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P450-Humanized and Human Liver Chimeric Mouse Models for Studying Xenobiotic Metabolism and Toxicity

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ABSTRACT

Preclinical evaluation of drug candidates in experimental animal models is an essential step in drug development. Humanized mouse models have emerged as a promising alternative to traditional animal models. The purpose of this mini-review is to provide a brief survey of currently available mouse models for studying human xenobiotic metabolism. Here, we describe both genetic humanization and human liver chimeric mouse models, focusing on the advantages and limitations while outlining their key features and applications. Although this field of biomedical science is relatively young, these humanized mouse models have the potential to transform preclinical drug testing and eventually lead to a more cost-effective and rapid development of new therapies.

Introduction

Translating a drug from discovery to human therapy relies heavily on data from preclinical studies in animal models. However, many drug candidates fail during clinical trials because the experimental animal models used in preclinical studies poorly predict human xenobiotic metabolism. Such failures are caused in part by species differences in drug-metabolizing enzymes (DMEs), which have evolved and adapted to the different metabolic conditions of each species. Mice, for instance, have an expanded set of DMEs referred to as members of the cytochrome P450 (P450) family. In mice, these DMEs are encoded by 72 functional genes, whereas humans possess only 27 (Nelson et al., 2004). Thus, drugs are often metabolized differently in mice and in humans. Reactive drug metabolites drive toxicity, and their presence or absence is determined by the species-specific set of DMEs. Therefore, preclinical testing using a "humanized" system would be desirable before starting a clinical trial with a new drug.

There is growing interest and practice in utilizing humanized mouse models to overcome species differences in drug metabolism; the list of available humanized mouse models is rapidly expanding. Thus, although the topic has been frequently reviewed (Strom et al., 2010; Shen et al., 2011; Yoshizato et al., 2012; Kitamura and Sugihara, 2014; Sanoh and Ohta, 2014; Scheer and Wolf, 2014; Stiborová et al., 2014; Gonzalez et al., 2015; Scheer and Wilson, 2016; Yamazaki et al., 2016), it is worthwhile to revisit and update in the context of this special issue on humanized models of xenobiotic metabolism and toxicity.

It is necessary to stress that humanized mouse models are not humans. Though many of the available mouse models are valuable for revealing important aspects of human xenobiotic metabolism and response, none can perfectly replicate the human response. The purpose of this mini-review is to provide a brief survey of currently available mouse models for studying human xenobiotic metabolism by outlining their key features, limitations, and application notes, in hopes of helping the readers to identify the appropriate models for their specific application. Efforts are made to discuss P450-humanized mouse models, where one or more members of the mouse P450 family are replaced by relevant members of the human P450 family, together with human liver chimeric mouse models, where much of the mouse liver is replaced by human hepatocytes, given the current debate on the relative merits of the two distinct types of models.

P450-Humanized Mouse Models

P450 humanization in mice typically involves cross-breeding of a human *CYP*-transgenic mouse model with a relevant mouse-*Cyp* knockout mouse model or knocking in the human genes to replace the mouse genes. The human transgene fragment may include a single *CYP* gene or multiple neighboring *CYP* genes that are in close proximity and may share regulatory

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ABBREVIATIONS: BAC, bacterial artificial chromosome; BaP, benzo(a)pyrene; CAR, constitutive androstane receptor; DME, drug-metabolizing enzyme; FAH, fumarylacetoacetate hydrolase; FIAU, fialuridine; HAC, human artificial chromosome; HSVtk, herpes simplex virus type 1 thymidine kinase; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; P450, cytochrome P450; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; PXR, pregnane X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; uPA, urokinase-type plasminogen activator; WT, wild-type.

elements. The relevant knockout mouse may have one or multiple mouse *Cyp* genes deleted. The tissue specificity and expression levels of the human CYP transgene are not always identical to the situation in humans for a given transgenic mouse model. Thus, an effective application of a P450-humanized mouse model for studying xenobiotic metabolism or toxicity requires detailed knowledge of the characteristics of each transgenic model as well as the mouse-*Cyp* knockout model used. Unfortunately, some of the reported mouse models have not been characterized as thoroughly as others, which limits their usefulness for broad application.

Most human P450 family members that are involved in xenobiotic metabolism, including members of the *CYP1-4* gene families, have been introduced into the mouse genome as a transgene, as shown in Table 1. The reported characteristics of these human *CYP*-transgenic mouse models are summarized here, including the origin of the human transgene used for model generation, the nature of the promoter and regulatory components that control the expression of the transgene, and the main tissue sites of reported transgene expression. Additional information, such as on transgene inducibility, occurrence of sexual dimorphism, and means of humanization, that is relevant to the usefulness or limitation of a given model is further considered. Where available, applications of P450-humanized mouse models in xenobiotic metabolism or toxicity studies are briefly described. Discussions are organized by *CYP* gene families and subfamilies.

CYP1

CYP1A1 and *CYP1A2* are neighboring genes arranged in a head-to-head orientation, which made it practical and necessary to produce a transgenic mouse that harbors both human genes (Jiang et al., 2005). Given the use of a bacterial artificial chromosome (BAC) clone containing ample regulatory sequences of the human genes, both CYP1A1 and CYP1A2 are expressed in the same tissues in mice as in humans and demonstrate the expected inducibility by aryl hydrocarbon receptor ligands (Jiang et al., 2005; Dragin et al., 2007; Shi et al., 2008). Humanization was made possible by crossing the *CYP1A*-transgenic mouse to the *Cyp1a1/1a2*-null mouse. Expression of the transgene supported effective disposition of oral benzo(a)pyrene (BaP) and provided partial rescue of BaP-induced toxicological phenotypes in the null mice (Dragin et al., 2007). This humanized mouse model has also been used to demonstrate the ability of the human CYP1A transgenes to mediate PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; a dietary carcinogen)-induced colon carcinogenesis; the

TABLE 1

Human P450-transgenic and P450-humanized mouse models

Model	Human P450 Transgene Structure	Promoter	Mouse Gene Knockout for Humanization	References
CYP1A1/1A2-transgenic	Gene (BAC)	Authentic	None	Jiang et al. (2005)
CYP1A1/1A2 humanized	Gene (BAC)	Authentic	*Cyp1a1_1a2*-null	Dragin et al. (2007)
CYP1B1-transgenic	cDNA	Tetracycline-regulated promoter	None	Hwang et al. (2001)
CYP1B1 humanized	Gene (BAC)	Authentic	*Cyp1b1*-null	Li et al. (2014a)
CYP2A6-transgenic	cDNA	Mouse transthyretin promoter and enhancer	None	Zhang et al. (2005)
CYP2A13/2B6/2F1-transgenic	Gene (BAC)	Authentic	None	Wei et al. (2012)
CYP2A13/2B6/2F1 humanized	Gene (BAC)	Authentic	*Cyp2a5*-null *Cyp2f2*-null *Cyp2abfgs*-null	Megaraj et al. (2014) Cruzan et al. (2013) Li et al. (2014b)
CYP2A13-humanized	Gene (modified BAC)	Authentic	*Cyp2abfgs*-null	Jia et al. (2014)
CYP2C8-transgenic	cDNA	Mouse Tie2 promoter and enhancer	None	Lee et al. (2010)
CYP2C9-humanized	cDNA	Mouse albumin promoter	*Cyp2c*-null	Scheer et al. (2012a)
CYP2C18/2C19-transgenic	Gene (BAC)	Authentic	None	Löfgren et al. (2008)
CYP2D6-transgenic	Gene (PAC)	Authentic	None	Corchero et al. (2001)
CYP2D6-transgenic	Gene (PAC)	Authentic	None	Cheng et al. (2013)
CYP2D6.N-humanized	Gene (BAC)	Authentic	*Cyp2d*-null	Scheer et al. (2012b)
CYP2D6.1-humanized	Gene (modified BAC)	Authentic	*Cyp2d*-null	Scheer et al. (2012b)
CYP2D6.2-humanized	Gene (modified BAC)	Authentic	*Cyp2d*-null	Scheer et al. (2012b)
CYP2E1-transgenic	cDNA	Mouse albumin promoter/enhancer	None	Morgan et al. (2002)
CYP2E1-humanized	Gene (BAC)	Authentic	*Cyp2e1*-null	Cheung et al. (2005)
CYP2J2-transgenic	cDNA	Mouse Tie2 promoter and enhancer	None	Lee et al. (2010)
CYP3A4-transgenic	Gene (BAC)	Authentic	None	Granvil et al. (2003)
CYP3A4-transgenic	cDNA	Human ApoE promoter	None	van Herwaarden et al. (2005)
CYP3A4-humanized	cDNA	Human ApoE promoter or mouse villin promoter	*Cyp3a*-null	van Herwaarden et al. (2007)
CYP3A7-transgenic	cDNA	Mouse MT-1 promoter	None	Li et al. (1996)
CYP3A4/3A7-transgenic	Gene (BAC)	Authentic	None	Cheung et al. (2006)
CYP3A4/3A7-transgenic	Gene (BAC)	Authentic (PXR humanized)	None	Ma et al. (2008)
CYP3A4/3A7-humanized	Gene (modified BAC)	Authentic (PXR and CAR humanized)	*Cyp3a*-null (except for *Cyp3a13*)	Hasegawa et al. (2011a)
CYP3A4/3A5*3/3A7/3A43-humanized	Gene (HAC)	Authentic (CYP3A5 not expressed)	*Cyp3a*-null	Kazuki et al. (2013)
CYP3A4/3A5*1/3A7/3A43-humanized	Gene (modified HAC)	Authentic (CYP3A5 expressed)	*Cyp3a*-null	Abe et al. (2017)
CYP4A11-transgenic	Gene (BAC)	Authentic	None	Savas et al. (2009)
CYP4B1-transgenic	cDNA	Human ApoE promoter	None	Imaoka et al. (2001)
CYP4F2-transgenic	cDNA (expressing His-tag)	Mouse KAP promoter	None	Liu et al. (2009)

Models that are derived via cross-breeding between two different human CYP-transgenic models are not included. Authentic, original human CYP promoter was contained in the transgene; KAP, kidney androgen-regulated protein; PAC, P1 phage artificial chromosome.

humanized model showed colon tumorigenesis under conditions that did not yield detectable tumors in wild-type (WT) mice (Cheung et al., 2011). More recently, the same model was used to show the role of human CYP1As in PhIP-induced prostate carcinogenesis (Li et al., 2012) and the inhibitory effects of different forms of tocopherol on prostate cancer development (Chen et al., 2016).

CYP1A1/CYP1A2-humanizd mice have also been applied to studying drug metabolism. In one study utilizing the human-CYP1A1/1A2-transgenic/*Cyp1a2*-null mouse (Derkenne et al., 2005), the role of the human P450 family members in theophylline disposition was demonstrated. Although the necessity to preinduce transgene expression with a CYP1A inducer [2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)] makes the study complicated to conduct and interpret, the fact that human CYP1A-specific metabolites are formed in vivo illustrated the potential utility of the model for in vivo drug metabolism studies. Two recent articles (Lin et al., 2017; MacLeod et al., 2018) also described the utility of a newly generated CYP1A1/CYP1A2-humanized model in studying the disposition of anticancer drugs; however, details regarding the mouse model were not provided.

Several issues may complicate the data interpretation or limit the application of the current CYP1A1/CYP1A2-humanized mouse model. Because of technical difficulties in separating human CYP1A1 and CYP1A2 proteins on immunoblots, the absolute levels of these proteins in various tissues of the transgenic or humanized mice have not been determined. The *Cyp1a1*-null mice were reportedly hyper-responsive to BaP-induced expression of *Cyp1b1*, which was not overcome by the transgenic expression of the human CYP1As (Dragin et al., 2007); it is unclear whether this property extends to the induction of *Cyp1b1* by other inducers. Given the overlapping substrate specificity between CYP1A1 and CYP1A2 and their known common responses to inducers, it would also be advantageous to have mouse models that express only one human CYP1A.

Although a human *CYP1B1* transgenic mouse with an inducible tetracycline promoter was reported many years ago (Hwang et al., 2001), the model was not used for studies on CYP1B1 function. A CYP1B1-humanized mouse model was recently generated by cross-breeding between a human *CYP1B1*-BAC transgenic mouse and a *Cyp1b1*-null mouse (Li et al., 2014a). Although this model contained the endogenous human promoter, constitutive expression of human CYP1B1 mRNA in the mouse liver and extrahepatic tissues was much lower than that of mouse CYP1B1; but human CYP1B1 protein was detectable in the liver after induction with TCDD (Madeen et al., 2017). The transgenic expression of human CYP1B1 was insufficient to drive dibenzo[def, p]chrysene-induced transplacental carcinogenesis according to comparisons of lung tumor data from WT, *Cyp1b1*-null, and CYP1B1-humanized mice (Madeen et al., 2017). Further characterization of the constitutive and inducible expression of the human *CYP1B1* transgene, particularly in extrahepatic target tissues, would facilitate application of the model in xenobiotic metabolism and toxicity studies.

CYP2

A *CYP2A6*-transgenic mouse model was developed using the CYP2A6 cDNA driven by a liver-specific murine transthyretin promoter/enhancer (Zhang et al., 2005). The CYP2A6 protein level in the transgenic mouse liver was within the range observed in human liver microsomes; however, the use of a surrogate promoter prohibits the utility of the mouse model to study the transcriptional regulation of the *CYP2A6* gene. The transgenic mouse liver displayed significantly higher coumarin 7-hydroxylation activities than WT mice, both in vitro and in vivo. Efforts are underway in the X. Ding laboratory to humanize CYP2A6, by intercrossing the *CYP2A6*-transgenic mouse with the *Cyp2abfgs*-null mouse, which has all *Cyp* genes in the mouse *Cyp2a*, *Cyp2b*, *Cyp2f*, *Cyp2g*, and *Cyp2s* subfamilies deleted (Li et al., 2014b). The resultant CYP2A6-humanized mouse should be valuable for studying the function of hepatic CYP2A6 in drug metabolism and chemical toxicity. Nonetheless, a *CYP2A6*-BAC transgenic mouse that demonstrates human-like CYP2A6 expression in hepatocytes as well as extrahepatic tissues such as the lung is still needed.

A *CYP2A13/CYP2B6/CYP2F1*-transgenic mouse model was developed using a BAC clone containing the three closely situated genes *CYP2A13*, *CYP2B6*, and *CYP2F1* (Wei et al., 2012). The tissue distribution of transgene expression closely resembled the known profile in humans, with a respiratory tract–selective expression of CYP2A13 and CYP2F1 and an hepatic expression of CYP2B6. Three humanized versions of this mouse model have been described. The initial study of a humanized model on a *Cyp2a5*-null background revealed the remarkable ability of the human transgenes to mediate lung tumorigenesis induced by the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Megaraj et al., 2014). The same model was used to demonstrate the ability of lung inflammation to suppress CYP2A13 expression in vivo (Wu et al., 2013; Liu et al., 2015). In contrast, studies on styrene lung toxicity, utilizing a CYP2A13/CYP2B6/CYP2F1-humanized model on the *Cyp2f2*-null background, suggested that the human transgenes did not play a significant role (Cruzan et al., 2013).

A newer version of the humanized mice was prepared on *Cyp2abfgs*-null background; this null mouse was resistant to NNK-induced lung tumorigenesis (Li et al., 2014b). Studies using the CYP2A13/CYP2B6/-CYP2F1-humanized model on the *Cyp2abfgs*-null background confirmed that human CYP2F1 is functional in the bioactivation of naphthalene, an acute lung toxicant and lung and nasal carcinogen, and that CYP2A13 and CYP2F1 are capable of mediating naphthalene toxicity in the lung and nasal mucosa in vivo (Li et al., 2017).

The polygenic nature of the CYP2A13/CYP2B6/CYP2F1-humanized model may limit the ability to identify specific roles of each of the *CYP* transgenes in the metabolism or toxicity of a given compound. This was overcome in part by the generation of a *CYP2A13* transgenic mouse model, in which the neighboring CYP2F1 and CYP2B6 genes were inactivated through targeted mutations in the BAC genomic clone, while preserving the regulatory sequences (Jia et al., 2014). A comparison of the CYP2A13-humanized model and CYP2A13/CYP2B6/CYP2F1-humanized model provided definitive data to support the role of CYP2A13 in NNK bioactivation in the lung (Jia et al., 2014) and the respective activities of CYP2A13 and CYP2F1 toward naphthalene (Li et al., 2017).

The hepatic expression, induction, and activity of CYP2B6 have also been characterized in the CYP2A13/CYP2B6/CYP2F1-humanized mouse models (Liu et al., 2015). The transgenic *CYP2B6* was highly induced by phenobarbital and dexamethasone, which are known inducers of CYP2B6 in human liver, although a sexual dimorphism in the induction was observed, with males being more responsive to the inducers. The transgenic *CYP2B6* was active in the metabolism of bupropion, a known CYP2B6 substrate drug; it also contributed to systemic clearance of nicotine. Notably, constitutive expression of CYP2B6 in the liver is low in this model (Li et al., 2018); studies on the in vivo roles of hepatic CYP2B6 in xenobiotic metabolism and toxicity should examine both naive and inducer-treated mice.

Three different human *CYP2C*-transgenic mouse models have been reported. One was a *CYP2C8*-transgenic mouse, which, together with a human *CYP2J2*-transgenic mouse, was developed using human cDNA driven by the murine endothelium–specific *Tie2* promoter (Lee et al., 2010). These mouse models, which were generated to study the role of renal P450 epoxygenase function in the regulation of blood pressure, have not been humanized (i.e., the corresponding mouse P450 family members have not been removed). A CYP2C9-humanized mouse model

was generated on a *Cyp2c*-null background; the *CYP2C9* transgene was driven by the liver-specific albumin promoter, whereas 14 of the 15 functional mouse *Cyp2c* genes (except *Cyp2c44*) were deleted (Scheer et al., 2012a). The transgenic *CYP2C9* was active toward the known CYP2C9 substrates tolbutamide and diclofenac (Scheer et al., 2012a). A *CYP2C18*/*CYP2C19*-transgenic mouse was generated using a BAC genomic clone containing the complete *CYP2C18*/*CYP2C19* genes (Löfgren et al., 2008). *CYP2C18* and *CYP2C19* mRNA expression exhibited sexual dimorphism in the transgenic mouse, in an apparent tissue-specific manner (Löfgren et al., 2008), and both genes were subject to regulation by androgen and growth hormones (Löfgren et al., 2009). Functional CYP2C19 protein was expressed mainly in the male mouse liver, as evidenced by microsomal immunoblot analysis and activity toward two known CYP2C19 substrates, *R*-omeprazole and *S*-mephenytoin; CYP2C18 protein was not detected in either liver or kidney. It is anticipated that a humanized version of the *CYP2C18*/*CYP2C19*-transgenic mouse would be useful for in vivo studies of CYP2C19-mediated xenobiotic metabolism or toxicity in male mice.

Several *CYP2D6* transgenic mouse models have been reported. The first *CYP2D6*-transgenic mouse was generated using a phage genomic clone containing the entire *CYP2D6* gene and flanking sequences (Corchero et al., 2001). The model was referred to as a humanized mouse, since the mouse CYP2D enzymes have little activity toward some of the human CYP2D6 substrates (e.g., debrisoquine). Transgenic *CYP2D6* was expressed in multiple mouse tissues, including liver, small intestine, and kidney, as in humans; but, unlike in humans, the transgenic CYP2D6 was not expressed in the brain (Corchero et al., 2001; Miksys et al., 2005). This mouse model was used extensively to study the in vivo role of CYP2D6 in the metabolism of drugs, such as debrisoquine, and of various endogenous compounds, particularly the role of CYP2D6 in the metabolism of amines like serotonin (Yu et al., 2004; Cheung and Gonzalez, 2008; Shen and Yu, 2009; Wu et al., 2009; Winter et al., 2011). It has also been used to study in vivo regulation of *CYP2D6* expression (Koh et al., 2014; Pan and Jeong, 2015; Pan et al., 2015, 2017; Kent and Jeong, 2017). A second *CYP2D6*-transgenic mouse model was generated using a P1 artificial chromosome clone containing the complete human CYP2D locus; it was found to have CYP2D6 expression in the brain as well as in other tissues (Cheng et al., 2013). However, as far as we know, this model has not been used for the study of xenobiotic metabolism. Neither model has been crossed to a *Cyp2d*-null background.

A series of three CYP2D6-humanized mice (encoding CYP2D6.1, CYP2D6.2, and a novel CYP2D6 variant, CYP2D6.N) were produced on a *Cyp2d*-null background, using an expression cassette derived from a *CYP2D6* BAC clone; the cassette consisted of 9 kilobase pairs from promoter region and the 3′-untranslated region, as well as all exons and introns, from a novel *CYP2D6* allele (Scheer et al., 2012b). The *CYP2D6.1* and CYP2D6.2 expression cassettes were produced via coding region modifications of the CYP2D6.N cassette. The CYP2D6.1 strain was characterized only on a heterozygous *Cyp2d*-null background, but the other two strains, particularly CYP2D6.2, were more thoroughly characterized. All three transgenic proteins were expressed in liver and intestine and metabolized known CYP2D6 substrates bufuralol or debrisoquine. One of these strains, CYP2D6.2, was used to study the role of CYP2D6 in the metabolism of primaquine (Potter et al., 2015) and the interaction between tamoxifen and several antidepressants, such as paroxetine, that are both substrates and inhibitors of CYP2D6 (MacLeod et al., 2017).

Two different human *CYP2E1*-transgenic mice have been reported. The first model was developed using a *CYP2E1* cDNA driven by the murine albumin enhancer/promoter (Morgan et al., 2002). Given the known regulation of CYP2E1 expression by post-translational mechanisms, it was not surprising that the transgenic protein was induced by chronic alcohol treatment. This overexpression model was used for studying the role of CYP2E1 in alcohol-induced liver injury and other pathologic changes (Butura et al., 2009; Kathirvel et al., 2009), but the model has not been humanized. Another *CYP2E1*-transgenic mouse model was developed using a BAC clone consisting of the complete *CYP2E1* gene; the model was then humanized on the *Cyp2e1*-null background (Cheung et al., 2005). The humanized model was used to demonstrate the ability of human CYP2E1 in mediating acetaminophen-induced liver toxicity (Cheung et al., 2005) and ethanol-induced liver injury (Cederbaum, 2010; Lu et al., 2010).

CYP3

Several versions of human *CYP3A*-transgenic mice have been reported. A *CYP3A4*-transgenic mouse model was developed using a BAC clone consisting of the complete *CYP3A4* gene (Granvil et al., 2003). CYP3A4 was expressed mainly in the small intestines of adult male mice in this model, which was used to demonstrate the importance of intestinal CYP3A4 in the first-pass metabolism of midazolam and the interaction between midazolam and ketoconazole (Granvil et al., 2003). Hepatic CYP3A4 expression was largely influenced by age and sex in this model (Yu et al., 2005), a phenomenon also observed in a BAC *CYP3A4*/*CYP3A7*-transgenic mouse (Cheung et al., 2006; Felmlee et al., 2008) and an intercrossed *CYP3A4*/*CYP2D6*-double transgenic mouse (Felmlee et al., 2008). The BAC clone that was used for the generation of the above *CYP3A4*-transgenic mice was further used to generate a new mouse model that harbored the *CYP3A4* and *CYP3A7* genes and are humanized for pregnane X receptor (PXR) (Ma et al., 2008). Notably, hepatic CYP3A4 expression in this mouse model still showed age-dependent suppression in adult males. Nonetheless, the incorporation of PXR humanization added a new dimension for application in drug metabolism research (Cheng et al., 2009, 2011; Holmstock et al., 2013).

None of the above *CYP3A4*-transgenic strains was on a *Cyp3a*-null background, in contrast to another type of model where all or most of the endogenous mouse *Cyp3a* genes have been removed, such as the "multiple humanized" *Cyp3a*-null (except for *Cyp3a13*) mouse model—the CYP3A4/CYP3A7/PXR/constitutive androstane receptor (CAR)-humanized mouse (Hasegawa et al., 2011a). In a further improvement, humanized CYP2C9, CYP2D6, and CYP3A4/7 models were combined with humanized PXR and CAR through sequential cross-breeding (Scheer et al., 2015). Both models have seen effective application (MacLeod et al., 2015; Chang et al., 2016; Ly et al., 2017; McMillan et al., 2018).

The transgenic expression and function of CYP3A7, which is restricted to the embryo in humans, have not been studied as extensively as those of CYP3A4. An early model using *CYP3A7* cDNA driven by the mouse metallothionein promoter supported CYP3A7 expression in adult tissues and demonstrated apparent activity toward aflatoxin B_1 (Li et al., 1996). The transgenic *CYP3A7* from the *CYP3A4*/*CYP3A7* BAC transgenic mouse was developmentally regulated by glucocorticoids (Pang et al., 2012). However, few studies have examined the role of CYP3A7 in fetal drug metabolism using these models.

In a different approach than BAC transgenesis, two similar lines of *CYP3A4*-transgenic mice were generated using CYP3A4 cDNA driven by the human Apolipoprotein E promoter (van Herwaarden et al., 2005). Transgenic *CYP3A4*, which was active toward cyclosporin and midazolam, was selectively expressed in the liver and, at lower levels, the kidney. A complementary model developed using CYP3A4 cDNA driven by the mouse villin promoter was also generated, which expressed CYP3A4 selectively in duodenum, jejunum, ileum, and colon (van Herwaarden et al., 2007). Both models were humanized on the *Cyp3a*-null background. The two humanized models are not amenable

for studies of transcriptional regulation of CYP3A4 or drug-drug interactions at the gene regulation step, as the CYP3A4 expression is controlled by heterologous promoters, rather than by the authentic human *CYP3A4* promoter. However, they are valuable for studying the respective contributions of CYP3A4 in the two organs for the disposition of a variety of drugs, as well as the impact of the interactions among the drugs on CYP3A4 enzyme function (van Herwaarden et al., 2007; van Waterschoot et al., 2009, 2010; Mitsui et al., 2014; Choo et al., 2015; Zhang et al., 2016).

A *CYP3A* (*CYP3A4*, *CYP3A5*, *CYP3A7*, *CYP3A43*) [human artificial chromosome (HAC)] transgenic mouse, humanized on a *Cyp3a*-null background, has also been reported; the human transgenes showed human-like tissue specificity and developmental regulation, as well as an ability to metabolize marker substrates for CYP3A4 and CYP3A7 (Kazuki et al., 2013). Notably, although this model does not show the age-dependent hepatic CYP3A4 suppression that was found in male *CYP3A4*-BAC transgenic mice (Yu et al., 2005), the CYP3A4 expression level was significantly lower in males than in females (Kobayashi et al., 2017). The fetal expression of functional CYP3A7 in this mouse model allowed it to be used for demonstrating the ability of the human transgenes to support thalidomide-induced embryonic toxicity (Kazuki et al., 2016). However, the *CYP3A5* transgene was of the nonexpressed *3 allele, which has since been converted using gene-editing technology to the *1 allele, yielding the first *CYP3A*-humanized mouse model with functional CYP3A5 protein in the liver and intestine (Abe et al., 2017).

CYP4

Few models of human *CYP4* transgenic mice have been reported. Two lines of *CYP4A11* transgenic mouse were generated using a BAC clone (Savas et al., 2009); the model has been used to study CYP4A11 regulation and protein modification, as well as the role of CYP4A11 in 20-hydroxyeicosatetraenoic acid–dependent hypertension (Savas et al., 2016; Albertolle et al., 2017). A *CYP4B1* transgenic mouse was prepared using CYP4B1 cDNA driven by the human apolipoprotein E gene promoter for hepatic expression; the transgenic *CYP4B1* was active toward lauric acid and 2-aminofluorene (Imaoka et al., 2001). A *CYP4F2*-transgenic mouse was prepared using CYP4F2 cDNA driven by a kidney androgen-regulated protein promoter for the production of an His-tagged CYP4F2 protein in the renal proximal tubule epithelial cells; the protein was active in production of 20-hydroxyeicosatetraenoic acid (Liu et al., 2009). None of these have been humanized or used for studies of drug metabolism.

Challenges of Genetic Humanization

Besides the many obvious advantages and successful applications, as described above, humanization by exchanging murine genes with their human orthologs (genetic humanization) also has some potential limitations.

Species Difference in Regulation of the Human P450 Transgene. Despite the efforts to humanize some relevant nuclear factors, such as PXR and CAR, the human *P450* transgenes may be regulated by other factors, some of which we may not know or may not be able to humanize. Such differences in regulation may lead to species-specific drug-drug interactions that result from drug-induced changes in P450 expression in humanized mice, but not in humans, or vice versa.

Incomplete Humanization of All Pathways and Events Related to Drug Absorption, Distribution, Metabolism, and Disposition. Species differences in competing phase I metabolism pathways, phase II metabolism, or transporters can affect in vivo pharmacokinetic outcome.

Challenges in Studying Genetic Variants. Genetic polymorphisms of human *P450* genes can be studied in the mouse models, but it may not be cost effective. With the rapid advancement of the gene-editing technology, it is easier than ever to generate mice that produce variant P450 proteins for functional analysis. However, the large number of these variants, the lack of complete understanding of the role of noncoding sequences in transgene expression, and the need to characterize each model once generated, can be cost and time intensive.

Lack of Complete Characterization. Most currently available models are incompletely characterized or documented. Some of these models were produced for a purpose other than xenobiotic metabolism, which impacted the extent to which the model was analyzed. Critical information regarding potential compensatory changes in the mouse DME genes is incompletely analyzed at best. At a minimum, the tissue distribution, levels of transgene protein expression, and inducibility at major sites of transgene expression should be determined and compared with those of humans. Information on developmental expression and sex differences in transgene expression, as well as activity of the transgenic human P450 toward known substrates, are also important to document.

Understanding the properties of a given model under study, including its limitations, will ensure proper experimental design. The factors to consider may include the selection of control mouse strains, the selection of proper genetic background, the inclusion of necessary in vitro studies and in vivo pharmacokinetic analysis of test compounds for efficacy studies, and the inducibility of the transgene by test compounds. Furthermore, the incorporation of the pharmacological inhibition approach for validation, as well as the confirmation of transgene expression in the specific conditions used for testing xenobiotic metabolism/toxicity are also useful. It may also be advisable to validate key findings in additional mouse models, such as the human liver chimeric mouse models described below.

Human Liver Chimeric Mouse Models

Development of Human Liver Chimeric Mice

An interesting alternative to the problems faced by genetic humanization is to develop human liver chimeric mice. Transplantation of entire human hepatocytes, instead of only replacing the gene of interest in mouse hepatocytes, leads to a cellular chimerism in the liver and circumvents some of the aforementioned challenges. Hepatocyte transplantation is usually performed via splenic injection of mice (Ponder et al., 1991). The hepatocytes migrate from the spleen into the liver where they integrate into the hepatic plates of the murine liver. Hepatocytes have also been transplanted into sites other than the liver such as the lymph nodes (Komori et al., 2012) or peritoneal cavity (Strom et al., 1999), but the need for proper drainage of the bile limits the long-term benefit of ectopic hepatocyte transplantation. Three different models of human liver chimeric mice have been described.

The *alb-uPA* Mouse. The principles of human liver chimerism were established using the transgenic *alb-uPA* mouse (urokinase-type plasminogen activator expressed under the albumin promoter), which was the first mouse model in which the liver could be repopulated with human hepatocytes (Dandri et al., 2001; Mercer et al., 2001). The *alb-uPA* mouse was developed as a coagulation model by the laboratories of Ralph Brinster (University of Pennsylvania) and Richard Palmiter (Howard Hughes Medical Institute and University of Washington) (Heckel et al., 1990). As expected, many of the *alb-uPA* mice died neonatally because of bleeding. About half of the transgenic pups survived but underwent liver failure a few weeks after birth. Interestingly, a few mice survived this second crisis and urokinase-type plasminogen activator (uPA) serum levels gradually returned to normal levels within 2 months. Analysis of these mice revealed healthy looking nodules within the liver, which expanded over months and eventually completely replaced the sick liver. Molecular analyses demonstrated

a genetic rearrangement that partially excised the toxic transgene (*alb-uPA*) within the nodules, which lead to clonal expansion of transgene-deficient hepatocytes (Sandgren et al., 1991). This natural selection phenomenon was subsequently recognized as a promising approach to efficiently expand transplanted healthy hepatocytes in a diseased liver. Rhim et al. (1994) were the first to prove this hypothesis in the *alb-uPA* mouse using healthy murine hepatocytes as a donor source. Eventually, two independent groups generated the first human liver chimeric mice, both using the *alb-uPA* strain (Dandri et al., 2001; Mercer et al., 2001). The *alb-uPA/SCID* mouse, which is also immune deficient to prevent donor cell rejection, can reach high human chimerism (Tateno et al., 2004).

The pioneering work done in the *alb-uPA* strain opened the door to hepatocyte repopulation and particularly to human liver chimerism. However, the *alb-uPA* mouse has several disadvantages, (see below and Table 2); therefore, updated versions of this strain (Weglarz et al., 2000; Suemizu et al., 2008; Tesfaye et al., 2013; Tateno et al., 2015) as well as new mouse genotypes amenable to human hepatocyte transplantation were developed (Washburn et al., 2011; Borel et al., 2017). We will limit this mini-review to human liver chimeric mice with a high degree of human chimerism (>70% of the liver), which is essential for many in vivo applications, including drug metabolism.

The *Fah*$^{-/-}$/*Il2rg*$^{-/-}$/*Rag2*$^{-/-}$ Mouse. Repopulation with nodules of healthy hepatocytes has also been observed in patients with hereditary tyrosinemia type I (Kvittingen et al., 1993). In this case, the point mutation underlying the genetic disorder in the fumarylacetoacetate hydrolase (*FAH*) gene reverted to the WT allele (Kvittingen et al., 1994) by somatic mutation. Analogous to the rearranged *uPA* transgenic hepatocytes, the corrected *FAH* hepatocytes possess a growth advantage over the mutant cells and clonally expand to form nodules. This information in hand, it was apparent that the *Fah*$^{-/-}$ mouse could be repopulated with WT murine hepatocytes (Overturf et al., 1996), as shown previously for the *uPA* mouse (Rhim et al., 1994). Although *Fah*$^{-/-}$ mice were similarly neonatally lethal, they had an advantage over the transgenic *uPA* predecessors: the toxicity resulting from the mutated *Fah* gene could be regulated by the small-molecule drug nitisinone. After *Fah*$^{-/-}$ mice receive transplantations, nitisinone is withdrawn to apply selection pressure for the transplanted, FAH-positive hepatocytes. In 2007, two groups independently established the *Fah*$^{-/-}$/*Il2rg*$^{-/-}$/*Rag2*$^{-/-}$ (FRG) mouse strain as a human liver chimeric mouse model (Azuma et al., 2007; Bissig et al., 2007). Their results were comparable and demonstrated robust repopulation of adult FRG mice with both fresh and cryopreserved human hepatocytes. In addition, Azuma et al. (2007) showed serial transplantation of human hepatocytes in FRG mice, whereas Bissig et al. (2007) demonstrated neonatal hepatocyte transplantation and viral transduction of transplanted human hepatocytes. Humanized FRG mice were healthy and could achieve high human chimerism (<95% human) (Bissig et al., 2010).

The TK-NOG Mouse. More recently, the TK-NOG mouse has been developed (Hasegawa et al., 2011b) for human liver chimerism. This strain contains the transgenic herpes simplex virus type 1 thymidine kinase (*HSVtk*) under the albumin promoter. HSVtk phosphorylates the drug ganciclovir, which leads to intoxication of the cell, whereas human hepatocytes without the transgene are not sensitive to the drug. Ganciclovir is administrated before the hepatocyte transplantation, inducing intoxication of murine hepatocytes. In principle, this murine-specific intoxication could be used repeatedly even after the human hepatocytes are transplanted; however, none of the publications on the TK-NOG mouse describe such a repeated application. This mouse strain has also been shown to reach high human chimerism both by immunostaining and by human albumin levels in the murine serum (Hasegawa et al., 2011b).

Utility of Human Liver Chimeric Mice for Drug Metabolism

Since the primary site of drug metabolism is the liver, human liver chimeric mice are an attractive alternative to conventional animal models when it comes to in vivo validation of a new drug. All three human liver chimeric mouse models discussed here have been used for drug studies: human toxicity (Yamamoto et al., 2007; Sato et al., 2008; Foster et al., 2012; Kakuni et al., 2012; Samuelsson et al., 2014; Xu et al., 2014, 2015); human drug metabolism profiling (Katoh et al., 2007; Inoue et al., 2008; Lootens et al., 2009a,b,c, 2011; Pozo et al., 2009; Samuelsson et al., 2012, 2014; Sanoh et al., 2012b,c; Schulz-Utermoehl et al., 2012; Yamazaki et al., 2012; Nishimura et al., 2013; Tanoue et al.,

TABLE 2

Comparisons among the three human liver chimeric mouse models

Strain	uPA	FRG	TK-NOG
Development/genotype	Dandri et al. (2001)[1] – alb-uPA/Rag2$^{-/-}$ Mercer et al. (2001)[2] – alb-uPA/SCID/Bg Tesfaye et al. (2013)[3] – mup-uPA/SCID/Bg Tateno et al. (2015)[4] – alb-cDNA-uPA/SCID	Bissig et al. (2007) Azuma. et al. (2007) FRG	Hasegawa et al. (2011b) Alb-HSVtk/SCID/Il2rg$^{-/-}$
Mechanism of murine liver injury	Intoxication by liver overexpression of uPA [1–4]	Accumulation of toxic tyrosine catabolites	Generation of a toxic ganciclovir metabolite by expression of HSVtk in the liver
Advantages	Most experience	Inducible system Hepatocyte transplantation at any age	Inducible system Hepatocyte transplantation at any age
Drawbacks	Noninducible murine liver injury due to a constitutive uPA expression [1–4] Narrow window of hepatocyte transplantation [1,2,4] High postnatal mortality due to internal bleeding [1,2,4] Spontaneous deletion of the uPA transgene decreases human hepatocyte repopulation and increases liver tumors incidence [1,2] Kidney disorders [1,2] Small body size [1,2] Female reproductive disorders [1,2] High human hepatocyte repopulation requires inhibition of host innate immune response and/or the human complement [1,2]	Human hepatocyte repopulation requires small molecular drug (nitisinone) Kidney disease due to FAH deficiency Cancer model with frequent liver tumors	Repopulation efficiency more hepatocyte donor dependent than other models Selection pressure only applied before hepatocyte transplantation Low breeding efficiency due to male sterility

2013; Bateman et al., 2014; Kamimura et al., 2015; Nakada et al., 2016; Wilson et al., 2018); drug-drug interaction (Hasegawa et al., 2012; Nishimura et al., 2013; Yamazaki et al., 2013; Suzuki et al., 2017; Uchida et al., 2018); human pharmacogenetic studies (Hu et al., 2013; Nishiyama et al., 2015); or other DME studies (Katoh and Yokoi, 2007; Inoue et al., 2009; Sanoh et al., 2012a, 2015; Schulz-Utermoehl et al., 2012; Tsukada et al., 2013; Suemizu et al., 2014; Nishiyama et al., 2015; Utoh et al., 2016; Kamimura et al., 2017; Shimizu et al., 2017; Yamazaki-Nishioka et al., 2018). A detailed analysis of all these studies is beyond the scope of this review; we will confine our review to a few examples illustrating the utility of human liver chimeric mice for drug studies.

A recent study by Xu et al. (2014) exemplifies the advantages of using human liver chimeric mouse models for hepatotoxicity over current preclinical models. In this study, the authors tested the dose response of the drug fialuridine (FIAU) in highly humanized TK-NOG mice. FIAU is a nucleoside analog that was developed to treat hepatitis B viral infections. FIAU did not present any toxicity in preclinical animal models but was terminated in phase II clinical trials because of lactic acidosis in patients after long-term exposure to the drug, which led to liver complications and pancreatitis (McKenzie et al., 1995). The authors showed a dose-dependent liver toxicity in the human liver chimeric mouse group as opposed to the nonhumanized control group. After 4 days of treatment with the highest dose of FIAU (400 mg/kg), only the chimeric group appeared lethargic and presented lactate and alanine aminotransferase elevations in plasma. Histologically, the liver tissue of these mice presented an accumulation of lipids in the human regions but not in the corresponding mouse areas. Furthermore, analysis by electron microscopy demonstrated mitochondrial abnormalities in the human hepatocytes, which were previously demonstrated to be the cause of liver failure in humans.

Another study by Nakada (2017) showed that chimeric mouse models were able to generate specific human metabolites that are not present in other species. The glucuronide metabolite M1 of YM543, a selective inhibitor of the sodium-glucose cotransporter, was detected only in the blood and urine of humans and humanized uPA/SCID mice, but not the other species tested (cynomolgus monkey, rhesus monkey, marmoset, beagle dog, NZW rabbit, Hartley guinea pig, golden hamster, and ICR mouse). Moreover, in vitro studies incubating human hepatocytes with YM543 did not show the presence of M1 metabolite, which demonstrates the ability of chimeric mouse models to predict human drug metabolites beyond those detected in cell culture platforms.

These two studies highlight the utility of human liver chimeric mice and their ability to identify "human-like" profiles for metabolites, toxicity, and drug-drug interactions. There is optimism that human liver chimeric mouse technology will become an essential element in preclinical drug development, since such models have several distinct advantages. In contrast to clinical trials, the effect of drugs can be tested in an isogenic context in human liver chimeric mice, meaning that the control groups are repopulated with hepatocytes from the same donor. Hence, the experimental readout is not biased by polymorphisms in P450 family members or other drug-processing enzymes. Alternatively, donor hepatocytes can be selected based on specific P450 family member profiles of particular relevance to the tested drug. From a practical standpoint, human liver chimeric mice offer the potential for gathering some unique data that are not available from clinical trials, in a controlled environment. The mice can be exposed to very high doses of an experimental drug, and all the while the human liver tissue can be harvested and analyzed at any time after exposure—both settings are unlikely to be approved in clinical trials from an ethical perspective, yet deliver valuable information for moving a drug forward into the clinic. Despite these advantages, the field of human liver chimeric mice is still young and there is a lot of room for optimization and standardization.

Challenges of Human Liver Chimeric Mice

In addition to the obvious benefits of using human liver chimeric mice for studying drug metabolism and toxicity, there are some general and model-specific limitations.

Variability within Human Liver Chimeric Mice. Contrary to inbred mice with a defined and well-characterized genotype and phenotype, human liver chimeric mice suffer from an increased variability. The variability is particularly related to the degree of human chimerism. It is difficult to control the degree of chimerism since many variables influence this outcome. Rigorous standard operating procedures can control some of this variation, but there is still much room for improvement in this area.

Remaining Murine Liver and Other Drug Metabolizing Murine Organs. Current evidence suggests that a 100% humanization of the murine liver is not possible. We hypothesize that species incompatibilities are the main reason for this phenomenon (e.g., multiple ligand receptor systems that cannot communicate across the species barrier). Differences in fibroblast growth factor (Naugler et al., 2015) or growth hormone (Masumoto et al., 2007) signaling have been shown, with many more species-specific protein interactions likely. Irrespectively, the consequence of this limitation is that the remaining murine liver cells express the full set of mouse DMEs. This is a significant problem since murine DMEs could be upregulated or have higher affinities for certain drugs than their human counterparts. Also, some DMEs, such as the P450 family members, have a high functional capacity; a reduction of the murine liver mass to 10% does not mean that the drug-metabolizing capacity of the murine liver component is reduced to 10%. Additionally, all three chimeric mouse models (particularly the FRG mouse) have a higher incidence of murine liver cancer than WT mice. Although there might be little normal murine liver remaining in older mice, large areas of neoplastic tissue can contribute to drug metabolism in liver toxicity assays. Extrahepatic murine drug metabolism can also be a confounding problem, depending on the DMEs involved in the metabolism of a given drug.

Immune Deficiency in Human Liver Chimeric Mice. The murine immune system has to be compromised to generate human liver chimeric mice. However, there are differences between mouse models. It seems clear that for all human liver chimeric mouse models T- and B-cell function needs to be eliminated, whereas there is a little bit more flexibility with natural killer cell function. For instance, the *alb-uPA* mouse has been crossed with the $Rag2^{-/-}$ (Dandri et al., 2001) strain (only T- and B-cell deficient) to successfully engraft human hepatocytes. This is in contrast to the $Fah^{-/-}$ mouse, which also requires the depletion of natural killer cells ($Il2rg^{-/-}$) for humanization (Bissig et al., 2007).

Many adverse drug reactions are mediated by the immune system. To detect such an event, a dual humanization with the hematopoietic system would be required. There are a few reports of such systems (summarized in Bissig et al., 2016); but further optimization is required to use these humanized mice for drug metabolism.

Strain-Specific Differences and Limitations. Human liver chimeric mice are bred on different genetic backgrounds. The importance of the mouse genetic background has been well documented and elaborated for human hematopoietic xenograft models; however, the situation is not clear for human liver chimerism.

The model-specific limitations are related to the knockout gene or inserted transgene. Table 2 summarizes these challenges, which are often technical. Every model has limitations, and no one model is clearly superior to another. There has been only one group comparing two different human liver chimeric mouse models (uPA and FRG) using the same drug, troglitazone (Schulz-Utermoehl et al., 2012; Samuelsson et al., 2014). That comparison was far from ideal, as the transplanted human hepatocytes were not from the same donor, the

human liver chimeric mice were produced by different groups, and, most importantly, the mean human liver chimerism was different in the two models. Nonetheless, analysis of troglitazone metabolites gave comparable results in humanized uPA and FRG mice (Schulz-Utermoehl et al., 2012; Samuelsson et al., 2014).

All of these challenges, in combination with reduced scalability, appear to dampen the enthusiasm to broadly implement human liver chimeric mice in the pharmaceutical industry. Nevertheless, there have been several promising advancements in recent years, and we expect that these kind of mouse models will increasingly be used.

Next Generation Human Liver Chimeric Mice

Increasing human chimerism is essential when using human liver chimeric mice for drug metabolism studies; but, as discussed above, there are natural boundaries. Another way of increasing human over murine drug metabolism is to functionally inactivate mouse DME without reducing the murine liver tissue mass. A first such attempt was recently taken by crossing the *Cyp3a* knockout mouse to the albuPA/SCID mouse (Kato et al., 2015; Nakada et al., 2016). Unfortunately, these mice upregulate the expression of several other CYP gene clusters, which defeats the purpose of the model. Therefore, we recently introduced a new humanized mouse strain in which we functionally inactivated all murine microsomal P450 family members. This was achieved by using a conditional knockout of the NADPH-P450 oxidoreductase ($Por^{c/c}$) (Gu et al., 2003; Henderson et al., 2003; Wu et al., 2003), the electron donor for all microsomal P450 family members. When combined with the $Il2rg^{-/-}$, $Rag2^{-/-}$, and $Fah^{-/-}$, the novel PIRF strain was generated (Barzi et al., 2017). Humanized PIRF mice demonstrate higher levels of human-specific drug metabolites when compared with humanized FRG or nonhumanized PIRF mice (Barzi et al., 2017). Ideally, the $Por^{c/c}$ technology would be combined with new mouse strains that do not have a higher incidence of murine liver cancer, but for the time being this is a safe way of inactivating neoplastic and residual mouse liver drug metabolism.

Perspectives

Advancements in genetics and techniques to manipulate the mouse embryo have allowed the generation of genetically humanized mouse models. Many P450 family member clusters have been humanized and used for a better understanding and prediction of human drug metabolism. The transcriptional and post-transcriptional regulation of these transgenic human P450 family members is complex and may not be identical to their regulation in human cells, even though some improved mouse models (with the inclusion of humanized transcriptional regulators) have been generated. In addition to further optimized P450-humanized mouse models that will be developed as a result of continued efforts to increase the degree of genetic humanization, human liver chimeric mice are emerging as a promising alternative. These cellular chimeras have a very similar DME expression profile as human livers (Tateno et al., 2004); but their utility will be at the cost of other technical challenges, particularly the high experimental variability, even in the hands of specialists. Nevertheless, overcoming these challenges is possible and a broader implementation of this technology seems likely.

In summary, there is clearly a need for better models for predicting human drug metabolism and toxicity. Currently available mouse models of genetic humanization and cellular chimeras both have their own advantages and limitations, and their usage should be determined by the scientific question under study. Recent advancements in gene cloning and editing technologies have accelerated the rate at which these models can be improved, and it is possible that humanized mouse models will transform preclinical drug testing in the near future.

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Wrote or contributed to the writing of the manuscript: Bissig, Han, Barzi, Kovalchuk, L. Ding, Fan, Pankowicz, Zhang, and X. Ding.

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