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After GWAS: mice to the rescue?

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Abstract

The genetics of human autoimmune diseases remain incompletely understood, despite significant progress from genome-wide association studies (GWAS). In this review we outline how mouse studies may help filling these knowledge gaps. Forward genetic approaches including mutagenesis screens and quantitative trait locus mapping can be used to identify candidate genes for in depth analysis in human patient populations. Reverse genetic approaches utilize genetically engineered mice to analyze the function of disease-associated genes and their variants. Inbred strains are a distinctive feature of mouse genetics and we discuss their history, advantages and disadvantages. Three factors need to be considered when comparing experimental results from studies in mice and humans. In addition to species-specific differences, phenotypes are affected by the genetic background of the mouse strain being analyzed, as well as by microbial factors. Despite of these complexities, mice are essential discovery tools in the post GWAS era.

Introduction

Immunology research has for several decades relied on the use of mouse models. Despite growing skepticism [1], this is unlikely to change any time soon. A plethora of reagents for analyzing the murine immune system, powerful techniques for genetic engineering, and general advantages of the mouse as a laboratory species such as small size and short generation time, assure a central role for the mouse in immunology research. In this context we ask what mouse research can offer for understanding the genetics of human autoimmune diseases.

The mouse genome

A first draft of the mouse genome sequence was published in 2002, shortly after the human genome [2]. A more complete version followed in 2009 [3]. 75% of mouse genes have 1:1 orthologs in the human genome and one can think of the mouse and human genomes as assemblies of reshuffled genomic blocks derived from a common ancestor 90 million years ago [4]. While mice have fewer chromosomes than humans (19 versus 22 autosomes), the total number of genes is about 5% higher. Most of the surplus genes in mice are the result of

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gene duplications and serve reproductive or olfactory functions, suggesting rodent-specific selective pressures as the driving force behind this gene expansion [3]. Immune-related genes are generally conserved - with some notable exceptions. Several inflammatory chemokines for example exist only in one species but not the other. This again is likely the result of lineage-specific selective pressures from exposure to different sets of pathogens [5]. The reference sequence of the mouse genome is derived from the commonly used C57BL/6 laboratory strain. Significant efforts have gone into cataloging the sequence and structural genomic differences between C57BL/6 and other inbred strains. The Sanger Institute recently finished sequencing of the complete genomes of 17 inbred strains [6,7].

Inbred mice

The geneticist Clarence Little introduced the practice of inbreeding mice about 100 years ago and most of the classical inbred strains like DBA, C57BL/6, C3H, or BALB/c were generated in the early 20th century [8]. Inbred strains are derived by brother × sister mating for at least 20 generations. Inbred strains are, with sufficient approximation, homozygous across all loci and members of the strain are genetically identical. This genetic homogeneity removes an important source of experimental variability while restricting the observation of outcomes to a single artificial representative of the mouse species. Inbreeding reduces genetic diversity through the stochastic loss of variants as well as negative selection of recessive alleles with reduced fitness [9]. Shared ancestry contributes further to the limited genetic diversity in the classical inbred strains. The founders of many of these strains originated from a mouse farm in Massachusetts owned by a Abbie Lathrop, who supplied mice to “mouse fanciers” as well as scientists like Clarence Little [10]. The fashion of breeding pet mice with appealing morphological characteristics (“fancy mice”) originated in Asia, and Abbie Lathrop's mouse stock had genetic input from all over the world. Sequence analysis has confirmed that the classical inbred strains are hybrids between the three major subspecies of *Mus musculus* found around the world (*M.m.domesticus*, *M.m.musculus*, and *M.m.castaneus*) with most of their genomes deriving from *M.m.domesticus* [11]. Because of their specific history, the classical inbred strains demonstrate a substantial degree of identity by descent across their genomes [12] and collectively capture only a fraction of the genetic diversity present in mice living in the wild.

Forward genetic approaches in mice

Forward genetics is classical genetics that attempts to identify the genetic variant causing an observed phenotype. Several well-known examples exist for single gene autoimmune disorders that arose spontaneously in laboratory mouse colonies. For some of these such as the *scurfy* mouse with a regulatory T cell defect due to a mutation in the *FoxP3* gene [13], corresponding mutations and disease phenotypes exist in humans. The spontaneous mutation rate can be enhanced by administration of the mutagen N-ethyl-N-nitrosourea (ENU) [14]. ENU induces germline point mutations at a rate of about 1 in 10⁶ base pairs [15]. ENU mutagenesis screens can be tailored to identify mutations resulting in specific phenotypes, such as an altered *in vitro* response of macrophages to stimulation with TLR ligands [16]. Two large-scale ENU mutagenesis programs directed by Chris Goodnow [17] and Bruce Beutler [18] were specifically designed to identify mutations in immune-related genes. These programs continue to identify genes that control important checkpoints in the development of the immune system or influence immune responses [19-22]. Almost all ENU-induced functional mutations affect mRNA/protein sequence, either by introducing missense or stop codons into exons, or by disrupting splice donor-acceptor sites [23]. Massive parallel exome sequencing has therefore been used – with great success – as a means to accelerate the identification of causative mutations [23-25].

Polygenetic diseases resembling human autoimmune disorders, such as the non-obese diabetic (NOD) mouse as a model for type I diabetes [26] and several lupus-prone mouse strains [27], have also arisen spontaneously. The standard approach to identify the loci controlling susceptibility or severity in polygenetic diseases in mice is the quantitative trait locus (QTL) analysis [28]. A typical QTL study analyzes the F2 intercross between an inbred strain with a disease phenotype of interest and a second strain without or with less severe disease. Typically, these QTLs are rather large, sometimes measuring dozens of Mbp. In the next step, congenic mice are created that carry the nonsusceptible QTL allele on the susceptible background or vice versa. Fine-mapping of the QTL is achieved by analyzing the disease phenotype in a series of subcongenic mouse lines with overlapping fragments of the original congenic QTL locus. A few examples exist where this approach, in combination with sequence analysis, expression profiling and functional studies, has led to the identification of individual candidate susceptibility genes (e.g. *IL-2* in the NOD susceptibility locus *Idd3* [29]). QTL mapping is laborious, causality can be difficult to prove [30] and the identification of causative variants has been the exception rather than the rule. Nevertheless, QTL studies in mice can detect gene-gene interactions, something much more difficult to demonstrate in heterogeneous human populations where susceptible and protective alleles at many loci segregate randomly [31].

A number of strategies have been devised to increase the mapping power for quantitative traits in mice. The Collaborative Cross is a large-scale effort to generate several hundred new genetically and phenotypically diverse inbred strains from a set of 8 carefully chosen founder strains. The inbreeding phase of the project is still ongoing [32]. Already available is the Hybrid Mouse Diversity Panel (HMDP), a panel of 29 classical inbred strains and 71 recombinant inbred strains, derived from crosses between C57BL/6 and 3 other strains [33]. The mapping resolution using these mouse panels is less than 1 Mbp and thus significantly improved over traditional two-strain comparisons [33,34]. An alternative approach is to study genetically heterogeneous or outbred mouse stocks [35-37]. This type of analysis resembles studies in humans, as mice from these colonies are genetically diverse and heterozygous at many loci.

Reverse genetic approaches in mice

Reverse genetics starts with a genetic modification and analyzes the resulting phenotype. Transgenic mice, generated by injection of a DNA construct into the pro-nucleus of a fertilized egg and random integration into the genome, were introduced more than 30 years ago [38]. Gene targeting through homologous recombination in embryonic stem cells (ES cells) followed just a few years later [39,40]. This technology has been used widely to delete genes (knockout) but also to replace genes with hypomorphic variants or reporter alleles (knockin). Today, genes can be conditionally switched on/off with drug-sensitive promoters or deleted using site-specific recombinases in a cell-type and time-sensitive manner. The most popular system for conditional deletion is cre-loxP, where transgenic expression of the cre recombinase results in excision of a genomic DNA segment flanked by two *loxP* recognition sites [41]. More than 500 different cre-transgenic mice have been generated [42]. Novel site-specific recombinase systems (e.g. FLP-FRT) can be combined with cre-loxP to generate even more complex experimental systems. Complementing the classical knockout approach, transposon and gene trap vector technologies [43,44] generate deletion mutants through random insertion into the genome. Using the integrated vector as a tag, targeted loci are easily identified by sequencing.

Several large programs in Europe and North America (the International Knockout Mouse Consortium, IKMC) are working toward the goal of generating a gene-deficient mouse for every protein-coding gene in the genome [45]. Similar programs exist for miRNAs [46,47].

As an extension of IKMC, the International Mouse Phenotyping Consortium (IMPC) was launched in 2011. Its goal is to comprehensively phenotype every gene-targeted mouse generated by IKMC according to standardized protocols within the next decade. This is an expensive yet important project to avoid potential investigator bias in describing phenotypes and to capture the pleiotropic effects of all genes [48].

Of mice and men

GWAS have significantly advanced our understanding of the genetics of human autoimmune diseases. However, for many disease-associated loci, the causative genetic variants are still unknown. Furthermore, the sum of the disease-associated loci identified to date typically explains only a fraction of the total genetic risk. Mouse genetics can help in two principal ways to fill these knowledge gaps.

The first approach is to use gene-targeted mice and murine disease models to analyze the function of GWAS candidate genes *in vivo*. A recent study by Liu et al. provides an example for the successful integration of mouse and human studies in following a GWAS lead. These authors set out to identify the nature of the association of SNPs in the *LRRK2* gene with Crohn's disease (CD) [49]. First they showed that *Lrrk2* knockout mice develop more severe colitis when exposed to DSS. Making ingenious use of a drosophila RNAi phenotype database, they identified NFAT as an interacting partner of LRRK2. Mechanistic studies in mice then demonstrated that LRRK2 regulates macrophage activation via inhibition of NFAT1 activity. The paper concluded with evidence that a CD-associated non-synonymous coding SNP in *LRRK2* conferred reduced LRRK2 protein stability and that macrophages from human subjects homozygous for this variant produced less cytokines upon stimulation *in vitro* than macrophages from controls.

A more refined method for the analysis of GWAS hits is the generation of knockin mice for human disease-associated variants [50]. Human artificial chromosomes are an emerging technology that permits the introduction of large human DNA segments into transgenic mice [51] which may be useful for the analysis of non-coding intervals with putative regulatory function. The real strength of exploring the function of candidate genes in mice however is the availability of powerful tools to address mechanistic questions - for instance by crossing in additional gene modifications. The Mouse Genome Database (<http://www.informatics.jax.org/>) provides an excellent resource of curated information on availability and phenotype of mutant mouse strains.

The second approach is to ask whether genes with disease-associated variants in mice predict similar associations in humans with autoimmune disease. This is clearly the case for monogenetic disorders as discussed for *FoxP3* deficiency. Kaser et al. demonstrated that this approach can also be successful in polygenetic diseases [52]. Their paper described that the conditional deletion of *Xbp1* in intestinal epithelial cells results in inflammatory bowel disease in mice. XBP1 is a transcription factor that mediates one arm of the unfolded protein response, a cellular stress response to protein overload in the endoplasmic reticulum. Linkage studies in human IBD had suggested an association with a locus close to the human *XBPI* locus. After confirming this association, Kaser et al. sequenced the *XBPI* gene in a cohort of IBD patients. Several rare non-synonymous coding SNPs in *XBPI* were identified that were only present in patients but not in controls. Importantly, these *XBPI* variants were hypomorphic when tested *in vitro*.

A related idea is to identify disease-relevant genes by comparing mouse QTLs with data from human GWAS. Johnsen et al. used this approach in a QTL analysis of arthritis severity in the K/BxN serum transfer model [53]. This study involved a large cohort of 570 heterogeneous stock mice and a genotyping panel of 1449 SNPs permitting the relatively

precise mapping of QTLs down to 2 Mbp in size. Multiple QTLs were identified, however, only 2/32 rheumatoid arthritis (RA) risk loci (containing the candidate genes *TRAF1/C5* and *PADI4*) demonstrated overlap with the mouse QTLs. Even though a comparative analysis with GWAS data has not been published yet, the overlap between risk loci in the NOD mouse model and human type 1 diabetes appears to be bigger (*MHC* region, *PTPN22*, *INS*, *CTLA4*, *IL2*, and others) [29,54,55]. This could reflect a greater degree of similarity between mouse and human autoimmune diabetes as compared to the K/BxN model and RA. It is also possible that a study that interrogates disease severity rather than disease development per se will necessarily identify a different set of genes, or that the genes controlling K/BxN arthritis are only relevant in subsets of RA patients. Either way, to fully realize the potential of mouse QTL mapping for the prediction of human disease genes will require higher resolution mouse QTLs as discussed above well as systems biology approaches that incorporates genetic, epigenetic and functional information.

“Mouse traps”

The disparate phenotypes caused by mutations in the *IL1RN* gene illustrate other challenges encountered in mouse-human genetic studies. *IL1RN* encodes the IL-1 receptor antagonist, an endogenous inhibitor of IL-1 signaling. Humans with homozygous deletion of *IL1RN* develop a multisystem autoinflammatory syndrome [56,57]. The phenotype of *IL1RN* knockout mice on a BALB/c background is dominated by spontaneous arthritis, whereas *IL1RN* knockout mice on a C57BL/6 background are protected [58]. The *IL1RN* example makes two important points. First, mice are not miniature humans. Species-specific differences in the function of the immune system exist and caution is advised when extrapolating from findings in a mouse disease model to the human disorder (and vice versa). Second, genetic background matters. Most genetically modified mice are analyzed on a single genetic background, typically C57BL/6. While this permits the comparison of different genetic modifications within a standardized genomic context, it also restricts the observable phenotypic spectrum. Ideally, one would like to examine the effects of any genetic modification in multiple backgrounds. However, embryonic stem cells for homologous recombination are only available for 129 and C57BL/6 mice [42]. Genetic intervals of interest can be transferred from one strain to another through repeated rounds of backcrossing, but this is a time consuming process, even with marker-assisted selection of the best breeders at every generation (“speed congenics”). Furthermore, the backcrossed congenic interval inevitably introduces donor DNA flanking the modification of interest into the new background, which may affect the phenotype if polymorphic regulatory elements or modifier genes happen to be located in the region [59].

Since the time of their creation, C57BL/6 and other classical inbred strains have evolved into genotypically and phenotypically distinct substrains [60]. This is the result of genetic drift caused by random mutations that became fixed in segregated breeding colonies. C57BL/6J is the C57BL/6 substrain maintained at the Jackson Laboratory that provided the template for the mouse reference genome [2]. C57BL/6N diverged from C57BL/6J in 1951. A recent study found that 12 of 1449 genome-spanning SNPs (0.8%) were discordant between C57BL/6N and C57BL/6J, evidence for the accumulation of significant genetic differences since their separation in 1951 [61]. This is of practical importance as the ES cell line JM8 used by IKMP for the generation of C57BL/6 knockout mice was derived from C57BL/6N and not C57BL/6J [62]. The genome of a C57BL/6 knockout mouse generated with C57BL/6N ES cells therefore differs from a C57BL/6J mouse purchased from the Jackson Laboratory in more than the targeted locus. To prevent further genetic drift in the classical inbred strains, the Jackson Laboratory replaces its breeders every five generations from frozen embryos [63]. However, genetic drift occurs in every mouse colony and

genomic differences may accumulate if knockout and wildtype mice are maintained as separate lines.

Another confounding factor in the analysis of genetically modified mice is the dependence of some phenotypes on the presence of specific microbiota. One example is the link between the CD susceptibility gene *ATGL16L1* and murine norovirus infection. Norovirus is a frequent contaminant in mouse colonies. Mice homozygous for a hypomorphic *ATGL16L1^{HM}* allele are indistinguishable from wildtype mice but develop CD-like Paneth cell abnormalities when infected with norovirus [64]. Leaving aside the question whether this *ATGL16L1*-norovirus interaction in mice models a similar interaction in humans, the CD-like phenotype in *ATGL16L1^{HM}* mice would have been missed in a norovirus-free animal facility. Importantly, such microbiota-dependent phenomena are not restricted to the intestine. It has been shown that the formation of arthritogenic autoantibodies in the K/BxN model depends on a Th17 immune response driven by segmented filamentous bacteria (SFB) [65]. SFB are commensals that inhabit the murine small intestine. SFB are present in the mouse stock at Taconic but not the Jackson Laboratory (two main suppliers of laboratory mice today) [66]. While K/BxN arthritis is strongly attenuated in the absence of SFB, the opposite effect is seen in NOD mice. Here, colonization with SFB reduces diabetes penetrance [67]. It is likely that many more such associations exist.

Conclusion

In this review we have attempted to provide an overview of the mouse genetics toolbox and to outline how it may help us in understanding the genetics of human autoimmune diseases. We have highlighted recent advances in the field and discussed potential pitfalls.

Genetically engineered mice have been and will continue to be instrumental in elucidating the function of the genes and gene variants associated with human autoimmune diseases. Large-scale efforts are underway to generate a knockout mouse for every gene in the murine genome and knockin technology permits the *in vivo* analysis of human disease-associated variants. The need for comprehensive phenotypic characterization of all gene-targeted mice has been recognized and data generated by the International Mouse Phenotyping Consortium will provide a comprehensive picture of global gene function over the next 10 years. Despite the almost limitless possibilities for engineering the mouse genome, further technological advances are needed. Some regions, in particular clusters of highly homologous, tightly arrayed genes may be difficult to target using currently available technologies [42]. There is also a need to generate identical genetic modifications in different genetic backgrounds. Zn finger nucleases [68] may be the technology of choice to address this problem.

Single gene mutant mice with autoimmune disease phenotypes can provide sequencing targets for patients with corresponding human diseases. Both ENU mutagenesis screens as well as the diligent phenotypic characterization of all genetically modified mice may provide candidate genes for this approach. QTL studies in mice have been somewhat disappointing with regard to the identification of individual genes relevant for human autoimmune disease. However, the combination of strategies for higher resolution mapping, bioinformatics and novel gene targeting approaches may change this in the future. The ability to detect gene-gene interactions is a major advantage of QTL studies over single-gene approaches.

When comparing phenotypes between humans and mice at least three sources of discrepancy need to be considered. In addition to species-specific differences between mice and humans, phenotypes may be affected by the genomic background of the mouse strain being studied and by the microbial environment. Given the significant impact these factors

can have, mice may be “lousy models for clinical studies” [1], they nevertheless are essential discovery tools in the post GWAS era.

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Highlights

- The contribution of mouse studies to understanding the genetics of human autoimmune diseases is reviewed.
- Classical inbred strains have limited genetic diversity, which limits the power of QTL studies.
- The possibilities for genetically engineering mice are (almost) without limits.
- Phenotypic differences between mice and humans are related to species, mouse strain and microbiota.

Figure 1.

The two main approaches for integrating human and mouse genetic studies: (1) Genes and gene variants discovered in humans GWAS are functionally interrogated in genetically engineered mice. (2) Genes and gene variants associated with disease phenotypes in mice are tested for disease association in human patient populations.