Washington, DC,20055

Cressman, D. E., Greenbaum, L. E., DeAngelis, R. A., Ciliberto, G., Furth, E. E., Poli, V., and Taubt, R. (1996). "Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice." *Science*, 274(5291), 1379-1383.

Cubellis, M. V., Andreasen, P., Ragno, P., Mayer, M., Dano, K., and Blasi, F. (1989). "Accessibility of receptor-bound urokinase to type-i plasminogen activator inhibitor." *Proceedings of the National Academies of Science USA*, 86, 4828-4832.

Cubellis, M. V., Wun, T.-C., and Blasi, F. (1990). "Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1." *The EMBO Journal*, 9(4), 1079-1085.

Degryse, B., Sier, C. F. M., Resnati, M., Conese, M., and Blasi, F. (2001). "PAI-1 inhibits urokinase-induced chemotaxis by internalizing the urokinase receptor." *FEBS Letters*, 505, 249-254.

Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995). "Mice lacking p21 CIP1/WAF7 undergo normal development, but are defective in G1 checkpoint control." *Cell*, 82, 675-684.

Dennler, S., Itoh, S., Vivien, D., Dijke, P. t., Huet, S., and Gauthier, J.-M. (1998). "Direct binding of Smad3 and Smad4 to critical TGFb-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene." *The EMBO Journal*, 17(11), 3091-3100.

DiFeo, A., Narla, G., Hirshfeld, J., Camacho-Vanegas, O., Narla, J., Rose, S. L., Kalir, T., ShenYao, Levine, A., Birrer, M. J., Bonome, T., Friedman, S. L., Buller, R. E., and A.Martignetti, J. (2006). "Roles of KLF6 and KLF6-SV1 in ovarian cancer progression and intraperitoneal dissemination." *Clinical Cancer Research*, 12, 3730-3739.

Dimova, E. Y., and Kietzmann, T. (2008). "Metabolic, hormonal and environmental regulation of plasminogen activator inhibitor-1 (PAI-1) expression: lessons from the liver." *Thrombosis and Haemostasis*, 100, 992-1006.

Donovan, J., and Slingerland, J. (2000). "Transforming growth factor-ß and breast cancer: cell cycle arrest by transforming growth factor-ß and its disruption in cancer." *Breast Cancer Research*, 2(2), 116-124.

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). "WAF1, a potential mediator of p53 tumor suppression." *Cell*, 75, 817-825.

Elferink, C., and Whitlock, J. (1994). "Dioxin-dependent, DNA sequence-specific binding of a multiprotein complex containing the Ah receptor." *Receptor*, 4(3), 157-173.

Evans, B. R., Karchner, S. I., Allan, L. L., Pollenz, R. S., Tanguay, R. L., Jenny, M. J., Sherr, D. H., and Hahn, M. E. (2008). "Repression of aryl hydrocarbon receptor (AHR) signaling by AHR repressor: role of DNA binding and competition for AHR nuclear translocator." *Molecular Pharmacology*, 73, 387-398.

Fausto, N. (2000). "Liver regeneration." *Journal of Hepatology*, 32(S1), 19-31.

Flaveny, C., Murray, I. A., Chiaro, C. R., and Perdew, G. (2009). "Ligand selectivity and gene regulation by the human aryl hydrocarbon receptor in transgenic mice." *Molecular Pharmacology*, 75, 1412-1420.

Flaveny, C., Reen, R. K., Kusnadi, A., and Perdew, G. H. (2008). "The mouse and human Ah receptor differ in recognition of LXXLL motifs." *Archives of Biochemistry and Biophysics*, 417(2), 215-223.

Forkert, P.-G. (1997). "Coexpression of Ah receptor and CYP1A1 in hepatocytes of C57BL/6J and DBA/2J mice." *Toxicology and Applied Pharmacology*, 142, 69-78.

Frueh, F. W., Hayashibara, K. C., Brown, P. O., and Jr., J. P. W. (2001). "Use of cDNA microarrays to analyze dioxin-induced changes in human liver gene expression." *Toxicology Letters*, 122, 189-203.

Fukunaga, B., Probst, M., Reisz-Porszasz, S., and Hankinson, O. (1995). "Identification of functional domains of the aryl hydrocarbon receptor." *The Journal of Biological Chemistry*, 270(49), 29270-29278.

Gassmann, K., Abel, J., Bothe, H., Haarmann-Stemmann, T., Merk, H. F., Quasthoff, K. N., Rockel, T. D., Schreiber, T., and Fritsche, E. (2010). "Species-specific differential AhR expression protects human neural progenitor cells against developmental neurotoxicity of PAHs." *Environmental Health Perspectives*, 118, 1571-1577.

Ge, N. L., and Elferink, C. J. (1998). "A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein. Linking dioxin signaling to the cell cycle." *Journal of Biological Chemistry*, 273(35), 22708-22713.

Geng, Y., Eaton, E. N., Picon, M., Roberts, J. M., Lundberg, A. S., Gifford, A., Sardet, C., and Weinberg, R. (1996). "Regulation of cyclin E transcription by E2Fs and retinoblastoma protein." *Oncogene*, 12, 1173-1180.

Gonzalez, F. J., and Fernandez-Salguero, P. (1998). "The aryl hydrocarbon receptor: studies using the AhR-null mice." *Drug Metabolism and Disposition*, 26(12), 1194-1198.

Gu, Y.-Z., Hogenesch, J. B., and Bradfield, C. A. (2000). "The PAS superfamily: sensors of environmental and developmental signals." *Annual Review of Pharmacology and Toxicology*, 40, 519-561.

Hankinson, O. (1995). "The aryl hydrocarbon receptor complex." *Annual Review of Pharmacology and Toxicology*, 35, 307-340.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993). "The p21 cdk-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependent kinases." *Cell*, 75, 805-815.

Harper, P. A., Riddick, D. S., and Okey, A. B. (2006). "Regulating the regulator: factors that control levels and activity of the aryl hydrocarbon receptor." *Biochemical Pharmacology*, 72, 276-279.

Harstad, E. B., Guite, C. A., Thomae, T. L., and Bradfield, C. A. (2006). "Liver deformation in AhR-null mice: evidence for aberrant hepatic perfusion in early development." *Molecular Pharmacology*, 69, 1534-1541.

Higgins, G., and Anderson, R. (1931). "Experimental pathology of the liver. I. Restoration of the liver of the white rate following partial surgical removal." *Archives of Pathology and Laboratory Medicine*, 12, 186-202.

Hirai, H., Roussel, M. F., Kato, J.-Y., Ashmun, R. A., and Sherr, C. (1995). "Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6." *Molecular and Cellular Biology*, 15(5), 2672-2681.

Hoffman, E., Reyes, H., Chu, F.-F., Sander, F., Conley, L., Brooks, B., and Hankinson, O. (1991). "Cloning of a factor required for activity of the Ah (dioxin) receptor." *Science*, 252(5008), 954-958.

Holmes, J., and Pollenz, R. (1997). "Determination of aryl hydrocarbon receptor nuclear translocator protein concentration and subcellular localization in hepatic and nonhepatic cell culture lines: development of quantitative

western blotting protocols for calculation of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein in total cell lysates." *Molecular Pharmacology*, 52, 202-211.

Huang, G., and Elferink, C. J. (2005). "Multiple mechanisms are involved in Ah receptor-mediated cell cycle arrest." *Molecular Pharmacology*, 67(1), 88-96.

Huh, C.-G., Factor, V. M., Sánchez, A., Uchida, K., Conner, E. A., and Thorgeirsson, S. S. (2004). "Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair." *Proceedings of the National Academies of Science USA*, 101(13), 4477-4482.

Hushka, D. R., and Greenlee, W. F. (1995). "2,3,7,8-tetrachlorodibenzo-p-dioxin inhibits DNA synthesis in rat primary hepatocytes." *Mutation Research*, 333, 89-99.

Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y., and Kawajiri, K. (1998). "Nuclear localization and export signals of the human aryl hydrocarbon receptor." *The Journal of Biological Chemistry*, 273(5), 2895-2904.

Imataka, H., Sogawa, K., Yasumoto, K.-i., Kikuchi, Y., Sasano, K., Kobayashi, A., Hayami, M., and Fujii-Kuriyama, Y. (1992). "Two regulatory proteins that bind to the basic transcription element (BTE), a GC box sequence in the promoter region of the rat P-4501A1 gene." *The EMBO Journal*, 11(10), 3663-3671.

Inuzuka, H., Wakao, H., Masuho, Y., Muramatsu, M.-a., Tojo, H., and Nanbu-Wakao, R. (1999). "cDNA cloning and expression analysis of mouse zf9, a kruppel-like transcription factor gene that is induced by adipogenic hormonal stimulation in 3T3-L1 cells." *Biochemica et Biophysica Acta*, 1447(2-3), 199-207.

Kang, H. J., Kim, H. J., Cho, C.-H., Hu, Y., Li, R., and Bae, I. (2008). "BRCA1 transcriptional activity is enhanced by interactions between its AD1 domain and AhR." *Cancer Chemotherapy and Pharmacology*, 62(6), 965-975.

Kawanishi, M., Sakamoto, M., Ito, A., Kishi, K., and Yagi, T. (2003). "Construction of reporter yeasts for mouse aryl hydrocarbon receptor ligand activity." *Mutation Research*, 540, 99-105.

Ke, S., Rabson, A. B., Germino, J. F., Gallo, M. A., and Tian, Y. (2001). "Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor- and lipopolysaccharide." *The Journal of Biological Chemistry*, 276(43), 39638-39644.

Kim, T. H., Mars, W. M., Stolz, D. B., Petersen, B. E., and Michalopoulos, G. K. (1997). "Extracellular matrix remodeling at the early stages of liver regeneration in the rat." *Hepatology*, 26(4), 896-904.

Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohman, L. A., and Koff, A. (1996). "Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27Kip1." *Cell*, 85, 721-732.

Kobayashi, A., Numayama-Tsuruta, K., Sogawa, K., and Fujii-Kuriyama, Y. (1997). "CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt)." *Journal of Biochemistry*, 122, 703-710.

Kogevinas, M., Becher, H., Benn, T., Bertazzi, P. A., Boffetta, P., Bueno-de-Mesquita, H. B., Coggon, D., Colin, D., Flesch-Janys, D., Fingerhut, M., Green, L., Kauppinen, T., Littorin, M., Lynge, E., Mathews, J. D., Neuberger, M., Pearce, N., and Saracci, R. (1997). "Cancer mortality in workers exposed to phenoxy herbicides, chlorophenols, and dioxins." *American Journal of Epidemiology*, 145(12), 1061-1075.

Kojima, S., Hayashi, S., Shimokado, K., Suzuki, Y., Shimada, J., Crippa, M. P., and Friedman, S. L. (2000). "Transcriptional activation of urokinase by the kruppel-like factor Zf9/COPEB activates latent TFG-ß1 in vasular endothelial cells." *Blood*, 95(4), 1309-1316.

Kolluri, S. K., Weiss, C., Koff, A., and Gottlicher, M. (1999). "p27(Kip1) induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells." *Genes & Development*, 13(13), 1742-1753.

Kremer-Tal, S., Narla, G., Chen, Y., Hod, E., DiFeo, A., Yea, S., Lee, J.-S., Schwartz, M., Thung, S. N., Fiel, I. M., Banck, M., Zimran, E., Thorgeirsson, S. S., Mazzaferro, V., Bruix, J., Martignetti, J. A., Llovet, J. M., and Friedman, S. L. (2007). "Downregulation of KLF6 is an early event in hepatocarcinogenesis, and stimulates proliferation while reducing differentiantion." *Journal of Hepatology*, 46, 645-654.

Kulkarni, P., Crespo, J., and Alfonso, C. (2008). "Dioxins sources and current remediation technologies - a review." *Environmental International*, 34(1), 139-153.

LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997). "New functional activities for the p21 family of CDK inhibitors." *Genes & Development*, 11, 847-862.

Lai, Z.-W., Pineau, T., and Esser, C. (1996). "Identification of dioxin-responsive elements (DREs) in the 5' regions of putative dioxin-inducible genes." *Chemico-Biological Interactions*, 100, 97-112.

Lalazar, A., Wong, L., Yamasaki, G., and Friedman, S. L. (1997). "Early genes induced in hepatic stellate cells during wound healing." *Gene*, 195, 235-243.

Lees, M. J., and Whitelaw, M. L. (1999). "Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein Arnt." *Molecular and Cellular Biology*, 19, 5811-5822.

Leow, C. C., Wang, B.-e., Ross, J., Chan, S. M., Zha, J., Carano, R. A. D., Frantz, G., Shen, M. M., Sauvage, F. J. d., and Gao, W.-Q. (2009). "Prostate-specific KLF6 inactivation impairs anterior prostate branching morphogenesis through increased activation of the Shh pathway." *Journal of Biological Chemistry*, 284 (31), 21057-21065.

Levine-Fridman, A., Chen, L., and Elferink, C. (2004). "Cytochrome P4501A1 promotes G1 phase cell cycle progression by controlling aryl hydrocarbon receptor activity." *Molecular Pharmacology*, 65(2), 461-469.

Lu, X. P., Koch, K. S., Lew, D. J., Dulic, V., Pinesll, J., Reed, S. I., Hunter, T., and Leffert, H. L. (1992). "Induction of cyclin mRNA and cyclin-associated histone H1 kinase during liver regeneration." *The Journal of Biological Chemistry*, 267(5), 2841-2844.

Ludlow, J. W., Glendening, C. L., Livingston, D. M., and DeCaprio, J. A. (1993). "Specifc enzymatic dephosphorylation of the retinoblastoma protein." *Molecular and Cellular Biology*, 13(1), 367-372.

Lundberg, A. S., and Weinberg, R. A. (1998). "Functional inactivation of the retinoblastoma protein requires sequential modification by a least two distinct cyclin-cdk complexes." *Molecular and Cellular Biology*, 18(2), 753-761.

Ma, Q., and James P. Whitlock, J. (1996). "The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin." *Molecular and Cellular Biology*, 16(5), 2144-2150.

Mandal, P. K. (2005). "Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology." *Journal of Comparative Physiology B*, 175(4), 221-230.

Marlowe, J. L., Knudsen, E. S., Schwemberger, S., and Puga, A. (2004). "The aryl hydrocarbon receptor displaces p300 from E2F-dependent promoters and represses S phase-specific gene expression." *The Journal of Biological Chemistry*, 279(28), 29013-29022.

Mars, W. M., Liu, M. L., Kitson, R. P., Goldfarb, R. H., Gabauer, M. K., and Michalopoulos, G. K. (1995a). "Immediate early detection of urokinase receptor after partial hepatectomy and its implications for initiation of liver regeneration." *Hepatology*, 21(6), 1695-701.

Mars, W. M., Liu, M. L., Kitson, R. P., Goldfarb, R. H., Gabauer, M. K., and Michalopoulos, G. K. (1995b). "Immediate early detection of urokinase receptor after partial hepatectomy and its implications for initiation of liver regeneration." *Hepatology*, 21(6), 1695-1701.

Mars, W. M., Zarnegar, R., and Michalopoulos, G. K. (1993). "Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA." *American Journal of Pathology*, 143(3), 949-958.

Masumoto, A., and Yamamoto, N. (1991). "Sequestration of a hepatocyte growth factor in extracellular matrix in normal adult rat liver." *Biochemical and Biophysical Research Communications*, 174(1), 90-95.

Masumoto, A., and Yamamoto, N. (1993). "Stimulation of DNA synthesis in hepatocytes by hepatocyte growth factor bound to extracellular matrix." *Biochemical and Biophysical Research Communications*, 191(3), 1218-1223.

McGuire, J., Coumailleau, P., Whitelaw, M. L., Gustafsson, J.-Å., and Poellinger, L. (1996). "The basic helix-loop-helix/PAS factor Sim is associated with hsp90." *The Journal of Biological Chemistry*, 270(52), 31353-31357.

McKay, G. (2002). "Dioxin characterisation, formation, and minimisation during municipal solid waste (MSW) incineration: review." *Chemical Engineering Journal*, 86, 343-368.

Michalopoulos, G. K. (2007). "Liver regeneration." *Journal of Cellular Physiology*, 213 (2), 286-300.

Michalopoulos, G. K., and DeFrances, M. C. (1997). "Liver regeneration." *Science*, 276(5309), 60-66.

Mimura, J., Ema, M., Sogawa, K., and Fujii-Kuriyama, Y. (1999). "Identification of a novel mechanism of regulation of Ah (dioxin) receptor function." *Genes & Development*, 13(1), 20-25.

Mimura, J., and Fujii-Kuriyama, Y. (2003). "Functional role of AhR in the expression of toxic effects by TCDD." *Biochimica et Biophysica Acta*, 1619(3), 263-268.

Mitchell, K. A., and Elferink, C. (2009). "Timing is everything: consequences of transient and sustained AhR activity." *Biochemical Pharmacology*, 77(6), 947-956.

Mitchell, K. A., Lockhart, C. A., Huang, G., and Elferink, C. J. (2006). "Sustained aryl hydrocarbon receptor activity attenuates liver regeneration." *Molecular Pharmacology*, 70(1), 163-170.

Mitchell, K. A., Wilson, S. R., and Elferink, C. J. (2010). "The activated aryl hydrocarbon receptor synergizes mitogen-induced murine liver hyperplasia." *Toxicology*, 276(2), 103-109.

Moennikes, O., Loeppen, S., Buchmann, A., Andersson, P., Ittrich, C., Poellinger, L., and Schwarz, M. (2004). "A constitutively active dioxin/aryl hydrocarbon receptor promotes hepatocarcinogenesis in mice." *Cancer Research*, 64, 4707-4710.

Naldini, L., Tamagnone, L., Vigna, E., Sachs, M., Hartmann, G., Birchmeier, W., Daikuhara, Y., Tsubouchi, H., Blasi, F., and Comoglio, P. M. (1992). "Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor." *The EMBO Journal*, 11(13), 4825-4833.

Naldini, L., Vigna, E., Narsimhan, R., Gaudino, G., Zarnegar, R., Michalopoulos, G., and Comoglio, P. (1991). "Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET." *Oncogene*, 6(4), 501-504.

Narla, G., DiFeo, A., Reeves, H. L., Schaid, D. J., Hirshfeld, J., Hod, E., Katz, A., Isaacs, W. B., Hebbring, S., Komiya, A., McDonnell, S. K., Wiley, K. E., Jacobsen, S. J., Isaacs, S. D., Walsh, P. C., Zheng, S. L., Chang, B.-L., Friedrichsen, D. M., Stanford, J. L., Ostrander, E. A., Chinnaiyan, A. M., Rubin, M. A., Xu, J., Thibodeau, S. N., Friedman, S. L., and Martignetti, J. A. (2005a). "A germline DNA polymorphism enhances alternative splicing of the KLF6 tumor suppressor

gene and is associated with increased prostate cancer risk." *Cancer Research*, 65, 1213-1222.

Narla, G., DiFeo, A., Yao, S., Banno, A., Hod, E., Reeves, H. L., Qiao, R. F., Camacho-Vanegas, O., Levine, A., Kirschenbaum, A., Chan, A. M., Friedman, S. L., and Martignetti, J. A. (2005b). "Targeted inhibition of the KLF6 splice variant, KLF6 SV1, suppresses prostate cancer cell growth and spread." *Cancer Research*, 65, 5761-5768.

Narla, G., Heath, K. E., Reeves, H. L., Li, D., Giono, L. E., Kimmelman, A. C., Glucksman, M. J., Narla, J., Eng, F. J., Chan, A. M., Ferrari, A. C., Martignetti, J. A., and Friedman, S. L. (2001). "KLF6, a candidate tumor suppressor gene mutated in prostate cancer." *Science*, 294, 2563-2566.

Narla, G., Kremer-Tal, S., Matsumoto, N., Zhao, X., Yao, S., Kelley, K., Tarocchi, M., and Friedman, S. L. (2007). "In vivo regulation of p21 by the kruppel-like factor 6 tumor-suppressor gene in mouse liver and human hepatocellular carcinoma." *Oncogene*, 26, 4428-4434.

Ortega, S., Malumbres, M., and Barbacid, M. (2002). "Cyclin D-dependent kinases, INK4 inhibitors and cancer." *Biochemica et Biophysica Acta*, 1602(73-87).

Ott, M. G., and Zober, A. (1996). "Cause specific mortality and cancer incidence among employees exposed to 2,3,7,8-TCDD after a 1953 reactor accident." *Occupational and Environmental Medicine*, 53, 606-612.

Pan, X.-c., Chen, Z., Chen, F., Chen, X.-h., Jin, H.-y., and Xu, X.-y. (2006). "Inactivation of the tumor suppressor Krüppel-like factor 6 (KLF6) by mutation or decreased expression in hepatocellular carcinomas." *Journal of Zhejiang University Science B*, 7(10), 830-836.

Paranjpe, S., Bowen, W. C., Bell, A. W., Nejak-Bowen, K., Luo, J.-H., and Michalopoulos, G. K. (2007). "Cell cycle effects resulting from inhibition of hepatocyte growth factor and its receptor c-Met in regenerating rat livers by RNA interference." *Hepatology*, 45(6), 1471-1477.

Pediaditakis, P., Lopez-Talavera, J. C., Petersen, B., Monga, S. P. S., and Michalopoulos, G. K. (2001). "The processing and utilization of hepatocyte growth factor/ scatter factor following partial hepatectomy in the rat." *Hepatology*, 34(4), 688-693.

Perdew, G. (1988). "Association of the Ah receptor with the 90kD heat shock protein." *Journal of Biological Chemistry*, 263, 13802-13805.

Philipsen, S., and Suske, G. (1999). "A tale of three fingers: the family ofmammalian Sp/XKLF transcription factors." *Nucleic Acids Research*, 27(15), 2991-3000.

Pitot, H. C., Goldsworthy, T., Campbell, H. A., and Poland3, A. (1980). "Quantitative evaluation of the promotion by 2,3,7,8-tetrachlorodibenzo-p-dioxin of hepatocarcinogenesis from diethylnitrosamine." *Cancer Research*, 40, 3616-3620.

Poland, A., and Knutson, J. (1982). "2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity." *Annual Review of Pharmacology and Toxicology*, 22, 517-554.

Pollenz, R., Popat, J., and Dougherty, E. J. (2005). "Role of the carboxy-terminal transactivation domain and activetranscription in the ligand-induced and ligand-independent degradation of the mouse Ah(b1) receptor." *Biochemical Pharmacology*, 70, 1623-1633.

Pollenz, R. S. (2002). "The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation." *Chemico-Biological Interactions*, 141, 41-61.

Polyak, K., Lee, M.-H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994). "Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals." *Cell*, 78, 59-66.

Probst, M., Reisz-Porszasz, S., Agbunag, R., Ong, M., and Hankinson, O. (1993). "Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action." *Molecular Pharmacology*, 44, 511-518.

Prokipcak, R. D., and Okey, A. B. (1990). "Downregulation of the Ah receptor in mouse hepatoma cells treated in culture with 2,3,7,8-tetrachlorodibenzo-p-dioxin." *Canadian Journal of Physiological Pharmacology*, 69, 1204-1210.

Puga, A., Barnes, S. J., Dalton, T. P., Chang, C.-y., Knudsen, E. S., and Maier, M. A. (2000a). "Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest." *The Journal of Biological Chemistry*, 275 (4), 2943-2950.

Puga, A., Maier, A., and Medvedovic, M. (2000b). "The transcriptional signature of dioxin in human hepatoma HepG2 cells." *Biochemical Pharmacology*, 60(8), 1129-1142.

Ramadoss, P., and Perdew, G. H. (2004). "Use of 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin as a probe to determine the relative ligand affinity of human versus mouse aryl hydrocarbon receptor in cultured cells." *Molecular Pharmacology*, 66, 129-136.

Ratziu, V., Lalazar, A., Wong, L., Dang, Q., Collins, C., Shaullian, E., Jensen, S., and Friedman, S. L. (1998). "Zf9, a Kruppel-like transcription factor up-regulated in vivo during early hepatic fibrosis " *Proceedings of the National Academies of Science USA*, 95, 9500-9505.

Reeves, H. L., Narla, G., Ogunbiyi, O., Haq, A. I., Katz, A., Benzeno, S., Hod, E., Harpaz, N., Goldberg, S., Tal-Kremer, S., Eng, F. J., Arthur, M. J. P., Martignetti, J. A., and Friedman, S. L. (2004). "Kruppel-like factor 6 (KLF6) is a tumor-suppressor gene frequently inactivated in colorectal cancer." *Gastroenterology*, 126(4), 1090-1103.

Ren, Z., and Zheng, M. (2009). "Impacts of human activities on dioxins emissions at national scale." *Chemosphere*, 76(6), 853-859.

Roberts, B. J., and Whitelaw, M. L. (1999). "Degradation of the basic helix-loop-helix/ Per-ARNT-Sim homology domain dioxin receptor via the ubiquitin/

proteasome pathway." *The Journal of Biological Chemistry*, 274(51), 36351-36356.

Rodriguez, E., Aburjania, N., Priedigkeit, N. M., DiFeo, A., and Martignetti, J. A. (2010). "Nucleo-cytoplasmic localization domains regulate kruppel-like factor 6 (KLF6) protein stability and tumor suppressor function." *PLoS ONE*, 5(9), 1-13.

Roselli, H. T., Su, M., Washington, K., Kerins, D. M., Vaughan, D. E., and Russell, W. E. (1998). "Liver regeneration is transiently impaired in urokinase-deficient mice." *American Journal of Physiology: Gastrointestinal and Liver Physiology*, 275, G1472-G1479.

Rowlands, J. C., and Gustafsson, J. A. (1997). "Aryl hydrocarbon receptor-mediated signal transduction." *Crit Rev Toxicol*, 27(2), 109-34.

Safe, S. (2001). "Molecular biology of the Ah receptor and its role in carcinogenesis." *Toxicol Lett*, 120(1-3), 1-7.

Safe, S. H. (1986). "Comparative toxicology and mechanism of action of polychlorinated dibenzo-p-dioxins and dibenzofurans." *Annu Rev Pharmacol Toxicol*, 26, 371-99.

Sato, Y., Nakatsuka, H., Yamamoto, S., Oya, H., Kobayashi, T., Watanabe, T., Kokai, H., Kurosaki, I., Shirai, Y., and Hatakeyama, K. (2008). "Coagulation and fibrinolytic systems during liver regeneration in the early period after adult living related partial liver transplantation." *Transplantation Proceedings*, 40, 2501-2502.

Schneiderman, J., Sawdey, M., Craig, H., Thinnes, T., Bordin, G., and Loskutoff, D. J. (1993). "Type 1 plasminogen activator inhibitor gene expression following partial hepatectomy." *American Journal of Pathology*, 143(3), 753-762.

Schroeder, J. C., DiNatale, B. C., Murray, I. A., Flaveny, C. A., Liu, Q., Laurenzana, E. M., Lin, J. M., Strom, S. C., Omiecinski, C. J., Amin, S., and Perdew, G. H. (2010). "The uremic toxin 3-indoxyl sulfate is a potent endogenous agonist for the human aryl hydrocarbon receptor." *Biochemistry*, 49(2), 393-400.

Serrano, M., Hannon, G. J., and Beach, D. (1993). "A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4." *Nature*, 366, 704-707.

Sewall, C. H., Lucier, G. W., Tritscher, A. M., and Clark, G. C. (1993). "TCDD-mediated changes in hepatic epidermal growth factor receptor may be a critical event in the hepatocarcinogenic action of TCDD." *Carcinogenesis*, 14(9), 1885-1893.

Sherr, C. J. (1993). "Mammalian G1 cyclins." *Cell*, 73, 1059-1065.

Sherr, C. J. (1996). "Cancer cell cycles." *Science*, 274(5293), 1672-1677.

Sherr, C. J., and Roberts, J. M. (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." *Genes & Development*, 13, 1501-1512.

Shimizu, M., Hara, A., Okuno, M., Matsuno, H., Okada, K., Ueshima, S., Matsuo, O., Niwa, M., Akita, K., Yamada, Y., Yoshimi, N., Uematsu, T., Kojima, S., Friedman, S. L., Moriwaki, H., and Mori, H. (2001a). "Mechanism of retarded liver regeneration in plasminogen activator-deficient mice: impaired activation of

hepatocyte growth factor after Fas-mediated massive hepatic apoptosis." *Hepatology*, 33(3), 569-76.

Shimizu, M., Hara, A., Okuno, M., Matsuno, H., Okada, K., Ueshima, S., Matsuo, O., Niwa, M., Akita, K., Yamada, Y., Yoshimi, N., Uematsu, T., Kojima, S., Friedman, S. L., Moriwaki, H., and Mori, H. (2001b). "Mechanism of retarded liver regeneration in plasminogen activator-deficient mice: impaired activation of hepatocyte growth factor after Fas-mediated massive hepatic apoptosis." *Hepatology*, 33(3), 569-576.

Silkworth, J. B., and Brown, J. F. (1996). "Evaluating the impact of exposure to environmental contaminants on human health." *Clinical Chemistry*, 42(8), 1345-1349.

Sogawa, K., Numayama-Tsuruta, K., Takahashi, T., Matsushita, N., Miura, C., Nikawa, J., Gotoh, O., Kikuchi, Y., and Fujii-Kuriyama, Y. (2004). "A novel induction mechanism of the rat CYP1A2 gene mediated by Ah receptor-Arnt heterodimer." *Biochemical and Biophysical Research Communications*, 318(3), 746-755.

Son, D.-S., and Rozman, K. K. (2002). "2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces plasminogen activator inhibitor-1 through an aryl hydrocarbon receptor-mediated pathway in mouse hepatoma cell lines." *Archives of Toxicology*, 76(7), 404-413.

Sorg, O., Zennegg, M., Schmid, P., Fedosyuk, R., Valikhnovskyi, R., Gaide, O., Kniazevych, V., and Saurat, J.-H. (2009). "2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) poisoning in Victor Yushchenko: identification and measurement of TCDD metobolites." *Lancet*, 374, 1179-1185.

Stolz, D. B., Mars, W. M., Petersen, B. E., Kim, T.-H., and Michalopoulos, G. K. (1999). "Growth factor signal transduction immediately after two-thirds partial hepatectomy in the rat." *Cancer Research*, 59, 3954-3950.

Suzuki, T., Yamamoto, T., Kurabayashi, M., Nagai, R., Yazaki, Y., and Horikoshi, M. (1998). "Isolation and initial characterization of GBF, a novel DNA-binding zinc finger protein that binds to the GC-rich binding sites of the HIV-1 promoter1." *Journal of Biochemistry*, 124, 389-395.

Thornton, A. J., Bruzdzinski, C. J., Raper, S. E., and Gelehrter, T. D. (1994). "Plasminogen activator inhibitor-1 is an immediate early response gene in regenerating rat liver." *Cancer Res*, 54(5), 1337-43.

Tian, Y., Ke, S., Denison, M. S., Rabson, A. B., and Gallo, M. A. (1999). "Ah receptor and NF-kB interactions, a potential mechanism for dioxin toxicity." *The Journal of Biological Chemistry*, 274(1), 510-515.

Toyoshima, H., and Hunter, T. (1994). "p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21." *Cell*, 78, 67-74.

U.S. Environmental Protection Agency, U. (1997). "Health Effects Assessment Summary Tables. FY1997 Update", O. o. H. a. E. A. Environmental Criteria and Assessment Office, Office of Research and Development, Cincinnati, OH.

Viluksela, M., Bager, Y., Tuomisto, J. T., Scheu, G., Unkila, M., Pohjanvirta, R., Flodstrom, S., Kosma, V.-M., Maki-Paakkanen, J., Vartiainen, T., Klimm, C., Schramm, K.-W., Warngard, L., and Tuomisto, J. (2000). "Liver tumor-promoting activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in TCDD-sensitive and TCDD-resistant rat strains." *Cancer Research*, 60, 6911-6920.

Vogel, C. F. A., Sciullo, E., Li, W., Wong, P., Lazennec, G., and Matsumura, F. (2007). "RelB, a new partner of aryl hydrocarbon receptor-mediated transcription." *Molecular Endocrinology*, 21(12), 2941-2955.

Vos, J. G., Moore, J. A., and Zinkl, J. G. (1974). "Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in C57B1/6 mice." *Toxicology and Applied Pharmacology*, 29, 229-241.

Walisser, J. A., Glover, E., Pande, K., LIss, A. L., and Bradfield, C. A. (2005). "Aryl hydrocarbon receptor-dependent liver development and hepatotoxicity are mediated by different cell types." *Proceedings of the National Academies of Science, USA*, 102(49), 17858-17863.

Wang, F., Wang, W., and Safe, S. (1999). "Regulation of constitutive gene expression through interactions of Sp1 protein with the nuclear aryl hydrocarbon receptor complex." *Biochemistry*, 38, 11490-11500.

Warner, M., Eskenazi, B., Mocarelli, P., Gerthoux, P. M., Samuels, S., Needham, L., Patterson, D., and Brambilla, P. (2002). "Serum dioxin concentrations and breast cancer risk in the Seveso women's health study." *Environmental Health Perspectives*, 110(7), 625-628.

Weinberg, R. A. (1995). "The retinoblastoma protein and cell cycle control." *Cell*, 81, 323-330.

Weiss, C., Kolluri, S. K., Kiefer, F., and Gottlicher, M. (1996). "Complementation of Ah receptor deficiency in hepatoma cells: negative feedback regulation and cell cycle control by the Ah receptor." *Experimental Cell Research*, 226(1), 154-163.

Wu, H., Wade, M., Krall, L., Grisham, J., Xiong, Y., and Dyke, T. V. (1996). "Targeted in vivo expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development and regeneration." *Genes & Development*, 10, 245-260.

Wu, L., and Whitlock, J. P. (1993). "Mechanisms of dioxin action: receptor-enhancer interactions in intact cells." *Nucleic Acids Research*, 21(1), 119-125.

Yamada, Y., Kirillova, I., Peschon, J., and Fausto, N. (1997). "Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptro." *Proceedings of the National Academies of Science USA*, 94, 1441-1446.

Zarnegar, R., DeFrances, M. C., Kost, D. P., Lindroos, P., and Michalopoulos, G. K. (1991). "Expression of hepatocyte growth factor mRNA in regenerating rat liver after partial hepatectomy." *Biochemical and Biophysical Research Communications*, 177(1), 559-565.

Zhang, W., Shields, J. M., Sogawa, K., Fujii-Kuriyama, Y., and Yang, V. W. (1998). "The gut-enriched kruppel-like factor suppresses the activity of the CYP1A1 promoter in an Sp1-dependent fashion." *The Journal of Biological Chemistry*, 273(28), 17917-17925.

Zheng, M.-H., Bao, Z.-C., Zhang, B., and Xu, X.-B. (2001). "Polychlorinated dibenzo-p-dioxins and dibenzofurans in paper making from a pulp mill in China." *Chemosphere*, 44(6), 1335-1337.

Zheng, Q., Tang, Z.-Y., Xue, Q., Shi, D.-R., Song, H.-Y., and Tang, H.-B. (2000). "Invasion and metastasis of hepatocellular carcinoma in relation to urokinase-type plasminogen activator, its receptor, and inhibitor." *Journal of Cancer Research and Clinical Oncology*, 126, 641-646.

Vita

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