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Epigenetics in Drug Disposition & Drug Therapy: Symposium Report of the 24th North American Meeting of the International Society for the Study of Xenobiotics (ISSX)

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Abstract

The 24th North American International Society for the Study of Xenobiotics (ISSX) meeting, held virtually from September 13 to 17, 2021, embraced the theme of “Broadening Our Horizons.” This reinforces a key mission of ISSX: striving to share innovative science related to drug discovery and development. Session speakers and the ISSX New Investigator Group, which supports scientific and professional development of student and early career ISSX members, elected to highlight the scientific content presented during the captivating session titled, “Epigenetics in Drug Disposition & Drug Therapy”. The impact genetic variation has on drug response is well established; however, this session underscored the importance of investigating the role of epigenetics in drug disposition and drug discovery. Session speakers, Drs. Ning, McClay, and Lazarus, detailed mechanisms by which epigenetic players including long non-coding RNA

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(lncRNAs), microRNA (miRNAs), DNA methylation, and histone acetylation can alter the expression of genes involved in pharmacokinetics, pharmacodynamics, and toxicity. Dr. Ning detailed current knowledge about miRNAs and lncRNAs and the mechanisms by which they can affect expression of drug metabolizing enzymes (DMEs) and nuclear receptors. Dr. Lazarus discussed the potential role of miRNAs on UDP-glucuronosyltransferase (UGT) expression and activity. Dr. McClay provided evidence that aging alters methylation and acetylation of DMEs in the liver, affecting gene expression and activity. These topics, compiled by the symposium organizers, presenters, and the ISSX New Investigators Group, are herein discussed, along with exciting future perspectives for epigenetics in drug disposition and drug discovery research.

Introduction

The ISSX New Investigator Group provides opportunities for student and early career members of ISSX to be actively engaged in the Society by organizing webinars, fireside chats, networking and mentorship, and career development events. The ISSX New Investigator Group selected a symposium from the 24th North American ISSX Meeting held virtually from September 13–17, 2021, to share the most up-to-date knowledge on a cutting-edge topic with unique insights from the speakers. Our objective is to provide a high-level summary from the different presentations, allowing for attendees to recall the session highlights while also promoting dissemination of learnings to those who were unable to attend. The ISSX New Investigator Group selected the “Epigenetics in Drug Disposition and Drug Therapy” symposium since it is a rapidly evolving expanding field that can be used as a tool in precision medicine (Rasool et al. 2015).

In studying genomics and pharmacogenomics, we look at the effects of specific mutations or polymorphisms in a gene; i.e., changes in the DNA sequence, which alter the expression level, localization, and/or activity of proteins such as a drug metabolizing enzymes (DMEs), and nuclear receptors (NR) (Sim et al. 2013; Ahmed et al. 2016). These genetic variations are broadly consistent across the lifespan and are generally considered unresponsive to environmental factors. On the other hand, the study of epigenetics does not refer to alterations to the DNA sequence itself; epigenetics refers to certain signature marks placed upon areas of the genome in response to a variety of environmental factors such as stress, diet, drug exposure, and changes across the lifespan (Heerboth et al. 2014). The epigenetic marks include DNA methylation of the carbon 5 position of cytosine residues (5-methylcytosine, 5mC) and several different posttranslational modifications to histone proteins, which make up the nucleosomes around which DNA is coiled. Histone modifications include methylation and acetylation of histone lysine residues. Typically, closed or “silent” chromatin results from the addition of 5mC and removal of histone acetylation marks. Conversely, open, active chromatin results from the addition of histone acetylation marks and the removal of 5mC (Handel et al. 2010; Stricker et al. 2017). MicroRNA (miRNA) and long noncoding RNA (lncRNA) regulation also fall under the umbrella of epigenetics, and there has been increasing interest in using circulating miRNAs and lncRNAs as disease biomarkers (Bolha et al., 2017; Condrat et al., 2020). Epigenetic modifications including DNA methylation, chromatin remodeling, histone modification, miRNAs, and lncRNAs contribute to global gene expression including genes relevant to

xenobiotic metabolism and transport, as well as genes involved in disease processes such as cancer.

The session chair, Ann Daly (Newcastle University, Newcastle upon Tyne, United Kingdom), recognized that although considerable progress has been made in the field, this is still a relatively poorly understood area, and that studying changes in epigenetic regulation due to disease and environmental factors are of particular importance for maximizing effective drug therapy. Drugs that epigenetically modulate gene expression, especially those relevant to oncology, are increasingly being investigated and approved, and the scientific community is just beginning to scratch the surface in terms of understanding implications on biological processes. Pharmaceutical companies have invested heavily into epigenetics research with a particular interest in oncology. However, epigenetics is also of increasing interest in disease areas outside of oncology including metabolic diseases such as metabolic associated fatty liver disease (Bayoumi et al. 2020), central nervous system pathologies including Alzheimer's Disease, and inflammatory diseases such as asthma (Prachayasittikul et al. 2017). The global epigenetics market was valued at $1.0 billion USD in 2020 and is projected to reach $4.1 billion USD by 2030, growing at a compound annual growth rate of 14.1% from 2021 to 2030 (Balkrishna and Sumant 2022). The major classes of epigenetic drugs currently in use are DNA methylation inhibiting drugs, bromodomain inhibitors, histone acetyltransferase inhibitors, histone deacetylase inhibitors, protein methyltransferase inhibitors, and histone methyltransferase inhibitors (Heerboth et al. 2014).

The speakers for the session represented diverse research expertise from both academia and government. Dr. Baitang Ning (National Center for Toxicological Research/FDA, Jefferson, Arkansas, USA) provided detailed current knowledge about how miRNAs and lncRNAs can regulate the expression of drug metabolizing enzymes and nuclear receptors. Dr. Philip Lazarus (Washington State University College of Pharmacy, Spokane, Washington, USA) discussed the potential role of miRNAs on UDP-glucuronosyltransferase (UGT) expression and activity. Dr. Joseph McClay (Virginia Commonwealth University, Richmond, Virginia, USA) described epigenetic profiles in drug response and provided evidence that DMEs in the liver are subject to epigenetic aging via altered DNA methylation and histone acetylation. Data presented by Dr. McClay suggests epigenetic state is a better predictor of drug metabolism than chronological age.

The symposium offered detailed presentations about the current landscape for epigenetics in drug disposition and drug discovery, and there was an engaging question and answer session after the presentations. The consensus was that there is still much more to be discovered about epigenetics to continue to advance human health, but current research is paving the way for these insights. The fast-developing advancements in genomics and epigenomics research provide the groundwork necessary to improve current drug treatment, while also breaking ground for novel therapeutics for difficult-to-treat diseases such as rare diseases, cancer, cardiovascular, and neurological diseases (Nguyen 2019). Similar to how far genomics research has helped advance drug discovery in the past 20 years (Russell et al. 2021), we anticipate that in the coming 20 years, epigenetics will play a critical role in discovering next-generation therapeutics with improved efficacy and safety, and will provide the ability to harness novel targets.

Noncoding RNAs Affect Expression of Cytochrome P450 and Nuclear Receptor Genes

Dongying Li, PhD and Baitang Ning, PhD

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Cytochrome P450 (CYP) expression can be influenced by many factors, including genetic and epigenetic elements. Previously, extensive research has demonstrated that various types of genetic variations affect *CYP* gene expression, such as pseudogenes, copy number variations, and single nucleotide polymorphisms (SNPs) (Savolainen et al. 1997; Agundez 2004; Zhou et al. 2009). Transcriptional regulators, such as transcription factors and co-regulators, play important roles in transcriptional control of the expression of CYPs and nuclear receptors (NRs). Many NRs serve as key transactivators for CYPs and NRs, including hepatic nuclear factor (HNF) 1A, HNF4α, pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AHR), and vitamin D receptor (VDR) (Honkakoski and Negishi 2000). It has been shown that epigenetic factors, including DNA methylation and histone modification, also have critical regulatory functions in *CYP* and *NR* gene expression (Zanger and Schwab 2013; Tang and Chen 2015). For example, DNA methylating agents may cause hypermethylation and widespread transcriptional inhibition of *CYP* genes in major organs such as the liver (Dannenberg and Edenberg 2006; Zanger and Schwab 2013; Cheng 2015; Habano et al. 2015; Li Y et al. 2018). In the past couple of decades, non-coding RNAs (ncRNAs) including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) have emerged as epigenetic regulators of *CYP* and *NR* gene expression. miRNAs and lncRNAs respond to environmental and pharmaceutical chemicals at their expression levels; meanwhile, they may influence xenobiotic metabolism and toxicity by regulating the expression of DMEs, transporters, and NRs (Ning et al. 2019) (Figure 1).

miRNAs are a type of small ncRNAs that primarily inhibit gene expression post-transcriptionally by binding to target mRNAs and recruiting RNA-induced silencing complexes (RISC) for mRNA degradation or translational repression (Huntzinger and Izaurralde 2011). In 2006, Tsuchiya et al. demonstrated that *CYP1B1* mRNA was targeted by miR-27b for gene suppression. This was a pioneering breakthrough in epigenetics of DMEs at the time, identifying that *CYP* genes can regulated by miRNAs (Tsuchiya et al. 2006). Following this study, more findings were reported on miRNAs regulating CYP expression by directly binding to the mRNAs of target genes (Yu et al. 2015; Jin et al. 2016; Chen Y et al. 2017; Wang Y et al. 2017; Zeng et al. 2017; Li D, Tolleson, et al. 2019; Ning et al. 2019). miRNAs can also mediate CYP expression by targeting transcriptional activators including NRs or repressors of CYP expression. For example, multiple miRNAs may downregulate CYP3A4 indirectly by targeting mRNAs of NRs that activate CYP3A4, such as HNF4α, PXR, and VDR (Takagi et al. 2008; Pan YZ et al. 2009; Yu et al. 2018). In contrast, miRNA-mediated silencing of a transcriptional repressor of CYPs will increase CYP expression. For instance, miR-142-3p targets and inhibits small heterodimer partner

(SHP), a repressor of CYP2D6, leading to an increase in expression levels of CYP2D6 in mice (Pan X et al. 2017). miRNA-mediated regulation of CYP and NR expression shows further complexity when considering the functions of lncRNAs, particularly as miRNA sponges.

LncRNAs modulate gene expression by various mechanisms and often by interacting with regulatory proteins; the regulatory effects of lncRNAs on gene expression depend on their subcellular localization and the functions of the proteins they associate with (Li D, Tolleson, et al. 2019). Emerging studies have reported regulatory roles of lncRNAs in CYP and NR expression. LncRNAs may regulate CYP expression in association with miRNAs and NRs. For instance, LINC00844 modulates the expression of CYP3A4 and CYP2E1 in a molecular network that consists of miR-486-5p, PXR, and HNF4α via multiple cross-talking pathways (Li D et al. 2020). Further, lncRNAs in neighboring genomic positions with NR genes may mediate CYP expression that is targeted by those NRs. For example, HNF1α-AS1 and HNF4α-AS1 regulate the expression of several transactivation targets of HNF1α and HNF4α, including several CYPs and NRs, and influence the susceptibility to hepatotoxicity induced by acetaminophen, as shown in HepaRG cells (Chen L et al. 2018; Chen L et al. 2020). Despite recent efforts, many questions remain regarding the roles of lncRNAs in influencing xenobiotic metabolism and toxicity via the regulation of CYPs and NRs (Li D, Knox, et al. 2019).

Increasing evidence shows that CYP expression is mediated by different regulators (e.g., miRNAs, lncRNAs, and NRs), at multiple levels (e.g., transcriptional and post-transcriptional), via diverse mechanisms (e.g., genetic and epigenetic), in different subcellular compartments, and with different regulatory outcomes (e.g., gene activation vs. suppression) (Li D, Tolleson, et al. 2019). Human CYP2E1 serves as a good example for the diversity and complexity of ncRNA regulation of CYP expression (Figure 2). miRNAs may regulate CYP2E1 expression by a) targeting different regions of *CYP2E1* DNA and mRNA (Mohri et al. 2010; Miao et al. 2016; Wang Y et al. 2017), b) silencing transactivators of CYP2E1 such as HNF1α and NR1I2 (Yu et al. 2018) and c) functionally interacting with lncRNAs that target CYP2E1 (Li D et al. 2020). The regulatory effect of miRNAs on CYP2E1 may be affected by genetic variations, such as SNPs residing in the miRNA response element (Nakano et al. 2015). NcRNA regulation of CYP2E1 expression is critical to xenobiotic-induced CYP2E1-mediated liver toxicity and carcinogenesis, although the underlying mechanisms are complex and remain elusive.

To better understand the complicated and diverse mechanisms of ncRNAs in regulating CYP and NR expression, an integrated strategy that combines *in silico*, *in vitro*, and *in vivo* approaches is needed to generate multi-dimensional, high-confidence data that support novel findings. Various computational analyses may greatly enhance the efficiency of target screening and tremendously reduce time, cost, and labor needed for validation and mechanistic investigation. Several databases and computational tools are freely available to the public for ncRNA target prediction, gene expression correlation, and functional characterization. To verify ncRNA targeting site and regulatory effects on CYPs and NRs, a series of *in vitro* molecular and cellular techniques can be used, including luciferase reporter assays, gain- and loss-of-function assays, fluorescent-based RNA electrophoretic

mobility shift assays, and cellular toxicity assays (Li D, Knox, et al. 2019; Yu et al. 2020). Animal models are particularly useful for examining ncRNA and target gene expression as well as systemic responses (e.g., blood biochemistry and organ histology) to assess chemical-induced toxicity at multiple levels *in vivo* (Li D et al. 2021).

Current evidence indicates that ncRNA regulation of CYP and NR expression is common across different species, even though regulatory potency or efficiency may vary. Great advances have been made in understanding miRNA regulation of some major CYPs and NRs; however, studies detailing lncRNA modulation of CYPs and NRs remain elusive due to the vastly diverse mechanisms that lncRNAs utilize for gene regulation. NcRNA regulation of CYP expression remains a critical research subject as it has significant clinical implications, particularly concerning drug-drug interactions (DDI) and drug-food interactions. In response to certain drug or food intake, the levels of ncRNAs may change, which in turn increases or decreases the expression of their target CYP and NR genes, thus influencing the efficacy and toxicity of drugs that are metabolized by the affected CYPs. Additionally, ncRNAs are being explored as drug targets and agents for human diseases as a new type of RNA therapeutics (Winkle et al. 2021; Zeng et al. 2021). In conclusion, ncRNAs show great promise in enhancing drug development and drug safety and risk assessment, and they hold a bright future to be further utilized in personalized medicine.

Potential Role of miRNA in the Regulation of UGT Expression and Activity

Philip Lazarus, PhD

microRNAs (miRNAs) are dynamic epigenetic regulators of gene expression and play a role in both normal tissue development as well as tumor biology. miRNA post-transcriptionally repress protein expression primarily by binding to target messenger RNA (mRNA), usually within the 3'-untranslated region (UTR), with target recognition predominantly driven by the hybridization of the miRNA 'seed sequence' to the mRNA target (Carthew and Sontheimer 2009; Guo et al. 2010). Acting as a genetic switch and/or fine-tuner of protein expression, miRNA regulation can account for not only mild (e.g., two-fold) protein repression, but can also cause mRNA destabilization to dramatically reduce protein expression (Baek et al. 2008; Selbach et al. 2008; Mukherji et al. 2011).

The impact of miRNAs on drug response has not been studied extensively. miRNAs regulate expression of several human cytochrome P450 (CYP) phase I DMEs, including the major drug and hormone metabolizing CYPs such as 3A4, 2E1, 1B1, and 24 (Tsuchiya et al. 2006; Komagata et al. 2009; Pan YZ et al. 2009; Mohri et al. 2010), however, fewer studies have aimed to characterize effects of miRNAs on additional phase I enzymes as well as phase II DMEs.

The UDP-glucuronosyltransferase (UGT) phase II metabolic enzyme family primarily consists of two large subfamilies, the UGT1As and 2Bs. The UGTs are responsible for the metabolism and excretion of numerous endogenous compounds including bilirubin (Bosma et al. 1994) and steroid hormones (Belanger et al. 1998), as well as exogenous compounds including drugs, chemotherapeutic agents, and carcinogens (Nagar and Remmel 2006;

Balliet et al. 2009; Sun et al. 2013). UGT family members exhibit extensive interindividual variability of expression that may contribute to variability in patient response and toxicity (Court et al. 2001). The focus of the present seminar was to describe the miRNAs that are potentially important in regulating the UGT 1A and 2B enzymes.

After *in silico* approaches suggested that several miRNAs potentially interacted with the UGT1A family of enzymes, the UGT1A 3'-UTR was cloned into the luciferase pGL3-promoter vector immediately 3' of the luciferase open reading frame. After co-transient transfection of this plasmid together with miR-491-3p miRNA mimic into HEK293 (human embryonic kidney) cells, luciferase activity was significantly repressed at both 1 nM ($P<0.05$) and 2 nM ($P<0.05$) miR-491-3p concentrations, as compared to the scrambled miRNA-transfected control. No significant difference in luciferase activity was observed between a miR-491-3p seed deletion mutant and the negative scrambled miRNA control, and no significant alteration in luciferase activity was observed in co-transfections with mimics of two other miRNA, miR-148a and miR-136, predicted *in silico* to bind the UGT1A 3'-UTR.

miR-491-3p demonstrated significant decreases in the endogenous mRNA levels of UGTs *1A1*, *1A3*, and *1A6* as compared to scrambled miRNA-transfected controls in HuH-7 (human hepatoma-derived) cells. This corresponded with significantly decreased formation of raloxifene glucuronides in HuH-7 cell homogenates after transfection with miR-491-3p mimic; raloxifene is a known substrate of several UGT1A enzymes including UGT1A1 (Sun et al. 2013). In contrast, endogenous *UGT1A1* mRNA levels were significantly elevated in the presence of a specific miR-491-3p inhibitor as compared to the scrambled miRNA inhibitor control. This increase corresponded with significant increases in formation of raloxifene-6-glucuronide and raloxifene-4'-glucuronide in HuH-7 homogenates with repressed miR-491-3p levels as compared to scrambled controls. No alteration in mRNA levels or enzyme activity was observed for endogenous UGT2B7, which has its own unique 3'-UTR different from the common UGT1A 3'-UTR and is not predicted to bind miR-491-3p, after transfection with either miR-491-3p mimic or the mMiR-491-3p-specific inhibitor.

Interestingly, an opposite trend was observed in a panel of normal human liver specimens, where a significant inverse correlation ($r = -0.487$; $P < 0.01$) was observed between *UGT1A6* mRNA and miR-491-3p expression levels in 37 normal human liver specimens that expressed *UGT1A6* (Figure 3). A significantly ($P < 0.05$) higher level of *UGT1A6* expression was observed in miR-491-3p non-expressing liver specimens as compared to miR-491-3p expressers. While similar results were also observed for other UGT1A enzymes including *UGT1A3*, no correlation existed between *UGT1A1* or *UGT1A9* mRNA levels and miR-491-3p expression in the same panel of liver specimens.

A similar approach was used to identify miRNA that potentially regulated UGT2B enzymes (Dluzen et al. 2016). *In silico* and luciferase data suggested the presence of a functional binding motif for miR-216b-5p within the 3′ untranslated regions of UGTs *2B4*, *2B7*, and *2B10*. Overexpression of a miR-216b-5p mimic significantly repressed *UGT2B7* and *UGT2B10* mRNA levels in HuH-7 cells, and *UGT2B4* and *UGT2B10* mRNA in Hep3B

cells. UGT2B7 protein levels were repressed in both HuH-7 and Hep3B cells in the presence of increasing miR-216b-5p mimic concentrations, corresponding with a significant decrease in glucuronidation of the UGT2B7-specific substrate, epirubicin. Inhibition of endogenous miR-216b-5p levels significantly increased *UGT2B7* mRNA levels in HuH-7 and Hep3B cells, and increased epirubicin glucuronidation by 85% and 50% for HuH-7 and Hep3B cells, respectively, compared to scramble controls. UGT2B4-mediated glucuronidation of codeine and UGT2B10-mediated glucuronidation of nicotine were significantly decreased in both HuH-7 and Hep3B cells after overexpression of a miR-216b-5p mimic.

To further characterize other miRNAs that may play a role in UGT2B regulation, a novel high resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was employed to measure UGT2B protein levels in a panel of human liver microsomal samples (n=62) (Sutliff et al. 2019). Concurrent *in silico* analysis identified eight candidate miRNAs as potential regulators of UGT2B enzymes. Comparison of UGT2B protein expression and candidate miRNA levels from human liver samples demonstrated a significant inverse correlation between UGTs 2B10 ($P = 0.047$, $r = -0.214$) and 2B15 ($P = 0.038$, $r = -0.228$) and one of these candidate miRNAs, miR-485-5p. A near-significant correlation was also observed between UGT2B7 and miR-485-5p expression. *In vitro* analysis using luciferase-containing vectors suggested an interaction of miR-485-5p within the 3′-UTRs of *UGT2B10* and *UGT2B7* but not with the 3'-UTR of *UGT2B15*. A significant reduction in luciferase activity was also observed for a luciferase vector containing the *UGT2B7* 3'-UTR; this was not observed for the *UBT2B15* 3'-UTR. Subsequently, miR-485-5p mimic was overexpressed in HuH-7 and HepB3 cells, resulting in decreased UGT2B10 and UGT2B7 activities measured using were probed using nicotine and aminobenzotriazole, respectively, and a significant decrease in glucuronidation activity were observed for both substrates in HuH-7 and Hep3B cells upon overexpression of miR-485-5p mimic.

In summary, using several different approaches, we demonstrated that several miRNAs may be important in the regulation of different UGT enzymes. It appears that several of these miRNAs may be exerting a coordinated regulation of multiple UGT genes, which is consistent with the high homology of 3'-UTR sequences observed for *UGT2B* genes and the fact that the 3'-UTR is the same for all *UGT1A* genes. Further studies are necessary to determine whether this regulation is important in individual response to drugs and carcinogen exposures *in vivo*.

Epigenetic profiles and drug response

Joseph L. McClay, PhD

In recent years, evidence has accumulated that epigenetic factors play a major role in the regulation of genes involved in xenobiotic metabolism (Fisel et al. 2016; Kronfol et al. 2017). There are many potential sources of variation in the levels of epigenetic marks such as DNA methylation (5-methylcytosine or 5mC) and histone acetylation. Environmental factors such as stress, diet and exposure to drugs and toxins may all affect epigenetic states (Feinberg 2007; Feil and Fraga 2012). In this regard, epigenetics differs substantially from DNA sequence, which is invariant in response to environmental factors except for

mutagens. Epigenetic states can also vary by sex and by age. Considering aging, it has been established for several decades that epigenetically-driven changes to gene expression occur in early development (*in utero* into childhood) and are intrinsic to processes such as cell differentiation (Li E 2002). However, more recently and particularly in the last decade, it has become apparent that sweeping changes occur to the epigenome through adulthood and into old age (Fraga and Esteller 2007; Horvath 2013; McClay et al. 2014; Benayoun et al. 2015). These changes 1) are non-random, 2) co-localize with binding sites of chromatin binding factors of regulatory importance, 3) occur in genes belonging to specific biological pathways and 4) often have significant effects on gene expression (McClay et al. 2014; Steegenga et al. 2014; Peters et al. 2015). These observations led us to hypothesize that expression of genes involved in xenobiotic metabolism may be affected by epigenetic aging. It is known that drug clearance, mediated by factors including altered DME activity, expression, or hepatic blood flow, changes with age and the 65+ age group is at higher risk of severe adverse drug reactions (Routledge et al. 2004; Budnitz et al. 2011). We decided to test this hypothesis by selecting genes with evidence of epigenetic aging and testing levels of 5mC and histone 3 lysine 9 acetylation (H3K9ac) at these genes for association with gene function and rates of drug metabolism in the liver.

Our first step in the process was to identify genes that show some evidence of epigenetic aging. At the time that we started this project, in 2016, there were no epigenome-wide studies of aging in the liver of humans or model organisms, to the best of our knowledge. However, several studies of epigenetic aging had been conducted in human blood and some other tissues. We therefore collected information on epigenome-wide studies of aging in humans and focused on those conducted using Illumina 5mC microarrays because of the ease of comparing results across studies. We found three studies where the *CYP2E1* gene changed with age (Horvath 2013; Steegenga et al. 2014; Peters et al. 2015). It is also notable that *CYP2E1* was one of the loci comprising the epigenetic clock of Horvath (Horvath 2013), a highly accurate multi-locus predictor of biological aging. To study epigenetic aging of *CYP2E1* in the liver, we examined the mouse ortholog *Cyp2e1* in liver tissue from genetically homogenous mice aged under controlled conditions in the National Institute on Aging rodent colonies. Using these mice minimizes extraneous genetic and environmental variation that could hamper our study if using human post-mortem tissue. Our mouse samples ranged from 4 to 32 months of age, spanning young adulthood to very old age (approximately 20 to 80+ years old in human terms).

We first confirmed that 5mC levels at the *Cyp2e1* promoter, specifically the mouse region homologous to the human region identified by Horvath (Horvath 2013), did in fact change with age in mouse liver. In the cohort of mice aged 4 to 32 months, we observed significant hypermethylation in the *Cyp2e1* promoter of aged mice (P=0.002). We then proceeded to assay *Cyp2e1* mRNA expression and protein expression levels in the same liver samples using quantitative polymerase chain reaction (PCR) and western blotting, respectively. Both mRNA and protein expression were significantly (P<0.05) suppressed in the older samples relative to the younger samples. This is the expected direction of effect given that 5mC, typically associated with gene repression, increased with age at the *Cyp2e1 promoter*. We also assayed histone 3 lysine 9 acetylation (H3K9ac) and histone 3 lysine 27 acetylation (H3K27ac) using chromatin immunoprecipitation and RT-qPCR. While no

effect was detected with H3K27ac, we observed significant changes with age in H3K9ac levels. Finally, we tested if these epigenetic changes were associated with Cyp2e1-mediated metabolism. Therefore, we extracted microsomes from the same livers and assayed the rate of chlorzoxazone metabolism. Chlorzoxazone is a muscle relaxant that is almost exclusively metabolized by CYP2E1 and is therefore used as a CYP2E1 probe substrate (Court et al. 1997). We measured the Cyp2e1-mediated conversion of chlorzoxazone to 6-hydroxychlorzoxazone and tested for association between age-related epigenetic changes (5mC and H3K9ac) at *Cyp2e1* and chlorzoxazone intrinsic clearance. We found significant ($P<0.05$) associations for both 5mC and H3K9ac. Notably the effect sizes were quite large, with *Cyp2e1* 5mC and H3K9ac levels showing correlations with chlorzoxazone intrinsic clearance of −0.3 and 0.5 respectively. Overall, this analysis demonstrated a substantial effect of epigenetic aging on the expression and function of CYP2E1 (Kronfol et al. 2020).

Following our analysis of the Phase I drug metabolism enzyme CYP2E1, we decided to focus our attention on enzymes involved in Phase II drug metabolism. Once again, we looked to human studies to identify candidate Phase II (conjugative) drug metabolism genes potentially subject to epigenetic aging. We identified sulfotransferase 1A1 (*SULT1A1*) as the human Phase II gene showing most evidence for epigenetic aging (Reynolds et al. 2014; Steegenga et al. 2014) and we tested its epigenetic states and expression in aged mouse livers, as carried out for *Cyp2e1* above. For *Sult1a1*, we found significant hypomethylation with aging coupled to increased H3K9ac. We also found that H3K9ac explained almost one quarter of the variation in *Sult1a1* expression levels across all ages. Therefore, we were once again able to detect a substantial influence of epigenetic aging on expression of this drug metabolism gene (Kronfol et al. 2021).

Our studies were limited to the mouse and a few specific genes. Future studies should repeat these analyses for the entire epigenome using human liver tissue, if available. Moreover, our studies were correlational and we were unable to manipulate the epigenetic levels to unambiguously show the causal influence of epigenetic marks. Nevertheless, the effect sizes of epigenetic aging on expression and function of drug metabolizing enzymes appear to be substantial and further work is warranted. Our aspirational goal for this research program is to identify epigenetic biomarkers of drug metabolism in aging that could be used to adjust dosing and reduce the incidence of adverse drug reactions in older patients (Kronfol et al. 2017).

Conclusion

Recent advances in the field of epigenetics have uncovered several regulatory mechanisms that control aspects of DMEs, including miRNA, epigenetic aging, and lncRNA.

Dr. Ning discussed the types of ncRNAs, such as miRNAs and lncRNAs, and their various roles in regulating ADME (absorption, distribution, metabolism, and excretion) processes. miRNAs repress gene expression via direct interaction with target mRNA thus leading to RISC-mediated degradation. For example, miR-27b targets *CYP1B1* mRNA, leading to lowered gene expression. Alternatively, lncRNAs modulate gene expression via several mechanisms, including direct interaction with regulatory proteins. For instance,

LINC00844 is known to alter gene expression of multiple CYP enzymes by interacting with regulatory proteins, miRNAs, and nuclear receptors. Additionally, lncRNAs and miRNAs may interact directly thus adding further layers of complexity to post-transcriptional epigenetic regulation.

Dr. Lazarus demonstrated that miRNA, specifically miR-491-3p, exerts a family-wide repressive effect on the expression of UGT1A enzymes due to a conserved 3'-UTR binding site across each protein isoform. The repressive effect results in measurable decreases in raloxifene glucuronidation *in vitro* and these results may translate to *in vivo*, thus partially explaining the variable UGT activity observed in the clinic. Additional miRNAs were also discussed that may similarly regulate other UGTs and DMEs.

Finally, Dr. McClay discussed epigenetic aging, which is the phenomenon of non-random epigenetic modification of specific genes associated with increased age that impacts the activity of certain biological pathways that occurs with increased age. CYP2E1 was cited as an example in mice because the *Cyp2e1* gene promoter region can be repressed or activated by methylation or acetylation, respectively. Gene promoter region hypermethylation was observed in elderly mice, resulting in decreased mRNA and protein expression. Across the entire mouse population, microsomal Cyp2e1 intrinsic clearance was positively correlated with promoter region acetylation and negatively correlated with promoter region methylation. Screening efforts also identified *Sult1a1* as a potential target of epigenetic aging with increased age being correlated with gene promoter hypomethylation. Further experimentation is required to identify additional DMEs impacted by epigenetic aging as well as assessing clinical relevance in humans. Overall, both Phase I and Phase II metabolism have been shown to be controlled, in part, by epigenetic modulation of gene expression. Future work incorporating *in silico*, *in vitro*, and *in vivo* models will help expand our understanding of the clinical relevance of epigenetics in drug ADME. These advances will enable more accurate predictions of drug ADME and safety in years to come.

Future perspectives

Different regulators (e.g., miRNAs, lncRNAs, and NRs), act via several mechanisms which can have different outcomes, such as gene activation or gene suppression (Dluzen and Lazarus 2015; Li D, Knox, et al. 2019; Wang J et al. 2020). As increasing evidence emerges about the mechanisms involved in the process of epigenetic regulation of DMEs, these processes will begin to be used in drug development to improve the drug screening and selection process. However, screening for and evaluating epigenetic modifications and their impact on ADME and pharmacokinetics is not currently routine practice. Furthermore, although the current conference report highlights epigenetics in DMEs, effects of epigenetics on drug transporters should be further studied to understand their influence in ADME processes.

As seen in the examples discussed herein and from other studies, *in vitro* evidence clearly demonstrates that epigenetic modifications contribute to the differential expression of DMEs. These epigenetic changes entail different mechanisms with various external and environmental influences (Kringel et al. 2021). Given the direct interplay between DME

expression and activity and pharmacokinetic parameters such as bioavailability, volume of distribution, and half-life, epigenetic modifications can alter clearance of drugs leading to variable exposure (Ingelman-Sundberg et al. 2013). Additional studies are required to evaluate the clinical relevance of such impacts on the pharmacokinetics of drugs. One barrier to directly study epigenetic signatures and their implications in pharmacokinetics and pharmacodynamics is that they are often tissue specific. Blood is commonly sampled as a surrogate tissue, and extracellular vesicles including exosomes and microvesicles released by cancer and immune cells containing epigenetic-related players may help serve as a biomarker of epigenetic regulation in disease or drug therapy (Lorico et al. 2015). However, it remains critical to obtain a biopsy from the tissue of interest to adequately assess epigenetic regulation of other specific genes (Bonder et al. 2014; Hannon et al. 2015; Lowe et al. 2015; Lauschke et al. 2019). Furthermore, an unmet need in drug development is to develop quantitative models to predict the magnitude of the effect epigenetic modifications will have on pharmacokinetics in specific patient populations. Although *in silico* and *in vitro* studies have been conducted to evaluate epigenetic mechanisms and their impacts on DME expression and activity, quantitative predictive models need to be developed to account for these changes during drug development and can allow for a precision medicine approach (Stern et al. 2016). Development of quantitative systems pharmacology (QSP) models or PKPD relationships which account for epigenetic mechanisms would be useful in predicting time-dependent changes in the human dose for compounds which modulate epigenetics. The predictive models would also be useful to evaluate the impact of factors which influence epigenetics like diet, age, and comedications, which will be useful in calculating personalized human dose. However, a current barrier is the lack of robust datasets detailing the different mechanisms and pathways that regulate DMEs through epigenetic mechanisms, which would be a prerequisite for development of QSP models. Epigenetic regulations which affect the expression levels of different DMEs as seen above might also explain the observed clinical variability in pharmacokinetics.

Certain clinical compounds which modulate epigenetics can lead to changes in DME expression which can cause and exacerbate DDIs. Evaluation of induction- and/or inhibition-mediated changes in DMEs have become a routine during drug development, which are useful to predict clinical drug-drug interactions. Reversible inhibition mediated DDIs are predicted with reasonable accuracy; however, prediction of irreversible and induction mediated DDIs are less accurate (Einolf et al. 2014; Fowler et al. 2017; Yadav J et al. 2018; Lu and Di 2020; Yadav J. et al. 2020). There are several factors that cause misprediction (Treyer et al. 2019; Tseng et al. 2021). Although *in vitro* studies have suggested that epigenetic mechanisms could potentially lead to DDIs, these have not been evaluated or remain poorly understood. Moreover, drugs which modulate enzyme activity via inhibition or induction could also modulate apparent activity through epigenetic mechanisms. Consideration of epigenetic mechanisms along with other mechanisms like induction and inhibition might be useful to improve the prediction accuracy. Since there is limited clinical evidence of DDIs due to epigenetic mechanisms, such evaluations are rarely performed. More systematic studies need to be performed to assess the role of epigenetic mediated changes in DDIs.

Despite current challenges, the future of epigenetics in drug discovery and development is bright. We anticipate key discoveries contributing to advancing the current understanding of epigenetic modulations and their downstream effects on pharmacokinetics and pharmacodynamics. As we continue to gather epigenomic data and understand its contribution to disease and pharmacotherapy, these learnings may also contribute to the understanding of drug resistance (Cascorbi and Schwab 2016). We foresee that the optimization and implementation of current epigenetic assays in drug discovery will help close knowledge gaps and contribute to more robust datasets, which will be used to train predictive models to understand implications of epigenetics on ADME genes, pharmacokinetics, drug response, and toxicity – working toward precision medicine to benefit all patients.

Abbreviations

5mC	5-methylcytosine, methylated cytosine typically associated with gene repression
ADME	absorption, distribution, metabolism, excretion
AHR	Aryl hydrocarbon receptor
CAR	Constitutive androstane receptor
CYP	Cytochrome P450, phase I drug metabolizing enzymes
DDI	Drug-drug interaction
DME	Drug metabolizing enzymes
H3K9ac	Histone 3 Lysine 9 Acetylation
H3K27ac	Histone 3 Lysine 27 Acetylation
HNF	Hepatic nuclear factor
HuH	human hepatoma-derived cells
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
lncRNA	long non-coding RNA
mRNA	messenger RNA
miRNA	microRNA
ncRNA	Non-coding RNA
NR	Nuclear receptors
PCR	Polymerase chain reaction
PK	Pharmacokinetics

PD	Pharmacodynamics
PXR	Pregnane X factor
QSP	Quantitative systems pharmacology
RISC	RNA-induced silencing complexes
SHP	Small heterodimer partner
SNP	Single nucleotide polymorphism
SULT1A1	Sulfotransferase 1A1
UGT	UDP-glucuronosyltransferases, phase II drug metabolizing enzymes
UTR	Untranslated region
VDR	Vitamin D receptor

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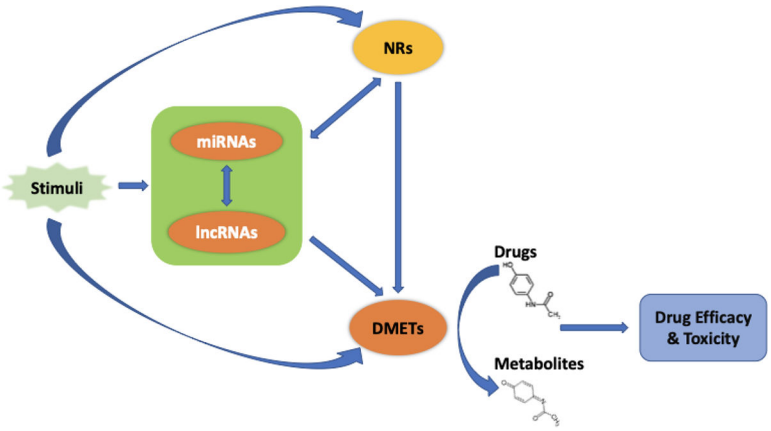

Figure 1.

Crosstalk: miRNAs, lncRNAs, NRs and DMETs work as a network. The interaction among miRNAs, lncRNAs, NRs and DMETs constitutes a network that works together to respond to endogenous and exogenous stimuli. The network is a key modulator contributing to drug efficacy and safety. Figure reproduced with permission from Elsevier.

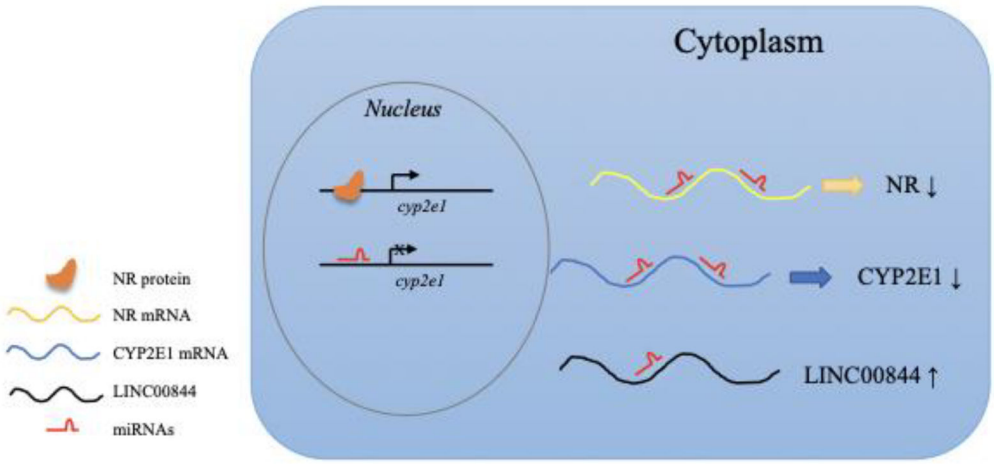

Figure 2.

Diverse mechanisms for ncRNA regulation of CYP2E1 expression. Multiple miRNAs may recognize and bind to different regions of CYP2E1 DNA and mRNA and thus directly inhibit or promote CYP2E1 expression. miRNAs may also downregulate CYP2E1 by directly binding to mRNAs of CYP2E1 transactivators such as HNF1? and PXR and repressing the expression of the transactivators. miR-486-5p indirectly increases the expression of CYP2E1 by upregulating lncRNA LINC00844.

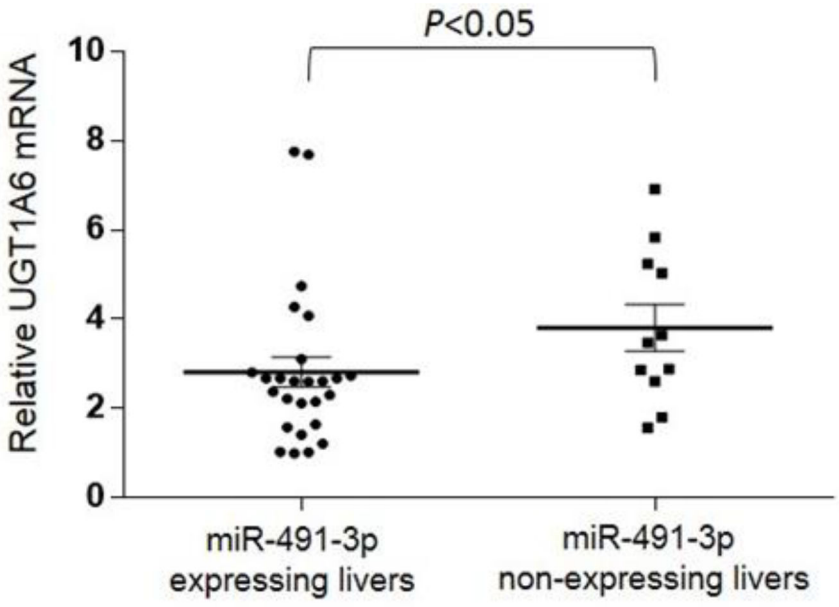

Figure 3.

Expression of UGT1A6 mRNA versus miR-491-3p in human liver specimens. mRNA levels of UGT1A6 (n=37 mRNA vs. miR-491-3p) were quantified in normal liver samples via RT-qPCR and normalized to RPLPO and RNU6B, respectively. UGT1A6 mRNA expression levels were examined in liver specimens stratified by expression vs. no expression of miR-491-3p. Dots represent the mean ± S.E. of three independent replicates. Reproduced with permission from Dluzen et al., 2014; J Pharmacol Exper Ther.

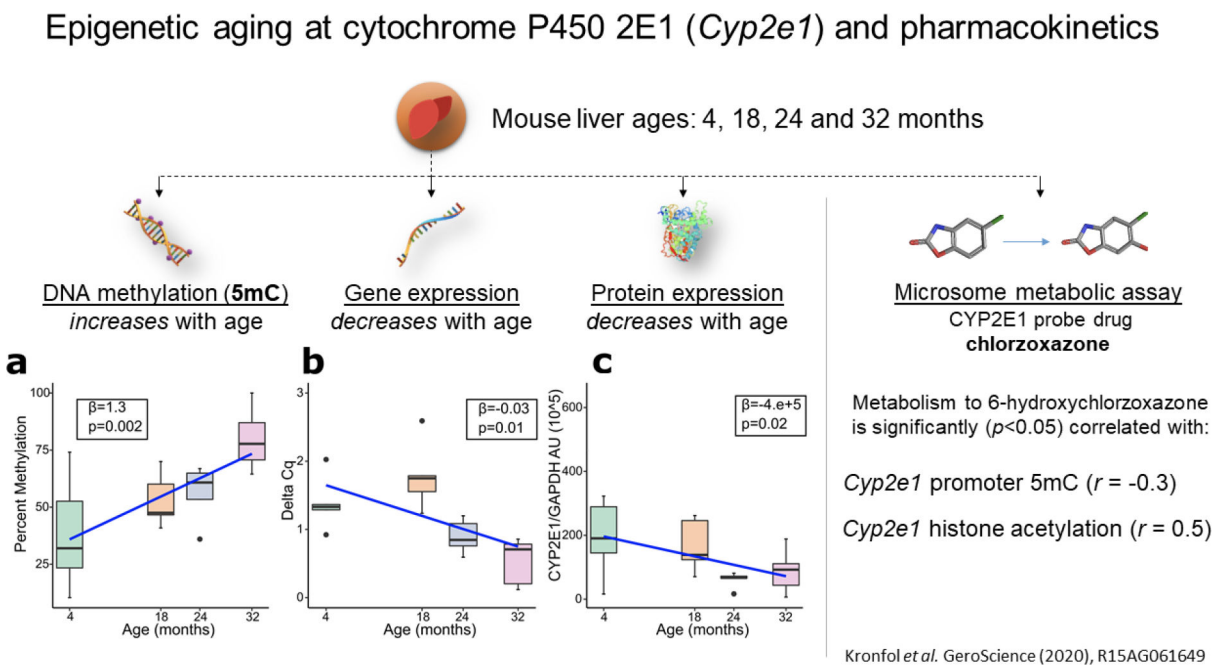

Figure 4.

Summary of a study of epigenetic aging effects on regulation of CYP2E1. Aged mouse liver samples were studied using several different assays. In panel a, we show that DNA methylation (5mC) levels significantly increased with age at the Cyp2e1 gene promoter in mouse liver, resulting in a concomitant decrease in gene (panel b) and protein (panel c) expression levels. We also assayed the rate of chlorzoxazone metabolism in microsomes from the same livers. Chlorzoxazone is a CYP2E1 probe drug, meaning that it is almost exclusively metabolized by that CYP450 enzyme. We found a substantial negative correlation between intrinsic clearance of chlorzoxazone and Cyp2e1 5mC levels and a substantial positive correlation with Cyp2e1 histone acetylation levels, indicating a substantial effect of epigenetic aging on the function of CYP2E1. Reproduced with permission from Kronfol et al., GeroScience (2020).