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# Mouse Population-Based Approaches to Investigate Adverse Drug Reactions

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## ABSTRACT

**Genetic variation is now recognized as a key factor in the toxicity of pharmaceutical agents. However, genetic diversity is not present in standard nonclinical toxicology models, and small clinical studies (phase I/II) may not include enough subjects to identify toxicity liabilities associated with less common susceptibility factors. As a result, many drugs pass through preclinical and early clinical studies before safety concerns are realized. Furthermore, when adverse drug reactions are idiosyncratic in nature, suggesting a role for rare genetic variants in the toxicity susceptibility, even large clinical studies (phase III) are often underpowered (due to low population frequency and/or small effect size of the risk factor) to identify associations that may be used for precision medicine risk mitigation strategies. Genetically diverse mouse populations can be used to help overcome the limitations of standard nonclinical and clinical studies and to model toxicity responses that require genetic susceptibility factors. Furthermore, mouse population-based approaches can be used to: 1) identify sensitive strains that can serve as a screening tool for next-in-class compounds, 2) identify genetic susceptibility factors that can be used for risk mitigation strategies, and 3) study mechanisms underlying drug toxicity. This review describes genetically diverse mouse populations and provides examples of their utility in investigating adverse drug response. It also explores recent efforts to adapt mouse population-based approaches to in vitro platforms, thereby enabling the incorporation of genetic diversity and the identification of genetic risk factors and mechanisms associated with drug toxicity susceptibility at all stages of drug development.**

## Introduction

Genetic variation plays an important role in drug response. It has been estimated that >95% of the pharmacokinetic and pharmacodynamic variability in drug response can be explained by genetics. Genetic variation that influences the pharmacokinetics and pharmacodynamics can also contribute to adverse drug response (ADR). Pharmacogenetic information has included the labeling of over 200 drugs approved by the US Food and Drug Administration (FDA), and many of these are included among warnings and precautions for ADRs (https://www.fda.gov/downloads/Drugs/ScienceResearch/UCM578588.pdf). For example, the drug label for warfarin describes polymorphisms in the drug target vitamin K epoxide reductase and metabolizing enzyme cytochrome P450 (CYP)2C9 that can increase the risk for bleeding.

Genetic variation that does not directly influence pharmacokinetics or pharmacodynamics has also been associated with rare but serious adverse drug reactions. Most notably, susceptibility to several idiosyncratic ADRs has been linked to variants in human leukocyte antigen(HLA) alleles, which are thought to contribute to off-target, immune-mediated events. There is growing interest in using this kind of genetic information for risk mitigating precision medicine strategies to keep important drugs on the market. Currently, there are at least two examples where this approach has already been translated to the clinic. The FDA–approved label for abacavir requires testing for HLA-B*57:01, which is associated with increased risk for hypersensitivity reactions, and the FDA–approved label for carbamazepine requires screening for HLA-B*15:02 in patients of Asian descent due to a high risk of serious and sometimes fatal dermatologic reactions.

Unfortunately, genetic diversity is not present in standard nonclinical toxicology models, and small clinical studies (phase I/II) may not include enough subjects to identify toxicity liabilities associated with less common susceptibility factors. As a result, many drugs pass through preclinical and early clinical studies before safety concerns are realized, and even large phase III studies may not have sufficient power to identify genetic associations that could be used for precision medicine risk mitigation strategies. This is likely due to several factors including small sample sizes in clinical studies, low population frequency and/or small effect size of the risk variant(s), and confounding factors such as comorbidities and exposure to other drugs among study subjects. Furthermore, recent reports suggest that 40% of the functional variability in drug response can be attributed to

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**ABBREVIATIONS:** ADR, adverse drug response; ALT, alanine aminotransferase; APAP, acetaminophen; CC, Collaborative Cross; DO, diversity outbred; GRP, genetic reference population; IDILI, idiosyncratic drug-induced live injury; IDR, idiosyncratic drug response; MDP, mouse diversity panel; QTL, quantitative trait loci; SNP, single nucleotide polymorphism; TCE, trichloroethylene.

rare variants (Kozyra et al., 2017), i.e., polymorphisms that would not be detected without sequencing-based approaches that are cost prohibitive at the whole-genome level.

Genetically diverse mouse populations can be used to help overcome the limitations of standard nonclinical and clinical studies and to model toxicity responses that are associated with even rare genetic variants (Fig. 1). Furthermore, the robust and controlled genetic diversity of mouse genetic reference populations (GRPs) can also support the identification of candidate risk factors and mechanisms for ADRs (Rusyn et al., 2010; Chiu and Rusyn, 2018). As a result, GRPs can help to enable a targeted, hypothesis-based investigation of human genetic data, where limited cases and the need for deep sequencing reduce the usefulness of global genetic approaches. Mouse GRPs have gained popularity in the study of complex traits because genotyping is required only once while replicate individuals can be produced indefinitely allowing for optimal case/control and gene-by-treatment designs (Collaborative Cross Consortium, 2012). GRPs are also attractive because over time the quantity and type of data associated with each strain increases, facilitating a systems-biology approach to investigating mechanisms of toxicity.

The objective of this review is to describe the application of mouse GRPs to the understanding and prediction of ADRs. The enhanced genetic architecture of newer GRPs combined with novel approaches for phenotypic analysis may support the identification of rare variants and even mechanisms of idiosyncratic reactions that are difficult to ascertain in clinical data. Furthermore, recent efforts to translate these populations to in vitro platforms may enable the rapid and cost-effective use of GRPs at all stages of drug development. Definitions of common terms used in mouse genetic research are provided in Table 1 to facilitate the reading of this review.

## Benefits of Incorporating Genetic Diversity in Nonclinical Safety Studies

Despite the potential for genetic polymorphisms to influence ADRs, it is not common practice to evaluate the role of such variation in nonclinical safety studies for new chemical entities. For example, standard rodent toxicology studies are performed using a single inbred strain or outbred stock (Festing, 2016). Using genetically identical animals within a single inbred strain is appealing because the homogeneity reduces noise in the measurement of pharmacological and toxicological endpoints. However, this comes at the risk of missing responses that are influenced by genetic traits. On the other hand, the use of common outbred stocks provides genetic diversity, but because it is not controlled, it only increases "noise" and therefore reduces the power to detect differences in response to a test article.

A strong argument has been made that nonclinical safety studies could be improved by the use of several genetically defined inbred strains (Festing, 2014, 2016). An example of this has been illustrated by a small

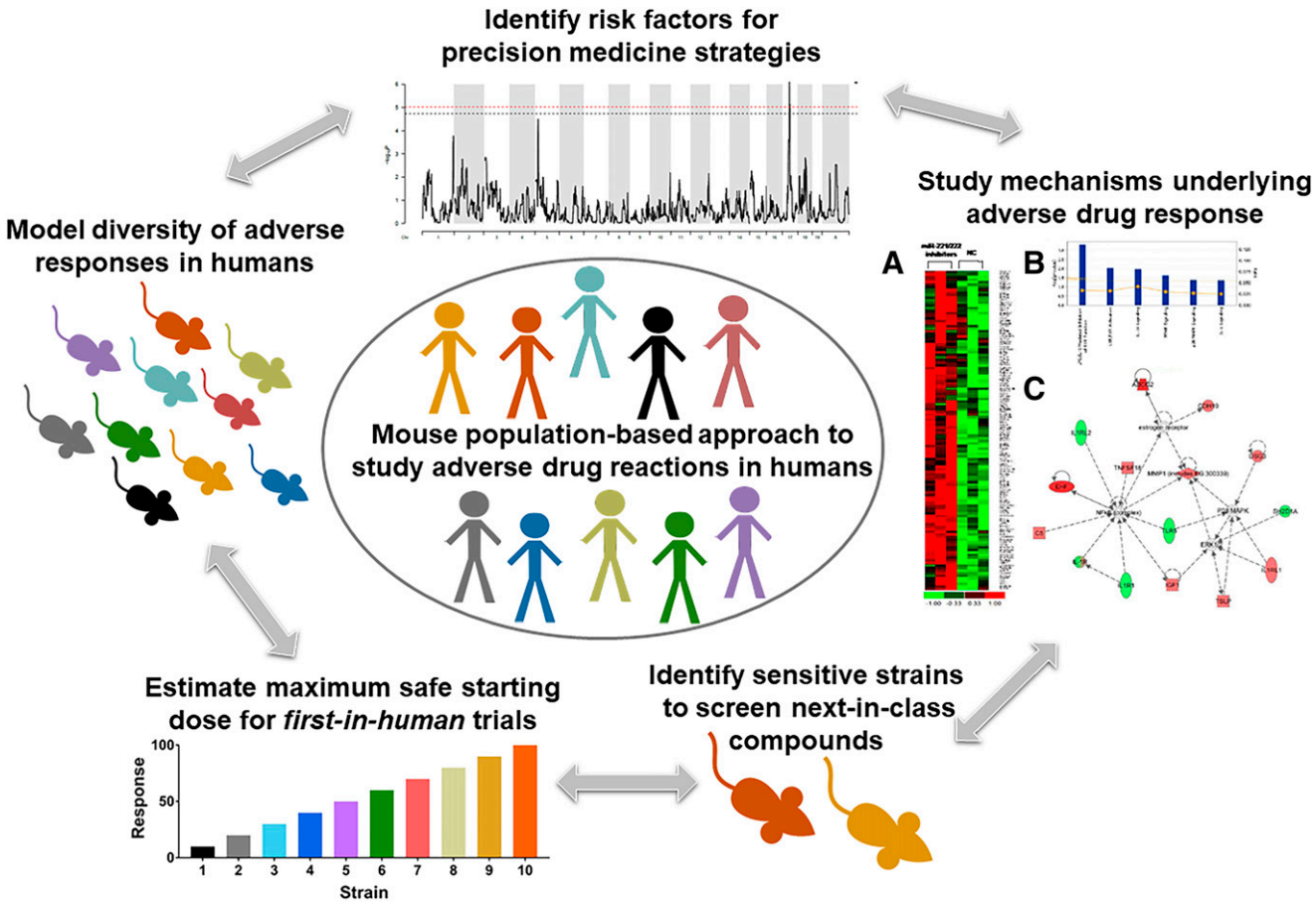


**Fig. 1.** Schematic illustrating mouse population-based approaches to study adverse drug reactions. Genetically diverse mouse populations can be used to better model human toxicity responses that require genetic susceptibility factors. As a result, mouse populations can aid in the screening of new drug candidates for adverse reactions and the estimation of maximum safe starting dose for first-in-human clinical trials. Quantitative data collected from genetically diverse strains can be used for genetic mapping to identify associations with toxicity susceptibility. These data may guide a hypothesis-driven interrogation of human genetic data and the identification of risk factors that can inform precision medicine risk mitigation strategies. The identification of risk factors in mouse studies may also inform mechanisms underlying the adverse drug response. Furthermore, the identification of sensitive strains can provide models to screen next-in-class compounds and perform additional mechanistic experiments.

TABLE 1

Definitions for common terms used in mouse genetic research

| Term | Definition |
|---|---|
| Inbred strain/line | All animals are genetically identical (isogenic) and homozygous at all gene loci due to many generations of inbreeding. |
| Outbred stock | All animals are genetically distinct (heterogenic) and will be heterozygous at many gene loci due to outbreeding. |
| Biparental population | Individuals derived from the mating (outcrossing) of two parental inbred strains; the parental strains are often chosen because they differ in phenotypes of interest. In a backcross, F1 animals are mated back to one of the parents; in an intercross, F1s are mated to each other. |
| GRP | A set of typically dozens to hundreds of inbred lines, each with fixed and known genomes and capable of producing replicates (indefinitely), intended for repeated use in genetic studies. In some cases, these are related by descent from a set of common ancestors (i.e., the founders). |
| Genetic mapping | A procedure whereby a phenotype measured in a genetically diverse population is tested for statistical association with a genetic variant in that population, the goal being to identify variants that influence the phenotype of interest. |
| Gene-by-treatment mapping | Genetic mapping performed to identify variants in the genome that influence a treatment-induced phenotype. |
| SNP | Variation in a single nucleotide that occurs at a specific position within the genome. |
| GWAS | Genetic mapping performed by testing for association with individual variants, typically using high-density SNP information. This is the primary mapping method used in human genetic studies. |
| Haplotype | A linked (i.e., contiguous) set of DNA variants (e.g., SNPs) on the same chromosome that are inherited together. These variants are in linkage disequilibrium. |
| QTL mapping | Typically refers to genetic mapping in a model organism, performed by testing for association with haplotypes. However, can sometimes be used to describe GWAS in model organisms and/or human GWAS performed on quantitative phenotypes. |
| eQTL mapping | QTL mapping performed in humans or model organisms using gene expression (baseline or fold change) as the phenotype. |
| QTL | A region of DNA that correlates (associates) with variation in a phenotype, as discovered using GWAS or QTL mapping. It is typically assumed that the underlying contributing variant(s) is within this region, although the exact location may be uncertain based on the available data. |
| QTG | A gene within a QTL that may affect the phenotype of interest. |

eQTL, expression QTL; F1, filial generation 1; GWAS, genome-wide association study, QTG, quantitative trait gene.

pilot study, where the effect of chloramphenicol on hemoglobin levels was compared between $N = 2$ mice per treatment and strain across four inbred strains and an equal number of CD-1 outbred mice (Festing, 2010). A better signal-to-noise ratio in the multi-inbred strain study design allowed for the identification of a statistically significant effect that was not observed in the CD-1 mice. Furthermore, this study demonstrated that some inbred strains are more sensitive to drug response than others, suggesting that an added benefit of this approach is the ability to identify individually sensitive animal models that could be used to study next-in-class compounds.

Using larger numbers of genetically different inbred strains such as those of a population model has been proposed as a way to further improve toxicity testing, particularly in chemical risk assessment (Harrill and McAllister, 2017; Chiu and Rusyn, 2018). Population-based approaches have a greater likelihood of achieving human relevant responses in part due to genetic diversity that equals or exceeds that of the human population (Ideraabdullah et al., 2004; Roberts et al., 2007). Unfortunately, the pharmaceutical industry has been hesitant to incorporate population models into nonclinical safety assessment for several reasons that are addressed subsequently in this review. As a result, data validating the ability of population models to predict toxicities that are not evident in standard preclinical models are limited in scope. However, there is significant evidence to support that once a liability is discovered, mouse GRPs can be used for genetic mapping to identify risk factors and mechanisms underlying toxicity susceptibility.

## Mouse Diversity Panel

The earliest studies demonstrating the utility of mouse population-based approaches to investigate ADRs were performed in an existing set of commercially available inbred strains referred to as the mouse diversity panel (MDP). As described by McClurg et al. (2007), these mouse strains were derived over many decades by crossing different mouse populations, thus providing the MDP with an overall greater genetic and phenotypic diversity than is found in a biparental population. Overtime, genotype data have been collected on these animals and stored in community databases that could be used to perform quantitative trait loci (QTL) mapping (Fig. 2) without the need to perform genotyping. Higher recombination rates and dense genotype maps have also helped to facilitate more precisely defined QTL regions and support the identification of quantitative trait genes.

An initial proof-of-concept for the use of the MDP in the investigation of ADRs was provided in a study of warfarin metabolism by Guo et al. (2006). Differences in the generation of 7-hydroxywarfarin among 13 inbred strains were found to correlate with genetic variation in the mouse Cyp2c enzymes. Because variants in *CYP2C9* had already been shown to impact the rate of warfarin metabolism in treated patients (Daly and King, 2003), the authors were able to demonstrate the ability of the MDP to identify human-relevant associations. Furthermore, they were able to interrogate the role of specific *Cyp2c* isoforms by characterizing the activity of recombinant enzymes from high and low metabolizing mice (Guo et al., 2006).

However, a more practical application was envisioned, whereby the MDP could be used to identify novel genetic associations that could inform a hypothesis-driven approach and thereby improve the power to identify risk factors in human genetic data. This was successfully demonstrated in a study of acetaminophen (APAP)-induced liver injury by Harrill et al. (2009). A range in serum alanine aminotransferase (ALT) elevations was observed at 24 hours postdose among 36 MDP strains treated with 300 mg/kg of APAP. Genetic mapping using ALT fold change values at 4 hours postdose identified a locus associated with the liver response, and polymorphisms were identified in four candidate genes within the QTL interval. Sequencing of these genes in genomic DNA collected from a clinical study where subjects were given a maximum daily dose of APAP for 14 days (Watkins et al., 2006) revealed a single nucleotide polymorphism (SNP) in *CD44* (rs1467558) associated with peak elevations in serum ALT. It was later independently shown that persons homozygous for the *CD44* polymorphism were overrepresented among the very rare

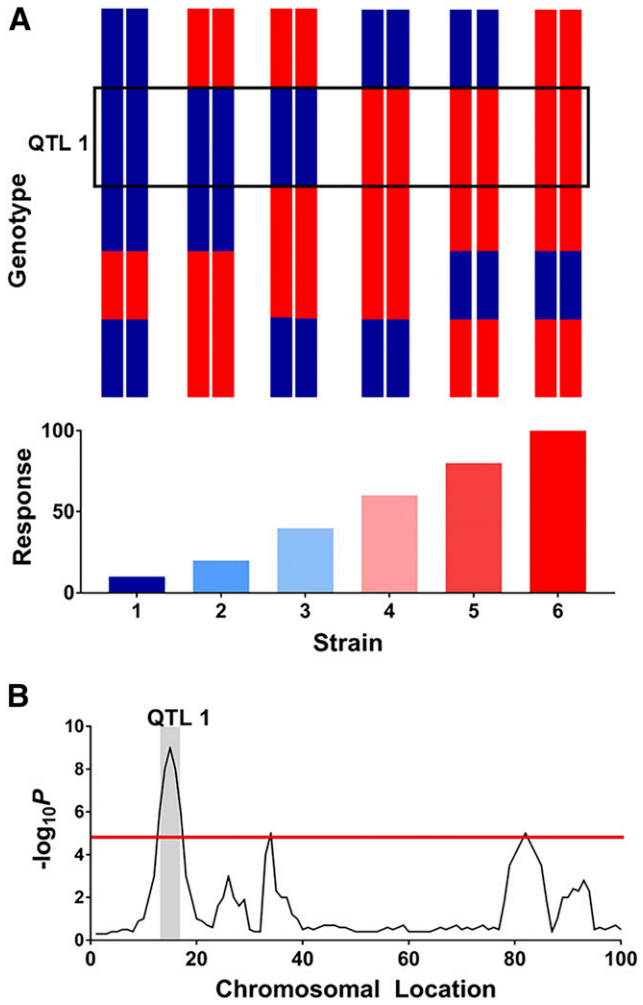


**Fig. 2.** Schematic illustrating basic principles of QTL mapping. (A) Inbred lines (shown here as strains 1–6) of genetic reference populations inherit segments of DNA (haplotypes) from founder strains. Differences at a variety of loci (e.g., QTL 1) contribute to differences in phenotypic response. (B) QTL mapping scans the genomes for loci where the DNA segments are shared by strains with a similar phenotypic response (i.e., genotype-phenotype correlation) and assigns statistical significance (e.g., $-\log_{10} P$) to the association. An empirically determined significance threshold is used to identify potentially informative loci, which can then be further interrogated for candidate quantitative trait genes.

patients who unintentionally develop acute liver failure from subtoxic doses of APAP when compared with patients who develop acute liver failure due to a suicidal overdose of APAP, patients with acute liver failure due to other causes, or a reference population (Court et al., 2014). In silico methods predicted that the effect of the relevant nonsynonymous coding SNPs would be a disruption in the protein function. The liver response to APAP was then compared between wild-type C57BL/6 and *Cd44*-null mice on a C57BL/6 background. *Cd44*-null mice exhibited significantly greater liver injury 24 hours following administration of 300 mg/kg APAP compared with their wild-type counterparts, confirming the functional relevance of the *CD44* polymorphism (Harrill et al., 2009).

The utility of the MDP to identify human ADRs that were not predicted by standard rodent models has also been demonstrated in a separate study by Harrill et al. (2012). Here, the authors showed a range in drug-induced elevations of kidney injury molecule-1 in the urine of 34 MDP strains treated with DB289. DB289 had shown efficacy in treatment of African sleeping sickness; however, development was terminated when several treated subjects presented with severe kidney injury, a liability not predicted from preclinical testing. Genetic analysis performed using kidney injury molecule-1 data identified several candidate risk factor genes for DB289 renal injury. This study also provided mouse strains that could be useful in screening renal injury liability for next-in-class compounds.

Several more recent studies have demonstrated the use of a systems-biology approach to not only inform risk factors but also identify mechanisms relevant to the response in the MDP. Using 34 strains, Mosedale et al. (2014) identified gene expression changes underlying strain-specific variation in the liver response to a ketolide antibiotic that caused elevations in serum liver chemistries in phase I clinical studies. Church et al. (2014) described the use of a multi-omics-based approach to identify transcriptional changes, metabolites, and gene variants that contribute to isoniazid-induced steatosis in 32 strains of the MDP. Similar efforts have also been pursued for environmental chemicals such as trichloroethylene (TCE) using fewer (14–16) strains (Bradford et al., 2011; Chiu et al., 2014).

Together, these studies have demonstrated the utility of mouse population-based approaches to both understand and predict ADRs. However, there are significant challenges to performing these types of studies in the MDP population. First, MDP studies are limited to around 30 strains, which may not provide sufficient power to identify relevant QTL. Second, the uncontrolled breeding process from which the MDP was derived has resulted in population structure (clusters of strains that are more related to each other than to other strains), which if not controlled for can lead to spurious associations. While several analytical approaches were developed to address these concerns (Pletcher et al., 2004; McClurg et al., 2006; Kang et al., 2008), they cannot completely resolve the problem. As a result, the use of the MDP has been largely abandoned in favor of the newer resources described subsequently.

## New Mouse Populations

The Collaborative Cross (CC) is an innovative and highly sophisticated GRP of multiparental recombinant inbred lines that was strategically designed to overcome limitations of classic inbred GRPs such as the MDP (Collaborative Cross Consortium, 2012). The CC lines were generated via a funnel breeding scheme that combined the genomes of eight inbred founder strains representing >90% of the genetic diversity in laboratory mice (Fig. 3). Three strategic outbreeding generations, followed by repeated generations of inbreeding through sibling mating, were performed to ensure the genetic variants are uniformly distributed across the population and genome. As a result, the CC population captures more diversity than other recombinant inbred panels but without population structure or blind spots of genomic variation (Collaborative Cross Consortium, 2012). CC lines have demonstrated a diversity of responses that is more extensive than the founder strains themselves (Kelada et al., 2014; Rutledge et al., 2014). Approximately 36 million SNPs have been reported in the genomes of the CC founder strains (Collaborative Cross Consortium, 2012), and the minor allele frequency in the CC population is relatively high for every SNP (12.5%–50%). This is in stark contrast to the human population, where 96% of nonsynonymous coding SNPs have allele frequencies of 0.5% or less, with more than half of these found only once in 2500 human genomes (Harrill and McAllister, 2017). Together, these features enable the CC to guide the investigation of complex traits and traits that are associated with rare variants in humans.

The genome of each CC line comprises a mosaic of DNA segments, inherited as haplotypes from the eight CC founders. Genetic mapping in the CC is most commonly performed using founder strain haplotype probabilities or the likelihood that a CC line has inherited DNA from a

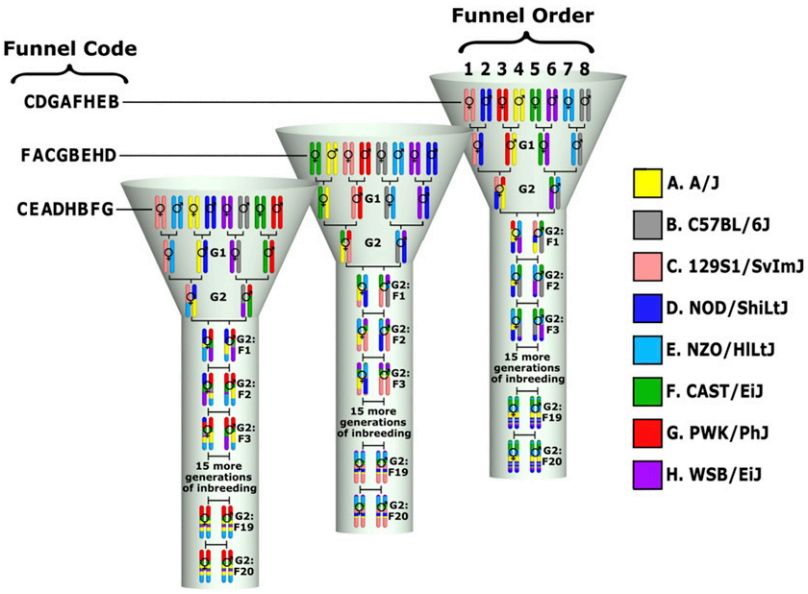


**Fig. 3.** Representative breeding scheme for three independent lines of the Collaborative Cross. Each mouse is represented by a pair of homologous chromosomes and a symbol denoting sex. Each line was generated from eight founder strains that capture >90% of the genetic diversity in laboratory mice. Founder strains were arranged in different positions of the breeding funnel (1–8) for three generations of outbreeding. Funnel order was randomized and not repeated across lines. Outbred animals (G2) were then used for repeated generations of inbreeding through sibling mating (F1–F20). Fully inbred Collaborative Cross strains (F20) have genetic variants that are uniformly distributed across the population and across the genome. The figure appears in Collaborative Cross Consortium (2012) and is used here with permission from the Systems Genetics Core Facility at the University of North Carolina at Chapel Hill.

founder strain at each genomic locus. The use of inferred haplotype composition rather than observed genotypes offers many important advantages when testing for genetic association (Zhang et al., 2014). Bioinformatics resources and methods for genetic mapping using the CC have been described in more detail elsewhere (Morgan and Welsh, 2015).

As a sufficient number of CC lines have been made available for use (>50 as of June, 2018), investigators have begun to show the power of this novel GRP for both understanding and predicting ADRs. For example, Nachshon et al. (2016) demonstrated substantial diversity in the baseline expression of hepatic expression drug disposition genes across 29 CC lines and the potential for using this resource to investigate the contribution of genetic variation to drug response. This has since been validated by several studies demonstrating extensive variability in metabolism and toxicity of small molecules such as tolvaptan in 45 lines (Mosedale et al., 2017), perchloroethylene in 45 lines (Cichocki et al., 2017), and butadiene in 60 lines (Hartman et al., 2017). Recently, Venkatratnam et al. (2017) performed a toxicokentic analysis of TCE in 50 CC lines and compared the data to a previous study conducted in the MDP (Bradford et al., 2011). While the overall study design was slightly different, Venkatratnam et al. (2017) reported differences in tissue trichloroacetic acid levels that varied by more than an order of magnitude across CC lines, whereas serum trichloroacetic acid levels reported in Bradford et al. (2011) varied only 4- to 6-fold across inbred strains of the MDP. A more recent paper by Venkatratnam et al. (2018) used samples from this same study to investigate transcriptomic dose-response effects for both TCE and trichloroacetic acid. While several known TCE-responsive pathways were identified among those affected across all CC lines and transcriptional perturbations were shown to be influenced by gene, dose, and gene-by-dose interactions, genetic mapping performed using dose-response data did not yield significant QTL. This may be due to several reasons including the polygenic nature of dose-response traits as well as the study design, which used only $N = 1$ animal per dose and CC line.

The tolvaptan study by Mosedale et al. (2017) demonstrates the importance of including multiple animals per CC line in each treatment group (e.g., $N = 4$) since variation in treatment-induced responses increases, even among genetically identical mice. This study also illustrated a unique design, where vehicle- and drug-treated animals within each CC line were treated in pairs and pairs within each line were randomized over the course of the study to minimize confounding effects of treatment date. Interestingly, elevations in plasma ALT were observed in three CC strains at clinically relevant doses of tolvaptan (Mosedale et al., 2017), whereas no liver injury was observed in traditional nonclinical models after multiple treatments with higher doses (Oi et al., 2011), supporting the potential of the CC to predict human-relevant ADRs. Another exciting application of the CC has been to explore the contribution of genetic variation on the success of lentiviral-vector-mediated hepatic gene delivery (Suwanmanee et al., 2017). In this study, the authors demonstrated line-specific differences in the overall success of transduction, vector biodistribution, and vector gene expression. These results highlight the potential contributions of the CC population in the emerging area of gene therapy.

A complementary resource called diversity outbred (DO) was derived from partially inbred CC lines in 2009. DO mice are maintained as a heterogenous stock and as a result the genetic variation and mapping resolution of this population are greater than the CC. Church et al. (2015) described an exciting application of DO mice to identify risk factors for susceptibility to hepatotoxicity associated with epigallocatechin gallate, the most abundant polyphenol in green tea and a major component of green tea extract. Severe hepatotoxicity was observed in a small fraction (43/272) of DO mice given intraperitoneal injections of epigallocatechin gallate (Church et al., 2015). QTL mapping using ALT fold change values at 24 hours postdose identified a locus on chromosome 4 associated with the liver response. Variants in 49 candidate genes identified in the mouse study were interrogated in genotyping data available for 15 patients judged to have experienced green tea extract–induced hepatotoxicity, and suggestive associations were observed for SNPs in three genes (Church et al., 2015).

More recently, DO mice have been used to identify genetic risk factors that influence chemotherapy-induced hematotoxicity (Gatti et al., 2018). In this study, DO mice were treated with three different chemotherapy drugs: doxorubicin (195 animals), cyclophosphamide (200 animals), and docetaxel (181 animals). Each drug resulted in distinct effects on blood-cell subpopulations that were associated with nonoverlapping genetic loci. DO mice have also been used to evaluate the population-based performance of novel biomarkers for drug-induced kidney injury (Harrill et al., 2018) and to investigate population-based

responses and genetic risk factors for hazardous environmental chemicals (French et al., 2015).

However, for the purposes of gene-by-treatment studies, the CC offers distinct advantages over the DO population. Outbreeding makes each DO mouse genetically unique and therefore only an $N = 1$ is available for each DO genotype. In contrast, an unlimited number of genetically identical animals are available for each CC line supporting the ability to examine biologic replicates and sex effects in this population. This would be particularly important for dose-response and toxicokinetic effects in studies where $N = 4$ mice may be required for multiple doses and/or time points of exposure. Furthermore, the complete genome sequence and/or high-resolution genotyping data for all CC lines are also freely available. As a result, QTL mapping can be performed immediately after phenotypic data analysis and without any additional cost for genotyping.

## Idiosyncratic Reactions

While mouse GRPs have demonstrated utility in understanding population variability associated with dose-dependent or intrinsic toxicity, perhaps the most important use of such tools will be to identify risk factors and mechanism associated with rare but serious idiosyncratic drug reactions (IDRs). IDRs are unpredictable, often life threatening, and cause a significant burden to patients, healthcare providers, drug developers, and drug regulators (Uetrecht and Naisbitt, 2013). Several studies support the contribution of genetic susceptibility factors to IDRs, and there is considerable interest in the development of genetic tests that may inform precision risk management strategies and enable a more accurate diagnosis, thereby improving patient safety and preventing abandonment of important drugs (Daly, 2013). It is possible that the CC may facilitate the identification of candidate risk factors and mechanisms for IDRs to enable a targeted, hypothesis-based investigation of human genetic data, where limited cases and the need for deep sequencing reduce the usefulness of global genetic approaches. However, an understanding of the pathology of IDRs and limitations of the GRP approach is important to facilitate the identification of clinically relevant results.

For example, drug-induced liver injury is one of the most common causes of adverse drug reactions, failed drug approval, and withdrawal of medications from the market (Watkins, 2011). It is widely accepted that most if not all idiosyncratic drug-induced liver injury (IDILI) reactions involve an adaptive immune attack on the liver (Mosedale and Watkins, 2017). As a result, it may be difficult to fully replicate serious IDILI events in nonclinical models, even with the use of a sophisticated mouse GRP such as the CC. However, significant evidence suggests that the cascade of events culminating in IDILI begins with some level of direct, drug-induced hepatocyte stress (Mosedale and Watkins, 2017). Much of the unexplained variability in toxicity susceptibility likely occurs at the hepatocyte level. Therefore, CC mice may be used to identify variants that impact the early events that are necessary but not sufficient to stimulate an adaptive immune attack (Fig. 4).

An important consideration here is the selection of IDILI-relevant endpoints to evaluate in the CC. For example, the drug-induced hepatocyte stress that initiates IDILI may not result in sufficient overt necrosis to be measurable by histology or serum ALT, but rather promote the release of danger signals, i.e., molecules that serve to activate an immune response (Momen-Heravi et al., 2015; Mosedale et al., 2018). Recent work suggests that these danger signals may travel in hepatocyte-derived exosomes, which owing to their small size (<150 nm) can be released from the liver and diffuse into circulation through the porous fenestrations in the sinusoidal endothelium (Wetmore et al., 2010; Holman et al., 2016). Therefore, it may be preferred to measure changes in circulating exosome number or content. Methods to assay for hepatocyte-specific exosome release in vivo have been proposed (Thacker et al., 2018), and it will be exciting to apply these to future CC studies.

In the meantime, transcript profiling of the liver after acute drug exposure can be used to assess early events in the hepatocyte that may precipitate an immune response (Laifenfeld et al., 2014; Leone et al., 2014). The evaluation of molecular signaling pathways can provide insight into mechanisms of drug toxicity, provide molecular phenotypes for eQTL mapping, and prioritize candidate genes identified by more traditional phenotypic QTL mapping (Kelada et al., 2014; Rutledge et al., 2014). For this purpose, an acute, high-dose exposure study design is recommended to give the greatest opportunity to identify transcriptional changes in the liver that may be indicative of events that initiate IDILI instead of those changes that are adaptive in nature (Laifenfeld et al., 2014; Leone et al., 2014). Additionally, it is important to consider the potential for confounding effects of genetic variation on gene expression analysis (Keane et al., 2011). For microarray-based approaches, investigators have described the removal of probes targeting SNP-containing regions across the founder strains of the CC population (Mosedale et al., 2017). A similar consideration, whereby RNA sequencing data are mapped to individual-specific genomes has also been described for DO mice (Chick et al., 2016).

## Challenges to Implementation

Unfortunately, the use of GRPs requires large in vivo studies that are time consuming and expensive. Furthermore, limited availability and

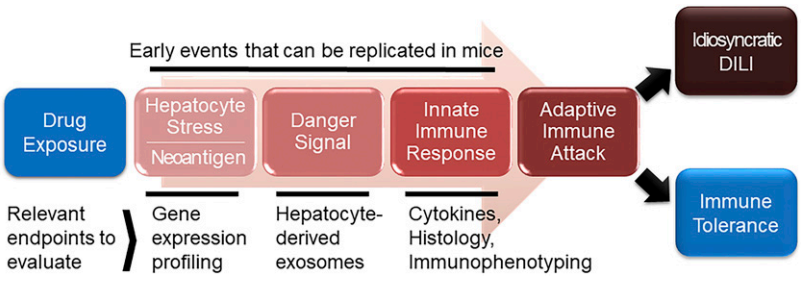


**Fig. 4.** Proposed steps leading to idiosyncratic drug-induced liver injury (DILI). Although most idiosyncratic DILI reactions are thought to involve an adaptive immune attack on the liver, significant evidence suggests that the cascade of events culminating in serious injury begins with some level of direct, drug-induced hepatocyte stress, the release of danger signals, and activation of the innate immune system. Much of the unexplained variability in toxicity susceptibility likely occurs during these early events, which can be replicated in genetically diverse mice. Because these events may not result in sufficient overt necrosis to be measurable by more traditional biomarkers of liver injury, additional endpoints should be considered. Hepatocyte stress can be evaluated with gene expression profiling. Danger signals can be quantified by assaying hepatocyte-derived exosomes. And an innate immune response can be measured via several approaches including cytokine analysis, histology, and/or immunophenotyping. The figure is adapted from Mosedale and Watkins (2017) and is used here with permission from the authors.

infrastructure to support commercial use, lack of historical reference data to support pathology, and unfamiliarity of drug developers and regulators with mouse population-based analyses are both an impedance to widespread acceptance and a result of limited utilization by the pharmaceutical industry. Population-based approaches have gained more traction in the investigation of liabilities identified late in clinical development, when a very large financial commitment has already been made to the development program. However, the requirement for multiple strains, treatments (drug and vehicle), and biologic replicates creates logistical restrictions on the ability to assay different exposures and endpoints. Therefore, genetic associations may be missed due to the selection of the wrong drug concentration, treatment regimen, and/or biomarker. Finally, once a candidate risk-factor gene is identified, it can be difficult to investigate its role in modulating the drug response using whole animal models.

## Innovations to Improve Utility

One way in which researchers are working to address the limitations of mouse population-based studies is by translating these models to a more rapid and cost-effective in vitro platform that will help enable the identification of genetic risk factors and mechanisms associated with drug toxicity at all stages of drug development (Frick et al., 2013). The adaptation of GRPs to an in vitro platform will substantially reduce the need to perform in vivo animal studies while simultaneously increasing: 1) the number of strains that can be reasonably assayed in a single experiment (i.e., power); 2) the ability to assay multiple concentrations, time points, and endpoints (i.e., content); and 3) efficiency in data collection and analysis (i.e., throughput). The use of cells cultured under identical conditions will also serve to increase reproducibility by reducing environmental variability that is commonly observed in vivo among animals in the same strain and treatment groups. Furthermore, in vitro systems will provide the ability to more closely control exposure, which is difficult to do in vivo due to complex traits influencing drug absorption, distribution, metabolism, and excretion (Mosedale et al., 2017).

Simple cell culture systems may also provide a more physiologically relevant model to study human ADRs. Most intrinsic drug toxicity is the result of a direct impact on the parenchymal cells of the target organ. For example, drug-induced liver injury is largely explained by events initiated at the level of the hepatocyte (e.g., the generation of reactive metabolites and subsequent oxidative damage). As a result, primary cells isolated from the genetically diverse strains should be an appropriate surrogate for whole animal drug toxicity studies and will more readily support the evaluation of early stress events that may occur in the absence of overt toxicity (Fig. 4). Furthermore, involvement of multiple cell types and steps in the cascade of events that occur after the initial parenchymal stress introduces more opportunities for species variation that can confound the interpretation of ADRs in preclinical models. Therefore, simple cell culture systems may in fact be better for translating responses from mouse to man. Finally, when genetic risk factors are identified, "knockdown" approaches can be performed in vitro much more easily than whole mouse "knockout" studies, allowing for a more rapid method to establish true causal links with candidate risk factor genes.

An early proof-of-concept for the use of in vitro GRP systems was provided by several studies demonstrating the utility of liver microsomal preparations from MDP strains to support the identification of gene variants influencing drug metabolism and toxicity (Guo et al., 2007; Zhang et al., 2011). Martinez et al. (2010) expanded on this work by demonstrating the successful culture of primary mouse hepatocytes isolated from MDP mice and the observation of strain-specific responses to toxicant exposure in vitro. Finally, Suzuki et al. (2014) adapted this approach to a high-throughput screen for gene-drug interactions by utilizing primary mouse embryonic fibroblasts derived from MDP mice in combination with cellular imaging methods. Here, the authors screened responses to 65 different compounds, performed a genome-wide association study using dose-response data, and validated the role of one candidate gene involved in rotenone sensitivity.

As the field has evolved to use newer mouse populations such as the CC and DO, so have efforts to create in vitro versions of these tools that are amenable to high-throughput measurements. On one end of the spectrum, investigators are generating embryonic stem cell lines from DO and CC mice and differentiating them into cardiomyoctes, neural progenitor cells, and other parenchymal cells that may support nonclinical safety testing (Harrill and McAllister, 2017). However, some embryonic stem–derived cell types remain phenotypically divergent from primary cells, limiting the ability to detect relevant toxicity responses (Goldring et al., 2017). Therefore, other efforts have focused on developing high-throughput organotypic culture models using primary cells isolated from CC mice. Primary mouse hepatocytes, for example, can be cultured in three-dimensional spheroids, which decreases the number of cells required per *N* while increasing the physiologic relevance of the in vitro model (Nautiyal et al., 2018). At a maximum expected number of hepatocytes per spheroid (1500 cells) and a minimum yield of hepatocytes per mouse (40 million), it may be possible to generate >25,000 spheroids per mouse. With cryopreserved cells, an investigator could generate thousands of spheroids from hundreds of lines and culture them on multiwell plates to allow for multiple concentrations, treatment regimens, and endpoints to be assayed across replicate wells in a single experiment (Fig. 5).

As noted by Harrill and McAllister (2017), published studies describing the use of these in vitro models are lacking as many of these resources are actively being developed. One challenge for investigators developing these tools will be the validation of findings from mouse population-based approaches in human genetic data. Even for in vivo studies, translation of the mouse findings has been difficult to do given the limited availability of relevant clinical genetic data. However, going forward this should be facilitated by more regular collection of DNA in clinical trials, the growing amount of data in publically available gene banks, and advancements in next-generation sequencing and associated analytical techniques.

## Conclusions

In conclusion, genetically diverse mouse population models are a powerful tool to understand and predict ADRs. Several studies have demonstrated the utility of mouse GRPs to 1) identify sensitive strains that can serve as a screening tool for next-in-class compounds, 2) identify genetic factors for toxicity susceptibility, and 3) provide new understanding of the mechanisms of ADRs. As a result, GRPs may help to inform precision medicine risk mitigation strategies to improve patient safety and prevent the abandonment of drugs that cause rare but serious reactions. With the right study design, GRPs may also facilitate the identification of risk factors and mechanism associated with rare but serious IDRs, and ongoing efforts to adapt mouse population-based approaches to in vitro platforms will enable incorporation of genetic diversity and the identification of genetic risk factors and mechanisms associated with drug toxicity susceptibility at all stages of drug development. Together, these insights will help to further reduce the cost of drug development and the potential for patient harm.

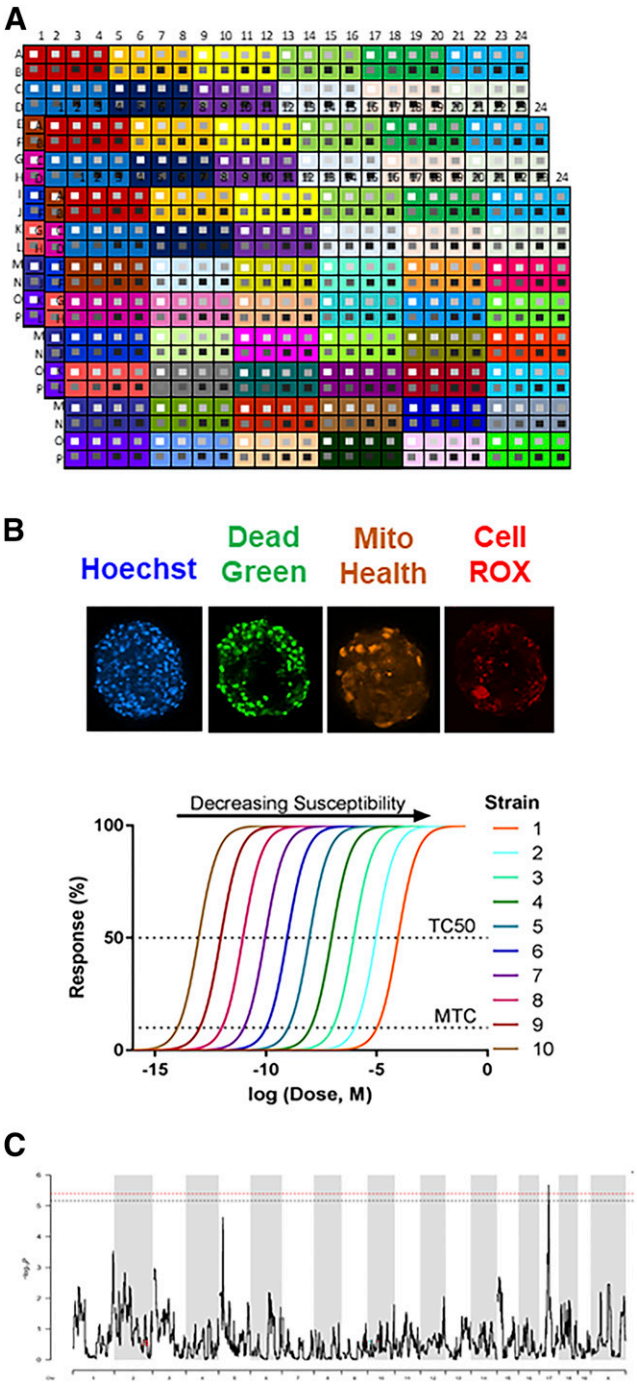


**Fig. 5.** Potential in vitro application of a mouse genetic reference population. (A) Cryopreserved primary hepatocytes from 48 collaborative cross strains (represented by different colors) are cultured in three-dimensional spheroids on a 384-well plate allowing for an eight-point dose-response curve to be assayed in triplicate across three plates. (B) Cellular imaging can be used to multiplex cell health and mechanistic endpoints measured by probes such as Hoechst, DEAD Green, MitoHeath, and CellROX. Dose-response curves reflect differential susceptibility across strains and can be used to calculate 50% toxic concentration (TC50) and minimum toxic concentration (MTC) values for each endpoint. (C) Genetic mapping using quantitative data from the initial screen identifies candidate risk factor genes, which can be validated by applying knockdown or overexpression in hepatocytes from sensitive and resistant strains.

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