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Role of extrahepatic UDP-glucuronosyltransferase 1A1: advances in understanding breast milk-induced neonatal hyperbilirubinemia

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Abstract

Newborns commonly develop physiological hyperbilirubinemia (also known as jaundice). With increased bilirubin levels being observed in breast-fed infants, breast-feeding has been recognized as a contributing factor for the development of neonatal hyperbilirubinemia. Bilirubin undergoes selective metabolism by UDP-glucuronosyltransferase (UGT) 1A1 and becomes a water soluble glucuronide. Although several factors such as gestational age, dehydration and weight loss, and increased enterohepatic circulation have been associated with breast milk-induced jaundice (BMJ), deficiency in UGT1A1 expression is a known cause of BMJ. It is currently believed that unconjugated bilirubin is metabolized mainly in the liver. However, recent findings support the concept that extrahepatic tissues, such as small intestine and skin, contribute to bilirubin glucuronidation during the neonatal period. We will review the recent advances made towards understanding biological and molecular events impacting BMJ, especially regarding the role of extrahepatic UGT1A1 expression.

Keywords

Bilirubin; UDP-glucuronosyltransferase 1A1; humanized *UGT1* mice; extra hepatic

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Conflict of interest

The authors declare that they have no conflict of interest.

Introduction

Bilirubin is the terminal metabolite of heme catabolism, is metabolized solely by UDP-glucuronosyltransferase (UGT) 1A1 through glucuronidation (Bosma et al., 1994), and accumulates in newborns because of a developmental delay in the expression of the UGTs (Burchell et al., 1989). Convincing evidence indicates that breast-fed infants have higher levels of total serum bilirubin (TSB) compared to bottle-fed infants (Arias et al., 1964; Schneider, 1986; Gourley, 2002; Stiehm and Ryan, 1965), suggesting that breast milk is a risk factor for the development of neonatal hyperbilirubinemia. While jaundice is usually benign, neonatal phototherapy treatment is often recommended in infants with severe jaundice. The complications of phototherapy include mother-infant separation that limits their interactions and has been linked to the development of postnatal depression (Crowell and Treboux, 1995; Onozawa et al., 2001; Coyl et al., 2002), but if not treated, infants with high levels of TSB become prone to unconjugated bilirubin-induced encephalopathy (Shapiro, 2003, 2005, 2010).

Arias et al. (1963) and Newman et al. (1963) first reported that breast milk was a contributing factor towards the development of neonatal jaundice. Substances in breast milk, including pregnane −3α,20β-diol, non-esterified fatty acid, β-glucuronidase, and other factors such as sub-optimal fluid intake, weight loss, increased enterohepatic circulation of bilirubin, and pre-mature birth were suspected of causing breast milk jaundice (BMJ) (Arias et al., 1964; Bevan and Holton, 1972; Gourley and Arend, 1986; American Academy of Pediatrics, 2004; Ip et al., 2004; Maisels et al., 2009). While these aforementioned factors may potentiate hyperbilirubinemia in jaundiced infants, key factors leading to BMJ have not yet been conclusively identified (Ramos et al., 1966; Constantopoulos et al., 1980; Gaffney et al., 1986). In 1979, Onishi et al. demonstrated that bilirubin UGT activity was repressed in human liver during neonatal development, suggesting the delayed development of hepatic UGTs leads to increased TSB levels. Since then, many lines of new evidence associated with neonatal jaundice and BMJ have been revealed; in particular, significant findings have been made in 2 areas, which are the main focus of this review: the important role of extrahepatic UGT1A1 expression and UGT1A1 polymorphism that is clinically relevant to hyperbilirubinemia.

UDP-Glucuronosyltransferase (UGT)

UGTs constitute a super family of endoplasmic reticulum (ER) bound proteins that form glucuronides by transferring glucuronic acid from UDP-glucuronic acid to the selective substrates (Dutton, 1980). The mammalian UGT gene superfamily, comprising four families, currently has more than 100 members (Mackenzie et al., 2005). In human, 19 UGTs had been identified, each being classified into the UGT1 (UGT1A1, -1A3, -1A4, -1A5, -1A6, -1A7, -1A8, -1A9 and -1A10) or UGT2 family (-A1, -A2, -A3, -B4, -B7, -B10, -B11, -B15, -B17, -B28) (Williams et al., 2004). The major UGTs involved in xenobiotic metabolism are UGT1A1, -1A3, -1A4, -1A6, -1A9 and -2B7 (Rowland et al., 2013), whereas a distinct yet overlapping set of UGTs is responsible for metabolism of various endogenous substances, such as bilirubin, estradiol, testosterone, 5-hydroxytryptamine, thyroid hormones (thyroxine and triiodothyronine), and bile acids (Tukey and Strassburg,

2000). It is now understood that the UGTs are expressed in a highly coordinated tissue specific fashion. The different complement of expressed UGTs in different tissues dictates the ability of each tissue to metabolize the large range of UGT substrates. With the number of UGTs well documented in different human tissues, many of them are expressed in the GI tract with great abundance and are subject to regulation by food derived nutrients and other substances that are consumed in the diet. With regard to newborns, these nutrients are being provided directly by breast milk or supplemented with formula.

While the UGTs exhibit broad substrate specificity, certain compounds are glucuronidated selectively. UGT1A1 is the sole UGT isoform responsible for the glucuronidation of bilirubin (Bosma et al., 1994). Since bilirubin is hydrophobic and exists in the blood mostly bound to serum proteins prior to its movement into cells and metabolism by UGT1A1 glucuronidation, certain physiological events can influence the relative saturation limits of serum proteins. In the event that bilirubin production exceeds the body's ability to maintain a steady-state balance with serum proteins, the circulating unconjugated bilirubin is free to move across cell membranes and concentrates in various tissues that are getting a rich supply of blood, such as the tissues of the central nervous system. In adults, the dense network of capillaries that form the blood brain barrier is well developed and serves as a barrier to prevent access of miscellaneous agents that are found free in the blood. But in newborns, the blood brain barrier is poorly defined and substances, such as unconjugated bilirubin if not bound to serum proteins, can accumulate in brain tissue[3]. If bilirubin is not metabolized, its accumulation can lead to the initiation of cellular inflammation and eventually gliosis of glial cells (kernicterus) (Shapiro, 2005; Yueh et al., 2014). The central molecular events involved in the homeostatic balance between conjugated and unconjugated bilirubin during neonatal development is the control and expression of the *UGT1A1* gene. Thus, developmental regulation of UGT1A1 is the rate limiting step in determining the severity of neonatal hyperbilirubinemia, and an appreciation of such regulation will play a significant role in preventing bilirubin induced neurological toxicities.

Metabolic pathway of bilirubin

Liver tissue plays an essential role in the metabolism of drugs, xenobiotics, and many exogenous compounds (Kutsuno et al., 2013 and 2014). In the liver, sinusoidal membrane transporters, such as organic anion transporting polypeptides (OATPs) and organic anion transporters (OATs), contribute to the uptake of bilirubin from blood to hepatocytes (Kamisako et al., 2000; Zhang et al., 2007), although passive diffusion may be substantial in the transport of bilirubin into liver cells. Characterization of the membrane-bound UGTs demonstrates that the majority of the protein, including its active site, is located on the luminal surface of the ER (Rowland et a., 2013; Shepherd et al., 1989; Vanstapel et al., 1988), indicating that, UGT substrates, including bilirubin, need to be transported to the luminal side of the ER to be metabolized. Subsequently, it is necessary to transport the glucuronides back to the cytoplasm following glucuronidation (Fig. 1). A study by Csala *et al*. suggested that transporter(s) mediate the movement of hydrophobic compounds and their glucuronides in the ER membrane (Csala et al., 2007). Reported accumulation of bilirubin glucuronides within the lumen of the ER, which was found in liver microsomes from a jaundiced patient (Waddell et al., 1995), might have resulted from a genetic deficiency of

such transporters in the ER membrane. Efforts have been made to identify ER membrane transporters that are responsible for the transport of bilirubin and its glucuronides across the ER membrane (Fujiwara and Itoh, 2014a and 2014b); however, such transporters have not been characterized to date. Protein interactions between UGT-UGT and -cytochrome P450 (CYP) might be involved in the transport of the substances across the ER membrane (Fujiwara et al., 2007a, 2007b and 2010; Nakajima et al., 2007; Finel and Kurkela, 2008; Ishii et al., 2007). Following bilirubin metabolism in liver cells, its glucuronides are transported out of these cells through the apical surface of the hepatocytes into the bile ducts by the multidrug resistance protein 2 (MRP2/ABCC2)(Fig. 1), leading to their movement through the GI tract where they eventually come into contact with microbes in the large intestine (Keppler et al., 1997; Kamisako et al., 2000). Bilirubin glucuronide is a substrate for microbial β-glucuronidase, which can cleave the glucuronide and liberate bilirubin for reabsorption through the basolateral surfaces of the intestines where it can undergo further metabolism or pass directly back into the circulation. This process, known as enterohepatic circulation, can extend the half-life of bilirubin while adding to the total serum bilirubin load. In addition, the presence of β-glucuronidase in breast milk has been linked to BMJ since it can directly increase the rate of enterohepatic circulation of bilirubin (Gourley, 2002).

Hereditary unconjugated hyperbilirubinemia

Bilirubin undergoes glucuronidation selectively by UGT1A1 (Bosma et al., 1994). Genetic polymorphisms in the *UGT1A1* gene can impair its enzymatic activity and cause hereditary unconjugated hyperbilirubinemia, specifically Crigler-Najjar syndrome (CN) type I (MIM #21880), type II (MIM #606785), and Gilbert's syndrome (MIM #143500) (Bosma et al., 1992, 1994, and 1995; Aono et al., 1993; Monaghan et al., 1996 and 1999). The severity of hyperbilirubinemia is based upon the degree of deficiency of UGT1A1 activity as determined by the mutations in the *UGT1A1* gene. Among over 40 mutations in the *UGT1A1* gene that are associated with inheritable unconjugated hyperbilirubinemia (Tukey and Strassburg, 2000; Strassburg, 2008), the majority of these mutations leads to one of three major hyperbilirubinemia syndromes: CN type I and type II and Gilbert's syndrome. Mutations in CN type I cause complete loss of UGT1A1 enzymatic bilirubin glucuronidation, resulting in a chronic, life-threatening condition of potential encephalopathy (Ritter et al., 1992 and 1993). Patients with CN type II have mutations that greatly reduce UGT1 activity and have moderate to high serum unconjugated bilirubin levels with rare occurrences of CNS toxicity (Crigler and Najjar, 1952; Arias et al., 1969; Robertson et al., 1991; Moghrabi et al., 1993). By comparison, Gilbert's syndrome is a common, mild form of hyperbilirubinemia with hepatic UGT1A1 activity being about 30% of the normal level (Strassburg, 2008; Burchell and Hume, 1999). Typically, it is often first noticed as intermittent mild jaundice during adolescence (Gilbert and Lereboullet, 1901). The prevalence of Gilbert's syndrome is approximately 3–9% of the population globally, with 1 in 3 affected patients being unaware that they have it (Owens and Evans, 1975; Sieg et al., 1987). The most common genotype of Gilbert's syndrome is the homozygous polymorphism of a TA insertion in the $(TA)_6TAA$ region of the UGT1A1 promoter (the 7/7 allele, also known as *UGT1A1*28*), whereas a missense mutation in coding exon 1 (G71R,

also known as *UGT1A1*6*) is more prevalent in Asian populations, especially in jaundiced Japanese newborns (Maruo et al., 1999 and 2004; Bosma et al., 1995; Beutler et al., 1998; Monaghan et al., 1999; Strassburg, 2008). Newborns that express the *UGT1A1*6* allele are at increased risk of developing BMJ.

Neonatal jaundice and breast milk-induced jaundice

While hyperbilirubinemia can develop at any age, physiological jaundice is common in newborns. One of the molecular mechanisms underlying the development of neonatal jaundice involves the delayed and reduced expression of hepatic UGT1A1 and the simultaneous increase in TSB levels following destruction of fetal hemoglobin shortly after birth. UGT expression is subject to developmental regulation (Onishi et al., 1979; de Wildt et al., 1999; Burchell, 1973 and 1974). Adults express abundant levels of hepatic UGT1A1, as confirmed by experimental data with mRNA and protein expressions and enzymatic activity (Ritter et al., 1999; Nakamura et al., 2008; Izukawa et al., 2009; Ohno and Nakajin, 2009; Strassburg et al., 1997, 1998a, 1998b, 1999a, and 1999b), whereas fetuses and neonates – in contrast – have very limited UGT1A1 expression and bilirubin glucuronidation activity in liver (Onishi et al., 1979; Strassburg et al., 2002). Through analysis of mutations in the *UGT1A1* gene it has been conclusively demonstrated that UGT1A1 is the sole enzyme responsible for bilirubin glucuronidation (Bosma et al., 1994), and its reduced expression (or absence) causes a correlated increase in levels of TSB during the neonatal period. With this statement, researchers logically assume that reduced UGT1A1 expression in the liver is the key factor determining the developmental onset of neonatal jaundice.

Accumulating evidence indicates that breast-fed infants have a higher risk for kernicterus development than formula-fed infants (Gourley, 1998 and 2002; Alonso et al., 1991; Bhutani et al., 2005), and the American Academy of Pediatrics (AAP) and the National Institute for Health and Clinical Excellence (NICE) of the U.K. have cited that breast-feeding is a risk factor for hyperbilirubinemia and potentially kernicterus formation. Components in breast milk, such as steroids, fats, cytokines, B-glucuronidase and the epidermal growth factor (EGF), have been tied to the accumulation of TSB through increased uptake of bilirubin by enterohepatic recirculation, a decrease in bilirubin excretion, or the inhibition of UGT1A1 activity (Arias et al., 1964; Bevan and Holton, 1972; Gourley and Arend, 1986; Shibuya et al., 2013). BMJ is defined as jaundice that occurs in neonates that consume adequate levels of breast milk. At the same time, inadequate breast milk-intake can lead to the onset of neonatal jaundice, which is called inadequate breastfeeding jaundice. In contrast to humans, experimental animals, such as mice or rats, do not naturally develop BMJ. Until recently, conclusive evidence explaining the mechanism of BMJ had not been obtained due to the lack of a suitable hyperbilirubinemia animal model.

The recent development of humanized *UGT1* (*hUGT1*) mice – expressing the entire human *UGT1* locus in a *Ugt1*-null background – has provided the first appropriate animal model to study the mechanisms associated with neonatal hyperbilirubinemia. The generation of humanized *UGT1* mice was accomplished in a few steps. First, a transgenic mouse line (*TgUGT1* mice), harboring the entire human *UGT1* locus and expressing all nine of the

UGT1A genes, was generated (Chen et al., 2005). Second, the entire murine *Ugt1* locus was inactivated (*Ugt1*$^{-/-}$ mice) through the introduction of a genetic mutation into exon 4, eliminating the nine mouse UGT1A proteins. Finally, crossing heterozygous *Ugt1*$^{+/-}$ mice with *TgUGT1* mice led to the generation of a hybrid mouse line (*TgUGT1/Ugt1*$^{+/-}$) and eventual *Tg(UGT1)Ugt1*$^{-/-}$ mice, which represent fully humanized *UGT1* (*hUGT1*) mice. The use of the heterozygous *Ugt1*$^{+/-}$ line was necessary because the *Ugt1*$^{-/-}$ mice exhibit the accumulation of lethal levels of TSB (Nguyen et al., 2008; Bortolussi et al., 2012), manifesting as an orange skin color early after birth, and most of them die within 7 days after birth (Fig. 2). Humanization with the *UGT1* locus, including the *UGT1A1* gene, rescues neonatal lethality pertaining to the *Ugt*1-null locus and the absence of the *Ugt1a1* gene. The expression pattern of the human *UGT1A* genes was nearly identical to that documented in human tissues (Tukey and Strassburg, 2000). Thus, *hUGT1* mice seem to be a relevant animal model for studying the molecular events regulating *UGT1A1* gene expression during development.

Shortly after birth, *hUGT1* mice develop severe hyperbilirubinemia, with peak levels of TSB being achieved approximately 14 days after birth and ranging from 12 to 15 mg/dL, compared with < 0.2 mg/dL in wild-type mic (Fig. 3). Overly extreme hyperbilirubinemia occurs in 5–10% of newborn *hUGT1* mice, with the TSB levels often reaching > 20 mg/dL, leading to the development of seizures and eventual death due to bilirubin accumulation in brain tissue. Mimicking the *hUGT1* model, children with CN type I also develop seizures as a result of severe hyperbilirubinemia. Studies examining the *UGT1A1* gene expression pattern and the regulatory role of xenobiotic receptors in *hUGT1* mice have revealed that the steady accumulation of TSB levels in the first two weeks after birth is attributed to significantly repressed expression of liver UGT1A.

We discovered that reduction in liver *UGT1A1* gene expression during development is a programmed event, with the pregnenalone X receptor (PXR) serving as a transcriptional co-repressor, as evidenced by the fact that humanized *UGT1* mice that are deficient in PXR do not develop extreme hyperbilirubinemia (Chen et al., 2012). While the reduction in hepatic *UGT1A1* continues 14 days after birth, we observed that TSB levels start to gradually decline and eventually reach normal levels as intestinal *UGT1A1* expression and activity coincidently progressively increase, implying the role of intestinal *UGT1A1* in bilirubin glucuronidation (Fujiwara et al., 2010).

It has been recognized for more than 50 years that newborns who are breastfed have a 3- to 6-fold greater probability of developing elevated TSB levels than formula-fed newborns (Newman and Gross, 1963; Arias et al., 1963; Schneider, 1986). Our descriptive observations that the timing of intestinal *UGT1A1* gene expression in *hUGT1* mice and the concordant reduction in TSB levels led us to examine the possibility that breast milk controlled expression of intestinal UGT1A1. When neonatal *hUGT1* mice that were 10 days of age were removed from nursing and placed on a formula diet, TSB levels dropped dramatically within 24 hours (Fujiwara et al., 2012). The reduction in TSB levels was a direct result of the formula, because when we fed *hUGT1* mice human breast milk (HBM), it had no impact on reducing TSB levels. This study suggested that breast milk, including HBM, directly suppresses expression of the *UGT1A1* gene. When gene expression patterns

were examined, formula feeding led to dramatic induction of intestinal *UGT1A1* gene expression. This induction did not occur when *hUGT1* mice were fed HBM. Importantly, formula treatment only induced intestinal UGT1A1 expression without affecting the expression patterns in liver. Two important conclusions were drawn from these studies. **First**, specific components in breast milk played an important role in repressing expression of intestinal UGT1A1. **Second**, regulation of intestinal UGT1A1 during neonatal development when liver gene expression was compromised appeared to play an important role in bilirubin metabolism and elimination.

During the course of these studies, selective gene profiling demonstrated that formula treatment led to induction of the mouse macrophage inflammatory protein-2 (*Mip-2*) gene, the homologue of human IL-8, as well as the *Cox-2* gene, both being classical NF-κB target genes. These findings were relevant since it had been reported previously that breast milk can inhibit nuclear factor κB (NF-κB)-dependent target gene expression (Minekawa et al., 2004). When neonatal *hUGT1* mice were treated orally with agents known to activate the IκB kinase (Ikk)/NF-κB pathway, intestinal *UGT1A1* gene expression was induced and TSB levels dropped. The correlation between Ikk/NF-κB activation by formula and induction of intestinal *UGT1A1* gene by other selective agents known to activate NF-κB led us to speculate that the Ikk/NF-κB pathway plays an important role in regulation of intestinal *UGT1A1* gene expression and the onset of BMJ. It is unclear how NF-κB is tied to regulation of intestinal UGT1A1, but we have speculated that NF-κB is important for intestinal *UGT1A1* gene expression and that inhibition of the Ikk/NF-κB pathway by possible agents present in breast milk contributes to the onset of BMJ (Fujiwara et al., 2012 and Fig. 4).

Possible factors in breast milk that cause neonatal hyperbilirubinemia

Identifying the components of breast milk that lead to the onset of BMJ has been elusive. Pregnane-3α, 20β-diol, a steroid in breast milk, was first suspected of being an inhibitor of bilirubin-conjugating activity (Arias et al., 1963). Indeed, this steroid had the potency to inhibit bilirubin glucuronide formation *in vitro* (Arias et al., 1964). However, other findings over 20 years later demonstrated that the levels of pregnane-3α, 20β-diol were barely detectable in breast milk (Murphy et al., 1981).

Breast milk contains a high amount of fatty acids, including oleic acid, linoleic acid, and docosahexaenoic acid, along with other C18 and C20 unsaturated fatty acids and has been shown to inhibit UGT activities *in vitro* (Bevan and Holton, 1972; Hargreaves, 1973; Shibuya et al., 2013), indicating that unsaturated fatty acids may contribute to the development of BMJ. Although these unsaturated fatty acids strongly inhibited UGT1A1 activity in vitro by using the recombinant UGT1A1 system, they induced both hepatic and intestinal *UGT1A1* expression and reduced TSB levels when they were treated to *hUGT1 neonates* (Shibuya et al., 2013). These findings suggest that the inhibitory potential of unsaturated fatty acids when being added directly to microsomes cannot mimic the actions seen in vivo, and the reason may be that UGT1A1 induction is secondary to the induction of cellular signaling by these fatty acids. One can speculate that the architecture of the

intestinal villi allows sufficient contact of the fatty acids with the rich layers of the epithelial cells where nuclear receptor activation and subsequent *UGT1A1* induction may occur.

Various cytokines are present in human breast milk, including interleukin (IL)-1β, IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)-α. In particular, the concentration of IL-1β in colostrum was statistically higher in mothers of infants with jaundice, compared with mothers nursing infants who did not develop jaundice (Zanardo et al., 2007). A similar observation was reported in 2012 showing that IL-1β concentrations were significantly higher in the breast milk of mothers whose infants had BMJ (Apaydin et al., 2012). Epidermal growth factor (EGF) has also been reported to be elevated in human breast milk of mothers whose infants developed BMJ (Kumral et al., 2009). While the molecular mechanism involving IL-1β- and EGF-induced jaundice is unknown, evidence has shown that through facilitating gastrointestinal development and promoting systematic responses, EGF and IL-1β might interrupt intestinal absorption and enterohepatic circulation of bilirubin. Moreover, it has been reported that IL-1β can inhibit the constitutive androstane receptor (CAR)-induced expression of hepatic *UGT1A1* gene expression (Assenat et al., 2004). However, research in our laboratory has demonstrated that knocking out CAR in *hUGT1* (*hUGT1/Car*$^{-/-}$) mice has no impact on the TSB levels in newborn mice (Fujiwara et al., 2012). It is interesting to note, however, that deletion of CAR seems to sensitize brain tissue to the elevated levels of unconjugated bilirubin, since over 50% of the *hUGT1/Car*$^{-/-}$ mice die shortly after birth. Thus, since breast milk contains higher concentrations of cytokines, including IL-1, IL-6, IL-10 and TNFα, inflammatory signaling may take place, leading to suppression of intestinal *UGT1A1* gene expression. This hypothesis, which is aligned with the previous findings associating the Ikk/NF-κB pathway and intestinal *UGT1A1* gene expression, needs further exploration (Fig. 4).

It was previously demonstrated that breast-fed infants had a higher prevalence of malnutrition compared to bottle-fed infants (Victora et al., 1984). Inadequate caloric intake or starvation has been implied as a cause of neonatal jaundice (Sato et al., 2013). While breast-fed newborns showed relatively severe hyperbilirubinemia, those newborns who received supplemental glucose along with breast milk showed significantly lowered TSB levels. Recently, the underlying mechanism of glucose-induced reduction in TSB levels was elucidated with a series of *in vitro* and *in vivo* studies. In *hUGT1* mice, supplemental oral glucose treatments specifically induced UGT1A1 in the small intestine but not in the liver (Aoshima et al., 2014). Glucose-mediated UGT1A1 induction was also observed in human intestinal Caco-2 cells. We further identified that Specificity Protein 1 (SP1) was the key transcriptional factor controlling the induction of intestinal UGT1A1 by glucose (Aoshima et al., 2014). These findings indicate that additional caloric intake through glucose supplementation induces expression of UGT1A1 in the small intestine, alleviating hyperbilirubinemia in breast-fed infants. Further research is required to understand the underlying mechanism through which glucose enhances UGT1A1 gene expression. Inadequate caloric intake is common in low birth weight preterm infants (Rodriguez and Rice, 2014), in whom a higher incident of jaundice has been reported (Watchko and Oski, 1992); therefore, inadequate caloric intake could be a causal factor of inadequate breastfeeding jaundice. The fact that large variability exists in both nutrients and caloric

content in human breast milk within mothers indicates that there may be other factors causing suppression of *UGT1A1* expression.

Role of extrahepatic UGT1A1 in bilirubin metabolism

While liver plays a major role in bilirubin metabolism, several key experiments have demonstrated that extrahepatic tissues contribute to bilirubin metabolism and clearance. Over 50 years ago, broken cell preparations of guinea pig gastrointestinal mucosa was used to demonstrate its enzymatic potential to form bilirubin glucuronides (Stevenson and Dutton, 1962), while Hartmann and Bissell (1982) demonstrated that bilirubin-UGT activity was qualitatively similar to that present in liver tissue by using cell-free extracts of small intestinal mucosa from rats or humans. Two independent studies, in which Gunn rats - deficient in the *Ugt1* locus and the *Ugt1al* gene - underwent small intestinal tissue transplantation from Wistar rats confirmed that intestinal tissue was capable of metabolizing bilirubin and reducing TSB levels (Takahashi et al. 1994; Medley et al, 1995). Since Gunn rats do not have the ability to conjugate bilirubin, it is assumed that the reduction of TSB levels in the small intestine-transplanted Gunn rat is associated with accelerated bilirubin metabolism and clearance in the tissue transplant. As described above, *hUGT1* neonates develop hyperbilirubinemia, and their TSB levels are inversely correlated with the expression level of UGT1A1 in the small intestine in the second half of the developmental period (Fujiwara et al., 2010). Furthermore, small intestine-specific induction of the *UGT1A1* gene resulted in a dramatic decrease in TSB levels in neonatal *hUGT1* mice (Fujiwara et al., 2012). Combined, these studies provide indirect evidence that intestinal tissue is capable of serving as the conduit for bilirubin glucuronidation and clearance.

Employing mouse genetics and the Cre-lox system, Chen *et al*. (2013) demonstrated that liver-specific deletion of mouse *Ugt1a1* resulted in only a slight increase in TSB levels during development. This is in stark contrast to whole animal deletion of the *Ugt1* locus ($Ugt1^{-/-}$), which has been shown to be a lethal mutation that results in dramatic increases in TSB levels shortly after birth (Nguyen et al., 2008). The dramatic increase in TSB levels in $Ugtl^{-/-}$ mice within the first week after birth leads to pronounced microglia neuroinflammation and severe brain impairment (Yueh et al., 2014). The targeted deletion of the *Ugt1a1* gene in liver tissue and the resulting mild increase in TSB levels is direct confirmation that bilirubin is conjugated and excreted by extra-hepatic tissues, such as the intestines, during neonatal development.

Exposing jaundiced newborns to sunlight has been associated with lowering TSB levels (Salih, 2001). Ultraviolet (UV)-B exposure will isomerize bilirubin leading to its excretion from the body (Salih, 2001). Therefore, it was originally hypothesized that the UVB-induced isomerization of bilirubin was the underlying mechanism for decreasing TSB levels in sunlight-exposed human infants. Another line of evidence indicates that UVB exposure photo-oxidizes L-tryptophan in the skin and generates 6-formylindolo[3,2-b]carbazole (FICZ), which is a known ligand for the aryl hydrocarbon receptor (AhR) (Wincent et al., 2009). Indeed, the *cytochrome P450 1A1* gene, a well-defined AhR target gene, is induced by FICZ in cultured cells (Sumida et al., 2013). Since *UGT1A1* is also a target gene of the AhR (Yueh et al., 2003), we hypothesized that induction of UGT1A1 in the skin can

contribute to bilirubin metabolism in sunlight-exposed neonates. Previous evidence has shown that selective UGT1A proteins, including UGT1A1, are expressed in human skin as well as in human skin derived keratinocytes (Sumida et al., 2013; Peters et al., 1987). Treatment of HaCaT keratinocytes with FICZ or UVB-exposed L-tryptophan resulted in an induction of UGT1A1 (Sumida et al., 2013). When *hUGT1* neonates were exposed to UVB, we further demonstrated that UVB exposure induced UGT1A1 in the skin, accompanied by a reduction of TSB levels (Sumida et al., 2013). Since the skin receives a large supply of blood, it is reasonable to speculate that UGT1A1 expressed in the skin plays an important role in metabolism and clearance of circulating bilirubin. Gene expression of *UGT1A1* has been detected in many extrahepatic tissues, including most of the GI tract (except esophagus), kidney, lung and brain (Nakamura et al., 2008; Shelby et al., 2003, Tukey and Strassburg, 2000). While the role of these tissues in the metabolic clearance of bilirubin is starting to be understood, we can speculate that UGT1A1 expression in the brain might exhibit protective effects against the accumulation of unconjugated bilirubin from bilirubin-induced brain damage.

Genetic polymorphisms in the UGT1A1 gene and their association with neonatal jaundice

Several lines of evidence indicate that polymorphisms in the *UGT1A1* gene are linked to BMJ. For example, the *UGT1A1*28* genotype in Gilbert's syndrome is linked to the onset of BMJ. In a secluded hospital-based nested case-control study in Northern India, Agrawal et al. concluded that the *UGT1A1*28* allele was a risk factor in newborns and was associated with TSB levels ≥ 18mg/dl, a condition requiring phototherapy. Other studies have documented that newborns homozygous for the Gilbert's syndrome A(TA)7TAA polymorphism serves as one of the factors contributing to neonatal jaundice (Laforgia et al., 2002). These findings, however, appear to be controversial since in other studies the probability of developing neonatal jaundice was not statistically different between neonates who carry the *UGT1A1*28* allele and those who carry the more common *UGT1A1*1* allele (Maruo et al., 1999; Babaoglu et al., 2006; Azlin et al., 2011; Huang et al., 2004; Travan et al., 2014). Data obtained with *hUGT1* mice also support the conclusion that the *UGT1A1*28* allele does not impact TSB levels in the neonatal period. Humanized *UGT1* mice that carry homozygous *UGT1A1*1* or the *UGT1A1*28* allele developed neonatal hyperbilirubinemia, and their TSB levels did not show statistical differences (Fujiwara et al., 2010).

In contrast, the *UGT1A1*6* allele associated with Gilbert's syndrome has been consistently linked to elevated TSB levels in newborns (Maruo et al., 1999 and 2000; Azlin et al., 2011; Huang et al., 2004; Chang et al., 2013; Sato et al., 2013) (Table 1). In a study with a large number of Japanese children, Maruo *et al.* (2014) reported that the allelic frequency of *UGT1A1*6* in the hyperbilirubinemia group (0.694) was significantly higher than that in the non-hyperbilirubinemia group (0.182). It was further demonstrated that the impact of the *UGT1A1*6* allele on TSB levels was significant in breast-fed infants (Maruo et al., 2014). The complete mechanism documenting the contribution of *UGT1A1*6* towards BMJ has not been fully elucidated; however, it has been suggested that delayed bilirubin glucuronidation

in newborns that express the *UGT1A1*6* allele results from inhibition of the p.G71R-UGT1A1 protein by the breast milk component 5α-pregnane-3α, 20β-diol (Ota et al., 2011).

In 1990, Newman *et al*. reported that there were statistically significant differences in the frequency of severe neonatal hyperbilirubinemia among various ethnic groups (Newman et al., 1990). For example, the incidence of hyperbilirubinemia was 31% in Asian infants and 9% in black infants. The frequencies of the *UGT1A1* polymorphisms that are associated with unconjugated hyperbilirubinemia were also very different amongst different racial groups worldwide (Table 1). Homozygous *UGT1A1*28* is the most prevalent genotype causing Gilbert's syndrome in Caucasians and Africans with frequencies of 0.36–0.40 and 0.42–0.48, respectively, which is significantly higher than that in East Asians (0.15, Japanese, Koreans, and Chinese) (Bosma et al., 1995; Monaghan et al., 1996; Maruo et al., 1999; Beutler et al., 1998). In contrast, the allelic frequency of *UGT1A1*6* was 0.16 in East Asians, whereas this polymorphism was not detected in Caucasians and Africans (Maruo et al., 1999). These findings indicate that the varied frequency of neonatal hyperbilirubinemia and BMJ among different races can be attributed in part to the different frequencies of genetic polymorphisms of the *UGT1A1* gene.

Conclusions

Along with epidemiological evidence that breast milk is a key factor in the development of neonatal hyperbilirubinemia, convincing new findings employing humanized and mouse genetics have demonstrated an important role for extrahepatic tissues in the metabolism of bilirubin during neonatal development (Table 2). Importantly, intestinal UGT1A1 plays a key role in bilirubin metabolism in BMJ and inadequate breastfeeding jaundice. These new experimental tools can be exploited to develop intervention therapies as needed in those extreme cases of severe neonatal hyperbilirubinemia.

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Abbreviations

BMJ	breast milk jaundice
UGT	UDP-glucuronosyltransferase
hUGT1	humanized *UGT1*
Tg	transgenic
OATP	organic anion transporting polypeptide
OAT	organic anion transporter
ER	endoplasmic reticulum
MRP2	multidrug resistance protein 2

AAP	American Academy of Pediatrics
NICE	National Institute for Health and Clinical Excellence
CAR	constitutive androstane receptor
FICZ	6-formylindolo[3,2-b]carbazole
AhR	aryl hydrocarbon receptor
UV	Ultraviolet
SP1	Specificity Protein 1
TSB	total serum bilirubin
EGF	epidermal growth factor
CN	Crigler-Najjar
PXR	pregnenolone X receptor
Mip-2	macrophage inflammatory protein-2
TNF	tumor necrosis factor
IL	interleukin
IKK	IκB kinase
NF-κB	nuclear factor κB

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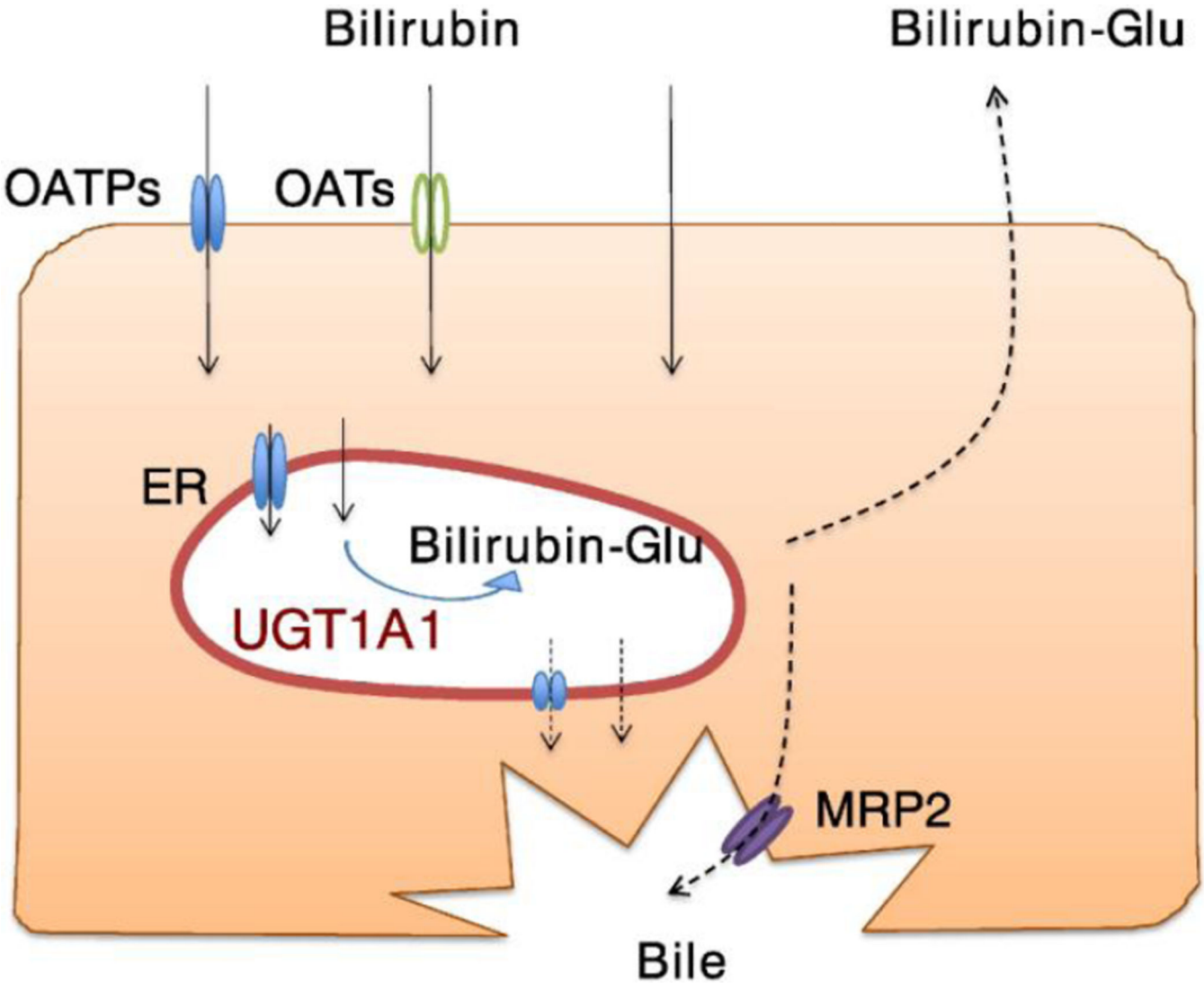

Fig. 1. Metabolic pathway of bilirubin in hepatocytes

OATPs and OATs uptake bilirubin into hepatocytes, while bilirubin is passively absorbed into the hepatocytes. Bilirubin is further transported into the luminal side of ER to be conjugated by UGT1A1. There might be a carrier that transport bilirubin and bilirubin-glucuronide across the ER membrane. Bilirubin-glucuronide is transported into the bile ducts by MRP2.

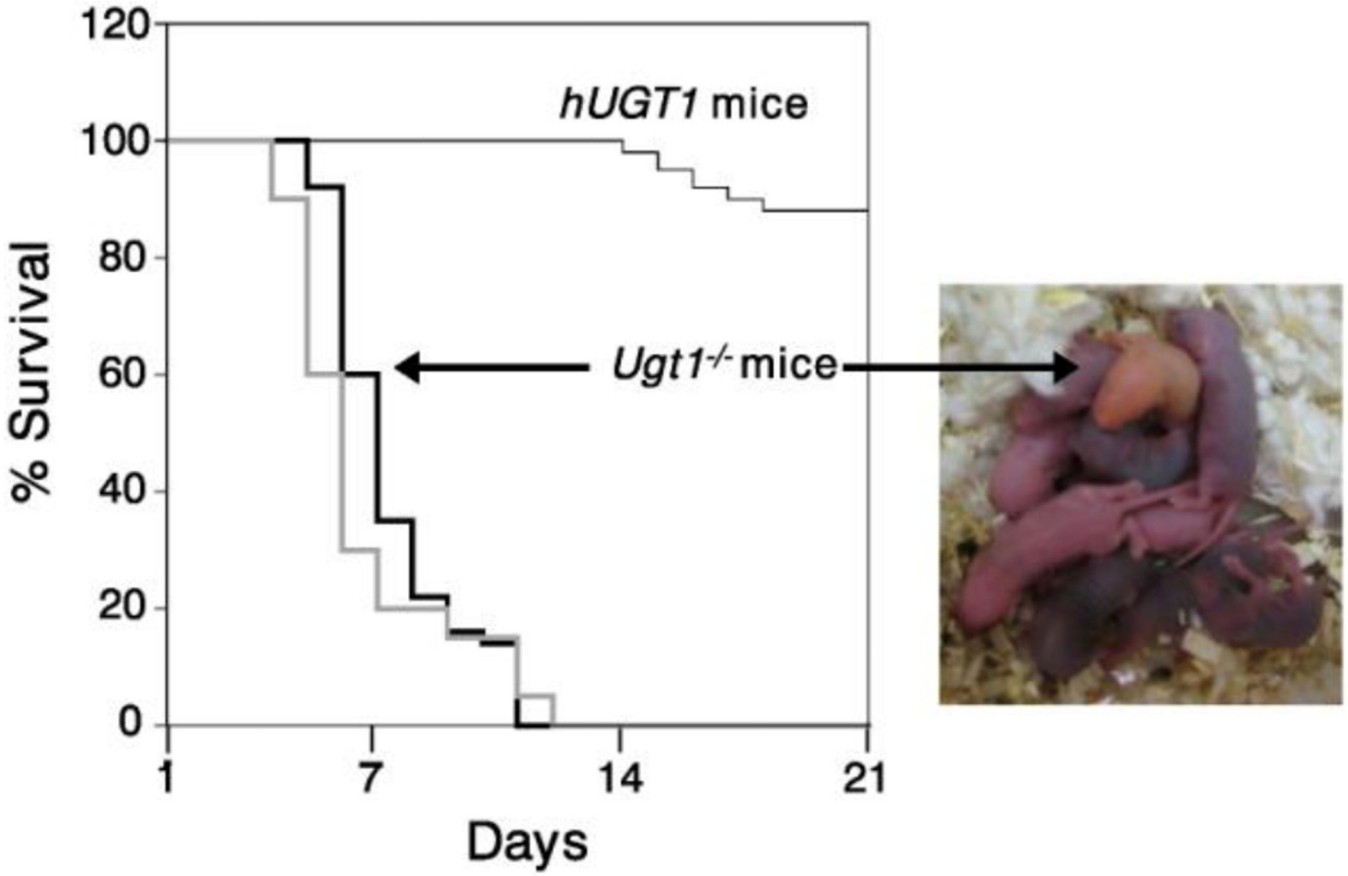

Fig. 2. Survival curves of *hUGT1* and *Ugt1*$^{-/-}$ mice

Due to the accumulation of lethal levels of TSB, most of *Ugt1*$^{-/-}$ mice die within 7 days after birth (thicker lines). Humanization with the UGT1 locus rescues neonatal lethality, although 5–10% of newborn *hUGT1* mice are still lethal (thin line). Black thick line indicates the survival curve of *Ugt1*$^{-/-}$ mice reported by Nguyen et al., (2008). Gray thick line indicates the survival curve of *Ugt1*$^{-/-}$ mice reported by Bortolussi et al., (2012).

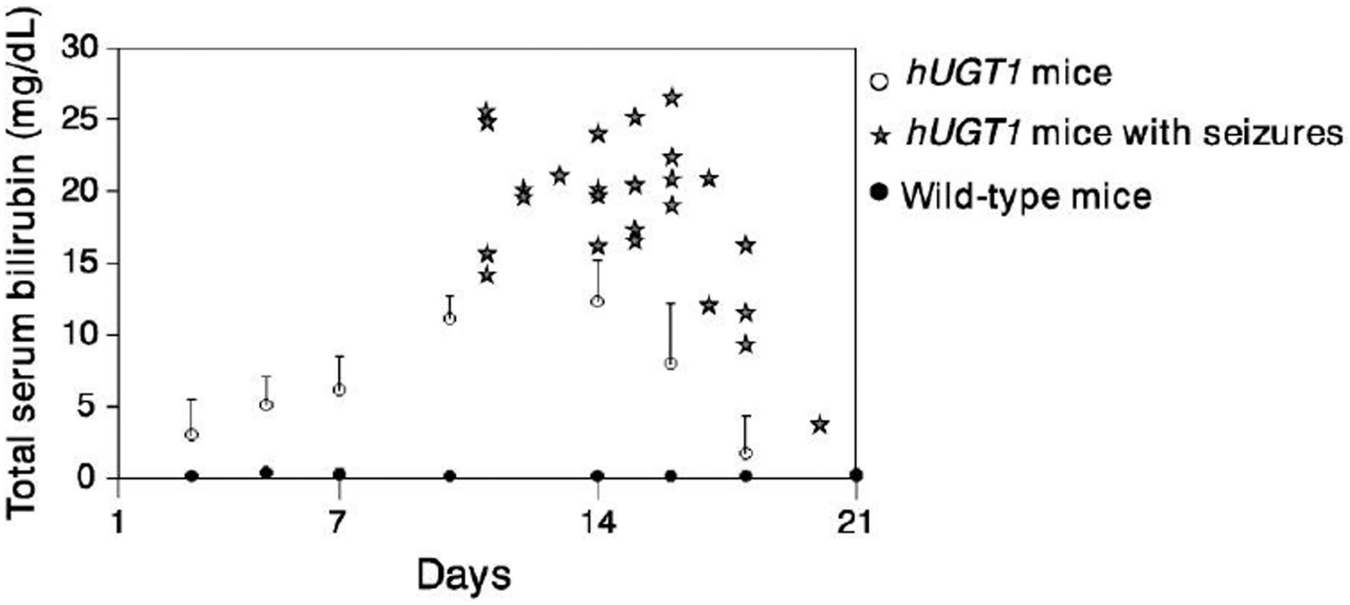

Fig. 3. Total serum bilirubin levels in neonatal *hUGT1* and wild-type mice

Mean TSB levels in neonatal *hUGT1* and wild-type mice were shown. Stars indicate the TSB levels of *hUGT1* mice that developed seizures.

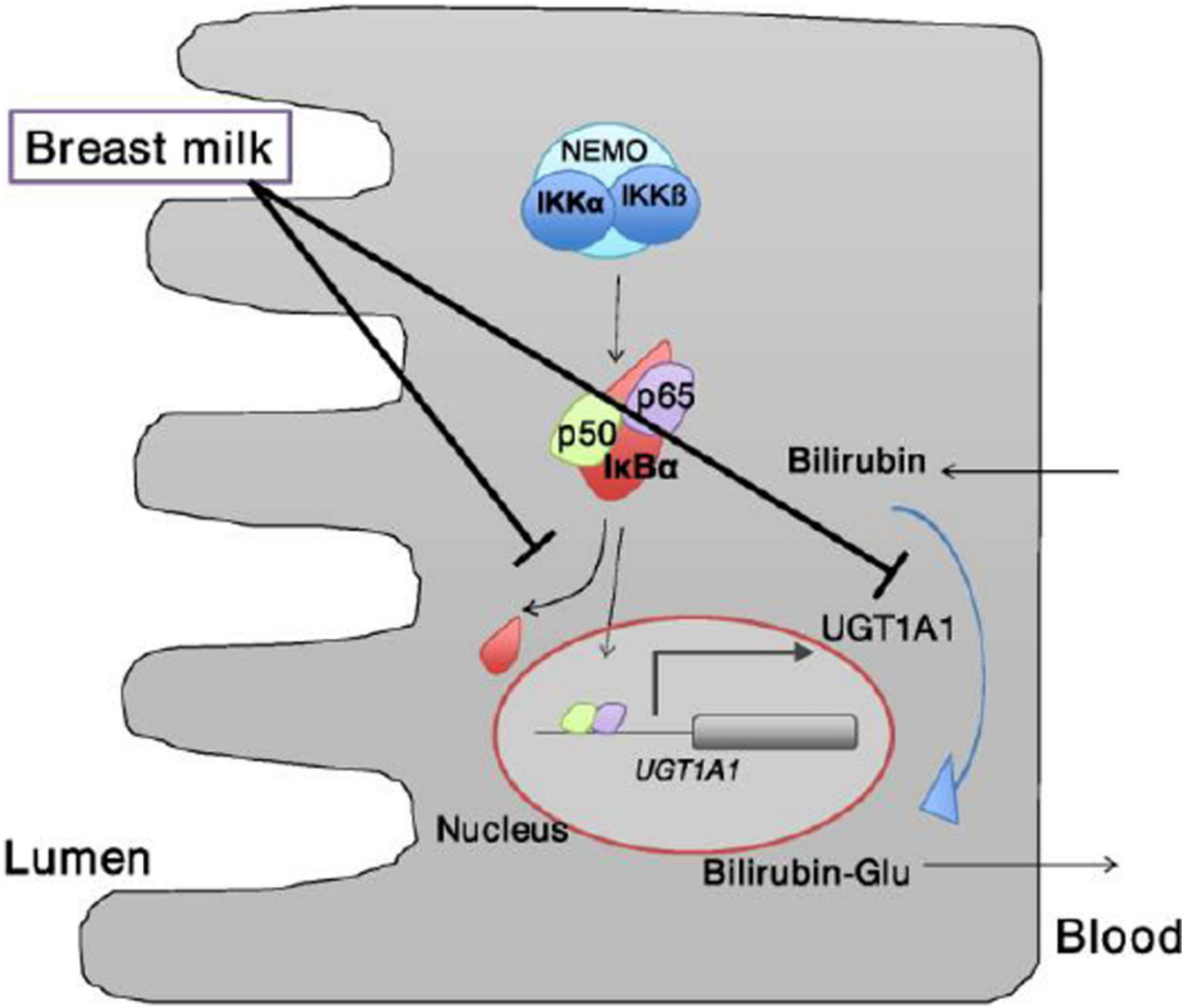

Fig. 4. Schematic representation of pathways in intestinal cells

In neonatal *hUGT1* mice, UGT1A1 metabolizes bilirubin in the gastrointestinal tract to prevent the accumulation of bilirubin. Intestinal UGT1A1 is under control by IKK/NF-κB (p50/p65) signaling. In the breast-fed neonates, breast milk can inhibit the NF-κB-mediated transcription of UGT1A1. Meanwhile, breast milk can also inhibit the bilirubin glucuronidation. NEMO, NF-κB essential modulator.

Table 1

Genetic polymorphisms in UGT1A1 and their impact on hyperbilirubinemia and neonatal jaundice

Polymorphism	Ethnic group	Frequency		Note	Reference
		Control*	Patient		
UGT1A1*6	Japanese	N.D.	0.68	Among 17 BMJ neonates, 8 neonates were homozygous and 7 were heterozygous.	Maruo et al., 2000
	Japanese	0.16	0.34	Allelic frequency was statistically higher in neonates with hyperbilirubinemia.	Maruo et al., 1999
	Malay	0.04	0.13	Allelic frequency was statistically higher in neonates with hyperbilirubinemia.	Azlin et al., 2011
		0.11	0.31	Allelic frequency was statistically higher in neonates with hyperbilirubinemia.	Huang et al., 2004
		0.13	0.23	Allelic frequency was statistically higher in neonates with hyperbilirubinemia	Chang et al., 2013
	Japanese	0.18	0.66	Allelic frequency was statistically higher in neonates with hyperbilirubinemia.	Maruo et al., 2014
UGT1A1*28	Japanese	N.D.	0.03	Among 17 BMJ neonates, just one neonate was heterozygous.	Maruo et al., 2000
	Japanese	0.15	0.04	Allelic frequency was lower in neonates with hyperbilirubinemia.	Maruo et al., 1999
	North Indian	0.29	0.487	Allelic frequency was higher in neonates with hyperbilirubinemia.	Agrawal et al., 2009
	Turkish	0.27	0.25	Allelic frequency was similar in neonates with hyperbilirubinemia.	Babaoglu et al., 2006
	Malay	0.001	0.035	Allelic frequency was not statically higher in neonates with hyperbilirubinemia.	Azlin et al., 2011
		0.065	0.02	Allelic frequency was lower in neonates with hyperbilirubinemia.	Huang et al., 2004
	Caucasia	0.41	0.41	Allelic frequency was similar in neonates with hyperbilirubinemia.	Travan et al., 2014
	Asian	0.16	N.D.		Beutler et al., 1998
	European	0.387	N.D.		Beutler et al., 1998
	Africa	0.426	N.D.		Beutler et al., 1998

*Allele frequency in neonates without hyperbilirubinemia.

Table 2

Impact of extrahepatic tissues on bilirubin metabolism

Tissue	Observations	Reference
Small intestine	UGT1A1 was highly expressed in small intestine	Nakamura et al., 2008 Tukey and Strassburg, 2000
	Transplantation of normal small intestine to Gunn rat resulted in decreased serum bilirubin levels	Takahashi et al., 1994 Medley et al., 1995
	Developmental expression of intestinal UGT1A1 correlated to serum bilirubin levels in neonatal *hUGT1* mice	Fujiwara et al., 2010
	Specific induction of UGT1A1 in small intestine resulted in a decrease in serum bilirubin levels in *hUGT1* mice	Fujiwara et al., 2012
	Breast milk suppressed UGT1A1 expression in small intestine of BMJ mice	Fujiwara et al., 2012
	Knockout of liver *Ugt1* locus and the *Ugt1a1* gene resulted in only a slight increase in TSB levels	Chen et al., 2013
Skin	UGT1A1 was expressed in human skin and human keratinocyte HaCaT cells	Sumida et al., 2013 Peters et al., 1987
	Expression of UGT1A1 was higher in the skin than that in the liver in neonatal *hUGT1* mice	Sumida et al., 2013
	UVB induced skin UGT1A1 and decreased serum bilirubin levels in *hUGT1* mice	Sumida et al., 2013