Daniel W. Nebert

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Gene-Environment Interactions: My Unique Journey

Daniel W. Nebert[1,2]

[1]Department of Environmental and Public Health Sciences and Center for Environmental Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

[2]Department of Pediatrics and Molecular Developmental Biology, Division of Human Genetics, Cincinnati Children's Hospital, Cincinnati, Ohio, USA; email: nebertdw@ucmail.uc.edu

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AHR-CYP1A1 axis, AHR transcription factor, aryl hydrocarbon hydroxylase, aryl hydrocarbon receptor, genetic differences in drug (or other environmental toxicant) response, pharmacogenetics, *Slc39a8* gene encoding ZIP8 metal cation influx transporter, standardized gene nomenclature, autobiography

Abstract

I am deeply honored to be invited to write this scientific autobiography. As a physician-scientist, pediatrician, molecular biologist, and geneticist, I have authored/coauthored more than 600 publications in the fields of clinical medicine, biochemistry, biophysics, pharmacology, drug metabolism, toxicology, molecular biology, cancer, standardized gene nomenclature, developmental toxicology and teratogenesis, mouse genetics, human genetics, and evolutionary genomics. Looking back, I think my career can be divided into four distinct research areas, which I summarize mostly chronologically in this article: (*a*) discovery and characterization of the AHR/CYP1 axis, (*b*) pharmacogenomics and genetic prediction of response to drugs and other environmental toxicants, (*c*) standardized drug-metabolizing gene nomenclature based on evolutionary divergence, and (*d*) discovery and characterization of the *SLC39A8* gene encoding the ZIP8 metal cation influx transporter. Collectively, all four topics embrace gene-environment interactions, hence the title of my autobiography.

INTRODUCTION

Family Origins

I was raised in modest surroundings: a 1.5-acre farm with dogs, cats, chickens, a cow, and a goat on a gravel road in rural Garden Home—a crossroads farming community southwest of Portland, Oregon. My father was an electrician foreman, my mother a homemaker. All four grandparents were German descendants from western Austria and Bohemia. I had a sister, eight years older than I, and two brothers, three and six years younger than I. My sister taught me to read before I entered first grade.

My earliest childhood memories include enjoying the moon, stars, constellations, and the Milky Way on warm summer evenings. I loved to read, especially science fiction. Because I had very white-blond hair and blue-gray eyes, and everyone else in the household had dark brown hair and brown eyes (okay, so my dad did have blue eyes), I was convinced that this was not my family but rather I had arrived from outer space. Early on, I wanted to be an astronaut.

At graduation from grade school, I was valedictorian of my eighth-grade class of 32 students. Among my class of 260 students at Beaverton High School, my grade point average was within the top ten. I never had any substantial homework; consequently, I was always busy doing my own projects and reading books. I could not sit still and was constantly asking questions. My dad often quipped, "Why d'you want to know, you writing a book?"

During the late winter of my third year of high school (1955), I was fascinated by a *Scientific American* article, which, in retrospect, became the fundamental theme of my entire career. The authors in a laboratory had grown bacteria in tryptophan-free medium; tryptophan was then added to the medium. The enzyme tryptophan pyrrolase was found to increase 80,000-fold—in a matter of minutes! To me, this observation was fascinating. How did the bacteria sense the sudden presence of this environmental signal? What was the mechanism by which the response to this signal resulted in huge increases in the enzyme(s) necessary to use tryptophan as an energy source? [Recall that the DNA double helix had just been resolved in 1953 and that protein synthesis (translation of messenger RNA into the protein gene product) was not understood until about a decade later.]

Higher Education and Beyond

With enthusiastic help from my high school American history teacher, Mr. Ted Van Buren, I was awarded a General Motors full scholarship to attend Wesleyan University (Middletown, CT). After three years of college and five years at University of Oregon Medical School (Portland), I had completed BA, MS, and MD degrees (1956–1964). My MS degree mentor was Howard S. Mason (1), codiscoverer with Osamu Hayaishi (2) in Japan of mixed-function oxidases or oxygenases, most of which were later determined to be cytochrome P450 monooxygenases.

After pediatric internship and residency at the University of California, Los Angeles Health Sciences Center—instead of being drafted as a physician into the war in Southeast Asia—I was fortunate to land a two-year fellowship in the National Cancer Institute (NCI) at the National Institutes of Health (NIH) in Bethesda, Maryland (partly because I already had seven publications prior to becoming a postdoc); in 1966, only one of every 1,500 applicants was selected! In 1969, I set up and began to direct basic and clinical research in the National Institute of Child Health and Human Development (NICHD), where I worked for the next two decades.

In December 1989, I became Professor in the Department of Environmental Health (DEH) at the University of Cincinnati College of Medicine and Adjunct Professor in the Human Genetics Division, Department of Pediatrics and Molecular Developmental Biology, at Cincinnati Children's Hospital Medical Center.

I chose to expand DEH research from twentieth-century classical toxicology to twenty-first-century environmental genetics, molecular biology, and genomics, thereby integrating DEH research with that of other departments in the College of Medicine. In 1992, I became Principal Investigator of a novel five-year Center of Excellence grant from the National Institute of Environmental Health Sciences (NIEHS). My Center for Environmental Genetics (CEG) was the first of its kind; by 2000, virtually every one of the approximately 25 NIEHS-funded Centers of Excellence and Marine Biology Centers included a gene-environment component. Today, the University of Cincinnati's CEG continues—more than three decades after its inception.

In July 2008, I was awarded a five-year NIEHS-funded Gene-Environment Interactions Training Program for teaching graduate students and postdoctoral fellows in this relatively new research field. Along with the in-house training program, I began an educational newsletter (or email blog), which expanded to several hundred recipients worldwide; amazingly, these Internet sharing-of-information and interactive discussions continue today, more than 15 years later. In June 2013, I became Professor Emeritus, although, to this day, I have not stopped writing or even slowed down. I am continuously driven—it is in my DNA.

I have published more than 600 papers in the numerous scientific fields listed above. In 2023, my Google Scholar h-index was 136, with more than 78,000 citations by my peers. Below I have divided my projects into four distinct areas and introduce them chronologically, roughly in the order in which they were developed.

AHR/CYP1 axis: ligand-bound activated AHR upregulates three CYP1 enzymes; CYP1A1 in particular provides negative feedback by degrading AHR's physiological ligands

Polycyclic aromatic hydrocarbons (PAHs): a class of environmental CH-containing chemicals that contain between two and seven aromatic rings; PAHs are uncharged, hydrophobic, and planar

MY SCIENTIFIC CAREER

Discovery and Characterization of the AHR/CYP1 Axis

Starting as an NCI postdoctoral fellow with Harry Gelboin (1966–1968), I decided to design a simple enzyme assay for measuring aryl hydrocarbon hydroxylase (AHH, also called benzpyrene hydroxylase) activity. In cell culture (3, 4), and then in monkeys, hamsters, rats, and mice as well as transplacentally in rodents (5), I showed that numerous planar polycyclic aromatic hydrocarbons (PAHs) (many of which are carcinogens) are inducers of AHH activity (**Figure 1**) and that the induced enzyme, in turn, metabolizes the incoming PAH inducer.

With Joseph Winker, an obstetrics-gynecology resident at the National Naval Medical Center, I collected placentas from 51 nonsmokers and 46 women who smoked cigarettes during pregnancy; AHH was undetectable in placentas of all nonsmokers, but a wide range of induced AHH activity was measurable in those of every smoker (6). An earlier study found similar results (7). These data indicate that the AHH induction observed in cell culture and intact mammals behaves similarly in humans.

Using inducible AHH activity in hamster fetal cell cultures, I showed that AHH activity likely represents a cytochrome P-450 monooxygenase and named it P_1-450 (now CYP1A1) (8). The mammalian CYP1 family was later found to include three PAH-inducible genes: *CYP1A1*, *CYP1A2*, and *CYP1B1* (9). My car license plate is "CYP1A1"; my wife's license plate is "CYP1A2"—because *CYP1A2* is evolutionarily the younger gene.

The beauty of this striking genetic difference between C57BL/6J (B6) and DBA/2J (D2) mice is that when one crosses these two inbred strains, AHH inducibility is found to be predominantly an autosomal dominant monogenic (Mendelian) trait, meaning that *Ahr*-nonresponsive D2 mice carry two autosomal recessive alleles (10). This is as simple as the human brown eyes (dominant) versus blue eyes (recessive) trait.

In 1974, I saw a publication by Alan Poland & Edward Glover (11) stating that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin; **Figure 1**) was about 30,000 times more potent than 3-methylcholanthrene at inducing chick embryo AHH activity. I telephoned Dr. Poland and

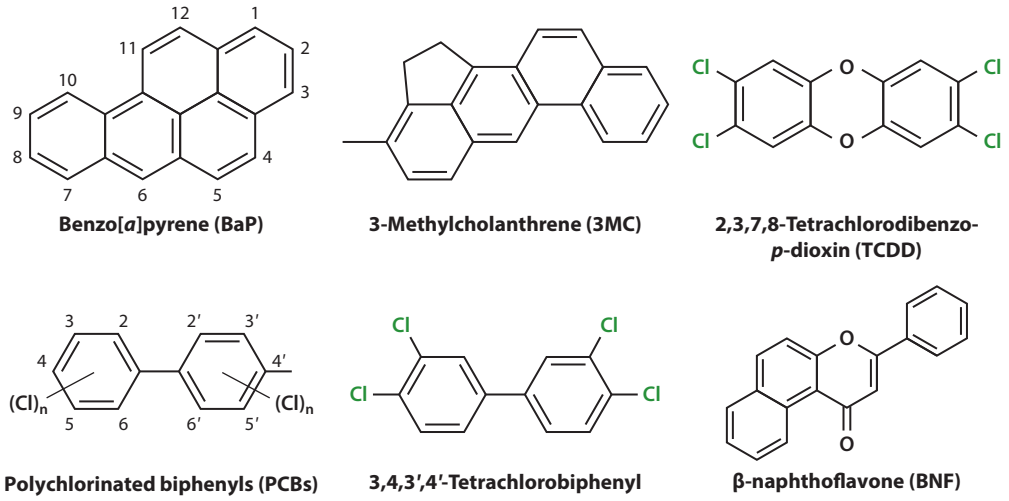


Figure 1

Illustration of the chemical structures most frequently discussed throughout this autobiography. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, often simply called dioxin) is planar in structure and is among the most potent ligands for aryl hydrocarbon receptor (AHR). Benzo[*a*]pyrene (BaP) is a prototypical polycyclic aromatic hydrocarbon (PAH) ubiquitously found in cigarette and cigar smoke, roofing and highway tar, and wood-burning or charcoal-cooking processes. BaP also has a planar structure, binds to AHR, and is commonly used as substrate in the aryl hydrocarbon hydroxylase (AHH) enzyme assay. 3-Methylcholanthrene (3MC) is not found in the environment but is commonly used as a prototypical planar PAH that is highly carcinogenic and is an excellent inducer of AHH activity in laboratory animals and cells in culture. Polychlorinated biphenyls (PCBs) are common environmental contaminants, and the two benzyl rings can contain anywhere from one to ten chloride atoms. PCBs often exist as coplanar structures, unless any three of the four 2,6,2′,6′ carbon atoms have bound chloride atoms, in which case the large halogen atoms distort the coplanarity; thus, the noncoplanar structure renders those PCBs unable to bind to AHR and induce AHH activity. 3,4,3′,4′-Tetrachlorobiphenyl is an example of a PCB that is a potent AHR ligand and inducer of AHH because it is coplanar. β-Naphthoflavone (BNF) is a noncarcinogenic prototypical AHR ligand and an effective planar inducer of AHH activity.

suggested that TCDD might be sufficiently potent to induce AHH activity in *Ahr*-nonresponsive D2 mice. I invited Alan to visit my lab, where we discovered a dose-response curve (**Figure 2**), consistent with receptor regulation of the P_1-450 structural gene (12, 13); we chose to name this putative regulatory gene aryl hydrocarbon receptor (*AHR*). Regarding PAH and TCDD binding, we proposed that genetically sensitive inbred mice such as B6 carry a high-affinity Ahr^b allele, whereas genetically resistant D2 mice have a poor-affinity Ahr^d allele (12).

Heterodimeric cotranscriptional partner: transcription factor that binds with a different one; the heterodimer then binds to a DNA response element and activates transcription (in contrast, homodimer denotes two identical transcription factors binding to a DNA response element)

In the Poland lab, following treatment with radiolabeled TCDD, hepatic cytosol accumulation of radiolabel was greatest in B6 mice, intermediate in $B6D2F_1$ mice, and lowest in D2 mice (14). In 1982, with Howard Eisen, we compared Ahr^b/Ahr^b and Ahr^b/Ahr^d *Ahr*-responsive mice with Ahr^d/Ahr^d *Ahr*-nonresponsive mice treated with radiolabeled TCDD (15) and showed that the movement of radioactivity from hepatic cytosol to the nucleus of *Ahr*-responsive mice was highly correlated with P_1-450 messenger RNA (mRNA) induction kinetics. In contrast, radioactivity in the hepatic cytosol and nucleus of Ahr^d/Ahr^d *Ahr*-nonresponsive mice was four- to fivefold lower. These experiments (14, 15) provided proof that AHR is first bound to TCDD in the cytosol, and, following migration to the nucleus, the ligand-bound receptor signal then initiates the enzyme induction response. Ten years later, Chris Bradfield's laboratory cloned the elusive mouse *Ahr* gene (16) and human *AHR* gene (17) and suggested (correctly) that aryl hydrocarbon receptor nuclear translocator (ARNT) is a heterodimeric cotranscriptional partner with AHR during gene activation (16).

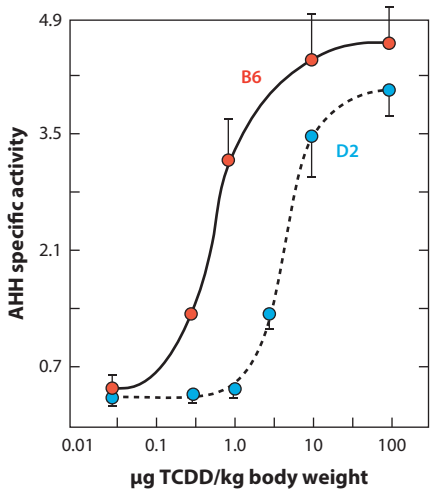


Figure 2

Mouse liver aryl hydrocarbon hydroxylase (AHH) activity as a function of an intraperitoneal dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 44 h. B6 represents sensitive or *Ahr*-responsive C57BL/6J mice, and D2 represents (relatively) resistant or *Ahr*-nonresponsive DBA/2J mice. The $B6D2F_1$ heterozygote is roughly as sensitive as the B6 inbred strain, indicating that the inducible AHH sensitivity in this genetic cross is largely an autosomal dominant trait, although modifier genes exist. Figure adapted from Reference 13, figure 2.

Twelve years later, it became apparent that AHR was the second-earliest-discovered member of the basic-helix-loop-helix/per-Arnt-sim (bHLH/PAS) gene subfamily (18–20); the BHLH gene superfamily encodes dozens of transcription regulators found throughout the Tree of Life. Human and mouse genomes both contain 109 (evolutionarily highly conserved) BHLH genes; the subfamily of 21 bHLH/PAS genes includes AHR and ARNT. The function of all BHLH genes is to sense innumerable extracellular and intracellular signals, including, for example, foreign chemicals, endogenous compounds, gas molecules, redox potential, oxidative stress, epigenetic stressors, photons (light), gravity, temperature, and osmotic pressure (J.N. Fisk, E.A. Bruford, V. Vasiliou, C.G. Bunick & D.W. Nebert, unpublished manuscript).

Considering evolution, I was certain that AHR had not evolved simply to bind PAHs and TCDD; that is, AHR must have arisen for one or more endogenous functions. In 1979, I discovered that the Ahr^b allele (*Ahr* responsiveness, high-affinity AHR) is associated with rapid resolution of ethanol-caused peritonitis, compared to slow resolution of peritonitis in *Ahr*-nonresponsive mice (20–23). We tried (and failed) repeatedly in 1982 to publish these data in the *Journal of Biological Chemistry*; the reviewers insisted that this finding was not possible! A 1983 report (24) then showed that in chick fetal liver, the nonsteroidal anti-inflammatory drug benoxaprofen decreased toxicity of planar 3,4,3′,4′-tetrachlorobiphenyl (a known AHR ligand; **Figure 1**). To me, this exciting finding, combined with the ethanol-caused peritonitis, indicated that AHR must participate in the arachidonic acid cascade involving inflammation.

I had proposed (20–23) that the AHR-mediated response to ethanol-induced inflammation was involved in lipid mediator (LM) second-messenger pathways (which now include prostaglandins, leukotrienes, eicosanoids, resolvins, neuroprotectins, and lipoxins). The strongest evidence to date in favor of my hypothesis was revealed when my lab collaborated with Charles N. Serhan in studies using multiple-reaction monitoring and liquid chromatography–ultraviolet coupled with tandem mass spectrometry–based LM metabololipidomics. We found (25) that statistically

Epigenetic: chromosomal effects (RNA interference, DNA methylation, histone modification, chromatin remodeling) that modify gene expression, independent of DNA sequence alterations

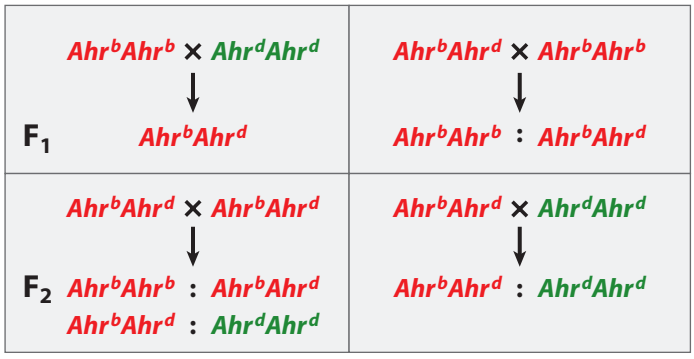


Figure 3

Exciting paradigm in which the $Ahr^{b/d}$ × $Ahr^{d/d}$ backcross (*lower right*) gives a 1:1 ratio of the two genotypes, and the $Ahr^{b/d}$ × $Ahr^{b/d}$ F_1 intercross (*lower left*) yields F_2 litters of the two genotypes in a 3:1 ratio. The test compound X is given to all mice in the same litter—in contrast to the usual typical experiment in which a test compound X is given to the experimental group, while controls receive vehicle only or no treatment at all. This autosomal-dominant versus autosomal-recessive trait is similar to brown eyes versus blue eyes in humans.

significant changes in levels of leukotriene B_4 and seven other LM metabolites are associated with AHR/CYP1-mediated proinflammatory and inflammation-resolution pathways during experimental zymosan-initiated peritonitis.

The importance of pharmacokinetics. PAHs are found in cigarette and cigar smoke, tar, and wood-burning or charcoal-cooking processes. Using mouse genetic differences in high- versus poor-affinity AHR (**Figure 3**), my lab and other labs—in dozens of studies between 1973 and 2000—showed differences in risk of PAH-, polychlorinated biphenyl (PCB)- (**Figure 1**), and TCDD-induced carcinogenesis (26, 27); bone marrow and immune toxicity (28, 29); teratogenesis (30); and mutagenesis (31; reviewed in 32, 33).[1]

Regarding teratogenesis (**Figure 4**), if the mother [bred to an *Ahr*-responsive (Ahr^{b}/Ahr^{d}) male] is *Ahr*-nonresponsive (Ahr^{d}/Ahr^{d}) and is given benzo[*a*]pyrene (BaP) intraperitoneally, the Ahr^{b}/Ahr^{d} genotype fetuses exhibit more stillborns and resorptions, decreased fetal weight, increased congenital anomalies, and enhanced P_1-450-mediated covalent binding of BaP metabolites to fetal protein and DNA when compared to the Ahr^{d}/Ahr^{d} genotype of fetuses from the same uterus (34). If the mother is *Ahr*-responsive (Ahr^{b}/Ahr^{d}), however, none of these parameters can be distinguished between Ahr^{b}/Ahr^{d} and Ahr^{d}/Ahr^{d} pups in the same uterus, presumably because enhanced BaP metabolism in maternal tissues and placenta overwhelms those differences between individual fetuses. Of particular interest in this study is the fact that both the mother and the father must be of a particular genotype before differences in teratogenesis among fetuses (due to their genotype) will be expressed. These data might help explain clinically why only one child is affected with an apparent drug-induced syndrome although the mother has taken the same dose of a particular drug (or was exposed to some environmental toxicant) during each of several pregnancies.

Zymosan: naturally occurring insoluble macromolecule from the cell wall of yeast (*Saccharomyces cerevisiae*); used as food, phagocytic stimulus, and immune potentiator

[1] Historically, *Ah* (for responding to aromatic hydrocarbons) was named as the mouse genetic locus in 1974; the "[*Ah*] gene battery" denoted a growing number of genes upregulated by the putative receptor between the mid-1970s and early 1990s. When that locus was unequivocally proven to encode a receptor in 1992, the official mouse gene name became *Ahr*. To minimize confusion to the reader, in **Figures 3–5** we have changed *Ah* to *Ahr* although the original published figures used the *Ah* nomenclature.

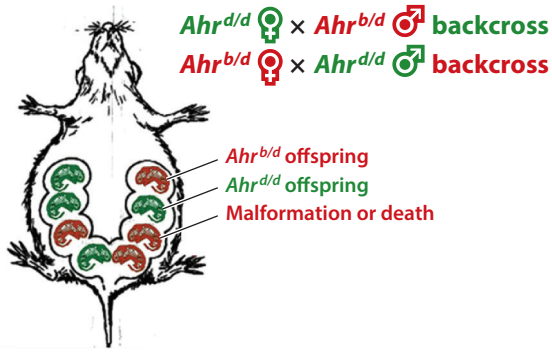


Figure 4

Genetic differences between pups in utero after the (relatively) *Ahr*-nonresponsive ($Ahr^{d/d}$) mother receives an intraperitoneal dose of the test compound benzo[*a*]pyrene (BaP). The backcross gives equal numbers of the two genotypes, whereas the intercross (not shown) would give a 3:1 ratio of responsive to nonresponsive, respectively. (In genetic crosses, female is always written first, male always second.) Treatment of the mother results in the same dose of test compound X reaching both genotypes equally; this protocol for teratogenesis studies is superior to comparing treated with vehicle-treated control or untreated control pregnant mothers. If the mother is *Ahr*-responsive ($Ahr^{b/d}$), however, none of these parameters can be distinguished between $Ahr^{b/d}$ and $Ahr^{d/d}$ pups in the same uterus (reviewed in 34, 35) because enhanced BaP metabolism in maternal tissues and placenta overwhelms genetic differences between individual fetuses. Figure adapted from Reference 35, figure 8, with permission from the Royal Society of Chemistry.

Our laboratory also realized the critical importance of the route of administration (**Figure 5**). The *Ahr*-responsive mouse—which is at increased risk for cancer caused by subcutaneous, topical, or intratracheal PAHs—is at decreased risk for bone marrow toxicity and leukemia caused by oral PAHs when compared with (relatively) *Ahr*-nonresponsive mice receiving the same dose of the same PAH (reviewed in 36). In other words, tissue sites in direct contact with the carcinogen [e.g., skin, subcutaneous tissue, lung, gastrointestinal (GI) tract] develop cancer in *Ahr*-responsive mice because of robust local metabolism of BaP by the induced P_1-450. Tissues in distal sites of the body may develop malignancy in *Ahr*-nonresponsive mice because more carcinogen reaches that tissue due to negligible P_1-450 induction at the site of administration and therefore there is decreased local BaP detoxication, but increased BaP metabolic activation at the distal sites. This experimental model system offers the hematologist and clinical toxicologist a means to study genetic differences in toxic chemical depression of the bone marrow as well as a potential model to study aplastic anemia and leukemia explainable on a single-gene basis. Numerous cancer and toxicity experiments confirmed the same paradigm (37, 38); if the PAH is given orally or topically, instead of intraperitoneally, association of the *Ahr* alleles (**Figure 5**) with cancer and toxicity is reversed.

These observations became further clarified in 2010. With Jake Shi and Nadine Dragin, we compared hepatocyte-specific CYP1A1 with GI epithelial–specific CYP1A1 conditional knockout mice (37). Once wild-type mice begin to ingest BaP (**Figure 6**), CYP1A1 is quickly induced in the proximal small intestine (PSI), resulting in BaP being metabolized and detoxified. Although liver CYP1A1 is induced by oral BaP for several hours early on, this induced level of hepatic CYP1A1 soon disappears because the inducer is cleared from the PSI before it is able to reach the liver. Because most laboratories use intraperitoneal PAH administration (as my lab also did, initially), the important effects of oral BaP detoxication are not seen. Oral BaP-induced CYP1A1 in the PSI also prevents CYP1B1-mediated BaP toxicity of immune tissues and cancer in distal tissues; on the other hand, when intraperitoneal BaP is administered to wild-type mice (**Figure 6**), it is

Conditional knockout mouse: animal having one or more genes that are able to be disrupted in a specific tissue or cell type within the body

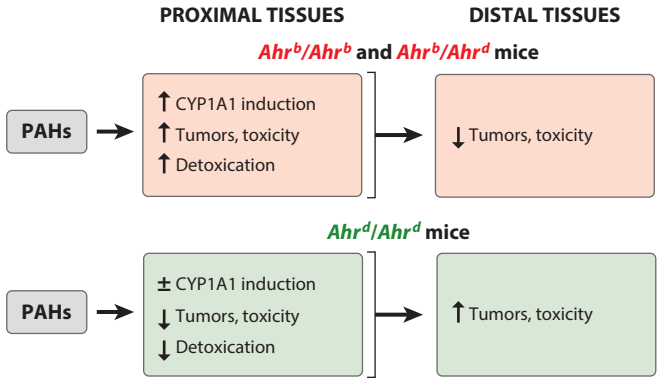


Figure 5

Summary of polycyclic aromatic hydrocarbon (PAH)-induced carcinogenesis and toxicity data that are associated with the *Ahr* genotype. The *Ahr*-responsive Ahr^b/Ahr^b wild type and Ahr^b/Ahr^d heterozygote (*upper row*) are at increased risk for tumors and toxicity in proximal tissues directly in contact with the administered PAH (e.g., skin, subcutaneous soft tissue, lung, proximal gastrointestinal tract). Increased PAH metabolism thus results in less PAH reaching distal tissues and therefore fewer tumors and less toxicity. In contrast, the (relatively) *Ahr*-nonresponsive Ahr^d/Ahr^d homozygote (*lower row*) is at decreased risk for tumors and toxicity in tissues directly in contact with the administered PAH (because of less CYP1A1 metabolism); this leads to more PAH reaching distal tissues (e.g., bone marrow, other immune tissues, placenta and in utero embryos, and preputial gland duct) and therefore more tumors and more toxicity (36, 37; reviewed in 36). Later studies with knockout mouse lines confirmed that it is induced CYP1B1-mediated metabolism that is responsible for the neoplasias and toxicity in distal tissues (bone marrow and other immune tissues); it was suggested (reviewed in 38) that induced CYP3A59 metabolism was most likely responsible for PAH-induced tumors of the preputial gland duct. Figure adapted from Reference 32, figure 20, with permission from Taylor & Francis.

the induced CYP1B1 metabolism in marrow and immune tissues that causes cancer and toxicity (reviewed in 38).

An intriguing project in my lab that linked TCDD to AHR/CYP1-mediated teratogenesis is worthy of mention. Exposure to Agent Orange, a defoliant biowarfare mixture, is associated clinically with phocomelia, cleft palate, hydronephrosis, and other birth defects (39); the active teratogenic contaminating chemical in Agent Orange is believed to be TCDD.

Phocomelia: congenital defect characterized by severe malformations of extremities; can be caused by TCDD or thalidomide exposure during pregnancy

Defoliant: any herbicide [e.g., tribufos, dimethipin, thidiazuron, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)] that causes the leaves of plants to fall off

Previous reports had indicated that human liver CYP1A2 [whose levels can range 50-fold among individuals (40)] is able to sequester TCDD (e.g., 41). We wondered whether TCDD sequestration by maternal CYP1A2 might protect embryos in utero. With Nadine Dragin and Tim Dalton, we compared $Cyp1a1^{(-/-)}$, $Cyp1a2^{(-/-)}$, and $Cyp1b1^{(-/-)}$ knockout mice with $Cyp1^{(+/+)}$ wild-type mice (42). TCDD was given by gavage on gestational day 10 (GD10); embryos were examined on GD18. Although the incidence of cleft palate and hydronephrosis was not significantly different in fetuses from $Cyp1^{(+/+)}$ wild-type mice or $Cyp1a1^{(-/-)}$ and $Cyp1b1^{(-/-)}$ knockout mice, fetuses carried by $Cyp1a2^{(-/-)}$ knockout mothers all died. This lethal dose of TCDD was found to be absolutely dependent on the maternal *Cyp1a2* genotype, and this effect was independent of the embryonic *Cyp1a2* genotype (42).

TCDD levels were more than sixfold higher in placenta and embryos from $Cyp1a2^{(-/-)}$ mothers compared to those from $Cyp1^{(+/+)}$ wild-type mothers. Fetuses from $Cyp1a2^{(-/-)}$ mothers exhibited approximately sixfold increased sensitivity to cleft palate, hydronephrosis, and lethality (42). Using our transgenic mouse that had mouse *Cyp1a2* replaced with the human *CYP1A2* gene, we observed no teratogenic effects of TCDD. In other words, maternal mouse CYP1A2, as

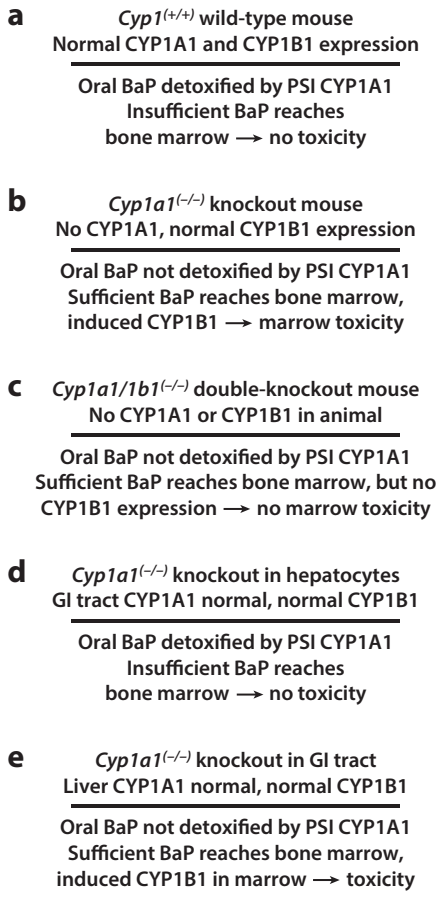


Figure 6

Diagram showing the importance of oral benzo[*a*]pyrene (BaP) pharmacokinetics. (*a*) *Cyp1*$^{(+/+)}$ wild-type mice exhibit massive CYP1A1 induction by oral BaP in the proximal small intestine (PSI), resulting in BaP detoxication and excretion; thus, negligible amounts of BaP reach the bone marrow, resulting in no marrow toxicity. [In the first several hours after oral BaP exposure, CYP1A1 in liver is induced; however, this quickly dissipates once induced levels of CYP1A1 are reached in the PSI, and BaP detoxication in the gastrointestinal (GI) tract becomes prominent.] (*b*) Loss of CYP1A1 in *Cyp1a1*$^{(-/-)}$ knockout mice leads to negligible BaP metabolism in the GI tract, resulting in large amounts of BaP reaching the marrow and inducing CYP1B1; BaP-induced CYP1B1-mediated metabolism causes marrow toxicity. (*c*) Absence of both CYP1A1 and CYP1B1 in *Cyp1a1/1b1*$^{(-/-)}$ double-knockout mice leads to no marrow toxicity because induced CYP1B1-mediated toxic metabolites are needed for marrow toxicity. However, because of the absence of CYP1A1, BaP is not detoxified; therefore, the mouse carries a large BaP burden throughout its body. (*d*) Removal of CYP1A1 only from liver hepatocytes (*Alb>Cre>1a1* mice) results in a phenotype similar to *Cyp1*$^{(+/+)}$ wild-type mice (i.e., negligible amounts of BaP reach the bone marrow, resulting in no marrow toxicity) because oral BaP detoxication in the PSI is what prevents sufficient amounts of BaP from reaching the marrow to cause CYP1B1-mediated toxicity. (*e*) Removal of CYP1A1 only from the GI tract enterocytes (*Vil>Cre>1a1* mice) leads to no substantial oral BaP detoxication by the PSI; therefore, large amounts of BaP reach the marrow, where CYP1B1-mediated marrow toxicity by BaP then occurs (37). These experiments demonstrate unequivocally that it is the CYP1A1 in the GI tract, and not the liver, that is most important in oral BaP detoxication and hence protection from BaP-caused immunosuppression, immunotoxicity, and early death. Figure adapted from Reference 38 with permission from ASPET Press.

well as human CYP1A2—by sequestering TCDD in liver and placenta and thus altering the pharmacokinetics—protects embryos from toxicity and birth defects (42). Clearly, this observation has clinical toxicological implications.

Global knockout mouse: animal having one or more exons of the target gene deleted in all cell types throughout the body, rendering the gene nonfunctional

Mutagenesis and risk assessment. In the early and mid-1970s, the Ames bacterial mutagenesis assay became very popular, and it remains so today; liver supernatant (S-9) fractions from 3-methylcholanthrene (3MC)- or β-naphthoflavone (BNF)-treated rats (**Figure 1**) are typically used (43). My lab used S-9 fractions from 3MC- and BNF-treated B6 versus D2 mice, plus the *Ahr*-responsive versus (relatively) *Ahr*-nonresponsive littermates from the B6 × D2 backcross (**Figure 3**), to demonstrate that AHR-mediated, P_1-450-generated metabolite formation is associated with in vitro mutagenicity of several PAH carcinogens (31).

The World Health Organization (WHO), as well as almost every other regulatory agency in the world, has expanded greatly upon our pioneering work on the AHR/CYP1 axis and AHR ligands, using toxic equivalency factors (TEFs) (44) to estimate the relative contribution of multiple dioxins, dibenzofurans, and PCBs to the risk of toxicity and cancer; this, in turn, generates toxic equivalency quotients (TEQs), which facilitate risk assessment and government regulations on air, water, and ground pollution; factory emissions; and clean-up levels (45).

However, this field is extremely complicated due to dramatic interspecies and intraspecies TEF and TEQ differences in AHR-binding affinities [e.g., AHR affinity in rats is 10,000-fold greater than that of guinea pig; AHR affinity varies more than thirtyfold among mouse strains; AHR affinity in mouse versus rat can be 100 times greater than that in human; and for certain dioxins and dibenzofurans, AHR affinity is much greater in rat than in human (46, 47)]. In fact, for particular dioxin-like planar PCB congeners, rat consensus TEF values have been shown to be more than 1,000-fold more potent than human consensus TEF values (48, 49), instead of using the WHO-based 2005 TCDD-treated rat TEF values (44). Amazingly, of the 12 dioxin-like PCB congeners studied in rats, only two (PCB81 and PCB126) have detectable binding activity toward the human AHR (49). Sadly, billions of dollars are spent each year in cleaning up PAH-contaminated sites, usually based on risk assessments using rat TEF values! Thus, the problem of species differences in AHR affinity and other environmental response genes is extremely important in the fields of risk-assessment policy and gene-environment interactions, but, in my opinion, it does not get nearly the consideration that it deserves.

Generation of knockout and knock-in mouse lines. My lab was the first to clone and sequence the mouse *Cyp1a1* and *Cyp1a2* copy DNAs (cDNAs) and genes (50) and the human *CYP1A1* (51) and *CYP1A2* (52) genes. With Frank J. Gonzalez, I helped create the first $Ahr^{(-/-)}$ global knockout mouse (53). With the help of Steve Potter, we produced the $Cyp1a2^{(-/-)}$ global knockout (54). Then, with Shige Uno, we made the $Cyp1a1^{(-/-)}$ global (55) and $Cyp1^{(-/-)}$ conditional (37, 56) knockouts, all three possible $Cyp1^{(-/-)}$ double knockouts (57, 58) and a viable but abnormal $Cyp1^{(-/-)}$ triple knockout (59).

Shared worldwide, these various global and tissue-specific knockout mouse lines continue to help many laboratories understand AHR/CYP1-mediated inflammatory pathways (20, 60–63) and realize that oral PAH-caused immunotoxicity—as well as the type and location of cancer risk—is highly dependent on the PAH's route of administration, dose, time, and target organ and the *Cyp1* genotype (38).

Embryonic cell CYP1A1 expression. The first hint of AHR/CYP1 signaling in embryos came from sister-chromatid exchange (SCE) studies. With Roger Pedersen, we showed that GD7.5 mouse embryo explant cultures in medium containing BaP and 5-bromodeoxyuridine caused SCEs that were associated more closely with the *Ahr*-responsive than with the (relatively)

Ahr-nonresponsive phenotype (64). Subsequently, with Anup Dey, we discovered in untreated pregnant mice that the zygote 12 hours after fertilization (but not the egg before) exhibited dramatically elevated levels of AHR-mediated *Cyp1a1* mRNA (65). This finding led to more than two decades of studies from many laboratories characterizing AHR/CYP1-mediated functions and signaling pathways in embryonic stem cell cultures and embryoid bodies (reviewed in 20). Today, AHR/CYP1 signaling pathways (**Table 1**) are known to be involved in an astonishing array of genetic networks and subcellular processes critical to life (reviewed in 20).

Today, we know that the AHR/CYP1 axis represents a ligand-bound activated AHR transcription factor that upregulates one or more CYP1 enzymes; mammalian CYP1A1 in particular provides negative feedback of itself by degrading AHR's physiological ligands (reviewed in 20, 33, 62, 63). The AHR/CYP1 axis might have developed this function evolutionarily as early as tunicates—such as sea squirts (*Ciona*), the very first chordate, which appeared on earth approximately 550 million years ago—because the tunicate genome carries both AHR and CYP1 genes.

Embryoid bodies: aggregates of pluripotent cells that are induced to differentiate by changing the culture medium and then grown in 3D structures

Genetic architecture: the underlying genetic basis of a phenotypic trait and its variational properties

Pharmacogenomics and Genetic Prediction of Response to Drugs and Other Environmental Toxicants

Following Arno Motulsky's (66) prediction in 1957, it quickly became appreciated by most scientists that each individual's response to any drug is largely dependent on that subject's genetic makeup (i.e., each person's genetic architecture). After the Human Genome Project was initiated in 1990, the term pharmacogenetics (gene-drug interactions) transitioned into pharmacogenomics (genome-drug interactions). Here, I use PGx to lump both together.

More than 30 years ago, I proposed that drug-metabolizing genes originated from animal-plant communications (67). Then—considering the length of evolutionary time for a new gene to appear, compared with the time since pharmaceuticals were first synthesized—it was reasonable to assume that every drug-metabolizing-enzyme gene originally arose to serve an endogenous function (68). In fact, if one considers how early in evolution metabolism genes must have appeared in animal genomes, the microbiome (20) would be among the most likely candidates. Before nematodes (which, during evolution, were likely the first animal to have a gut), the ambulacral groove of animals in the phylum Echinodermata or classes Asteroidea and Edrioasteroidea—and even the internal cavitations of animals in the phylum Cnidaria (e.g., sponge, jellyfish, sea anemones, and corals)—digested food; thus, even these organisms likely have a microbiome (69).

From the 1970s onward, I regarded many of the reviews by others on the topic of genetic contribution to drug response as too simplistic. Considering the holistic nature of each unique human being, an individual's response to virtually every drug would most likely be far more complex than being caused by just one or several single-nucleotide variants (SNVs) among a haploid genome of approximately three billion bases. From my earliest invited review on the topic (70) to some of my later reviews (71–73) and my latest journal review with Ge Zhang (74), our understanding of the complexity (that would be required to predict individual drug response) has evolved, along with our growing knowledge of the complexity of the human genome. Variability in interindividual drug response can be classified as (*a*) monogenic (Mendelian) traits, typically influenced by one or a few (typically) rare coding variants; (*b*) predominantly oligogenic traits that usually represent variability that is largely elicited by a small number of major pharmacogenes; and (*c*) complex PGx traits, which are produced mostly by innumerable small-effect variants (74).

As concluded in my recent book chapter with Ge Zhang (75), each patient's response to a drug (or environmental toxicant) is now considered to reflect the combination of (*a*) genetics, (*b*) epigenetic effects, (*c*) endogenous influences, (*d*) environmental exposures, and (*e*) each individual's microbiome. Except for genetics (i.e., each person's germline DNA sequence), the other four categories are not constant but rather continuously changing throughout one's lifetime.

Table 1 Participation of AHR/CYP1 signaling pathways and networks

Fundamental physiological processes	Phenotypes exhibited by AHR/CYP1 signaling pathways and networks
Metabolism, mutagenesis, and oxidative stress	Metabolic activation and detoxication of many foreign chemicals (recall that "studying AHH induction" was the origin of the AHR discovery) (3, 4, 20) DNA-adduct formation involving mutagenic or toxic metabolites, mutagenesis Mitochondrial ROS formation, as well as antioxidant protection against ROS formation and mitochondrial H_2O_2 production (33)
Fundamental early-embryogenesis processes	Cell division, adhesion, migration, cell-cycle regulation, germ-cell apoptosis, the MID1-PP2A-CDC25B-CDK1-signaling pathway that regulates mitosis, ectoderm-to-epithelium transition, transmesoderm-to-osteoblast transition, cavity formation during the morula-to-blastula transition, cardiomyocytogenesis, activator of Rho/Tac GTPases, WNT-signaling pathways, homeobox-signaling pathways, angiogenesis, and DNA synthesis and repair
Organogenesis	Formation of brain and central nervous system Formation of the gastrointestinal tract, pancreas, liver, heart, respiratory tract, and kidney Formation and development of immune system, male and female sex organs, cochlea of inner ear, and the eye's ciliary body
Development of blood cell–forming system	Hematopoiesis Activation, as well as suppression, of erythroid development
Hypoxia signaling	Crosstalk between hypoxia and HIF signaling pathways
Bone	Bone formation and osteoclastogenesis (to generate cells that break down bone)
Nervous system	Neurogenesis, creation of specific neuronal cell types, disruption of GABAergic transmission defects, and circadian rhythmicity
Immune system	Participation in the immune response, innate immunity, proinflammatory and postinflammation responses, and immunomodulatory effects
Brain-gut-microbiome	Participation in the brain-gut-microbiome network Barrier-protective roles of AHR in hematopoietic, as well as nonhematopoietic, cells within the intestinal microenvironment (62)
Cardiovascular and beneficial as well as unwanted metabolic effects	Cardiovascular physiology, atherogenesis (plaque formation), hypertension, pancreatic beta-cell regulation, glucose and lipid metabolism, hyperlipidemia, and hepatic steatosis
Sex organs	Serum testosterone levels, spermatogenesis, fertility and degenerative changes in the testis, mammary gland duct–cell epithelial hyperplasia, and endometriosis
Skin	Skin-barrier physiology, atopic dermatitis Combination of activated AHR and TLR genes leads to IL-22-induced production of antimicrobial molecules and defensins—thereby aiding in host defense and barrier function in gut, lung, and skin (63)
Growth factors	Transforming growth factor signaling pathways as well as growth suppression, tumor initiation, and tumor promotion
Epigenetics	Transgenerational inheritance Epigenetic effects (e.g., DNA methylation, RNA interference, histone modifications, and chromatin remodeling) Aging-related and degenerative diseases

For a detailed review of this information, the reader is referred to Reference 20. Abbreviations: AHR, aryl hydrocarbon receptor; CYP1, cytochrome P450 family 1 (but mostly CYP1A1); HIF, hypoxia-inducible factor; ROS, reactive oxygen species; TLR, Toll-like receptor.

Standardized Drug-Metabolizing Gene Nomenclature—Based on Evolutionary Divergence

Phytoalexins: low–molecular weight chemicals produced by plants in response to biological and physical stresses, which play an important role in the plant's defense system

During the 1970s, with the purifying of many P450 proteins and generating of polyclonal antibodies to these proteins, each P450 laboratory independently gave each P450 enzyme its own pet name. By the early 1980s, I realized that this haphazard approach would soon lead to chaos; it would be confusing not only to graduate students and postdoctoral fellows just entering the field but also to established investigators in other fields.

In the early to mid-1980s, clones of P450 cDNAs (formed by reverse transcription of mRNA isolated with polyclonal antibodies) began to be sequenced, and from these the amino acid sequences could be deduced. Perceptively, I noticed that if one aligned amino acid sequences of P450 proteins from *Pseudomonas*, yeast, and eight vertebrates, including humans, an approximately 10-amino-acid-long cysteinyl-containing peptide located in the heme-binding enzyme active site was highly conserved (76).

Given my fervent interest in evolution, I suggested that genes in families and subfamilies might be named using a nomenclature system based on evolutionary divergence. The root symbol for each gene in each superfamily should be identical to the original ancestral gene. The other challenge was to convince principal investigators of all major P450 labs to agree to common gene names, so I invited them to be coauthors on a paper proposing standardized gene nomenclature.

P450 was the first root chosen for the gene superfamily, as described in the first nomenclature paper with 13 coauthors (77). Subsequently, we decided the gene root name should be only letters; CYP was agreed upon in the second cytochrome P450 nomenclature update (78).

Gene families and subfamilies needed to be categorized within the superfamily. The original cutoff for P450 proteins within one family was more than 40% identity; P450 protein sequences having less than 40% similarity would represent *CYP* genes of different families. The original cutoff for members within one subfamily was more than 60% similarity (9, 77–80). Many complications and overlaps arose, and each new gene had to be manually curated before decisions could be made. The earliest method of visualizing DNA or protein sequence similarities/differences was the unweighted pair group method with arithmetic mean, a simple agglomerative (bottom-up) hierarchical clustering method (**Figure 7**). Today, many new algorithms are being developed in this rapidly expanding field of evolutionary divergence analysis (reviewed in 69).

Gene symbols should be all capital letters for human and most vertebrate genomes, that is, *CYP1A2*, *CYP1B1*, *CYP24A1*, *CYP51A1*, and so on. Mice deviate from this model due to historical contingency of an earlier nomenclature, capitalizing only the first letter; hence, the mouse orthologous genes are named *Cyp1a2*, *Cyp1b1*, *Cyp24a1*, *Cyp51a1*, and so on.

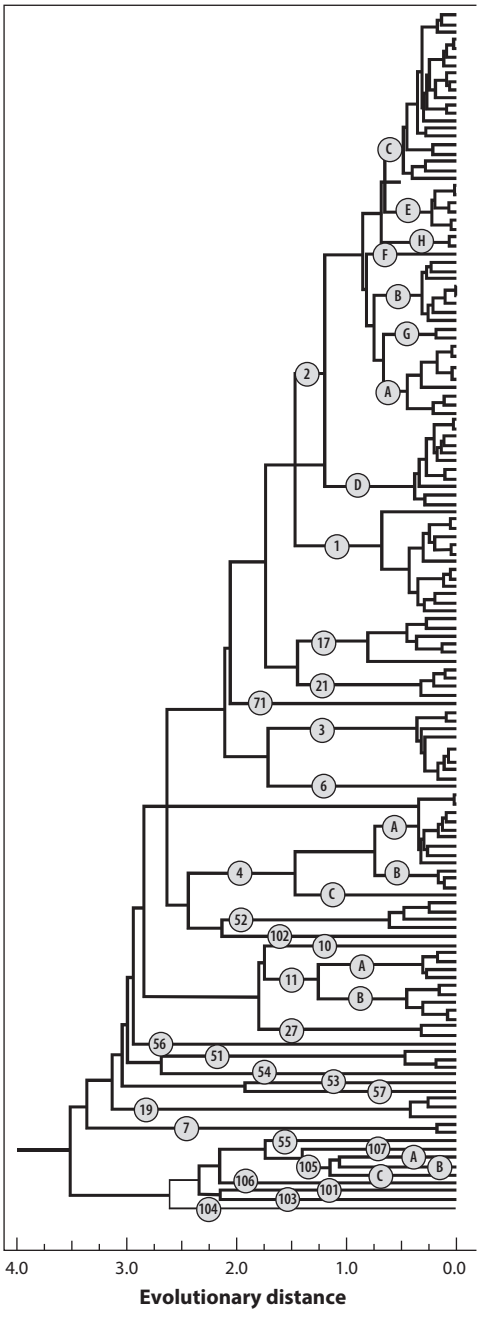
In the process of naming the CYP gene families, they were originally somewhat arbitrarily divided into different classes of organisms based on our naïve assumption that the number of P450 genes likely to exist in all animals on the planet would not exceed 50. However, this assumption has proven to be a striking underestimate.

As of June 15, 2023, a total of 146,529 CYP genes in 9,877 CYP gene families—among animals, plants, fungi, protozoa, bacteria, archaea, and viruses—have been named; in animals alone, a total of 38,690 CYP genes in 1,911 families have been named (D.R. Nelson, personal communication). David Nelson has established his intended numerical format for the assignment of CYP gene families in animals, yeast and fungi, plants, lower eukaryotes, and bacteria and viruses (reviewed in 69, table 4).

In general, genomes of many individual plant species carry far more P450 genes than do animal genomes, most likely because P450-mediated pathways in plants are critical for virtually all life processes: growth, differentiation, defense (phytoalexin formation), fruit production, flower color, and generation of the attractive (and repulsive) scents of flowers (9, 78–80).

Figure 7

The CYP gene superfamily, as of December 1990. The dendrogram is plotted via an unweighted pair group method with arithmetic mean analysis. Arabic numbers denote gene family designations, capital letters gene subfamily names. The abscissa denotes evolutionary time before the present, which is arbitrary and not linear. The first 50 CYP numbers include animal P450 genes [e.g., *CYP10A1* is the first gene discovered in the CYP10 family and exists in the great pond snail (*Helix stagnalis*)]. Reference 9 describes species of organisms that carry each P450 gene family; for example, the *CYP55* gene family was assigned to the fungus *Fusarium oxysporum*, and the *CYP104* gene family was characterized in the pathogenic bacterium *Agrobacterium tumefaciens*.



Lastly, gene names of many other drug-metabolizing gene families and subfamilies have also been developed along the lines of evolutionary divergence (reviewed in 69, table 3). I was invited by some of these committees to help as a coauthor.

Discovery of *Slc39a8*, Encoding the ZIP8 Metal Cation Influx Transporter

In 1973, Ben Taylor and colleagues (81) reported that resistance to subcutaneously administered cadmium (Cd^{2+})-caused testicular necrosis in mice segregated as an autosomal recessive trait. Taylor named this fascinating trait the *Cdm* locus; by mouse genetic crosses with known loci, Ben was able to localize the *Cdm* locus between two previously mapped genes, amylase-1 (*Amy1*) and varitint-waddler (82).

Isolation of the *SLC39A8* gene and determination of its function. Given the many more sophisticated genetic tricks available in the late 1990s, I decided it might be fun if our lab corroborated and then extended Taylor's studies from 25 years earlier. By using Cd-sensitive versus Cd-resistant inbred mouse strains and 26 recombinant inbred BXD/Ty lines, plus taking advantage of available polymorphic satellite markers, we narrowed down the *Cdm* locus on chromosome 3 from more than 24 cM to 0.64 cM (83).

The next step in this 0.64-cM region (which represented ~4.96 Mb) was to find the gene responsible for the Cd-responsive trait. Using SNV analysis of this region, plus comparing two Cd-sensitive and two Cd-resistant inbred mouse strains, along with the recombinant inbred BXD14/Ty line, Tim Dalton found a 400-kb haplotype block associated with the Cd-induced toxicity phenotype (84). Within this block we found the complete gene for *Slc39a8*, encoding ZIP8, a member of the solute-carrier transporter superfamily; at that time, the only homologous genes in the Tree of Life database were the putative zinc-responsive (ZRT)- and iron-responsive transporter (IRT)-like protein-8 (ZIP8) in plant and yeast genomes.

Using mouse fetal fibroblast cultures, we showed ZIP8 expression to be associated with large increases in Cd^{2+} influx, accumulation, and toxicity—so, we knew we were on the right track! By in situ hybridization, ZIP8 mRNA was found to be prominent in testicular vascular endothelial cells of Cd-sensitive, but not of Cd-resistant, strains of mice. ZIP8 expression was subsequently found to be highest in kidney, lung, and testis and was ubiquitously expressed to varying degrees throughout all tissues examined (84).

With Bin Wang and Tim Dalton, we then created a transgenic mouse line (85), which carried a bacterial artificial chromosome (BAC) containing the entire autosomal dominant allele of the *Slc39a8* gene from the 129/SvJ Cd-sensitive mouse; this was inserted into the Cd-resistant C57BL/6J mouse genome. The bacterial transgenic mouse (BTZIP8-3) was found to contain five *Slc39a8* copies in its genome—three (dominant) from the BAC, plus the two (recessive) wild-type copies. ZIP8 mRNA and protein levels were shown to be located in the same tissues but expressed approximately 2.5-fold higher in BTZIP8-3 compared with wild-type mice. Cd treatment of BTZIP8-3 mice reversed the Cd resistance trait (seen in nontransgenic littermates) to Cd sensitivity; reversal of the testicular necrosis phenotype therefore confirmed that the *Slc39a8* gene unequivocally represents the *Cdm* locus (85).

We clearly had discovered an influx transporter in mouse cells that mediates transport of Cd^{2+} into cells. A homolog of this transporter also exists in plants and yeast, implying that the substrate for this evolutionarily highly conserved transporter must be a cation(s) important to the survival of many diverse organisms in the Tree of Life. With Lei He, we therefore used stable retroviral infection of the ZIP8 cDNA in mouse fetal fibroblast cultures (rvZIP8 cells) to study divalent cation–uptake kinetics and Michaelis-Menten constant (Km) values to assess binding affinity. As

Varitint-waddler: short for "variable tint" (coat color) and gait of mouse—now known to represent the *Mcoln* gene (mucolipin TRP cation channel-3)

Recombinant inbred lines: two parent inbred strains are crossed and then brother-sister pairs are mated for 20 generations to create multiple isogenic lines; used for mapping quantitative trait loci

Polymorphic satellite markers: repeated segments within a DNA microsatellite sequence that often vary among people; helpful in criminology or in studying family inheritance patterns

Haplotype block: region of genome having no evidence of recent genetic recombination; thus, genes in this DNA fragment tend to be inherited together

physiological substrate for ZIP8, Mn^{2+} was found to be better than Zn^{2+} (86). Fe^{2+} and Co^{2+} have also been suggested as ZIP8 substrates (87).

In ZIP8-expressing *Xenopus* oocyte cultures, electrogenicity studies revealed an influx of two bicarbonate (HCO_3^-) anions per one Zn^{2+} (or Mn^{2+} or Cd^{2+}) cation; that is, an $M^{2+}/(HCO_3^-)_2$ electroneutral complex acts as a substrate for this symporter (88). Subsequently, selenite ($HSeO_3^-$)—a form of selenium that can be taken up by cells—was shown to require Zn^{2+} and (HCO_3^-) and to be transported by ZIP8 (89); thus, the most likely electroneutral complex, $Zn^{2+}/(HCO_3^-)(HSeO_3^-)$, was proposed but has not yet been proven.

The ZIP8 eight-transmembrane protein is largely internalized during Zn^{2+} treatment, as well as during homeostasis. We discovered that ZIP8 moves predominantly to the cell-surface membrane (via trafficking) when Zn^{2+} is depleted in culture medium (88).

Again, fascinated by evolution, I suggested that we examine the homology of amino acid sequences among the 14 *Slc39a* gene subfamily members in the mouse; we found that *Slc39a14* was most closely evolutionarily related to *Slc39a8*. We subsequently cloned and characterized the *Slc39a14* gene (90). ZIP14 exhibits similar transporter properties to ZIP8, but tissue specificity of ZIP14 differs from that of ZIP8 (90, 91). Alignment of mouse and human *SLC39A* members showed a very high degree of evolutionary conservation among each of the 14 orthologs (91).

Previous studies had found that ZIP8 is expressed in gastrula and in visceral endoderm at GD7.5 (92). In fact, ZIP8 had been proposed (93) as a potential indicator of cell differentiation (self-renewal-related signaling) in embryonic stem cells. Learning this, I postulated [and, with Bin Wang, we confirmed (94)] that a $Slc39a8^{(-/-)}$ global knockout would likely be embryolethal very early.

While generating the global knockout, we accidentally produced an intriguing knockdown mouse line (95). This $Slc39a8^{(neo/neo)}$ hypomorph expresses ZIP8 mRNA and protein levels that are approximately 15% of those in all wild-type tissues examined. The hypomorph is viable—at least until GD16.5—with some pups surviving until postnatal day 1. Here, then, was an experimental model that provided us with a way to study ZIP8 functions in placenta, yolk sac, and fetal tissues in utero.

The $Slc39a8^{(neo)}$ allele was shown to be associated with diminished Mn^{2+}, Zn^{2+}, and Fe^{2+} in mouse fetal fibroblast and liver-derived cultures. Levels of these cations were also decreased in several tissues of $Slc39a8^{(neo/neo)}$ newborns. $Slc39a8^{(neo/neo)}$ homozygotes—from GD11.5 until death—are extremely pale (95) and stunted in growth, with hypomorphic limbs (**Figure 8**). Additional abnormalities include severely hypoplastic spleen and substantially reduced organ size (liver, kidney, lung, and brain, both cerebrum and cerebellum). Histologically, $Slc39a8^{(neo/neo)}$ neonates show decreased numbers of hematopoietic islands in yolk sac and liver. Low hemoglobin, hematocrit, red blood cell count, serum iron, and total iron-binding capacity confirmed a severe anemia (95).

In an attempt to explain the $Slc39a8^{(neo/neo)}$ phenotypic pleiotropy, with Jing Chen and Marina Gálvez-Peralta, we carried out bioinformatics (RNA-seq) analysis of the transcriptome in GD13.5 yolk sac and placenta and in GD16.5 liver, kidney, lung, heart, and cerebellum, comparing $Slc39a8^{(neo/neo)}$ with $Slc39a8^{(+/+)}$ wild-type mice (96). Based on transcription factor (TF) profiles and a search for enriched TF-binding sites, we identified numerous genes encoding zinc-finger proteins and other TFs associated with hematopoietic stem cell functions. We concluded that in $Slc39a8^{(neo/neo)}$ mice, deficient ZIP8-mediated divalent cation transport—predominantly in yolk sac—affects zinc-finger protein TFs (e.g., GATA) and other TFs interacting with GATA proteins (e.g., TAL1). These RNA-seq data (96) strongly support the in utero phenotypes observed in the hypomorph, including dysmorphogenesis, dysregulated hematopoietic stem cell fate, and anemia (95).

Trafficking: process by which proteins and other macromolecules are distributed throughout the cell and released to (or internalized from) the extracellular space

Gastrula: in vertebrates, the embryonic stage following the blastula (a ball of cells) when it becomes a hollow cup-shaped structure

Hypomorph: organism having decreased gene function (diminished expression of mRNA or protein or lowered functional performance), but not a complete loss

Pleiotropy: two or more apparently unrelated effects (traits, phenotypes) produced by the same gene

Transcriptome: sum total of all messenger RNA molecules—expressed at any moment in time—from the genes of an organism; studied using RNA-seq analysis

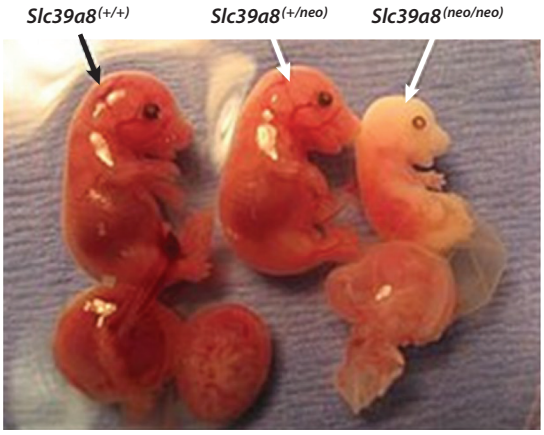


Figure 8

Gross appearance of *Slc39a8*$^{(+/+)}$ wild-type, heterozygote, and knockdown homozygote fetuses on GD16.5 in utero (placentas are shown, below each fetus). Although the *Slc39a8*$^{(+/neo)}$ can be seen as slightly pale and smaller compared with the *Slc39a8*$^{(+/+)}$ wild type, the *Slc39a8*$^{(neo/neo)}$ homozygote is strikingly anemic with stunted growth, which we are able to observe easily from GD11 onward (95). Figure adapted from Reference 96 with permission from Springer Nature.

Clinical relevance of the SLC39A8 transporter. The *SLC39A8* gene was originally discovered in human monocytes (97) and named BIGM103. ZIP8 function was shown in human lung and in cell cultures to protect against inflammation (98–101). In addition, numerous genome-wide association studies (GWASs) have identified human *SLC39A8* variants correlated with a wide spectrum of clinical pleiotropic phenotypes: heart disease, lipid profile, and blood pressure regulation (102–108); schizophrenia (109, 110); osteoarthritis (111, 112); Crohn's disease (113); and retinal iron accumulation (114).

SLC39A8 variants are also associated with a wide variety of dysmorphogenesis and birth defects: congenital deformed skull, cerebellar atrophy, profound psychomotor and mental retardation, severe seizures, short limbs, and hearing loss defects (115, 116). ZIP8 deficiency was found to impair Mn^{2+}-dependent enzyme function, which severely affects posttranslational glycosylation (115–117); this finding of defective glycosylation likely explains many of the ZIP8-mediated pleiotropic clinical effects. The latest update on the SLC39A8 transporter can be found in Reference 118, and the latest review on the SLC39A8 role of Mn^{2+} versus Zn^{2+} in host immune defense has recently appeared (119).

Legacy

Reverse genetics is an experimental approach that begins with the gene (or genetic locus) and searches for a trait (or traits) caused by that DNA sequence. Forward genetics, on the other hand, starts with a phenotype (trait) and then seeks to find the gene (or genetic basis) responsible for that trait.

During my career spanning more than five decades, I unknowingly chose two forward-genetics approaches beginning with two exciting phenotypes: AHH induction by PAHs and TCDD, and Cd^{2+}-induced testicular necrosis. For both traits, I took advantage of large genetic differences between inbred mouse strains. For both projects, I used foreign agents (i.e., PAHs and TCDD, and Cd^{2+}) to perturb genetic pathways in living cells. In both cases, this led to the discovery of genes (*AHR* and *CYP1A1*, and the *SLC39A8* gene) that were unexpectedly expressed in mammalian embryonic stem cells.

Posttranslational glycosylation: process resulting in covalent attachment of glycans or monosaccharides to proteins; about 50% of all cellular proteins are glycosylated

REFLECTIONS AND CONCLUDING REMARKS

Did I Plan My Career?

Growing up, I was very naïve about where I was and what I was going to do next. In high school, I had planned to go to college, but the opportunity to go to a New England liberal arts university on a large scholarship was a pleasant surprise. I began college, leaning toward a career in either academic theology or basic science, and I deliberately steered clear of "premed cliques." I was intrigued by seven-year MD-PhD programs, but nationwide, there were very few in the late 1950s. I had never heard of a five-year MS-MD program, but I wound up doing just that at the University of Oregon Medical School in Portland.

I discovered that I enjoyed basic laboratory bench research very much, but I also decided that becoming board qualified or certified in a clinical subspecialty might become necessary someday—if research funds were ever to dry up due to a world war or other international disaster. I chose pediatrics because treating young children so that they might become lifetime healthy, successful, future contributors to society seemed most worthwhile; moreover, I found that I enjoyed evolution very much and later I realized that developmental biology was simply an extension of evolution ("evo-devo" research).

Winding up in an NCI lab, and choosing to study AHH induction, was completely serendipitous. Choosing to move from my two-year NCI fellowship to setting up my own lab in the NICHD for two decades was a no-brainer. Transitioning from NIH to academia for 23 more years reflected my desire to pursue more clinical research projects, interact with greater numbers of diverse colleagues, and attract more grad students as well as postdocs, compared with what was possible at NIH.

In conclusion, without much forethought, I stumbled forward from one exciting challenge to the next. On May 24, 2023, Research.com, a leading academic platform for researchers, notified me of the 2023 edition of their ranking of best genetics scientists. I am ranked 133 in the world and 72 in the United States. I have also been recognized with their Genetics Leader Award for 2023. And this is ten years after I became Professor Emeritus!

What Might Be the Best Future Directions in My Research Projects?

For my first and major project, I believe that an important direction will be further resolution of the role of the AHR/CYP1 axis in LM second-messenger pathways (discussed above), which will also likely involve the intestinal barrier against foreign agents by way of the brain-gut-microbiome network (62). Moreover, bone marrow–derived macrophages produce IL-22 following activation by AHR when cells are activated through the Toll-like receptor family (63), which offers a bridge to immunology; upstream of this, it is proposed that the LM second-messenger pathways are participating. I predict these research areas will benefit in the near future from single-cell multiomics studies in several of the most relevant tissues.

For the field of PGx, when analyzing GWASs of large cohorts, polygenic risk scores (120, 121) have perhaps helped a bit in attempting to predict the risk of complex human disease traits (as well as some PGx drug, or environmental-toxicant, response traits) in the individual patient; however, the holistic nature of each person's genetic makeup makes this goal extremely challenging (if not impossible). As very recently reviewed (122), there is a substantial gap in pharmacy school curricula regarding PGx and personalized medicine training, which, coupled with the slow rate of implementation of these concepts into clinical practice, seems to restrain students' aspirations to further pursue this area of research. Perhaps artificial intelligence algorithms, coupled with machine learning, might offer key breakthrough advances in the near future.

With respect to gene nomenclature in all species on the planet, this topic has been covered extensively and pragmatically in our recent review (69) on understanding the creation of a gene family or group (slow paralog evolution) versus evolutionary blooms (rapid paralog evolution).

With regard to the SLC39A8 influx transporter, we know that Mn^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, and selenite ($HSeO_3^-$) can be substrates; but how does *SLC39A8* or the ZIP8 transporter protein determine which is the preferred cation substrate in any particular organ, tissue, or cell type? Perhaps single-cell multiomics studies might discover that individual cells within a tissue contain ZIP8 proteins that specifically uptake one, but not another, metal cation?

Also, what is the mechanism by which the SLC39A8 transporter moves predominantly to the cell-surface membrane (via trafficking) under conditions of Zn^{2+} depletion in culture medium (88)? Do different cations behave similarly in different cell types? If so, how does the cell do this?

Six human *SLC39A8* variants are associated with many pleiotropic developmental disorders, whereas just one variant (p.Ala391Thr) is correlated with an amazing array of at least 22 traits (reviewed in 118). An understanding of these observations will require further studies—perhaps including 3D modeling, structural biology, and physical (Mn, Zn, Fe, Se, and Co) ion-binding properties of each ZIP8 transporter variant. Single-cell multiomics studies again are needed to examine each of the seven *SLC39A8* mutant alleles compared to the wild-type allele—as separate cDNA constructs individually introduced into more than ten cell types (described in 118). In each cell type, each *SLC39A8* variant should be tested separately against wild-type *SLC39A8* cDNA for Mn, Zn, Fe, Se, and Co uptake. Each of these metals is an important nutrient. Would any substantial differences in metal ion uptake be discovered, depending on the cell type? If so, how is this determined?

What Fundamental Pharmacological and Toxicological Rules Did I Realize?

First, evolution was always first and foremost in my mind. Each time a trainee discovered a new finding, I tried to emphasize, "Why did Mother Nature design this phenomenon in this (particular) way?" "Why didn't this mechanism evolve in the other way that we had hypothesized?"

Second, there is a fundamental rule that each species evolved on this planet with genes that allowed that particular species to find food, avoid predators, and reproduce. Toxic agents (toxicology) and drugs (pharmacology) came later. Students must accept that every xenobiotic (including nonessential metal ions) enters the cell and elicits beneficial or detrimental effects or disrupts endogenous pathways using endogenous molecules that evolutionally preceded the foreign agent.

Third, clinical expression of a gene in stem cells means that this gene will become ubiquitous in (most, if not all) cell types and thus is absolutely essential in critical life processes and health in the organism. Expression of any gene in stem cells also connotes that SNVs (de novo mutations) in such a gene [or disruption in the genetic pathway(s)] will usually lead to embryolethality and/or numerous serious clinical disorders (reviewed in 20, 118). Because AHR, CYP1A1, and SLC39A8 are important in many critical life processes, it is generally not a good idea to consider developing drugs to overexpress, downregulate, or totally block such genes. Genes expressed in stem cells are not the best candidates for drug targets; developing a new drug to stimulate or block a gene expressed in pluripotent embryonic stem cells will likely result in a narrow window of beneficial effect, plus a high likelihood of serious side effects because of undesirable drug interactions with off targets.

Lastly, attempting to falsify data is completely antiscience. Laboratory and clinical science are fun. Each finding of something that Mother Nature has developed over millions of years is always far more exciting than any human's preconceived idea!

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