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Review
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Positional cloning of rat mutant genes reveals new functions of these genes

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Abstract: The laboratory rat (*Rattus norvegicus*) is a key model organism for biomedical research. Rats can be subjected to strict genetic and environmental controls. The rat's large body size is suitable for both surgical operations and repeated measurements of physiological parameters. These advantages have led to the development of numerous rat models for genetic diseases. Forward genetics is a proven approach for identifying the causative genes of these disease models but requires genome resources including genetic markers and genome sequences. Over the last few decades, rat genome resources have been developed and deposited in bioresource centers, which have enabled us to perform positional cloning in rats. To date, more than 100 disease-related genes have been identified by positional cloning. Since some disease models are more accessible in rats than mice, the identification of causative genes in these models has sometimes led to the discovery of novel functions of genes. As before, various mutant rats are also expected to be discovered and developed as disease models in the future. Thus, the forward genetics continues to be an important approach to find genes involved in disease phenotypes in rats. In this review, I provide an overview the development of rat genome resources and describe examples of positional cloning in rats in which novel gene functions have been identified.
Key words: disease model, forward genetics, mutation, positional cloning, rat

Introduction

The laboratory rat (*Rattus norvegicus*) is a key animal for biomedical research. Since 1970, nearly 1,700,000 papers using rats have been published based on a PubMed search using "rat" as a keyword on May 30, 2022. The rat has a long history as an experimental animal and can be subjected to strictly genetic and environmental control. The rat's large body size is suitable for various manipulations, including surgical operation and measurement of many physiological parameters. Because of these advantages, rat models have proven useful in biochemistry, neurobiology, nutrition, pharmacology, physiology, and other fields. Numerous models for genetic diseases and disorders have been identified among more than 200 inbred rat strains. These include models for alcohol preference, autoimmunity, behavior, cancer, diabetes, epilepsy, eye disorders, hematological disorders, hypertension, metabolic disorders, neurobiology, renal failure, and toxicology. In particular, in the field of chronic diseases such as cancer, diabetes, hypertension, neurology, and obesity, useful rat strains have been established such as ACI (stomach cancer, prostate cancer), BB (insulin-dependent diabetes), Dahl rat (hypertension), Eker rat (renal cancer), KDP (insulin-dependent diabetes), NER (epilepsy), OLETF (non-insulin-dependent diabetes, obesity), GK (non-insulin-dependent diabetes), SHR (hypertension), and SHRSP (hypertension, stroke). Once causative genes have been identified in the rat, underlying pathophysiological mechanisms can be extrapolated to the corresponding human disorder.

Identification of causative genes in these models is

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one of the most important steps to understand etiology of the diseases. Forward genetics is a proven approach for identifying the causative genes. It primally depends on chromosomal position of a causative gene but not on any preconceived ideas on the diseases. In addition, various mutant rats are also expected to be discovered and developed as disease models in the future. Thus, the forward genetics continues to be an important approach to find genes involved in disease phenotypes, even when the genome editing technology is available in rats.

In this review, I will provide an overview of the development of the rat genetic infrastructure that enables the identification of causative genes using rat disease models. I will also describe some examples of the forward genetics approach using rats which led to the discovery of novel gene functions.

Rat Genome Resources

Genetic markers

SSLP markers: Simple sequence length polymorphism (SSLP) markers, also called microsatellite markers, are widely used as genetic markers in various species. SSLP markers are developed by tagging simple sequence repeats, such as (AC)n, (AG)n, and (GT)n, distributed across the entire genome. The dinucleotide repeat number usually varies among individuals and length polymorphisms due to differences in repeat number can be detected by PCR. Thus, SSLP markers are ideal genetic markers, which allow us to genotype them quickly, easily, and correctly.

SSLP markers were first developed in rats in 1991 to map genetic loci associated with blood-pressure regulation in an SHR model [1]. In 1992, sufficient numbers of SSLP markers were developed and mapped to every chromosome for chromosomal mapping purposes [2]. To date, nearly 50,000 rat markers are available and information about allelic sizes in different strains have been stored in the 4,461 markers common to them (Rat Genome Database, Medical College of Wisconsin, Milwaukee, WI, USA (URL: http://rgd.mcw.edu/) [June, 2022]).

SNP markers: Single nucleotide polymorphisms (SNPs) are one-nucleotide differences in DNA sequence. When these differences occur within the coding sequence of a gene, some cause the amino acid sequence of the gene product to be altered (missense mutation) while others can cause it to be truncated (nonsense mutation). SNPs are very common and are found throughout the genome. SNP genotyping can be carried out with high-throughput systems at relatively low cost and the resultant genotypes are simple to process. Thus, SNP markers are ideal genetic markers.

In 2008, almost three million SNPs were newly identified in rats. A subset of 20,238 SNPs was genotyped across 167 distinct inbred rat strains and two rat recombinant inbred panels. High-density genetic maps have been generated using 81% of these SNPs [3]. To date, almost 14.5 million variants from whole-genome sequencing of 48 strains have been mapped against the most recent rat assembly, mRatBN7.2 (Rat Genome Database, Medical College of Wisconsin. (URL: http://rgd.mcw.edu/) [June, 2022]). These variants have been used to identify disease-related loci and to perform haplotype mapping [4, 5].

Genome sequencing

A rat genome sequencing project was launched in the United States by the National Human Genome Research Institute and the National Heart, Lung and Blood Institute. This project, led by Baylor College of Medicine's Genome Sequencing Center, yielded a high-quality "draft" sequence covering over 90% of the Brown Norway rat genome in 2004. Upon release of the draft sequence, the rat genome size was predicted to be 2.75 Gb. The numbers of predicted transcripts and genes were 28,516 and 20,973, respectively [6]. The latest rat genome assembly was released as mRatBN7.2 in 2020. The total sequence length is 2,647,915,728 (GenBank assembly accession: GCA_015227675.2) and 42,129 genes have been mapped on the assembly. In addition, the complete genomes of more than 20 individual strains were produced by using next-generation sequencing technologies that have identified over 80 million variants [7, 8]. This expansion in genomic data has helped investigators to identify causative genes in various rat models of human diseases.

Bioresource center

Rat bioresources include inbred strains; mutant strains; recombinant inbred strains; genetically modified strains, including knock-out, knock-in, and transgenic; and outbred rats. Information on these bioresources is available in the Rat Genome Database, Rat Resource and Research Center, and the National BioResource Project for the Rat in Japan.

Rat Genome Database (RGD): RGD (https://rgd.mcw.edu) was established in 1999 and is managed by the Medical College of Wisconsin. The RGD is the premier site for rat genomic and phenotype data [9]. The RGD also provides tools for mining, analyzing, and visualizing this data. Additionally, the RGD provides official nomenclature for rat genes, QTLs (quantitative trait loci), and strains. RGD provides easy access to comprehensive human and mouse data, including genes, variants, and

QTLs. The RGD genome browser (https://rgd.mcw.edu/jbrowse/) provides access to multiple rat genome assemblies, which enable the physical position of a gene of interest across different assemblies to be found. The Variant Visualizer tool can identify variants among strains of interests. Investigators can search for strain variation based on either an individual gene or a gene list within a region defined by a genomic position and two flanking genetic markers located on the same chromosome.

Rat Resource and Research Center (RRRC): RRRC (http://www.rrrc.us/) is managed by the University of Missouri. The RRRC's major services are to curate valuable rat lines for cryopreservation and rederivation, as well as to distribute rat strains. The Center's research is focused on improving cryopreservation and *in vitro* fertilization, generating new rat models, and characterizing the gut microbiota of rat models. The RRRC also provides colony management and breeding services, genetic testing, and microinjection services. Recently, a total of ten Cre rat lines were generated, including eight for neural-specific and two for cardiovascular-specific Cre expression [10].

National BioResource Project for the Rat in Japan (NBRP-Rat): NBRP-Rat (http://www.anim.med.kyoto-u.ac.jp/NBR/) was established in 2002 and is managed by Kyoto University, Japan. The NBRP-Rat was formed to collect, maintain, and preserve rat strains, to characterize them both genetically and phenotypically, and to develop and maintain a publicly available database with information about deposited strains [11]. As of May 2022, over 900 rat strains have been deposited. NBRP-Rat also offers training courses on master reproduction technology, including superovulation, embryo manipulation, *in vitro* fertilization, and gene editing.

NBRP-Rat has characterized a variety of phenotypes for many deposited strains [12]. Measurements were performed on six individual male or female rats between the ages of five and 10 weeks to obtain 109 parameters that cover a range of behavioral, morphological, and physiological phenotypes. Comparing individual parameters among strains enables investigators to select strains most suitable for their research as well as to know the mean and standard deviation across different rat strains.

Forward Genetics Approach for Identifying Mutated Rat Genes

The forward genetics approach, also called positional cloning, is a classical genetic approach used to identify mutations in model organisms, including rats. Forward genetics consists of two major steps: genetic mapping of the disease locus/loci to the specific regions of chromosomes and identification of the mutation within the disease locus (Fig. 1). To facilitate the forward genetics

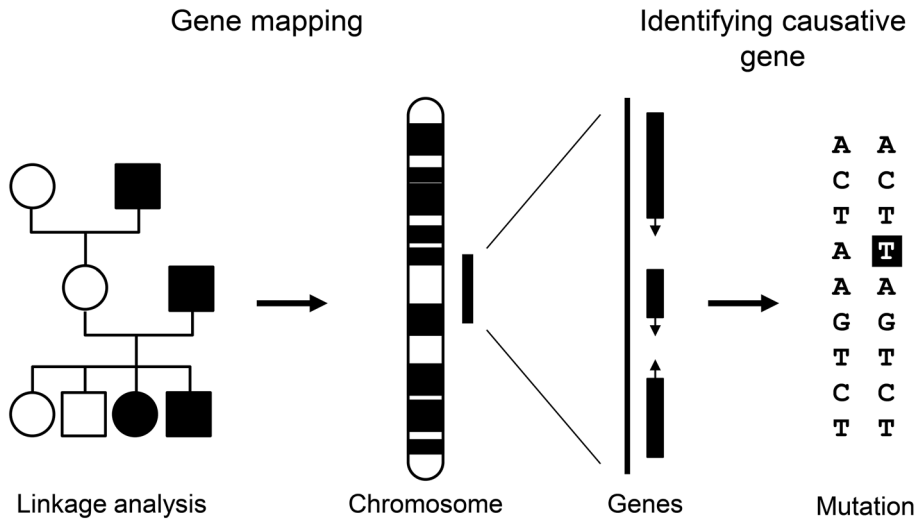

Fig. 1. Outline of positional cloning of a mutant gene. Positional cloning of a mutant gene consists of two major steps. The first is the identification of the gene locus associated with the mutant phenotype. The locus (vertical bar along the chromosome) is usually identified by linkage analysis with backcross progeny or F2 intercross progeny. The subsequent step is the identification of the causative gene. Genes already mapped within the locus are screened for the mutation. In theory, every gene within the locus can be considered a candidate gene. Among candidate genes, some are selected for sequencing based on differences in transcript expression between mutant and wild-type. Others are selected when they are known to express in organs or tissues where mutant phenotypes are observed. Finally, the mutation specific to the mutant strain is identified (white letter). To complete positional cloning efficiently, it is preferable to map the causative locus to as narrow a chromosomal region as possible in the first step. Such mapping is accomplished by using large numbers of both genetic markers and phenotype-segregated animals.

approach, an ample number of genetic markers and genome sequence information are needed. In rats, the accumulation of genetic markers and release of the draft genome sequence paved the way for mutated genes responsible for monogenic traits to be identified. The first instance of positional cloning of the causative gene in a rat disease model was achieved by our group in 2001 [13]. As of 2022, at least 108 monogenic disease-related genes are identified by the forward genetics approach using rat disease models [14, 15].

Identifying genes controlling complex traits is much more difficult. Linkage analysis of such traits leads to the localization of QTLs, which are too large for the causative gene to be identified definitively. Complementary strategies are therefore required to narrow down the list of candidate genes, including the generation of congenic lines or the use of heterogenous stocks. The first complex-trait gene identified in rats is *Cd36*, which causes insulin resistance, hyperlipidemia, and hypertension in a spontaneously hypertensive rat (SHR) [16].

Since some disease models are more accessible in rats than mice, the identification of causative genes in these models has sometimes led to the discovery of novel gene functions. Below, I describe some examples of how positional cloning in rats was used to discover new gene functions. These studies were carried out by my research group. I also provide a list of rat strains, available from the NBRP-Rat, in which 57 causative genes have been identified by positional cloning (Supplementary Table 1).

The attractin (*Atrn*)

Atrn gene was identified as a causative gene for hypomyelination in the zitter mutant rat [13]. The zitter rat was originally found in a Sprague-Dawley colony as a tremorous mutant. This phenotypic abnormality is caused by mutation of an autosomal recessive gene, zitter (*zi*) [76, 77]. The tremor develops spontaneously at three weeks of age and flaccid paralysis of the hind limb is observed at around six months of age [78]. The main pathological findings are progressive hypomyelination and vacuolation in the central nervous system (CNS), although the initiation of myelination and the fundamental structures of the myelin sheaths are unaltered [79]. Thus, the zitter rat is a prominent animal model for studying the maintenance of myelin sheaths.

To identify the *zi* mutation, we mapped it to a 0.1-cM interval between *Ptpra* and *D3Mgh15* on rat chromosome (Chr) 3. We subsequently localized 10 genes within this interval. Expression analysis of these candidate genes revealed a marked reduction in the measured level of a gene that encodes the membrane-bound ATRN isoform in the brain of zitter rats. Sequence analysis of the *Atrn* gene in zitter rats uncovered an 8-bp deletion at the splice donor site of intron 12, which is predicted to result in aberrant and unstable *Atrn* transcripts [13].

Atrn is known to play multiple roles in regulating physiological processes that are involved in interactions between monocytes and T-cells, agouti-related hair pigmentation, and control of energy homeostasis [80–82]; however, *Atrn* is not known to function in myelination. Although mouse *Atrn* encodes a membrane-associated form only, rat *Atrn* encodes two isoforms, secreted and membrane-associated forms as its transcript undergoes alternative splicing like the human *ATRN* mRNA. The *zi* mutation at the *Atrn* locus causes darkened coat color when introduced into agouti rats, as also seen in mahogany (*mg*) mice that carry the homozygous mutation at the *Atrn* locus. Furthermore, transgenic rescue experiments showed that membrane-associated *Atrn*, but not secreted *Atrn*, complements both neurological alteration and abnormal pigmentation in zitter rats. Therefore, we concluded that in addition to immune response, hair pigmentation, and energy control, membrane-associated *Atrn* has a critical role in myelination in the CNS [13].

We also found a deletion of the *Atrn* gene in the myelin vacuolation (*mv*) rat [58] that exhibits body tremors and an insertional mutation in the *Atrn* gene in the black tremor hamster that exhibits body tremor and darkened coat color [83]. These findings support the result obtained in the zitter rat and confirmed the involvement of *Atrn* in myelination and coat color regulation. In 2021, a novel rat strain harboring a missense mutation in the *Atrn* gene that resulted in G505C amino acids substitution was generated using the CRISPR/Cas9 system [84]. Behavioral analyses of these rats indicated that *Atrn* is involved in spatial learning and memory. As described above, these *Atrn*-mutant rats exhibit a distinct phenotype, namely neurological impairments. Thus, they can serve as a good animal model for investigating the mechanism by which mutant ATRN induces neurodegenerative changes.

The MRS2 magnesium homeostasis factor (*Mrs2*)

The *Mrs2* gene, which encodes a mitochondrial magnesium transporter, was identified as a causative gene of demyelination in the *dmy* mutant rat [72]. The *dmy* mutation was first discovered in a Sprague-Dawley rat colony and causes severe myelin breakdown after normal postnatal completion of myelination [85]. By positional cloning, we found a G-to-A transition 177 bp downstream of exon 3 of the *Mrs2* (MRS2 magnesium homeostasis factor (*Saccharomyces cerevisiae*)) gene. The substitution generates a novel splice acceptor site that

causes functional inactivation of the gene. *Mrs2* encodes an essential component of the major Mg^{2+} influx system in the mitochondria of both yeast and human cells [86]. We demonstrated that *dmy* rats exhibit elevated lactic acid concentration in cerebrospinal fluid, reduction of plasma ATP, and increased mitochondrial numbers in oligodendrocytes. *Mrs2* is expressed in neurons rather than oligodendrocytes and has been observed in the inner mitochondrial membrane. Thus, *dmy* rats suffer from a mitochondrial disease. These findings suggest that myelin maintenance has a different mechanism from that of initial myelin formation and that mitochondrial Mg^{2+} influx is essential for myelin maintenance.

Two *Mrs2*-knockout mouse strains were recently developed using CRISPR/Cas9. The *Mrs2*$^{em1(IMPC)Bay}$ allele harbors an exon deletion and homozygous mice exhibited decreased locomotor activity and preweaning lethality with incomplete penetrance [87]. In the *Mrs2*^{em1Muma} allele, an alanine codon (GCA) at position 280 (p. A280X) was substituted with a premature stop codon (TAA). Hepatocytes from these *Mrs2* KO mice exhibit abnormal mitochondrial shape and physiology [88]. Myelination pathology was not examined among these KO mice, but fewer homozygous mice were born than expected from Mendelian ratios, which also suggests an important role for MRS2 during development.

The hyperpolarization-activated cyclic nucleotide-gated 1 channel (*Hcn1*)

Hcn1 gene was identified as a causative gene for essential tremor (ET) in TRM/Kyo mutant rats [66]. The TRM/Kyo rat, which is homozygous for the tremor (*tm*) mutation mapped on Chr 10, is a tremulous mutant that was originally isolated from a Kyo:Wistar rat colony [89] and serves as an animal model of ET. The TRMR rat also carries a homozygous *tm* mutation but shows no tremors, which suggests that another genetic component(s) may exist in the TRM rat genome. Positional cloning using (TRM × TRMR)F1 × TRM backcross progeny mapped a genetic component on Chr 2 of TRM rats and identified it as a missense mutation in the *Hcn1* gene that resulted in A354V amino acid substitution. The HCN1 A354V mutant protein failed to conduct hyperpolarization-activated currents *in vitro*, indicating that the mutation causes loss of function of the gene. An HCN1 channel blocker evoked tremors when administered to non-tremulous TRMR rats.

Interestingly, a rat strain carrying the A354V mutation alone showed no tremors. Body tremors, however, were evident when the two mutant loci, *tm* and *Hcn1*^{A354V}, were combined in a rat ET model.

The *tm* mutation is a deletion spanning a nearly 240-kb genomic region on Chr 10, to which 13 genes have been mapped [90, 91]. To identify the causative gene among these candidates, we produced a genome-edited KO rat strain deficient in the Aspartoacylase (*Aspa*) gene because some *Aspa*-KO mouse strains are known to show tremors. Although our *Aspa*-KO rats do not exhibit tremors, *Aspa*/*Hcn1* double mutant rats show tremors resembling those of TRM rats. The tremor was suppressed by drugs used to treat ET but not by drugs for Parkinsonian tremors. These findings indicate that *Aspa* gene deficiency causes tremors when combined with the *Hcn1* mutation in the rat model [67]. We therefore proposed that oligogenic inheritance may be responsible for the genetic heterogeneity of ET, and is likely to be digenic.

The RI*β* subunit of the cAMP-dependent protein kinase (PKA) (*Prkar1b*)

Prkar1b was identified as a causative gene of body tremors in furue mutant rats [73]. The WTC-*furue* rat is a spontaneous mutant that exhibits body tremor after weaning as well as small body size. The causative gene is *furue*, which means “tremor” in Japanese. Positional cloning of the *furue* mutation identified a ~5.9-kb deletion in the *Prkar1b* gene. *Prkar1b*-deficient rats produced by CRISPR/Cas9 exhibited body tremors similar to those of *furue* rats, which supports *Prkar1b* as the causative gene for the tremors shown by *furue* rats. Since *Prkar1b*-deficient mice exhibit no body tremors [92], we demonstrated a new function for *Prkar1b* in the expression of tremors using positional cloning of the *furue* mutation. In addition, *Prkar1b*-deficient rats exhibit defects in the hippocampal LTP and in hippocampus-related behaviors observed in the fear conditioning and open field tests, which suggest a role for the RIβ subunit of PKA in LTP generation in the rat hippocampus.

As described above, *Prkar1b*-deficient rats exhibit tremors and abnormal behaviors associated with hippocampal functions. By contrast, *Prkar1b*-deficient mice are not known to exhibit such behaviors [92, 93]. Thus, examination of phenotypic discrepancies between mice and rats in which the orthologous gene is disrupted highlights the importance of cross-species comparisons when researching both novel and established gene functions.

Summary

During the last few decades, genome resources including genetic markers, genome sequences, bioresource centers have been developed in rats. They enable us to identify genes responsible for disease-related phenotype using the forward genetics approach. Since the laboratory rat is a key animal for biomedical research and will

remain so in the future, rat forward genetics continues to be important for revealing new functions of genes.

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