Special Section – New Models in Drug Metabolism and Transport—Minireview

Humanized UGT1 Mice, Regulation of UGT1A1, and the Role of the Intestinal Tract in Neonatal Hyperbilirubinemia and Breast Milk-Induced Jaundice

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ABSTRACT

Neonatal hyperbilirubinemia and the onset of bilirubin encephalopathy and kernicterus result in part from delayed expression of UDP-glucuronosyltransferase 1A1 (UGT1A1) and the ability to metabolize bilirubin. It is generally believed that acute neonatal forms of hyperbilirubinemia develop due to an inability of hepatic UGT1A1 to metabolize efficiently bilirubin for clearance through the hepatobiliary tract. Newly developed mouse models designed to study bilirubin metabolism have led to new insight into the role of the intestinal tract in controlling neonatal hyperbilirubinemia. Humanization of mice with the UGT1 locus (hUGT1 mice) and the UGT1A1 gene provide a unique tool to study the onset of hyperbilirubinemia since the human UGT1A1 gene is developmentally regulated during the neonatal period in hUGT1 mice. A new mechanism outlying developmental expression of intestinal UGT1A1 is presented and its implications in the control of neonatal hyperbilirubinemia discussed. New findings linking breast milk protection against necrotizing enterocolitis and intestinal control of UGT1A1 may help explain the contribution of breast milk toward the development of neonatal hyperbilirubinemia. Our findings outline a new model that includes an active intestinal ROS/IκB kinase/nuclear receptor corepressor 1 loop that can be applied to an understanding of breast milk-induced jaundice.

Introduction

UDP-glucuronosyltransferase (UGT) 1A1, the only transferase capable of conjugating bilirubin (Bosma et al., 1994), is developmentally delayed in newborn children (Burchell et al., 1989). Thus, approximately 80% of newborns have some form of hyperbilirubinemia (Watchko, 2009; Maisels, 2015). Most cases have a benign outcome, except in situations when the rapid onset of severe neonatal hyperbilirubinemia (SNH) is not monitored nor prevented. Shortly after birth, an increase in red blood cell turnover occurs where heme is released from hemoglobin and further degraded by heme oxygenase to carbon monoxide and biliverdin, which is further reduced by biliverdin reductase to bilirubin (Tenhunen, 1976; Dennerly et al., 2001; Ayer et al., 2016) (Fig. 1). Once in the circulation, unconjugated bilirubin, which is hydrophobic, is readily absorbed into tissues such as the liver. Hyperbilirubinemia develops when a rise in total serum bilirubin (TSB) levels exceeds the capacity of hepatic UGT1A1 to drive bilirubin conjugation, an event that leads to the excretion of the glucuronide by multidrug resistance protein 2 (Kullak-Ublick et al., 2000) into the biliary channels for deposit in the gastrointestinal tract. Thus bilirubin glucuronidation by UGT1A1 is the rate limiting step in bilirubin excretion.

Major risk factors that lead to the onset of SNH include accelerated hemolysis (Wong and Stevenson, 2015) brought on by Rhesus disease and ABO incompatibility, glucose-6-phosphate dehydrogenase deficiency, infections, in addition to premature birth and breast milk (Bhutani et al., 2013; Maisels et al., 2014; Olusanya et al., 2015; Wong and Stevenson, 2015; Cunningham et al., 2016). As bilirubin accumulates early after birth and exceeds the capacity to bind to serum proteins, unconjugated bilirubin accumulates in brain tissue.
limiting step. Therefore, insufficient UGT1A1 enzyme activity may lead to the rapid build-up of free bilirubin, a condition called hyperbilirubinemia or jaundice.

Bilirubin is transported through OATP transporters. In the hepatocyte, bilirubin is metabolized through UGT1A1-catalyzed glucuronidation to form either mono- or di-glucuronides. The conjugates are secreted into bile via the multidrug resistance protein 2 (MRP2), and they in turn can be hydrolyzed by β-glucuronidase in the intestinal tract and then undergo enterohepatic circulation. Conventionally, liver is considered as the organ for bilirubin detoxification. It is worth noting that bilirubin is metabolized solely by UGT1A1, which becomes the rate limiting step. Therefore, insufficient UGT1A1 enzyme activity may lead to the rapid build-up of free bilirubin, a condition called hyperbilirubinemia or jaundice.

Extreme levels of TSB can lead to early or acute bilirubin encephalopathy (ABE) (Watchko, 2016), presented early as lethargy and poor feeding, but can progress to hypo- and hypertonia, high-pitched crying, muscle spasms, opisthotonus, seizures, and even death (Bhutani and Johnson-Hamerman, 2015). The more chronic form, which proceeds ABE, is termed kernicterus (Kaplan et al., 2011). Affected individuals are characterized by choreoathetotic cerebral palsy, dystonic/athetoid movement disorders, hearing loss, ocular motor defects, hypotonia, and ataxia, and it has been linked to cerebellar involvement (Dennergy et al., 2001; Tabarki et al., 2002; Kaplan et al., 2011; Bhutani and Johnson-Hamerman, 2015). Kernicterus was originally classified as the pathologic finding of yellow staining (icterus) of deep nuclei in the brain. Improved imaging technology has allowed for visualization of bilirubin damage to specific regions, e.g., the globus pallidus and subthalamic nucleus. Thus, there are two major periods after birth where SNH can lead to toxicity. ABE is designated for the early signs of toxicity, and chronic bilirubin encephalopathy (CBE) covers the long-term consequences of an acute but resolved process. Because the clinical signs of CBE are broad, it has been recommended to abandon the use of CBE for the term kernicterus spectrum disorder (KSD) (Le Pichon et al., 2017).

SNH, which leads to ABE and KSD, has been observed worldwide (Kaplan et al., 2011), with the highest incidences being recorded in sub-Saharan Africa and South Asia (Bhutani et al., 2013; Olausanya et al., 2016). A report from the Nigerian Society of Neonatal Medicine suggested that extreme hyperbilirubinemia accounts for at least 5% of all neonatal mortalities in Nigeria (Olausanya et al., 2016). Recent evidence shows that SNH, estimated to impact over 1 million children every year, is associated with substantial mortality and permanent morbidities (Bhutani et al., 2013; Olausanya et al., 2018). SNH accounted for over 1300 newborn deaths per 100,000 globally, ranking as the seventh most dangerous contributor toward infant deaths (Olausanya et al., 2018). If global deaths linked to SNH in children under 5 years of age are estimated, the mortality rate in Indonesia is calculated to exceed 40% of all deaths. While conventional therapies entail aggressive blue light therapy or blood transfusions once extreme hyperbilirubinemia has been diagnosed (Dennergy et al., 2001), the inability to conjugate effectively bilirubin resulting from developmental delay in expression of UGT1A1 can ultimately give rise to ABE and other KSDs (Johnson et al., 1999; Yueh et al., 2017). While genetic predisposition and environmental influences play key roles in driving TSB levels to potentially toxic levels, an understanding of the developmentally regulated molecular and cellular mechanisms that underlie the regulation of UGT1A1 expression have not been elucidated. With the development of new animal models designed to examine expression of the human UGT1A1 genes, progress has been made in characterizing the regulatory events that underlie the expression of the UGT1A1 gene during development. Our understanding of the events that control expression of the human UGT1A1 gene has provided new insight toward the mechanisms leading to hyperbilirubinemia and disease.

**Results**

**Humanized UGT1 Mice and Neonatal Hyperbilirubinemia.** Cellular and molecular events that underlie the regulation of the UGTs, especially those encoded by the human UGT1 gene family, have been studied in humanized transgenic mice that express the human UGT1 locus (TgUGT1 mice) in a Ugt1-null background (Ugt1−/− mice) (Fujikawa et al., 2010). In humans, there are nine UGT1A genes, including UGT1A1, that are encoded by the UGT1i locus (Clarke et al., 1997; Mackenzie et al., 1997; Gong et al., 2001). To examine the importance of the human UGT1 locus and the UGT1A1 gene in control of serum bilirubin, an inactivating mutation was engineered into exon 4 of the murine Ugt1ai gene, generating Ugt1−/− mice (Nguyen et al., 2005). The UGT1A proteins are encoded by five exons, with coding sequence for exons 2–5 being identical. Located on chromosome 1, the organization of the murine Ugt1 locus is encoded by >150 kDa of DNA, with exons 2–5 located at the 3′-end and flanked consecutively by an array of nine cassette exons that encode the N-terminal portion of each UGT1A protein. Thus the UGT1i locus in humans and the Ugt1 locus in mice encodes nine functional UGT1A proteins, each composed of a unique N-terminal region encoded by one of the 5-flanking exons while sharing an identical C-terminal region encoded by exons 2–5. When heterozygous Ugt1+− mice were bred, offspring with a Ugt1−/− genotype were born with a striking orange skin color that resulted from severely elevated UCB levels that developed into lethal SNH between 4 and 7 days after birth. To recover the lethality resulting from nonfunctional murine UGT1A1 expression, TgUGT1 mice that carried

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**Fig. 1.** Bilirubin production and metabolism. Bilirubin is a byproduct of heme degradation when phagocytic cells, primarily macrophages, remove aged red blood cells (RBC) from the circulation. The production of bilirubin through heme oxygenase is the first major step, after which bilirubin is produced through biliverdin reductase. Bilirubin, on the other hand, is oxidized to biliverdin to form the biliverdin reductase cycle that functions as an important antioxidant in protecting lipid oxidation. Depending on the part of the body in which the breakdown occurs, such as Kupffer cells of the liver or cells in the spleen and bone marrow, the produced bilirubin eventually is released into the circulation, where bilirubin is taken up by plasma albumin protein and transported throughout the body. Once bilirubin reaches the liver, bilirubin enters the hepatocyte either passively or actively through OATP transporters. In the hepatocyte, bilirubin is metabolized through UGT1A1-catalyzed glucuronidation to form either mono- or di-glucuronides. The conjugates are secreted into bile via the multidrug resistance protein 2 (MRP2), and they in turn can be hydrolyzed by β-glucuronidase in the intestinal tract and then undergo enterohepatic circulation. Conventionally, liver is considered as the organ for bilirubin detoxification. It is worth noting that bilirubin is metabolized solely by UGT1A1, which becomes the rate limiting step. Therefore, insufficient UGT1A1 enzyme activity may lead to the rapid build-up of free bilirubin, a condition called hyperbilirubinemia or jaundice.
and expressed the entire UGT1 locus (Chen et al., 2005) were crossed with Ugt1+/− mice to generate TgUGT1/Ugt+/− mice. These founders were then backcrossed to generate TgUGT1/Ugt+/− mice or humanized UGT1 (hUGT1) mice. Expression of the human UGT1A1 gene rescued the lethality attributed to SNH in Ugt1−/− mice (Fig. 2).

Developmental and tissue specific regulation of the UGT1A genes in hUGT1 mice (Chen et al., 2005; Seneke-Effenberger et al., 2007; Fujiwara et al., 2010; Yueh et al., 2017) has been shown to reflect similar patterns of expression as characterized in human tissues (Strassburg et al., 1997a,b, 1998a,b; Tukey and Strassburg, 2000), allowing us to leverage the use of hUGT1 mice in experiments designed to characterize the mechanisms leading to tissue specific, inducible and developmental expression of the human UGT1A genes. An important discovery was the finding that the human UGT1A1 gene, which is developmentally delayed in newborn children, is also developmentally delayed or repressed in hUGT1 neonatal mice (Fujiwara et al., 2012). Since UGT1A1 is the sole enzyme responsible for the metabolism of serum bilirubin, this delay in hepatic UGT1A1 gene expression leads to SNH in neonatal hUGT1 mice, with lethality affecting 10% of the newborns, as displayed by dramatic motor impairment with seizure-like movements. The brains of hUGT1 mice that develop seizures concentrate bilirubin in the tissues, which is evident by an intense yellow or icteric appearance (Fujiwara et al., 2010). The icteric pattern is not regionally localized as the tissues, which is evident by an intense yellow or icteric appearance.

The presence of the human UGT1A1 gene locus and UGT1A1 from the germ line is lethal, which results from the accumulation of unconjugated bilirubin. The loss of the Ugt1 locus and UGT1A1 from the germ line is lethal, with all the Ugt1−/− mice dying within 2 weeks after birth. When we crossed mice carrying the entire human UGT1A1 gene locus as a transgene with mice carrying the Ugt1-null allele, humanized UGT1 mice (hUGT1) were generated. The presence of the human UGT1A1 gene rescues the lethality associated with the absence of murine UGT1A1. All newborn hUGT1 mice develop severe neonatal hyperbilirubinemia (SNH). Total serum bilirubin (TSB) levels in hUGT1 newborns increase after birth, reach peak levels at approximately 2 weeks after birth with the levels returning to a normal range after weaning. The majority of the hUGT1 mice can mature to adulthood. Approximately 10% of newborn hUGT1 mice develop seizures and die. These mice accumulate high concentrations of bilirubin in brain tissue. The development of SNH results from delayed liver UGT1A1 expression. UGT1A1 expression in the small intestine is associated with saving the lethality of hUGT1 mice and lowering of TSB levels.

![Figure 2](image-url)
induction of liver or intestinal UGT1A1 followed by reductions in TSB levels. The reduction in TSB levels early in the neonatal window assures that the mice avoid bilirubin-induced neurologic dysfunction (Fig. 3).

**PXR is a Steroid Target, Repressor Protein, and Regulator of UGT1A1.** The importance of PXR as a regulator of endocrine control, drug metabolism, and hyperbilirubinemia started to emerge when we predicted that PXR controlled UGT-directed drug metabolism during gestation. For example, circulating TSB levels are lower in women during pregnancy, indicating that bilirubin metabolism is accelerated through induced UGT1A1 (Bacq et al., 1996). Labetalol, an antihypertensive drug that is metabolized primarily by UGT1A1, shows increased clearance in the second and third trimesters of pregnancy compared with the postpartum period (Jeong et al., 2008). Pregnancy results in dramatic surges in hormones during gestational development, with increases in steroids such as estrogens, progestins, and the glucocorticoids. Since surges in hormones during gestational development, with increases in the postpartum period (Jeong et al., 2008). Pregnancy results in dramatic surges in hormones during gestational development, with increases in steroids such as estrogens, progestins, and the glucocorticoids. Since surges in hormones during gestational development, with increases in surges in hormones during gestational development, with increases in

-1A3, -1A4, -1A6, and -1A9 gene expression in hUGT1/Pxr−/− mice are greatly reduced during the later stages of gestation. When the glucocorticoid dexamethasone (DEX) was administered to hUGT1 mice, each of the five UGT1A genes expressed in the liver was induced. Although activation of the glucocorticoid receptor by DEX was shown previously to activate UGT1A1 reporter gene constructs, DEX had no effect on induction of UGT1A1 in hUGT1/Pxr−/− mice, indicating that the induction of the UGT1A genes by glucocorticoids is facilitated solely by activation of PXR in vivo.

**Transcriptional Silencing of the UGT1A1 Gene.** In breeding experiments to create hUGT1/Pxr−/− mice, analysis of TSB levels in neonatal hUGT1 mouse demonstrated that PXR plays a key role in controlling TSB levels during development. In neonatal hUGT1/Pxr−/− mice, liver UGT1A1 is induced when compared with its expression in hUGT1 mice (Chen et al., 2012). The increased levels of liver UGT1A1 in hUGT1/Pxr−/− mice lead to reduced TSB levels. This finding suggests that in the absence of endogenous ligand, the physiologic role of PXR leads to repression of the UGT1A1 gene during early development. More importantly, blocking activation of the UGT1A1 gene by PXR is controlled in liver through neonatal developmental processes, since its deletion in adult hUGT1/Pxr−/− mice has no impact on UGT1A1 gene expression, except for pregnant mice. Thus PXR is involved in transcriptional silencing of the UGT1A1 gene (Fig. 3).

In combination with nuclear receptors, transcriptional silencing, or repression is largely achieved with two prototypical nuclear repressor corepressors, the silencing mediator of retinoid and thyroid hormone...
receptor (SMRT), and the nuclear receptor corepressor 1 (NCoR1) (Francis et al., 2003; Mottis et al., 2013), suggesting that NCoR1 and/or SMRT participate in control of UGT1A1 expression during development. Mechanistically, corepressor proteins interact with XNRs, such as RXR, LXR, PPAR, and PXR, to control collectively gene expression patterns by recruiting chromatin-modifying enzymes that limit nucleosomal DNA accessibility and transcriptional activation. In the absence of either the nuclear receptor or the corepressor, additional coactivators cooperate with a different set of chromatin-modifying enzymes to promote transcriptional activation. In experiments targeted to delete NCoR1/SMRT in various tissues or cell lines, deletion of the corepressor spontaneously leads to nonligand activation of the XNRs, facilitating transcriptional activation of XNR target genes (Li et al., 2013). Often transcriptional activation of downstream target genes by spontaneously activated XNRs provides a genomic signature that can be used to identify the exact XNR involved in the response. To determine if spontaneous activation of UGT1A1 expression provides a genomic signature that can be used to identify the exact XNR involved in the response. To determine if spontaneous activation of UGT1A1 expression provides a genomic signature that can be used to identify the exact XNR involved in the response. To determine if spontaneous activation of UGT1A1 expression provides a genomic signature that can be used to identify the exact XNR involved in the response. To determine if spontaneous activation of UGT1A1 expression provides a genomic signature that can be used to identify the exact XNR involved in the response. To determine if spontaneous activation of UGT1A1 expression provides a genomic signature that can be used to identify the exact XNR involved in the response.

An important role for NCoR1 in regulating UGT1A1 gene expression in neonates has emerged (Chen et al., 2017). We generated floxed Ncor1 mice under a hUGT1 background (TgUGT1/UGT1△/Ncor1F/F), named as F/F△ mice, and then further knocked out NCoR1 in liver (ΔHEP△ mice) and intestinal tissue (ΔIEC△ mice) of hUGT1 mice. When TSB levels were examined in newborn mice, wild-type F/F△ and ΔHEP△ mice exhibited similar patterns of neonatal hyperbilirubinemia as observed in hUGT1 mice. Deletion of NCoR1 in liver tissue had no impact on hepatic UGT1A1 gene expression. Strikingly, following intestinal-specific deletion of NCoR1, the development of neonatal hyperbilirubinemia was completely diminished in ΔIEC△ mice. The impact of deleting intestinal NCoR1 led to the derepression of intestinal UGT1A1 expression with significant induction of protein occurring in both the longitudinal axis (i.e., all sections of the small intestine (SI)) as well as the crypt-villi axis. Mechanistically, derepression or induction of UGT1A1 following NCoR1 knockout was observed only during neonatal developmental, since derepression of UGT1A1 did not occur in adult ΔIEC△ mice. This interesting observation indicates that NCoR1 plays a key role as a corepressor protein in developmentally important processes linked to intestinal homeostasis. These processes may be directly, or indirectly, linked to derepression of the UGT1A1 gene (Fig. 4).

When we conducted transcriptional profiling of intestinal tissue by implementing RNA-seq analysis of F/F△ and ΔIEC△ mice, gene ontology analysis identified the metabolic pathway as the most significantly altered pathway in ΔIEC△ mice, followed by oxidative phosphorylation and drug metabolism. Deleting NCoR1 promoted expression of PPARα and LXRα target genes, indicating that NCoR1 is docked on these genes during neonatal development. NCoR1 deletion had an extensive impact on fatty acid and energy metabolism, in addition to lipid and cholesterol metabolism. Messenger RNA levels of TCA cycle genes and oxidative phosphorylation genes were coordinately increased. These accelerated metabolic pathways indicated that NCoR1 was repressing key pathways essential for epithelial cell maturation and synthesis. In ΔIEC△ neonatal mice, the average length of the small intestine was repressed as mean ± SEM, ***p<0.001, ****p<0.0001, Student’s t test. Two-way ANOVA analysis for ΔIEC△ vs F/F△ (p<0.0001).
Intestines was ~8% longer and 15% heavier than in F/F<sup>+</sup> littermates, indicating increased intestinal proliferation. Histology and electron microscopy findings indicated greatly matured brush border and microvilli in ΔIEC<sup>+/+</sup>. When we examined cellular proliferation signals, immunostaining of Ki-67, a cellular marker for proliferation, was increased in both ΔIEC<sup>+/+</sup> duodenum and jejunum. In support of this finding, bromodeoxyuridine staining, used to detect proliferating cells, showed a progressive increase in positive epithelial cells. These findings demonstrated that NCoR1 deletion was the catalyst to accelerate cell proliferation and promote epithelial cell proliferation. In support of cellular proliferation, upregulation of intestinal maturation Sucrase isomaltase, a brush border glucosidase that only exists in differentiated intestinal tissue and is a marker for enterocyte maturation, was significantly induced in ΔIEC<sup>+/+</sup> mice, along with other maturation markers that included the duodenal-specific Akp3 gene and the jejunal-specific Krt20 gene. These studies confirmed that neonatal NCoR1 expression serves to repress gene expression, including UGT1A1, while mechanistically controlling the developmental changes underlying intestinal maturation from the neonatal stage to the adult tissue.

**NCoR1 Regulation and Expression of Intestinal UGT1A1.**

Intestinal NCoR1 may be the centerpiece in controlling bilirubin metabolism in intestinal epithelial cells (IECs) during the neonatal window. The functional ability of NCoR1 to promote its repressive properties with nuclear receptors as well as its potential to activate gene transcription have been linked to phosphorylation events (Mottis et al., 2013; Jo et al., 2015), controlled by a number of different kinases (Fernández-Majada et al., 2007; Huang et al., 2009; Choi et al., 2013). For example, the loss of nuclear NCoR1 in malignant melanoma was associated with IKK-dependent phosphorylation of specific NCoR1 serine residues (Gallardo et al., 2015). When NCoR1 is phosphorylated, it dissociates from chromatin and migrates to the cytoplasm, a move that stimulates transcriptional derepression events. Findings from our studies have also established close ties between IKK activation, NCoR1 function, and reductions in TSB levels in hUGT1 mice (Chen et al., 2017). Intestinal IKKβ is critical in controlling basal UGT1A1-dependent metabolism of bilirubin. When IKKβ is deleted in IECs in hUGT1 mice (hUGT1/ikk<sup>B<sup>ΔIEC</sup></sup> mice), basal expression of intestinal UGT1A1 in neonatal mice drops, leading to elevations in TSB levels that are lethal (Liu et al., 2016). When NCoR1 is dephosphorylated, as potentially exists when IKKβ is deleted, we hypothesize that the NCoR1/NR complex tightens its binding to chromatin to improve transcriptional repression, as observed by greater reduction in IEC expression of UGT1A1. This hypothesis was tested by overexpressing active IKKβ in IECs, an event that led to derepression of intestinal maturation genes, like the aforementioned responses we documented when NCoR1 was deleted (Chen et al., 2017). The ability of constitutively activated IKKβ to mimic the actions of NCoR1 deletion was also demonstrated when hUGT1 neonatal mice were orally treated with cadmium (Cd<sup>2+</sup>), acting both as a potent inducer of oxidative stress and an activator of the IKKβ (NF-κB pathway (Liu et al., 2016). Cadmium treatment resulted in intestinal induction of UGT1A1, a sharp reduction in TSB levels, and induction of intestinal maturation genes, events that are observed when NCoR1 is deleted. Cd<sup>2+</sup>-dependent induction of intestinal UGT1A1 could be driven by activation of IKK, through which it can directly, or through alternative mechanisms, contribute to the production of reactive oxygen species (ROS). Interestingly, both processes could play a role in targeting the phosphorylation of NCoR1, an event that would result in activation of the intestinal UGT1A1 gene.

**Oxidative Stress and Regulation of UGT1A1.**

Knowing that constitutive expression of intestinal IKKβ is critical in maintaining adequate expression levels of the UGT1A1 gene, most likely through activating and modifying NCoR1, we examined the impact of intestinal oxidative stress toward induction of UGT1A1. The rationale for this experiment stemmed from findings that the IKKβ/NF-κB pathway responds to redox changes, such as alterations in ROS. To carry out this experiment and eliminate the role of IKKβ, hUGT1 and hUGT1/ikk<sup>B<sup>ΔIEC</sup></sup> mice were treated orally with either Cd<sup>2+</sup> or arsenic (As<sup>3+</sup>), both known to induce significant oxidative stress (Santra et al., 2000; Ercal et al., 2001; Li et al., 2002; Liu et al., 2003; López et al., 2006). Untreated hUGT1/ikk<sup>B<sup>ΔIEC</sup></sup> mice displayed reduced intestinal UGT1A1 expression and elevated TSB levels, with most of the mice dying from SNH (Liu et al., 2016). When neonatal hUGT1 mice were orally treated with either Cd<sup>2+</sup> or As<sup>3+</sup>, intestinal UGT1A1 was induced and TSB levels were lowered (Liu et al., 2016; Yoda et al., 2017). Likewise, when hUGT1/ikk<sup>B<sup>ΔIEC</sup></sup> mice were treated with Cd<sup>2+</sup> or As<sup>3+</sup>, oxidative stress was induced in the intestines, commensurate with induction of UGT1A1 and a lowering of the TSB levels. While untreated neonatal hUGT1/ikk<sup>B<sup>ΔIEC</sup></sup> mice accumulated toxic levels of TSB that were lethal, oral treatment with Cd<sup>2+</sup> or As<sup>3+</sup> led to induction of intestinal UGT1A1 and lowering of TSB levels (Fujitaka et al., 2012; Liu et al., 2016). Arsenic, an inducer of oxidative stress, leads to the rapid activation of p38 mitogen-activated protein kinase (Yoda et al., 2017) and extracellular signal-regulated kinase, both sensors of oxidative stress. Interestingly, oral As<sup>3+</sup> treatment confirmed that IEC maturation marker genes were also induced. In addition, expression of Gsh1, Nqo1, and Lrtp2 genes, which are downregulated at the suckling to weaning transition, was reduced, mimicking the same pattern observed in ΔIEC<sup>+/+</sup> mice. The activation of MAPK and extracellular signal-regulated kinase by As<sup>3+</sup> may be stimulating kinase activity in the IECs, promoting derepression of gene expression in an NCoR1-dependent fashion.

A preemptive assumption of oxidative stress in tissues and cells is the existence or evolution of a potent defense system to counter the potential toxicities associated with the generation of ROS. The formation of oxidative stress, induced by natural diet or through environmental exposure, sets into motion a unique signaling cascade that activates gene families that regulate cytoprotective proteins to provide an active defense against cellular damage. The central mediator of this defense is the nuclear E2-factor related factor 2 (Nrf2), which exists as an Nrf2-Keap1 complex that is sensitive to ROS (Wasserman and Fahl, 1997). Activation of Nrf2 sets into motion transcriptional activation of target genes following binding to unique enhancer sequences identified as antioxidant response elements (AREs) (Wasserman and Fahl, 1997; Nguyen et al., 2000). The ARE sequences are clustered with the AhR xenobiotic response element flanking the UGT1A1 gene and bind activated Nrf2 (Yueh and Tukey, 2007). The antioxidant response can target the UGT1A1 gene, in addition to other genes responsive to ROS, such as NAD(P)H:quinine oxidoreductase 1 (McWalter et al., 2004), glutathione-S-transferase, and heme oxygenase (Alam et al., 1999). Thus induction of these genes following oxidative stress provides a molecular footprint that can be leveraged to identify substances that activate the Nrf2-Keap1 complex in providing antioxidant protection.

**CAR, Oxidative Stress, and UGT1A1.**

Some of the more potent inducers of oxidative stress are the isothiocyanates (ITCs). Regarded as an important component of our diet, cruciferous vegetables (e.g., broccoli, Brussels sprouts, cauliflower, watercress) have been linked to a lower incidence of cancer development (Murillo and Mehta, 2001) because they produce antioxidative metabolites that induce cytoprotective proteins, such as the UGTs, believed to protect tissue from dietary or environmental toxicity such as cancer. These vegetables are rich in glucosinolates, which are hydrolyzed by plant myrosinases following chewing or by microflora in the gastrointestinal tract to form ITCs (Shapiro et al., 2001a,b, 2006), the active anticarcinogenic metabolites.
Isothiocyanates activate the Nrf2-Keap1 complex, initiating a series of events leading to induction of ARE-containing target genes (Nguyen et al., 2003). We rationalized that ITC treatment would induce intestinal and potentially liver UGT1A1 in hUGT1 mice and serve as an efficient modulator of neonatal hyperbilirubinemia by inducing UGT1A1 and lowering TSB levels. When neonatal hUGT1 mice were treated orally with phenethyl isothiocyanate (PEITC), TSB levels were reduced to normal after 24 hours (Yoda et al., 2017). Induction of UGT1A1 was dramatic in both liver and small intestines, but the impact on the classic Nrf2-target genes Nqo1 and Gsta2 was not significant. We showed previously that oral As3+, which generates a potent antioxidant response, has a dramatic impact on Gsta1 and Gsta2 induction while also upregulating several of the cytochrome P450 2 and 4 subfamilies (Liu et al., 2016), indicating that the antioxidant response potentially initiated by PEITC treatment could be influencing the activation of additional signaling pathways. A screen of potential target genes induced by XNRs resulted in prominent induction of Cyp2b10/CYP2B10 in both liver and SI, indicating that CAR may be involved in the PEITC induction of UGT1A1 (Fig. 5).

To examine the component of CAR linking induction of UGT1A1 and CYP2B10 by PEITC, hUGT1/Carp−/− were bred to generate CAR knockout hUGT1/Carp−/− and heterozygous hUGT1/Carp−/+ litters. Both hUGT1/Carp−/− and hUGT1/Carp−/+ neonatal mice develop hyperbilirubinemia at the same rate. When treated with an oral dose of PEITC, UGT1A1 and CYP2B10 were induced in liver and SI of hUGT1/Carp−/− mice, with a total reduction in TSB levels. The induction of hepatic UGT1A1 was significant and most likely drove the metabolism of bilirubin, leading to the reduction in TSB levels. In liver tissue from PEITC-treated hUGT1/Carp−/− mice, there was no expression of CYP2B10 or UGT1A1, indicating that CAR was essential for induction by PEITC. In SI, the induction of CYP2B10 was also absent, yet TSB levels were significantly reduced. In the absence of CAR, there was induction of UGT1A1 in the SI, which we expect was responsible for the reduction in TSB levels. While the induction by PEITC in SI could be attributed to activation of Nrf2, there was no significant transcriptional activation of other ARE target genes. The possibility exists that PEITC induction of intestinal UGT1A1 in neonatal mice is being driven by a mechanism that is independent from CAR and Nrf2 activation (Fig. 5).

Confirmation that CAR underlies CYP2B10 and UGT1A1 induction by PEITC was validated in adult hUGT1/Carp−/− and hUGT1/Carp−/+ mice. In liver and SI, oral PEITC treatment induced CYP2B10 in a strictly CAR-dependent fashion. Significant induction of CYP2B10 by PEITC in these tissues was generated only in hUGT1/Carp−/− mice, with no response in hUGT1/Carp−/+ mice in either tissue, leaving us to speculate that induction of CYP2B10 by PEITC involved activation of CAR. A different pattern of induction was observed with UGT1A1 expression. In liver, UGT1A1 expression was greatly reduced in hUGT1/Carp−/− mice, clearly linking CAR activation with UGT1A1 expression. However, in SI, PEITC treatment effectively induced UGT1A1 in both hUGT1/Carp−/− and hUGT1/Carp−/+ mice, indicating that alternative CAR processes, such as activation of Nrf2, are responsible for the induction. In addition, Nrf2 target genes were induced in both liver and SI, confirming that the actions of PEITC are also initiating an ARE response. These findings indicate that two important signaling events, the activation of CAR and Nrf2, are linked to induction of UGT1A1 (Fig. 5).

Independently, the activation of CAR by ligands or the activation of Nrf2 by oxidative stress can facilitate the induction of UGT1A1. However, with PEITC treatment, evidence suggests that CAR and Nrf2 are linked in the induction of liver UGT1A1. To separate an antioxidative response initiated by PEITC from activation of CAR and induction of UGT1A1, we first administered adult hUGT1 mice N-acetylcysteine (NAC) for 3 weeks in their drinking water. Cysteine is the rate-limiting substrate for the synthesis of glutathione (GSH), and thus supplementation of NAC promotes the resynthesis of GSH and the neutralization of free radicals (Rizzardini et al., 1994). GSH has antioxidant properties in addition to facilitating the activity of enzymatic antioxidant systems, including GSH peroxidase and GSH reductase. After 2 weeks of NAC exposure, the mice were treated with PEITC, and antioxidative responses were measured. In the absence of NAC exposure, PEITC treatment leads to robust induction of Nrf2 target genes Nqo1, Gsta1, and Gsta2, along with UGT1A1 and Cyp2b10. In mice exposed to NAC and treated with PEITC, Cyp2b10 gene expression was completely suppressed, along with Nqo1, Gsta1, and Gsta2. While PEITC induces oxidative stress that targets Nrf2 target genes, induction of UGT1A1 and CYP2B10 is dependent upon CAR. These findings indicate that induction of liver UGT1A1 by PEITC is dependent upon both Nrf2 and CAR, while the generation of oxidative stress is a prerequisite toward CAR activation and induction of UGT1A1 and CYP2B10 (Fig. 5).

Breast Milk-Induced Hyperbilirubinemia. Breast milk (BM) plays a significant role in neonatal hyperbilirubinemia (Newman and Gross, 1963; Fujiwara et al., 2015), termed breast milk jaundice (BMJ) and is...
characterized by prolonged increases in unconjugated bilirubin. Infants with BM have elevated TSB levels above 10 mg/dl that can reach levels above 20 mg/dl, approaching the range that can induce bilirubin-induced neurologic toxicities. The prolonged increases in TSB levels during the neonatal window in hUGT1 mice indicate that breast milk may be participating in the development of hyperbilirubinemia. When neonatal hUGT1 mice were fed human breast milk, the mice continued to develop jaundice (Fujiwara et al., 2012). Clinical studies have demonstrated that newborns with jaundice given a formula diet have lower levels of TSB than breast-fed newborns, indicating that breast milk facilitates the repression of UGT1A1 (Schneider, 1986; Huang et al., 2004). To examine if we could replicate this effect in hUGT1 mice, neonatal mice were given a diet of baby formula for 72 hours (Fujiwara et al., 2012).

After formula treatment, TSB levels were reduced over 95% compared with breast-fed mice. Formula induced UGT1A1 in intestinal tissue, which correlated with the reductions in TSB levels. Of significance, formula also induced the CAR target gene Cyp2b10 and PXR target gene Cyp3a11, indicating that formula targeted activation of CAR and PXR. When formula was given to neonatal hUGT1/Car−/− or hUGT1/Pxr−/− mice, intestinal UGT1A1, Cyp2b10, and Cyp3a11 were induced in both strains, indicating formula was not activating either CAR or PXR. However, formula feeding led to activation of IkBa/NF-κB target genes, linking activation of NF-κB with induction of intestinal UGT1A1. Given the finding that formula leads to activation of NF-κB and induction of intestinal UGT1A1 in neonatal hUGT1 mice, it can be speculated that the repressive effects of BM are tied with inhibition of the IKK/NF-κB pathway.

While IkBa kinase (IKK) is an upstream component of NF-κB signaling, it is linked to propagating the cellular response to inflammation (Karin, 2009) and plays an important role in neonatal intestinal UGT1A1 expression (Fujiwara et al., 2012). Human breast milk, especially colostrum, is rich in human milk oligosaccharides (HMOs) (Bode, 2015; Lin et al., 2017) that bind to Toll-like receptors (TLRs) and prevent activation of the receptors by pathogen-associated molecular patterns (PAMPs), which are expressed by microflora (He et al., 2016a; Newburg et al., 2016). When TLRs are activated, they initiate intracellular signaling events that result in regulation of IKK and NF-κB. In comparison with term babies and adults, preterm children express higher concentrations of TLRs, and if activated, could induce an IKK/NF-κB inflammatory response that results in sepsis or necrotizing enterocolitis (NEC) (He et al., 2016a,b; Niño et al., 2016). Components of breast milk block intestinal TLR activation by PAMPs, which in turn blocks downstream IKK phosphorylation. Since we have demonstrated that targeted deletion of intestinal IKKB in hUGT1 mice represses newborn intestinal UGT1A1, we can speculate that IKK activity is directly linked to intestinal UGT1A1 expression. This concept is further supported by findings that when intestinal TLRs are activated in neonatal hUGT1 mice by agents, such as LPS, or IKK is directly activated by Cd2+, intestinal UGT1A1 is induced followed by a reduction in TSB levels. Thus the delayed expression of intestinal UGT1A1 can be traced to breast milk inhibition of TLR activation, which controls downstream activation of IKK. This concept can be highly relevant toward understanding the underlying mechanism associated with BM, since NCoR1 activity controls intestinal maturation and is directly linked to induction of intestinal UGT1A1. This also may explain why a diet of formula, which lacks TLR binding glycans, allows downstream activation of the IKK/NCoR1 loop, induction of intestinal UGT1A1, and a reduction in TSB levels. Equation would naturally dilute the concentrations of the TLR binding glycans allowing activation of the TLRs by PAMPs, followed by derepression of NCoR1 and induction of intestinal UGT1A1.

**Discussion**

The human UGT1A1 gene has evolved as a key target for naturally produced humoral agents as well as dietary and environmental compounds through expression in both liver and intestinal tissues to control the levels of accumulating TSB in newborns. While current dogma has produced an image that UGT1A1 plays a principle role in drug metabolism, expression of the human UGT1A1 gene as a transgene in mice has allowed the development of experiments to document its role as a key regulator of TSB levels. The importance of the UGT1A1 gene cannot be underestimated since silencing mutations in the gene are lethal, leading to the accumulation of toxic levels of TSB that result in encephalities followed by neurologic damage that is eventually lethal. While most of mutations in genes associated with drug metabolism could be classified as “disease enhancing mutations” (Evans and Relling, 1999, 2004; Relling and Evans, 2015), meaning that they are not detrimental to the host but under stress conditions or in the presence of saturating substrates can result in toxicity, the mutations in the UGT1A1 gene can be classified as “disease causing mutations,” indicating that the UGT1A1 gene is essential for life. Since the hundreds of mutations in the other 19 UGT genes can be classified as disease enhancing mutations (i.e., not important for life), UGT1A1 can be thought of as the only relevant UGT needed for survival. Studies in hUGT1 mice have concluded that regulation of the UGT1A1 gene can be controlled by virtually all the key XNRs, which include PXR (Xie et al., 2003; Chen et al., 2005, 2012), CAR (Sugata et al., 2005; Cai et al., 2010), PPARα (Senekeo-Effenberger et al., 2007), LXRα/β (unpublished observations), as well as the environmental sensors AhR (Yueh et al., 2005; Bonzo et al., 2007) and Nrf2 (Yueh and Tukey, 2007). In addition to this diversity, changes in the redox state of the tissue that result in oxidative stress and activation of sensors, such as ROS-sensitive MAPKs, are directly linked to induction of the UGT1A1 gene (Fujiwara et al., 2012; Liu et al., 2016; Yoda et al., 2017). There are few other genes that have been linked to drug metabolism that command this range of regulation by humoral and environmental stimuli. An understanding of these events may lead to a greater appreciation of the contribution BM plays in the etiology of BM-induced jaundice.

An important finding that will shed light on the mechanisms leading to neonatal BM is the importance of UGT1A1 expression in the intestines and its contribution to the metabolism and clearance of accumulating serum bilirubin. The expression of UGT1A1 is abundant through the intestinal tract (Tukey and Strassburg, 2000, 2001; Tukey et al., 2002), and that conserved expression is observed in hUGT1 mice (Chen et al., 2005). Given that the intestines become introduced immediately after birth with breast milk, this provides a conduit for the colonization of microflora that produces humoral agents as well as dietary and environmental compounds through expression in both liver and intestinal tissues to control the levels of accumulating TSB in newborns. While current dogma has produced an image that UGT1A1 plays a principle role in drug metabolism, expression of the human UGT1A1 gene as a transgene in mice has allowed the development of experiments to document its role as a key regulator of TSB levels. The importance of the UGT1A1 gene cannot be underestimated since silencing mutations in the gene are lethal, leading to the accumulation of toxic levels of TSB that result in encephalities followed by neurologic damage that is eventually lethal. While most of mutations in genes associated with drug metabolism could be classified as “disease enhancing mutations” (Evans and Relling, 1999, 2004; Relling and Evans, 2015), meaning that they are not detrimental to the host but under stress conditions or in the presence of saturating substrates can result in toxicity, the mutations in the UGT1A1 gene can be classified as “disease causing mutations,” indicating that the UGT1A1 gene is essential for life. Since the hundreds of mutations in the other 19 UGT genes can be classified as disease enhancing mutations (i.e., not important for life), UGT1A1 can be thought of as the only relevant UGT needed for survival. Studies in hUGT1 mice have concluded that regulation of the UGT1A1 gene can be controlled by virtually all the key XNRs, which include PXR (Xie et al., 2003; Chen et al., 2005, 2012), CAR (Sugata et al., 2005; Cai et al., 2010), PPARα (Senekeo-Effenberger et al., 2007), LXRα/β (unpublished observations), as well as the environmental sensors AhR (Yueh et al., 2005; Bonzo et al., 2007) and Nrf2 (Yueh and Tukey, 2007). In addition to this diversity, changes in the redox state of the tissue that result in oxidative stress and activation of sensors, such as ROS-sensitive MAPKs, are directly linked to induction of the UGT1A1 gene (Fujiwara et al., 2012; Liu et al., 2016; Yoda et al., 2017). There are few other genes that have been linked to drug metabolism that command this range of regulation by humoral and environmental stimuli. An understanding of these events may lead to a greater appreciation of the contribution BM plays in the etiology of BM-induced jaundice.
but is a controlled event, with repression of the intestinal UGT1A1 gene being influenced by breast milk. In addition, we now know that induction of intestinal UGT1A1 during the neonatal period can be achieved by any means (i.e., oxidative stress, ligand-activated nuclear receptors, formula) will lower TSB values, adding support for a mechanism that implicates BM in facilitating reduced expression of UGT1A1. The establishment of the microflora starts immediately following birth. Pathogen-associated molecular patterns (PAMPs) can activate TLRs and induce an inflammatory response and gene transcription through NCoR1 derepression. This response may lead to important health concerns including necrotizing enterocolitis (NEC), a devastating disease that affects mostly premature infants. Countering this, premature infants on breast milk have demonstrated less NEC in comparison with formula feeding.

![Diagram of TLR function](image)

**Fig. 6.** NCoR1 as a potential target for both neonatal hyperbilirubinemia and necrotizing enterocolitis (NEC). Intestinal UGT1A1 is essential in bilirubin detoxification and is directly linked to neonatal hyperbilirubinemia. We demonstrated that NCoR1 represses intestinal UGT1A1 gene expression in a developmental-dependent manner that is limited within the breastfeeding stage, suggesting that NCoR1 may function as an important underlying mechanism for BMJ. It is known that human milk oligosaccharides (HMOs) inhibit Toll-like receptors (TLRs), a family of membrane receptors upstream of NCoR1. When activated, TLRs activate IKKs and c-Jun signaling cascades, leading to turnover of NCoR1 from the target promoters and subsequent gene transcription. It hints at the possibility that inhibition of TLR function by HMOs leads to the heightened NCoR1 repression on the UGT1A1 gene, resulting in an increase in TSB levels. In addition, we now know that induction of intestinal UGT1A1 during the neonatal period by any means (i.e., oxidative stress, ligand-activated nuclear receptors, formula) will lower TSB values, adding support for a mechanism that implicates BM in facilitating reduced expression of UGT1A1. The establishment of the microflora starts immediately following birth. Pathogen-associated molecular patterns (PAMPs) can activate TLRs and induce an inflammatory response and gene transcription through NCoR1 derepression. This response may lead to important health concerns including necrotizing enterocolitis (NEC), a devastating disease that affects mostly premature infants. Countering this, premature infants on breast milk have demonstrated less NEC in comparison with formula feeding.

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