Experimental precision medicine: Mouse models in which to test precision medicine

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Abstract

We require experimental populations in which to test precision medicine interventions before they can be translated to humans. Mouse models can provide immortal genometypes that can be resampled at any stage, in either sex, and under any experimental conditions to model genome-by-environment interactions and test genome-phenome predictions. This allows for experimental precision medicine—systems in which causal models of complex interactions among DNA variants, phenotype variation at many levels, and innumerable environmental factors can be built. In this chapter, I review some of the challenges of precision medicine and how mouse populations can overcome these challenges. Lastly, I provide specific suggestions and tools for future development of experimental precision medicine.

Key points

- The study of human populations can pose drawbacks for advancing precision medicine.
- Rodent experimental systems can help overcome some of these drawbacks.
- Replicable, isogenic inbred mouse genomes can be produced and tested in varied environments.
- Predictions can be made and then directly tested.

Introduction

Precision medicine can perhaps be summed up when a patient asks, "What is going to happen to me?" The task for precision medicine providers and scientists is to take information about an individual's genome, environment and lifestyle and make accurate predictions for that particular individual: What diseases will the patient get? How can they stay healthy? When will their illness progress? What treatments will work best for them?

Answering these questions is hugely constrained by the nature of the human population and has been difficult to resolve using conventional animal model systems. In this chapter I will focus on three problems in human precision medicine—the N-of-1 problem, the inability to run counterfactuals, and the complexity of genome-environment interactions—show how these challenges are relevant for both human and animal research, and then introduce animal models which can help solve these problems by advancing experimental precision medicine.

The N-of-1 problem

Each patient is unique—an N-of-1. Even monozygotic twins differ in their environments (Schüssler-Fiorenza Rose et al., 2019; Li and Auwerx, 2020). This N-of-1 problem has made it impractical, if not impossible, to accurately predict, at the individual level, disease risk or best treatment options for most common diseases, especially across populations (Martin et al., 2017; Fisher et al., 2018; Kim et al., 2018; Adolf and Fried, 2019; Medaglia et al., 2019; Mostafavi et al., 2020)—although we can make within population generalizations. Even for high-impact Mendelian disorders, physicians cannot give an exact time-course of the disease, and only offer generalizations and expectations. Many other alleles across the genome can influence disease outcomes, for example, age of onset (Lopera et al., 1997; Axelman et al., 1998; Larner and Doran, 2006; Long et al., 2018).

This N-of-1 problem has been thought of as easy to overcome in model organisms through inbreeding. All individuals of an inbred strain are (almost) completely genetically identical (isogenic). Model organisms can be raised in tightly controlled environments, where practically every aspect of their life is controlled by the experimenter. Further, each of these genometype-environment combinations can be replicated an arbitrarily large number of times to reduce technical and stochastic noise.

This allows us to carry out experiments that are practically or ethically impossible in humans, for example examining whole brain transcriptomes every month throughout the life of an animal and correlating these profiles with later life behavior in the same genetic and environmental 'individual'. Many tissues pertinent to human disease are poorly accessible in living patients.

Many pre-clinical studies carry out all experiments in a single genometype, and as such, this is still fundamentally an N-of-1, as we are only looking at a single genome. The C57BL/6J strain is often used to represent the entire mouse species (Johnson, 2012; Miller, 2016; Selman and Swindell, 2018), when in fact phenotypes can differ even from their closely related C57BL/6N sub-strain (Simon et al., 2013), and vastly between different strains. For example, the effect of caloric restriction, a commonly suggested intervention for extending lifespan, is hugely dependent upon genetic background (Garcia et al., 2008; Mulvey et al., 2014, 2021; Mitchell et al., 2016; Selman and Swindell, 2018). In the human research setting if we only looked at a single individual and their identical twin and assumed that our findings in this one genome extrapolated to all humans, the work would never be published. But in animal research, using a single genome has been the standard for decades. This may explain some of the failures to translate effects in mice to those seen in humans, as only a single (genetic) individual is being examined, with the expectation that the results can be extrapolated to the highly genetically diverse human population.

This has led to claims that data collected in mouse models do not translate to humans (Seok et al., 2013; Pound and Bracken, 2014), when the more accurate assessment should be that data collected in a single genome does not translate to a population with varied genomes. In a specific example, it was claimed that mouse models poorly mimic genomic response to human inflammatory diseases (Seok et al., 2013), however, when looking at the study one finds that a single sex of a single genome in a single environment was used, and then compared against a mixed population of eight, male and female humans. In sharp contrast, when the data were reanalyzed, using several strains, several environments and appropriate statistics, it was concluded that mouse models greatly mimic human inflammatory disease (Takao and Miyakawa, 2015).

This somewhat mirrors the debate around using a single sex of animal in experimental studies. For decades, most experiments were carried out only in males, and the results presumed to be applicable to females. Years of work have established that the microglial BDNF receptor is key for sensing pain—until it was realized that this only applies to males, not females, who likely use a T-cell dependent mechanism instead (Mapplebeck et al., 2016, 2018). We therefore know that genetic background has a huge impact on the phenotypes expressed after interventions—whether those interventions are genetic or environmental.

Large collaborations have taken this N-of-1 approach (e.g., the International Mouse Phenotyping Consortium and the Allen Brain Atlas) using only a single genome (C57BL/6N and C57BL/6J respectively) (Siddiqui et al., 2005; Lein et al., 2007; Meehan et al., 2017; Cacheiro et al., 2019). Although these groups have generated a huge amount of valuable data, they are still limited by this N-of-1 approach. We know that, for example, gene expression is highly variable between strains of mice (Morris et al., 2010). We therefore need to go beyond the N-of-1 genometype and use many genometypes in our experiments to be able to accurately mirror the human population, while still being experimentally tractable.

Counterfactuals

Every individual changes with time—a patient you see at age 20 will be very different at age 60. We cannot go backwards in time and see what would have happened to our 60-year-old patient if they had made a change to their lifestyle at age 20—we cannot run a counterfactual, we can only observe what actually happened.

In human cohorts we have to imagine a counterfactual (e.g. what would have happened had the patient been treated with drug X rather than drug Y?) rather than the fact (the patient was given drug Y, and had outcome Z), and a number of statistical and machine learning techniques have been developed to formally do this (Vanderweele and Vansteelandt, 2009; Pearl and Mackenzie, 2018; Rijnhart et al., 2021; Kühne et al., 2022; Schrod et al., 2022). However, in isogenic strains we can effectively run this counterfactual—nearly perfect genomically and environmentally matched individuals can be phenotyped with only a single environmental perturbation of interest between them. Even better, we can have multiple duplicates of these identical genometypes within each arm of the study, allowing a reduced effect of unwanted environmental perturbations, increasing power to detect true associations (Belknap, 1998), or to take different, mutually exclusive, measurements at many timepoints. That is, we can change interventions between environmentally and genetically identical individuals. In isogenic model systems this means that we can

make predictions, and then test those predictions in effectively the same individual—something that is not possible in a human. This is fundamentally the experimental part of experimental precision medicine.

One must, however, take into account the above detailed N-of-1 problem—and make sure that the counterfactual is broadly applicable; what works for me, might not work for you. The simple solution is to study these counterfactual scenarios across many different, but replicable genometypes of animals—to establish the parameters under which genetic and/or environmental context intervention X is most effective.

Complexity of genome-environment interactions

The scientific community often tries to understand disease in terms of one-to-one relationships—one gene leading to one disease, for example, or one drug having one effect. However, most traits are complex, influenced by many genes and the environment (Williams and Auwerx, 2015). Efforts to understand the outcomes of complex relationships between the genome and the environment have taken many forms and many names, including population genetics (Fisher, 1953), statistical genetics, quantitative genetics (Green, 1954; Damerval et al., 1994), complex trait analysis (Camussi, 1988; Frankel, 1995; Threadgill, 2006), genetical genomics (Jansen and Nap, 2001), systems genetics (Morahan and Williams, 2007; Schughart and Williams, 2017), systems medicine (Tao et al., 2004; Berlin et al., 2017), personalized medicine (Langreth and Waldholz, 1999), predictive medicine, and precision medicine (Wagner, 2009; Lloyd et al., 2015).

The modern formulation of this can perhaps be stated as an approach to exploring genotype-phenotype interactions, with the goal of understanding the whole biological system, not simply making an association between a genetic locus and a phenotype. In this approach, we can follow a causal pathway from the variant, through transcript expression, proteomics, metabolomics, and whole tissue changes. By integrating layers of 'omics data, we can build a comprehensive representation of the whole biological network, and how this is perturbed, either genetically or environmentally. This is a key element needed to advance experimental precision medicine.

Over the past few decades, sample sizes for genome-wide association studies have grown exponentially. This increase has made it undeniable that any single commonly segregating variant is likely to have a very small impact on disease risk (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Wood et al., 2014; Huan et al., 2015; Andrews et al., 2023). This has led to the proposal of an omnigenic model, whereby variants in every gene are likely to affect every phenotype (Boyle et al., 2017; Mathieson, 2021).

We therefore know that millions of variants across the genome are influencing our phenotypes of interest. Not only that, these variants interact with each other and with every aspect of our environment—from our mother's diet during gestation, to the month we were born in, to the amount of exercise we take. Every human creates their own environment over their lifetime. Each one of us is a complicated biological system, with phenotypes at many levels, including behavior, organ systems, cells, proteins, metabolites, and mRNAs, all interacting, all the time. We need to understand gene-gene (epistatic), gene-age, gene-sex, gene-treatment, and gene-environment interactions and all their combinations (Williams, 2009; Auffray et al., 2010; Hood and Friend, 2011).

How can we hope to understand these many-to-many-to-many relationships to achieve real precision medicine? I submit that appropriately designed animal models can give us the experimental tools needed to achieve this goal (Ashbrook and Lu, 2021; Ashbrook et al., 2021). In this chapter, I discuss different types of populations that can be used as experimental models for precision medicine, concentrating on rodents, and specifically, mouse models.

What mouse models are available?

Many different mouse populations are available, and choices have to be made when designing an experiment using animal models. Genetic reference populations have allowed systems genetics analyzes in many species and create the foundation for experimental precision medicine (Williams and Williams, 2017; Ashbrook et al., 2021; Ashbrook and Lu, 2021; Swanzey et al., 2021).

As noted earlier, the use of a single, fully inbred strain (one isogenic genometype) is often a poor solution due to the challenges posed by N-of-1. The question is how best to include the required genetic diversity in experimental designs? For several reasons, the BXD recombinant inbred (RI) family is the system our group has used for systems genetics and experimental precision medicine. I will first discuss recombinant inbred (RI) families and the BXDs in depth, and then introduce and contrast with other mouse model systems, and their relative advantages and disadvantages.

Recombinant inbred families

RI populations are a simple idea: Two inbred strains are crossed, and their F1 progeny are then crossed again to produce an F2. Pairs of these F2 animals are mated, and new lines are established through many rounds of sibling mating (Fig. 1A). By generation F20, we have a population of 99% inbred strains, in which each strain is a unique mosaic of homozygous genomic regions from both the parents, and for which an arbitrarily large set of genetically identical individuals can be produced anywhere and at any time (Bailey, 1971; Crow, 2007). This combination of genetic variability between strains but identical genome (isogenic) within strains allows the mapping of linkage between genotype and phenotype. It also overcomes the N-of-1 problem—as many genometypes can be

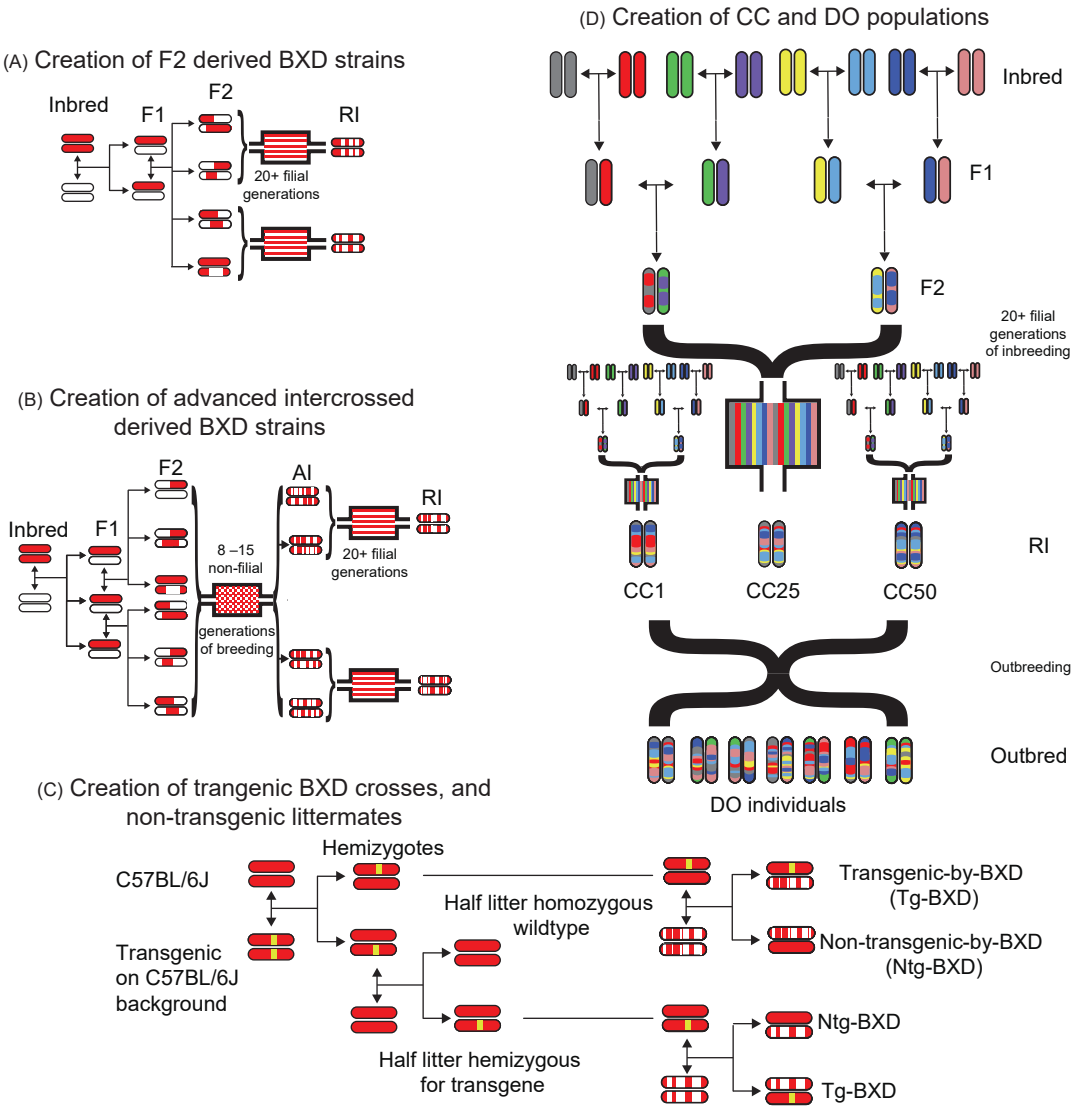


Fig. 1 Creation of recombinant inbred (RI) families. Approximately half of the BXD strains are from a two parent F2 (A), and approximately half of the BXD strains are from advanced intercrosses (B). Transgenic and non-transgenic crosses can be produced by crossing hemizygous transgenic mice to RI individuals (here represented by the BXDs), to produce litters containing both genotypes (C). The transgene is represented in yellow. The Collaborative Cross family (CC) are derived from eight parental strains, which were crossed and then inbred (D). The Diversity Outbred (DO) population were created by crosses between CC strains, and then outbreeding. Regions of the genome coming from different parental strains are represented by different colors of chromosome. Solid arrows have been used to represent a single generation of breeding. Adapted from Peirce JL, Lu L, Gu J, Silver LM and Williams RW (2004) A new set of BXD recombinant inbred lines from advanced intercross populations in mice. *BMC Genetics* 5: 7. https://doi.org/10.1186/1471-2156-5-7; Williams EG and Auwerx J (2015) The convergence of systems and reductionist approaches in complex trait analysis. *Cell* 162: 23–32. https://doi.org/10.1016/j.cell.2015.06.024; Ashbrook DG and Lu L (2021) Recombinant inbred mice as models for experimental precision medicine and biology. In: Purevjav E (ed.), *Preclinical Animal Modeling in Medicine.* Rijeka: IntechOpen. https://doi.org/10.5772/intechopen.96173; Ashbrook DG, Arends D, Prins P, Mulligan MK, Roy S, Williams EG et al. (2021) A platform for experimental precision medicine: The extended BXD mouse family. *Cell Systems* 12: 235–247.e9. https://doi.org/10.1016/j.cels.2020.12.002.

tested—and the counterfactual problem—many replicates can be produced for each genometype and subjected to different environmental perturbations. Therefore, we have a high-powered system for experimental precision medicine.

The RI design has been expanded in a variety of ways (Teuscher and Broman, 2007), such as increasing the number of parental strains (e.g. the 8 founder Collaborative Cross (CC) population) (Churchill et al., 2004; Shorter et al., 2019b) to increase the number of variants that segregate in the population, or using multiple rounds of crossing before inbreeding, producing so-called Advanced Intercross RI strains (AI-RI) to increase the number of recombinations, and therefore, the precision of mapping (Fig. 1) (Peirce et al., 2004). Although RI strains were first developed in mice, and it is mice that are the focus of this chapter, the design has been used for a wide variety of organisms, including *Arabidopsis* (Lister and Dean, 1993; El-Din El-Assal et al., 2001),

Zea mays (maize) (Pan et al., 2017), barley (Yin et al., 2005), *Drosophila melanogaster* (Ruden et al., 2009), *Drosophila simulans* (Cochrane et al., 1998), *Caenorhabditis elegans* (Snoek et al., 2019) and rats (Printz et al., 2003).

Several mouse RI populations are available (Table 1; Fig. 2), however, these families are often small and difficult to acquire. I will focus on one of the most commonly used families, the BXD, and contrast it with two other large families, the CC and the ILSXISS.

The BXD mouse family

The BXD family was among the first RI populations to be produced (Taylor et al., 1973; Morse et al., 1979; Crow, 2007). This work was started by crossing female C57BL/6J (B6 or B) and male DBA/2J (D2 or D) strains—hence BXD (Fig. 1A). The first sets of BXDs were intended for mapping Mendelian loci (Taylor et al., 1973, 1999), but the family was also used to map complex traits such as cancer and cardiovascular disease (McGinnis et al., 1993; Lee et al., 1995; Grizzle et al., 2002; Koutnikova et al., 2009), variations in CNS structure (Belknap et al., 1992b; Zhou and Williams, 1999; Seecharan et al., 2003; Rosen et al., 2009), and behavioral and pharmacologic differences (Weimar et al., 1982; Belknap et al., 1992a, 1993; Rodriguez et al., 1994; Grisel et al., 1997; Phillips et al., 1998; Jones et al., 2006; Palmer et al., 2006; Ashbrook et al., 2018b; Knoll et al., 2018). New strains derived from advanced intercross (AI) progeny that had accumulated chromosomal recombination events across 8–14 generations were added in the late 1990s (Fig. 1b) (Darvasi, 1998; Peirce et al., 2004). These AI-derived BXDs incorporate roughly twice as many recombinations between parental genomes than conventional F2-derived BXDs (Darvasi, 1998; Williams et al., 2001a; Parker et al., 2012a, 2012b; Pandey and Williams, 2014; Ashbrook et al., 2022). This improves mapping precision nearly two-fold. Alleles discovered in the BXD have been successfully translated into medical applications in humans, such as stratified preclinical testing based on glaucoma risk alleles revealed in the BXDs (Chen et al., 2017; Ibrahim et al., 2019).

Two things now set the BXD family apart from all other recombinant inbred populations: the number of extant strains within the family, and the deep, coherent phenome that has been collected for them.

The largest mammalian recombinant inbred family

The BXD family is the largest mammalian RI population available, having expanded from ~20 strains to a total of 198 strains with data on GeneNetwork.org (Taylor et al., 1973, 1999; Peirce et al., 2004). There are over 120 BXD strains currently distributed by The Jackson Laboratory (JAX) (Ashbrook et al., 2021). This expanded number of easily accessible strains increases the power and precision of linkage studies, and our ability to carry out experimental precision medicine (Ashbrook et al., 2021, 2022).

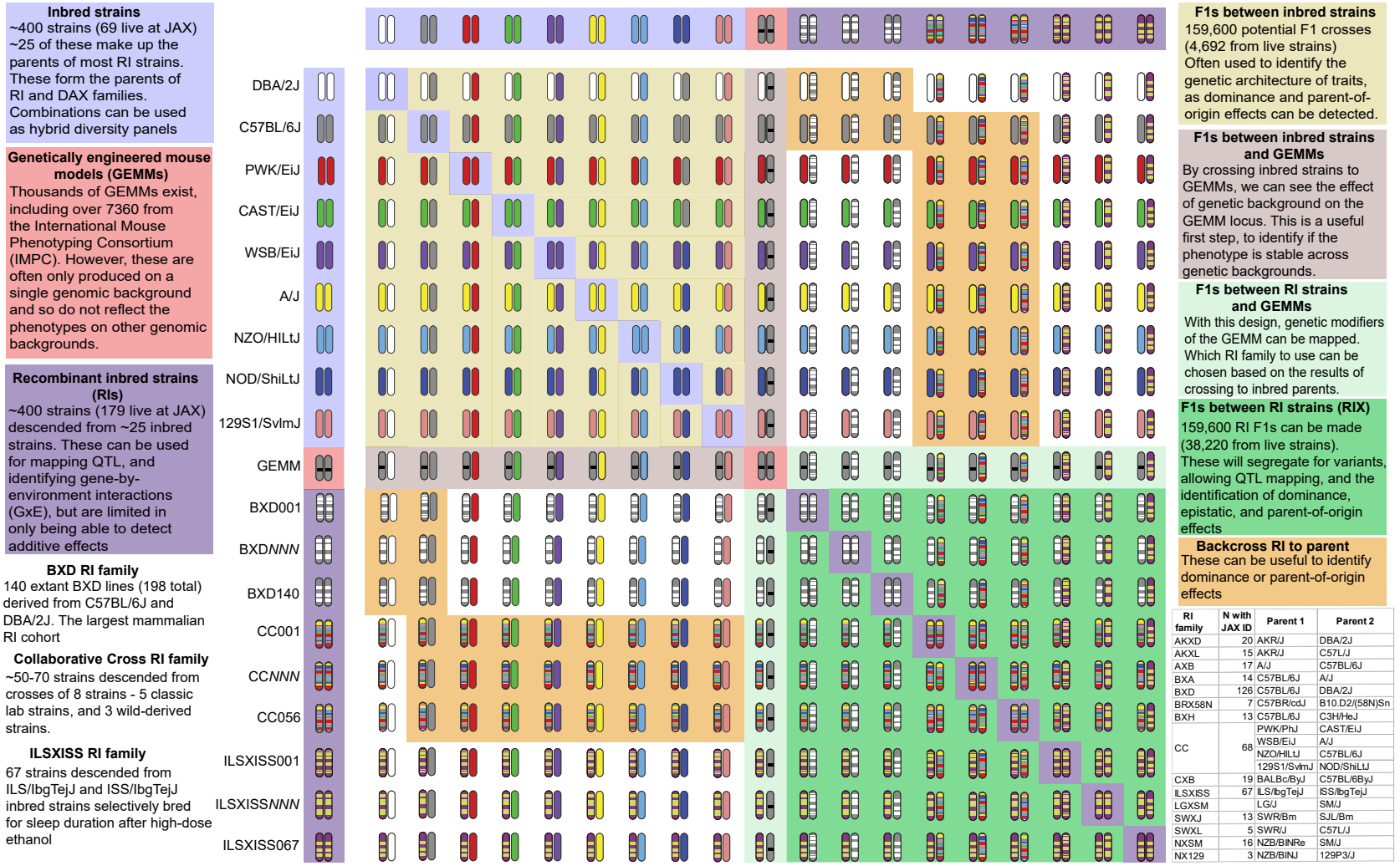
We have demonstrated that when using approximately half of the family (60–80 strains), precision is close to 1 Mb for many traits (Ashbrook et al., 2021). This is also partially due to two other features of the family. The first, common to all RIs, is that the effective heritability of the trait can be increased by resampling the same genometype (Belknap, 1998), and the second, that because there are two parents in the population, there is a well-balanced distribution of the two haplotypes across the genome.

When we examine power in the BXD family, we see a fact which might seem counter-intuitive to some: power is always increased more by increasing the number of strains compared to increasing the number of within strain biological replicates, even when heritability is low (Belknap, 1998; Andreux et al., 2012; Ashbrook et al., 2021). Therefore, as the largest RI family, the BXD has the greatest power to detect genotype-phenotype linkage.

Table 1 Recombinant inbred (RI) mouse families, the number of which have Jackson Laboratory identifiers, and the parental strains for the family.

RI family	*N with JAX ID*	*Parent 1*	*Parent 2*
AKXD	20	AKR/J	DBA/2J
AKXL	15	AKR/J	C57L/J
AXB	17	A/J	C57BL/6J
BXA	14	C57BL/6J	A/J
BXD	126	C57BL/6J	DBA/2J
BRX58N	7	C57BR/cdJ	B10·D2/(58N)Sn
BXH	13	C57BL/6J	C3H/HeJ
CC	68	129S1/SvImJ	A/J
		C57BL/6J	NOD/ShiLtJ
		NZO/HlLtJ	CAST/EiJ
		PWK/PhJ	WSB/EiJ
CXB	19	BALBc/ByJ	C57BL/6ByJ
ILSXISS	67	ILS/IbgTejJ	ISS/IbgTejJ
SWXJ	13	SWR/Bm	SJL/Bm
SWXL	5	SWR/J	C57L/J
NXSM	16	NZB/BINRe	SM/J
NX129	3	NZB/BINJ	129P3/J

Note the eight parental strains for the Collaborative Cross (CC).



RI family	N with JAX ID	Parent 1	Parent 2
AKXD	20	AKR/J	DBA/2J
AKXL	15	AKR/J	C57L/J
AXB	17	A/J	C57BL/6J
BXA	14	C57BL/6J	A/J
BXD	126	C57BL/6J	DBA/2J
BRX58N	7	C57BR/cdJ	B10.D2/(58N)Sn
BXH	13	C57BL/6J	C3H/HeJ
CC	68	PWK/PhJ	CAST/EiJ
		WSB/EiJ	A/J
		NZO/HlLtJ	C57BL/6J
		129S1/SvlmJ	NOD/ShiLtJ
CXB	19	BALBc/ByJ	C57BL/6ByJ
ILSXISS	67	ILS/IbgTejJ	ISS/IbgTejJ
LGXSM		LG/J	SM/J
SWXJ	13	SWR/Bm	SJL/Bm
SWXL	5	SWR/J	C57L/J
NXSM	16	NZB/BINRe	SM/J
NX129	3	NZB/BINJ	129P3/J

Fig. 2 Massive Diallel Crosses. Diallel crosses (DAXs) can be created from any F1 cross between two inbred strains. Each genome created in the DAX is isogenic (and therefore replicable). Different types of parental strain produce DAXs with different uses, but these can be combined together into a massive diallel cross. Information about different types of population are given in the colored boxes, and examples of these are colored in the central figure. Regions of the genome coming from different parental strains are represented by different colors of chromosome.

We should also note that the effect sizes seen in two-parent RIs (such as the BXDs), appears high, and this is in fact correct, as effect size is highly dependent upon the population being studied. Effect sizes measured in families of inbred lines are typically much higher than those measured in an otherwise matched analysis of other mouse or human populations. Two factors contribute to this higher level of explained loci variance. The first reason is due to replicability. When effect size is treated as the proportion of total genomic variance explained by the quantitative trait locus (QTL), effect size will increase as environmental effects decrease due to replication. That is, resampling decreases the standard error of the mean, suppressing environmental "noise" (Belknap, 1998). This is in addition to the increase in heritability above (i.e., an increase in total variance explained by the total genomic variance).

The second reason is that nearly all loci in inbred panels are homozygous, and so the same number of sampled animals will account for twice as much genetic variance as in an F2 cross, and four times as much variance as in a backcross (Belknap, 1998). When phenotyping with fully homozygous strains, we are only examining the extreme ends of the genomic distribution at that allele (i.e. no heterozygotes), providing a boost in power to detect additive effects. The downside is that we cannot detect non-additive effects—such as dominance or parent-of-origin effects—as these require heterozygotes.

The deepest phenome for any family

BXD mice are also the most deeply phenotyped RI family, with 50 years of data now openly and publicly available at GeneNetwork.org, thus providing an unrivaled resource. This dense and well-integrated phenome consists of over 10,000 classical phenotypes (Ashbrook et al., 2018a). The phenome begins with Taylor's 1973 analysis of cadmium toxicity, through to recent quantitative studies of addiction (Mulligan et al., 2013; Dickson et al., 2016, 2019), behavior (Philip et al., 2010; Carhuatanta et al., 2014; Graybeal et al., 2014; Mulligan and Williams, 2015), vision (Geisert and Williams, 2020), infectious disease (McKnite et al., 2012; Hayes et al., 2014; Wang et al., 2020), epigenetics (Baker et al., 2019; Sandoval-Sierra et al., 2020), and even indirect genetic effects (Ashbrook et al., 2015a, 2017; Baud et al., 2017). BXDs have been used to test specific developmental and evolutionary hypotheses (Seecharan et al., 2003; Hager et al., 2012; Oren et al., 2015). They have allowed the study of gene-by-environmental interactions, with environmental exposures including alcohol and drugs of abuse (Wang et al., 2013; Mulligan et al., 2018; Zhou et al., 2018; Dickson et al., 2019; Théberge et al., 2019), infectious agents (Boon et al., 2009, 2014; Nedelko et al., 2012; Russo et al., 2015; Chella Krishnan et al., 2016), dietary modifications (Fleet et al., 2016; Reyes Fernandez et al., 2016; Jones and Jellen, 2017; de Rodrigues et al., 2017; Jha et al., 2018a, 2018b; Roy et al., 2021), and stress (Jung et al., 2017; Diessler et al., 2018). The consequences of interventions and treatments as a function of genome, diet, age, and sex have been quantified (Philip et al., 2010; Fleet et al., 2016; Williams et al., 2016, 2020; Sandoval-Sierra et al., 2020; Roy et al., 2021), and gene pleiotropy has been identified (Wang et al., 2016a).

Beyond this, there is now extensive omics data for the BXD. Both parents have been fully sequenced (Keane et al., 2011; Wu et al., 2014; Wang et al., 2016b), and deep linked-read and long-read sequencing of 152 members of the BXD family is underway (Ashbrook et al., 2022). The family segregate for over 6 million common variants, similar to many human populations (1000 Genomes Project Consortium et al., 2015). Over 100 transcriptome datasets are available (e.g., (King et al., 2015; Li et al., 2018)), as well as more recent miRNA (Parsons et al., 2012; Mulligan et al., 2013), proteome (Williams et al., 2016, 2018, 2020), metabolome (Wu et al., 2014; Williams et al., 2016; Li et al., 2018), epigenome (Baker et al., 2019; Sandoval-Sierra et al., 2020), and metagenome (McKnite et al., 2012; Perez-Munoz et al., 2019) profiles. As each of these new datasets is added, they are fully coherent with previous datasets, multiplicatively increasing the usefulness of the whole phenome.

Websites to access and analyze this plethora of data are freely available and open-source, allowing users to download the data, or to make use of powerful statistical tools (e.g. GeneNetwork.org, bxd.vital-it.ch, and Systems-Genetics.org) (Sloan et al., 2016; Williams and Williams, 2017; Li et al., 2018).

It cannot be overstated how important it is that those using BXDs gain access to coherent genomes and quantitative phenomes generated under diverse laboratory and environmental conditions (Williams and Auwerx, 2015; Ashbrook et al., 2018a). New data can be correlated and co-analyzed with thousands of publicly available quantitative traits, and with each addition, the number of network connections grows quadratically—enabling powerful multi-systems analysis for all users (Chesler et al., 2003; Andreux et al., 2012; Williams et al., 2016; Jha et al., 2018a, 2018b; Li et al., 2018). Within minutes of finding a gene of interest, a researcher can look for correlations between its expression and thousands of other genes across dozens of tissues. Enrichment analyzes can then be carried out, suggesting pathways and networks that a gene of interest may be associated with. Correlations can be found between the expression of that gene and over 10,000 phenotypes, giving suggestions of its role at the whole-organism level. Shared QTLs, where both the gene-expression and a phenotype of interest are associated with the same locus, provide strong evidence of causation. Using GeneNetwork.org we can build biological networks, moving from genetic variant, to expression difference, to protein expression, to whole-system outcomes, with just a few keystrokes, and without touching a lab bench (Andreux et al., 2012; Mulligan et al., 2017; Parker et al., 2017; Watson and Ashbrook, 2020). Entire manuscripts can be written without leaving a web browser (Ashbrook et al., 2014a; Ashbrook and Lu, 2021; Chunduri et al., 2022). This is a massive step forward that is under-appreciated by many.

The BXD can help us achieve our goal of predictive modeling of disease risk and the efficacy of interventions (Hood and Flores, 2012). Indeed, the family has already been used to test specific functional predictions of behavior based on neuroanatomical variation (Yang et al., 2008). The BXD family is well placed to address questions that encompass both high levels of genetic variation and gene-environment interactions—our complexity challenge.

Other RI populations

As noted above, there are other RI panels. The most widely used after the BXD is the CC. These are ∼50–70 strains (63 currently available, of which 51 breed well; http://csbio.unc.edu/CCstatus/index.py?run=availableLines) that are descended from crosses of eight strains—five classic laboratory, and three wild-derived strains (Table 1; Figs. 1D and 2) (Threadgill et al., 2002; Churchill et al., 2004; Valdar et al., 2006a; Morgan and Welsh, 2015; Schughart and Williams, 2017; Shorter et al., 2019b). The CC RI strains are capable of even better mapping precision than standard RIs for two reasons. First, the number of recombinations in the CC is ∼1.75 times higher than a standard two-parent RI, due to the repeated intercrossing that was required to merge all eight genomes. Secondly, these multi-parental genomes allow fine grain analysis of haplotype contrasts, which can reduce haplotype intervals, and potentially get to quantitative trait genes (QTGs), rather than loci.

The increased genetic diversity comes at a cost though—minor allele frequencies are lower, decreasing the power for loci detection. The CC were originally planned to be 1000 strains, but difficulties in breeding have reduced the family to less than 100 strains (Noll et al., 2019). Their creation did lead to the Diversity Outbred population, described below.

The ILSXISS are 67 strains with a more complicated history. Two lines, ILS/IbgTejJ (**i**nbred **l**ong **s**leep) and ISS/IbgTejJ (**i**nbred **s**hort **s**leep) were selected and inbred from a heterogenous stock derived from an 8-way-cross (A, AKR, BALB/c, C3H/2, C57BL, DBA/2, IS/Bi and RIII), based on their duration of loss-of-righting response following high dose intraperitoneal administration of ethanol (Williams et al., 2004). The ILSXISS RI panel was later initiated from the two inbred ILS/IbgTejJ and ISS/IbgTejJ strains, and the RIs were inbred for over 22 generations before being made available from JAX (Williams et al., 2004). For a long time, the ILSXISS were the largest mouse RI family, and have been widely used, particularly to study metabolism, aging, and longevity (Liao et al., 2010, 2013; Rikke et al., 2010; Stöckli et al., 2017). However, they do not have the depth of well recorded phenotypes that the BXD have acquired, nor is their genome as intentionally designed and well understood as the CC.

Diversity panels

Panels of inbred strains provide a complementary solution—rather than using a single inbred strain, or even a single family of inbred strains, panels use many. Over the past century, many strains of mice have been inbred, sometimes to intentionally capture extremes of phenotypes (like the ISS and ILS above). Over 200 inbred strains are available from the Jackson Laboratory, and many now have genome-wide sequencing available (Lilue et al., 2018, 2019; Arslan et al., 2021; Ferraj et al., 2022). These can be used together to create so called diversity panels. In this case they have captured genetic diversity, and also replicate the same genome to reduce noise, or to test environmental interventions.

The Hybrid Mouse Diversity Panel (HMDP) (Ghazalpour et al., 2012; Bennett et al., 2015) is a collection of common inbred and RI strains, that have been phenotyped together and used as a joint mapping resource (Williams et al., 1996, 1998; Overall et al., 2009; Ghazalpour et al., 2012). They offer several advantages. Causal gene mapping precision is often higher than the RI alone, each strain is replicable (and therefore we can run counterfactuals), and we can assemble large phenomes (in which we can run gene by environment analyzes and address our complexity problem).

There is no formal membership list for the HMDP, and so different studies have used different specific strains, reducing our ability to combine studies as coherent datasets. Another downside is that finding causal genes in these panels is often difficult, as the same phenotype could be influenced by different biological pathways in the different strains—as such, power is low and false discoveries are high (Williams and Williams, 2017). However, haplotype contrasts, similar to those used for the CC, can be used by exploiting combinations of RI families, inbred strains, and F2 crosses (Taylor et al., 1973; Williams et al., 1998; Malmanger et al., 2006; Furlotte et al., 2012).

Outbred populations

A commonly used solution for many is the use of outbred animals. Like humans, each individual in an outbred population is genetically unique. Some of these, such as the Outbred Stock (OS; often referred to as Swiss Webster stock), are from very small founder populations (Lynch, 1969; Chia et al., 2005; Yalcin et al., 2010), whereas others, such as the Diversity Outbred population or heterogeneous stocks (HS) are from larger foundation populations. The Northport HS (HS-Npt)—made by intercrossing A/J, AKR/J, BALBc/J, CBA/J, C3H/HeJ, C57BL/6J, DBA/2J, and LP/J—has been used for high precision QTL mapping (Hitzemann et al., 1994; Valdar et al., 2006b). The DO is derived from the eight founders of the CC, three of which—PWK/Ph, CAST/Ei, and WSB/Ei—are inbred strains derived from highly diverse wild *Mus musculus* subspecies, meaning that, similar to the CC, the DO population segregates for well over 40 million common sequence variants – 4 or more times the number of common variants in the human population (Fig. 1D) (Churchill et al., 2012; Svenson et al., 2012; Smallwood et al., 2014).

This large genetic diversity is useful for several reasons: their genetic complexity equals or exceeds that of human populations; they accumulate large numbers of recombinations that allow high precision genotype-to-phenotype mapping; high genetic diversity ensures that phenotypes will be highly variable and that most regions of the genome will be polymorphic; and they usually have excellent breeding performance. This makes them excellent models for answering some questions—we can ask about the extremes of response to a drug, or what side effects may occur in only a small proportion of the population.

Despite the many advantages—hybrid vigor, more genetic diversity, loss of direct or indirect selection effects etc. —there are corresponding disadvantages. Like human populations, a very large sample size is required to make associations for non-Mendelian traits, and every individual must be genotyped. Specifically, they do not solve the N-of-1 or counter-factual problems detailed

above. Again, as in humans, every individual is unique, and therefore we cannot go back to that same individual and ask what we could have done differently or determine if an extreme phenotype had genetic, environmental, or stochastic causes—thus requiring the investigator to work at the population level. They are, therefore, not a good tool for *experimental* precision medicine, although they can accurately model human precision medicine. Many of the tools designed for precision medicine analyzes in humans can be applied and used in an outbred mouse population, but with the advantage of tighter environmental control.

F2 intercrosses and backcrosses

F2 intercrosses, effectively the grandchildren generated from between two and four mice, have been widely used (Damerval et al., 1994; Schadt et al., 2003, 2005). Their main advantage is the ability to make large numbers of progeny quickly from almost any stock—usually inbred strains. F2 intercrosses and N2 backcrosses have a structure that makes mapping and the analysis of covariance among traits simple. There is no need to correct for population substructure—a problem that arises in the multi-generation crosses described above. It is practical to enhance the complexity and utility of an F2 intercross for systems genetics and for standard QTL mapping by making a four-way F2—for example by crossing AXB F1s to CXD F1s to produce ABXCD F2 progeny. This type of F2 was used to create the UM-Het3 population (Miller et al., 2007).

The UM-Het3 are created by crossing CByB6F1/J females and C3D2F1/J males together. Therefore, each individual mouse contains approximately equal numbers of segregating alleles from the BALB/cByJ, C57BL/6J, C3H/HeJ, and DBA/2J inbred strains. Further, each individual shares approximately half their nuclear genome with each other UM-Het3 individual, making them, genetically, siblings (Jackson et al., 1999; Miller et al., 2007).

The UM-Het3 lie as a halfway house between HMDP/RIs and outbred strains. Like outbred strains, each individual mouse is unique (invoking our N-of-1 and counterfactual problems and requiring that each one be genotyped), but populations of very similar animals (containing the same four haplotypes in approximately the same population distribution) can be made quickly and tested under many conditions and across laboratories. This is the approach taken by the National Institute on Aging (NIA) Interventions Testing Program (ITP), and they have provided many exciting insights into environmental and sex effects on lifespan and potential drugs to extend longevity (Miller et al., 2007; Nadon et al., 2008; Cheng et al., 2019). In addition to testing interventions, current work is ongoing to test gene-by-environment interactions in this population, identifying genetic loci which increase longevity, and which influence response to interventions.

Combining populations together

Although I have presented the above populations separately, these are not either/or decisions. These families can be used together in the same study (as with the HMDP), or meta-analyzed to improve power and precision, increase the number of variants tested, and/or to increase the number of potential GxE interactions (Furlotte et al., 2012; Kang et al., 2014; Williams and Williams, 2017). For example, the CC and the DO have the same parents and therefore segregate for the same variants, the BXD, HMDP and UM-Het3 all contain C57BL/6J and DBA/2J alleles allowing them to be easily used in unity, and any set of isogenic mice can be combined into a massive diallel cross.

Massive diallel crosses

A diallel cross (DAX) is another experimental model that has been used for nearly 70 years (Hayman, 1954; Griffing, 1956; Kempthorne, 1956). A DAX is the set of all possible matings between several isogenic genometypes (Fig. 2). For example, for the C57BL/6J and DBA/2J there are two reciprocal F1s, and these have been used to study parent-of-origin effects and to estimate heritability (e.g. (Ashbrook et al., 2018b)). As the number of parental strains increases, the number of potential diallel crosses increases exponentially, and tools have been developed to deal with large DAXs (Lenarcic et al., 2012). Although we have learned much about the genetic architecture of traits (Airey et al., 2002; Lenarcic et al., 2012; Crowley et al., 2014; Percival et al., 2015; Ashbrook et al., 2018b; Maurizio et al., 2018), QTL mapping has been more difficult, given the relatively small number of strains used (Williams et al., 2001b).

We can make a DAX out of any set of isogenic strains—such as the BXD family. Using 120 easily available BXDs, there are 14,280 replicable F1s which are isogenic but not inbred. Each of these F1s is a reproducible genome, and so can be used for experimental precision medicine—predictions can be made in one part of the DAX, and then tested in another part.

Using a DAX, the number of genomes phenotyped can be increased massively to increase power to detect loci with even the weakest of effect sizes (Williams et al., 2001b). Precision can also be enhanced, as F1s can be produced that segregate for a narrow region of the genome, producing a small QTL interval containing fewer genes. All the data collected in these F1s can be coherently integrated into the phenome already aggregated for the parental populations—every new phenotype measured adds quadratically to the phenome and then every user of the DAX has access to decades of prior data.

A DAX also has an advantage over an RI or diversity panel as it allows detection of non-additive effects, such as dominance and parent-of-origin effects. This is because they are isogenic but not inbred—i.e., they will be heterozygous at some loci. Small DAXs of mouse strains have identified parent-of-origin effects, epistasis, and dominance, but have been unable to map the loci causing these effects (Green, 1962; Airey et al., 2002; Lenarcic et al., 2012; Crowley et al., 2014; Percival et al., 2015; Ashbrook et al., 2018b; Shorter et al., 2019a). By using reciprocal crosses of inbred strains (e.g. BXD1xBXD2F1 vs BXD2xBXD1F1), we can produce isogenic

litters, the members of which are all genetically identical, and whose only differences are due to parent-of-origin effects (Ashbrook and Hager, 2013) (Fig. 2). Mapping of these non-additive loci is a complete dark zone in fully homozygous inbred populations, and almost impossible to identify in human populations.

The most important role of these large DAXs is their application as a population for testing predictions, as there would be sufficient power to make gene-phenotype, environment-phenotype, and gene-environment-phenotype associations with high power in one set of F1s, and a sufficiently large population to then test those predictions in a different set of F1s. From RI and inbred strains, there are hundreds of thousands of potential F1s that are easily available and reproducible (Fig. 2). An additional expansion of this design is to cross RI families to genetically engineered disease models.

Diallel crosses to genetically modified strains

Genetically engineered mouse models (GEMMs), including humanized, transgenic, and knockout mouse models, have been a vital piece in uncovering genotype-phenotype associations. However, these models often suffer from the N-of-1 problem, with a transgenic produced on a single genetic background. There is over two decades of evidence that an identical genetic modification can have a different phenotype on different genetic backgrounds (Threadgill et al., 1995; Phillips et al., 1999; Sanford et al., 2001; Buchner et al., 2003; Hahn et al., 2004; Doetschman, 2009; Acevedo-Arozena et al., 2011; Heiman-Patterson et al., 2011; Cowin et al., 2012; Sisay et al., 2013; Fetterman et al., 2013; Sittig et al., 2016; Nair et al., 2019; O'Connell et al., 2019; Tabbaa et al., 2023). As a specific example from one of these studies, Sittig and colleagues crossed two different transgenic modifications to 30 different isogenic mouse strains, and found that diametrically opposing conclusions could be drawn in different genetic backgrounds. In the *Tcf7l2* heterozygous knockout on a C57BL/6J background they found no effect on open field activity, whereas on a DBA/2J background open field activity was halved (Sittig et al., 2016).

Increasing the number of genetic backgrounds tested is difficult, as the increase in effort per strain is nearly linear. However, in biomedical research it is important and necessary to be able to draw accurate, generalizable conclusions that are relevant to the human condition. A DAX can help with this problem, as each genetically modified isogenic line can be added into the DAX, and now any of hundreds of genetically defined isogenic F1s can be phenotyped (Figs. 1C and 2). Given that there are now thousands of knockout strains available (e.g. from the International Mouse Phenotyping Consortium) (Cacheiro et al., 2019; Lloyd et al., 2020), creating a DAX is a relatively cheap and quick method by which to test the effects of genetic background (Lifsted et al., 1998; Nnadi et al., 2012; Bennett et al., 2015; Dorman et al., 2016; Sittig et al., 2016). By using a DAX to an RI population, we can map the location of modifier loci, and use existing data in that RI population to infer causality (Hunter et al., 2001; Yang et al., 2005; Crawford and Hunter, 2011). These hypotheses can then be tested in other crosses to the RI population.

The Alzheimer's disease BXD (AD-BXD) panel developed by Neuner et al. (2019a, 2019b) provide an exemplar for this approach. The team crossed C57BL/6J-congenic females hemizygous for the humanized 5XFAD transgene (JAX Stock No. 008730) to males from BXD strains. Half of each of the resulting litters had the 5XFAD transgene (transgenic-AD-BXD), and half did not have the 5xFAD transgene (non-transgenic-BXD). The important note is that the entire litter is genetically and environmentally identical except for the presence of the transgene, giving an immediate and directly comparable control (Fig. 1C). By crossing the humanized 5XFAD strain on a single genetic background to a diverse, but defined set of BXDs, the study produced a population that incorporated high levels of sequence variation mirroring that of humans. These studies mapped genetic and molecular causes of cognitive loss (Neuner et al., 2015, 2017, 2019a, 2019b; Hyman and Tanzi, 2019; O'Connell et al., 2019), including a broad spectrum of cognitive loss similar to that of humans with familial and late-onset AD (Neuner et al., 2015). The human transgenes in the 5XFAD strain (Oakley et al., 2006) sensitizes BXD hybrids to a greater or lesser degree—some begin to lose conditioned fear memory as early as 6 months; others well after a year (Neuner et al., 2019a), demonstrating a gene-by-gene-by-age interaction. Variation is highly heritable, and causal loci can be identified. This is a powerful means by which to define genetic causality and mechanisms of memory and non-cognitive loss and resilience to loss, and in which to test interventions—a great model for experimental precision medicine.

This model has also been used for 'reverse translation'—moving from human genomic data to mouse phenotypes (Neuner et al., 2019a). A polygenic genetic risk score was generated using 21 genes whose human homologs increase Alzheimer's disease risk. This showed that the allele dosage was significantly associated with cognitive outcomes in the AD-BXD mice. This confirms that naturally occurring variation in these networks has overlapping effects in mice and humans, and that the gene-phenotype associations found in the AD-BXD translate across species.

Given that phenotypes from genetically engineered mice on a single genetic background cannot be reliably generalized to other mouse genetic backgrounds (Sittig et al., 2016), it is unsurprising that there are difficulties in generalizing to other species. By crossing genetically modified lines to RI strains to produce a DAX, we overcome this problem and allow the integration and translation of data to other populations and other species.

Translation to humans

Compared to F2s or outbred stock, large, two-parent, RI panels are particularly advantageous when the heritability of a trait is moderate or low. This is because the genetic signal can be greatly boosted by resampling members of the same isogenic strain many times (Belknap, 1998). The drawback of these two-parent panels, such as the BXDs, are lower precision, and a decreased amount of variation in the population compared to multiparent RI populations (such as the CC), and a consequent decrease in the total phenotypic variance (Abu-Toamih Atamni and Iraqi, 2019). However, this may not be a significant drawback, as medically relevant

phenotypes are variable in the BXD family, and sub-centimorgan precision QTL mapping is possible (Ashbrook et al., 2021). This is a sufficient level of precision to then transition from QTLs to causal genes, variants, and the study of mechanisms by taking advantage of complementary resources. These include sets of other murine mapping resources (e.g. HMDP), efficient in vitro and in vivo screens (Houtkooper et al., 2013; Smemo et al., 2014; Williams and Auwerx, 2015), and human genome-wide association study (GWAS) data.

Large GWASs are now routine, and these can be combined with mouse QTL studies for mouse-to-human translation (Koutnikova et al., 2009; Ashbrook et al., 2014b, 2015b; Wang et al., 2016b; Chintalapudi et al., 2017; Jha et al., 2018a, 2018b; Li et al., 2018). Human GWAS data can be used to refine QTLs found in mice, e.g. taking advantage of the power to detect associations in an experimental mouse population, and then using the precision of human GWAS to identify a candidate gene in the syntenic region (Ashbrook et al., 2014b, 2015b, 2019). Approximately 80% of mouse genes can be mapped to a single orthologous gene (i.e. 1:1 orthologues) within the human genome and >99% map to one or more orthologous genes (Mouse Genome Sequencing Consortium et al., 2002).

More importantly for experimental precision medicine, mouse data can be used to determine the function and causal pathway for associations made in humans, and to test hypotheses of causality. The only significant limitation in making variant-phenotype associations in human GWASs is collecting phenotypes from a sufficiently large and diverse population. However, determining the function of these variants, and how they alter the phenotype of interest, is far more difficult, given the vast variability in human environments and the small effect size of variants. Mouse models provide a method to carry out experimental precision medicine for reverse-translation from human-to-mouse—finding out how a variant found in humans effects the phenotype, and how it might respond to interventions. In these models we can separate different aspects of disease—for example age of onset, rate of progression, physical, and cognitive symptoms—that are difficult to identify in human GWAS, where criteria are often simply presence or absence of disease.

Conclusions

To fully achieve the potential of precision medicine for population level interventions, we need experimental systems in which to test human observations—experimental systems which do not share the limitations of working with a human population (including N-of-1, lack of counterfactuals, and inability to control complex genomes and environments). In this chapter, I have presented how mouse models can be used to achieve a high level of experimental precision medicine.

In the model systems described, the genetic architecture of traits can be dissected and causal relationships among networks can be explored (Roy et al., 2021). The ability to resample genomes across a stable reference family enables the expansion of data in almost any direction—studies and populations can be combined across laboratories and across time. Publicly accessible and well documented FAIR data (Wilkinson et al., 2016) enables cross-species translation, and expands the utility of other successive studies using that same population. This enables more diverse researcher communities to engage in replicable trans-disciplinary studies of genome-phenome prediction—that is, the ability to predict what is going to happen in a particular individual and then test that prediction.

Using these mouse model systems, we can generate large genetically complex resources with matched multi-scalar and multi-system phenome data. These systems allow us to do something that is currently not possible in the human population—we can identify the complex interplay among sets of variants, constellations of phenotypes, and different treatments and environments (Ashbrook et al., 2014a; Mulligan and Williams, 2015; Williams and Auwerx, 2015; Williams et al., 2016). Complex biological processes are now amenable to prediction (Li et al., 2008; Miyairi et al., 2012; Wang et al., 2016b) and we can define and test progressively more sophisticated computational models as a function of genometype, age, and exposure.

For precision medicine to continue to flourish, we need to understand all gene-by-gene-by-environment-by-age-by-sex-by-treatment interactions (Nair et al., 2019), and animal models are likely the only way to do this at scale (Brown, 2021). The importance of using genetically diverse mice has often been overlooked, leading to difficulties with translation. RI families, such as the BXDs and their expansions (Williams and Williams, 2017), including DAXs and reduced complexity crosses (Bryant et al., 2018, 2020), overcome this problem and are a vital experimental step towards accurate, individualized, predictive medicine. These systems give us replicable populations with the same intrinsic genetic complexity and admixture as humans, overcoming many of the challenges of precision medicine outlined in the introduction, and without many of the challenges inherent to clinical research—high cost, marginal compliance and control, confidentiality, and ethical constraints on designs and interventions. Mouse populations are ideally positioned to be replicable and extensible experimental testbeds with which to test the power, accuracy, and limits of precision medicine.

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