Rat genetics: the next episode

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More than a thousand quantitative trait loci (QTLs) relevant to many aspects of complex human disease have been identified in a wide range of rat inbred lines over the past few decades. With the complete rat genome available, it is now time for the next stage in rat genetic research: the identification and causal confirmation of underlying polymorphisms and genes. Recent developments in single nucleotide polymorphism-based genetic markers and technologies to manipulate the rat genome will undoubtedly be important tools in this next episode.

The rat as a model for complex human disease

The laboratory rat (Rattus norvegicus) is one of the most extensively studied model organisms for a range of aspects of human health and disease, physiology, toxicology, neurobiology and for drug development. For several diseases, some clinical aspects are best mimicked in the rat, particularly neurodegenerative diseases and disorders affecting higher brain function, such as schizophrenia, anxiety, depression and addiction. In April 2004, the rat joined the mouse and human as the third mammal for which the complete genome sequence with >90% coverage had been determined [1]. The complete rat genome sequence is of great value for comparative genomics, integrating different types of genomic data from multiple species [2,3], and it will undoubtedly also facilitate genetic studies.

Selective breeding and thorough phenotypic characterization has resulted in the establishment of >200 rat inbred strains that model many features of common human diseases [4]. Most phenotypes in these models are complex traits, where multiple functional elements contribute to the establishment of the pathological state. In crosses between inbred rat strains that specifically model a disease, nearly 1000 quantitative trait loci (QTLs) have been identified using QTL-mapping strategies (Figure 1) [5]. These QTLs range in size from 10 kbp to 190 Mbp (according to the Rat Genome Database (RGD), http://rgd.mcw.edu/) and contain hundreds or even up to a thousand genes. The availability of the rat genome sequence will undoubtedly accelerate genetic studies in these models, because it enables researchers to determine the positions of genetic markers and QTLs accurately and to identify candidate genes located in these intervals.

However, QTL mapping in rats has its limitations in terms of marker density and recombination events. Detailed maps of rat microsatellite markers or simple sequence length polymorphisms (SSLPs) have been developed [6–8], as well as automated, multiplexing genotyping technology for SSLP genome scanning [9], resulting in >17 000 entries stored in the SSLP database of RGD. However, the major drawback is the relatively moderate density of informative markers between strains of interest in a particular QTL region. The generation of genome-wide high-density single nucleotide polymorphism (SNP) maps and the construction of a rat haplotype map (which shows blocks of linked SNPs) might address this issue [10]. Although haplotype-based mapping has been used successfully in mouse [5,11,12], it remains to be proven if the organization of the rat genome is comparable to the mouse, or to what extent haplotype-based approaches can help to reduce the size of candidate regions, because blocks can be relatively large given the close relatedness of rat inbred strains.

Currently, the underlying gene has been identified for only a handful of rat QTLs. The successful gene identification procedures that have been applied after initial QTL mapping include further positional cloning using congenic strains and recombinant inbred (RI) lines, followed by candidate gene approach (Figure 1) [13,14], expression analysis of candidates [15], transgenic complementation [16,17] and even the use of the mouse candidate gene knockout phenotype [18]. Recently, an alternative approach to get to candidate genes was developed: the use of integrated genome-wide transcriptional profiling in combination with linkage analysis in RI strains. Taking advantage of physiological QTLs (pQTLs) that are already known and the newly identified expression level QTLs (eQTLs), this approach yielded an impressive list of likely candidate genes for hypertension [19].

Although QTL mapping is continuously ongoing in rats and techniques to narrow down the QTL region are being developed further [5,20] and should eventually lead to identification of many additional candidate genes (Figure 1), it becomes increasingly important to outline the next stage in the deciphering of complex traits. Identification of the responsible genomic region or gene is an important finding in itself, but the final stage would be the identification and causal proof of the underlying polymorphisms (Figure 1). Discriminating functional from non-functional polymorphisms from sequence and gene information alone is usually not sufficient, so genetic evidence is necessary and consequently tools that manipulate the genome are needed. Although such tools for the rat genome are lagging far behind those for the mouse, recent developments, such as transgenesis, gene silencing using RNA interference (RNAi), knockout technology and...
nuclear transfer, are promising and are expected to meet the emerging needs. Here, we describe the current state-of-the-art advances and their importance for the next episode in rat genetic research.

**Transgenesis**

The most straightforward way to assay the causality of a potential (perhaps partial) loss-of-function polymorphism is functional rescue of the phenotype by the introduction of a transgene expressing the wild-type gene into the mutant background. Conversely, gain-of-function polymorphisms can be studied by overexpression in a wild-type background. Occasionally, dominant-negative constructs can be used to mimic loss of function in a wild-type background; in which case, proper temporal and spatial expression of the transgene (Box 1) could be a problem. Rat transgenesis can be accomplished by microinjection of DNA into the pronucleus of the fertilised egg [21] or by infection of fertilised oocytes with lentiviral vectors [22] (Box 1), the latter approach being more favorable because of its greater efficacy and versatility, making it more cost effective [23].

Transgenesis has predominantly been used to overexpress gene constructs to generate rat models that mimic the clinical features of human diseases, such as hypertension [24], Alzheimer’s disease [25], Huntington disease...
Classical transgenesis by pronuclear microinjection of a DNA construct has been available since the early 1990s [21]. Once present in a fertilised oocyte, copies of DNA constructs can integrate randomly (usually as tandem arrays) at a single site in the genome. The method requires manual micromanipulations of fertilised eggs that are collected from a superovulated female. The transgenic construct is injected into the pronucleus using a glass micropipette and injected embryos are placed back into the oviduct of a pseudopregnant surrogate mother by microsurgery (Figure 1).

Alternatively, lentiviral vector-mediated transgenesis can be used, which has proven to be much more efficient and less labour-intensive [22]. Single-celled embryos can be infected with recombinant lentiviral vectors carrying the transgene under the control of a specific promoter. So far, perivitelline injections and co-incubation with denuded embryos (without zona pellucidae) have been used as infection methods, the latter being better because it does not require any micromanipulation. Infected oocytes are placed into oviducts of pseudopregnant foster mothers. Subsequently, offspring carrying the integrated construct are selected by Southern blot analysis or PCR and intercrossed to obtain F1 and F2 generation animals that stably express the transgene.

Finally, transgenic rats have also been generated by lentiviral transduction of male germline stem cells [86]. A population of cells enriched for SSCs were transduced with lentiviral vectors while in culture on a laminin matrix and transplanted to a single testis of recipient males. It was found that ~30% of the total pups produced contained the transgene.

The major complications of transgenesis procedures are associated with random integration, copy number effects, and concatemerised integration including vector sequences. As a result, a transgene can be highly transcribed, partially or fully silenced, or it might influence the expression of neighbouring genes, depending on the genomic locus or loci of integration. To allow for these potential effects, multiple independent lines need to be generated and functionally analysed. A clear advantage of transgenic methods is that, if the researcher wishes, a transgene can be expressed in a tissue-specific or time-specific manner, by using tissue-specific or stage-specific promoters, respectively, and local injections [87]. Conditional transgenics, a tool to turn on and off genes, are used in mice and can also be implemented in the rat [84]. In addition, transgenesis by microinjection is not restricted to certain strains, although greater efficiencies of reimplantation have been found in outbred rat strains [88].

Figure 1. Transgenesis by pronuclear injection. A superovulated female is mated with an untreated male to yield numerous fertilised oocytes. A custom made DNA construct is injected into the pronucleus of fertilised oocytes, which are subsequently placed into the oviduct of a pseudopregnant foster mother. The resulting F1 pups are screened by Southern blot or PCR analysis for presence of the transgene.

Knockdown by siRNA-mediated gene silencing

A second approach to prove the contribution of a gene to a QTL is by knockdown in a wild-type background. Gene knockdowns in vivo can be achieved by RNAi-mediated gene silencing through the application of short interfering RNAs [siRNAs, 20–25 nucleotide (nt) single-stranded (ss)RNA molecules] or short hairpin RNAs [shRNAs, double-stranded (ds)RNA molecules that are processed endogenously into 20–25 nt ssRNA molecules; Box 2 [29,30]. This technology is complementary to the traditional approaches of permanent gene knockouts and transgenics, because it can provide a temporal and/or tissue-specific gene knockdown while being relatively time- and cost-effective. A drawback of the method is that it might not work equally efficiently for all mRNAs in all tissues and, depending on its administration, its effect can be transient.
It is now well established that genes can be silenced in vivo using siRNAs or shRNAs. The silencing mechanism works by RNAi, a cell-surveillance mechanism that destroys all cytoplasmic mRNAs with sequence complementation to the dsRNA trigger [89]. Endogenous genetic knockdowns in mammals, in vivo, can be accomplished by introduction of the dsRNA trigger into the animal.

Antisense technology has been used for several decades to silence endogenous genes. For instance, in a brain-specific angiotensinogen knockdown study in rats, 90% silencing was obtained using antisense RNA of 200 bp [90]. However, because dsRNAs that are longer than 30 nt can induce an undesirable interferon response resulting in cell death [91,92], either short synthetic RNA molecules of 21–23 nt or a fold-back, stem-loop structure of ~19 perfectly matched nucleotides connected by various spacer regions and ending in a 2-nt 3’ overhang (shRNAs) can be used to induce RNAi efficiently [30]. Nowadays, dsRNAs or siRNAs can be supplied directly to the target tissue or shRNA molecules can be produced from transgenic DNA expression constructs to induce RNA interference in a tissue- or time-specific fashion (Box 1). Briefly, an enzyme called Dicer processes long dsRNA or shRNAs into the previously described siRNA duplexes (Figure I). These small molecules are assembled in the RISC complex, which directs target mRNA silencing by cleavage and degradation, by translational repression or by chromatin modification [30].

There are two major points of concern using this technology in vivo: avoiding off-target effects and efficient delivery. First, the risk of side-effects is reduced by the use of the machinery of a cell for directing sequence-specific antisense technologies, such as morpholinos [31] or ribozymes [32]. In addition, a proper control for specificity would be the use of several siRNAs or shRNAs directed against the same mRNA, which should result in the same phenotypic outcome.

Second, efficient delivery of the dsRNA trigger to the target tissue in vivo remains a serious obstacle. Various delivery systems for transient silencing have been tested in mice, such as infusion into the brain [33], electroporation into the retina [34], intravenous or local injection [35,36] and more. Recent studies, also in mice, have shown that lentiviral vectors can be used to transfect adult brains and accomplish stable RNAi, although only specific brain regions were efficiently targeted by this approach [37–40].

Long-term gene silencing has been demonstrated in vivo in mice using genetic mosaics, constructed by stem cell engineering with shRNA constructs and subsequent repopulation of the target tissue with these stem cells [41,42]. Germline-modified animals also exhibit long-term RNAi. Strains of mice have been constructed by standard pronuclear injection, engineered ES-cell chimaeras, or by subzonal injection of fertilised eggs with recombinant lentiviruses, to heritably suppress a target gene by shRNA expression [43–46]. These long-term silencing approaches might be most efficient, but because the preceding engineering steps take time, the advantage of rapidity of the RNAi-mediated knockdown approach in general can be limited.

Although there are no technical limitations on the implementation of the above-mentioned techniques for the rat, many fewer reports on in vivo RNAi in the rat have been published so far. Transient delivery by local injection [47–49] or infusion [50,51] of naked siRNA, sometimes followed by electroporation [52], has been used successfully. In addition, viral-mediated long-term siRNA production has been achieved by local injection [53,54] and used for generating transgenic animals from transfected preimplantation embryos [44]. All this work, in addition to the simultaneous production of lentiviral transgenic mice and rats [22], illustrates that ES-cell-independent technology is easily transferable from the mouse into the rat model system.

**Genetic knockouts by ENU mutagenesis**
The generation of stable knockouts by homologous recombination in embryonic stem (ES) cells, as is common for the mouse [55], is not available for most higher
Dawley and Wistar tend to give the best mutation frequency dose-dependent [63–65]. Outbred strains such as Sprague mutagenesis efficiency for the rat was found to be strain- and throughput of the mutation screening methodology. The factors: the induced mutation frequency and the efficiency successfully established for the rat (Box 3) [63,64].

Induced Local Lesions in Genomes), was recently also target-selected mutagenesis or TILLING (Targeting [56], maize [61], and high-throughput dideoxy resequencing [65]). Altogether, these studies resulted in eight knockouts, 68 missense mutations and several less interesting silent and non-coding mutations.

Once mutations are identified, interesting mutant animals are selected and outcrossed to a wild-type background to cross out other randomly induced mutations. Elimination of closely linked mutations can be problematic, but with the current mutation frequency of one in 1.2 million bp, the chance that an adjacent mutation ends up in and is deleterious to a nearby gene is extremely small. There are several procedures, however, to prove that a mutation causes a phenotype. First, genotyping in backcross progeny should result in full linkage between the mutation and the phenotype. Second, a second loss-of-function or reduction-of-function mutation in the gene should result in a similar phenotype, and this can be used to create heteroallelic animals that also must have the same phenotype. However, it should be mentioned that identification of two independent null alleles in a single gene is not realistic in the rat owing to the current low mutation rates and the limited sizes of libraries made from mutant animals. Finally, rescue of the phenotype by introduction of a wild-type copy of the gene using transgenesis (Box 1) or phenocopying by gene-specific RNAi (Box 2) could firmly establish the phenotype–genotype relationship.

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| Target-selected mutagenesis, also known as gene-driven mutagenesis or TILLING, is a powerful approach to manipulate a genome and its coding capacity permanently. In the rat, the approach starts with random mutagenesis of the male germline by intraperitoneal injection of the small chemical and mutagenic agent ENU, which introduces random DNA damage in SSCs that becomes fixed as point mutations in the DNA during point spermatogenesis. After a period encompassing a full round of spermatogenesis (~60–70 days) mutagenised males are mated with untreated females to generate a large population of F1 animals that harbour many random heterozygous point mutations in their genomes (Figure 1). Next, DNA is extracted from each F1 individual, which is subsequently screened for induced mutations in the coding or regulatory regions of genes of interest. These exonic mutations could be silent, change an amino acid (missense), or induce a premature stop codon (nonsense). In rats, mutations have been retrieved by using a yeast-based truncation assay [64], Cel I-mediated heteroduplex cleavage [63], and high-throughput dideoxy resequencing [65]. Altogether, these studies resulted in eight knockouts, 68 missense mutations and several less interesting silent and non-coding mutations.

![Figure 1. Target-selected ENU mutagenesis. The male germ-line is mutagenised with ENU. F1 progeny are generated by mating mutagenised males with untreated females, resulting in a population of animals that harbours many independent random heterozygous point mutations in their genomes. DNA samples are isolated for every F1 individual and these samples are screened in a high-throughput fashion for induced mutations in genes of interest. Interesting mutants are outcrossed and bred to homozygosity.](https://www.sciencedirect.com)

organisms, including the rat, because of the lack of pluripotent ES cells. An alternative approach to make genetic knockouts in particular genes has been developed for various model organisms, for example Caenorhabditis elegans [56], Drosophila [57,58], zebrafish [59], Arabidopsis [60], maize [61] and Lotus [62]. This method, known as target-selected mutagenesis or TILLING (Targeting Induced Local Lesions in Genomes), was recently also successfully established for the rat (Box 3) [63,64].

The efficacy of the procedure is dependent largely on two factors: the induced mutation frequency and the efficiency and throughput of the mutation screening methodology. The mutagenesis efficiency for the rat was found to be strain- and dose-dependent [63–65]. Outbred strains such as Sprague Dawley and Wistar tend to give the best mutation frequency and have superior reproduction characteristics compared with inbred strains, making them the most suited strains for N-ethyl-N-nitrosourea (ENU) mutagenesis-driven knock-out technology. However, for many experiments, the knockout allele is needed in a different genetic background: QTL characteristics are usually associated with specific strains because of genetic background or epistatic effects. This would require extensive backcrossing, which is usually necessary, because mutant animals resulting from this approach have additional induced mutations randomly across their genome.

Although these additional mutations seem disadvantageous, the advantage is that mutant collections can be used to screen for mutations and knockouts in multiple genes in parallel. The most commonly used methods for
mutation discovery are denaturing high-performance liquid chromatography [60,66,67], Cel I-mediated heteroduplex cleavage [62,63,68,69] and high-throughput resequencing [59,65,70,71]. The first rat knockouts, however, were produced using a yeast-based screening assay that identifies only protein-truncating mutations, thereby focusing on highly probable deleterious mutations but neglecting potentially interesting amino-acid substitutions [64]. An alternative approach using cost-efficient dideoxy resequencing of genes of interest identified >120 ENU-induced mutations, including six nonsense knockout mutations and 56 potentially valuable missense mutations [65]. The molecular mutation frequency deduced from the latter experiment turns out to be \( \sim 1 \) per \( 1.2 \times 10^6 \) bp, which is about four times lower than for zebrafish [69] but is similar to the mutation frequency obtained for the mouse [66,67,70,71].

Although already proven successful for the rat, the target-selected mutagenesis approach needs further optimization to become a routine method for knockout production. The mutation rates and/or throughput of the mutation discovery step need to be increased. Both the Cel I-based and resequencing approaches are well suited for automation and high-throughput processing, but emerging mutation detection and discovery technologies, such as microarray-based [72] and massively parallel sequencing approaches [73,74], are promising alternatives.

Because considerable numbers of mutagenised rats and expensive infrastructure are needed, the target-selected ENU mutagenesis screens are performed in only a few laboratories worldwide [63,64] (http://pga.mcw.edu; http://www.ingenium-ag.com), and knockouts are still produced at a relatively low rate. However, the technology might become more attractive for more laboratories when the same ENU-mutagenised animals are used in systematic forward genetic screens, similar to what has been done for the mouse [75,76]. Proof of principle for this approach in the rat was demonstrated by the identification of phenotypic mutants in ENU-mutagenised progeny [63,64] and the recent positional cloning of a nonsense mutation in \( myosin7a \) in the ENU-induced tornado mutant [77]. This resulted in a unique rat model for the Usher Syndrome Type 1b.

Phenotype-based screens in the rat might have good prospects, because years of phenotypic characterization of rat models have resulted not only in the identification of large numbers of clinically relevant phenotypes but also in sensitive phenotyping methods and assays. These tests could now be modified for setting up a comprehensive test battery. When combined with large libraries of mutant animals, systematic phenotypic screening can result in the identification of novel genetic factors that contribute to human disease and are difficult to recognize in other model organisms.

**Box 4. Somatic cell nuclear transfer**

Cloning, the asexual reproduction of individuals by nuclear transfer, has already been accomplished for many species, such as sheep, cow, pig, mouse, and recently also for the rat [79]. Practically, the process of nuclear transfer consists of four steps (Figure I). First, the haploid nucleus of the oocyte is removed physically or chemically. The oocyte has to endure in vitro culturing. A diploid nucleus from a somatic donor cell is collected, which is usually a nucleus from a foetal fibroblast-derived cell line. This donor nucleus is then microinjected into or electro-fused with the enucleated oocyte and, finally, the oocyte is placed back in a pseudopregnant foster mother [82]. All of these procedures demand a lot of endurance from the cell, which makes the method very inefficient. However, the problem of spontaneous activation of the oocyte, which appeared to be a particular problem for the rat, has recently been circumvented by using a protease inhibitor that reversibly blocks metaphase–anaphase transition [79].

Once rat cloning becomes a routine procedure, efficient homologous recombination in the donor fibroblast must be available to ensure generation of knockout animals. Occurrence of homologous recombination in somatic cells for cloning purposes has already been demonstrated for pigs [80,81].

![Figure I](https://www.sciencedirect.com)

**Figure I.** Cloning of an animal using SCNT can be accomplished by injecting a somatic donor nucleus into an enucleated oocyte. Cloned zygotes are placed back into oviducts of pseudopregnant foster mothers. By targeting interesting genes in the somatic donor nucleus (e.g. by homologous recombination), it is potentially possible to generate knockout rats.
Gene targeting by homologous recombination

As mentioned earlier, traditional gene knockouts by homologous recombination in pluripotent embryonic stem cells, analogous to the mouse, are not possible in the rat. Despite the quest that is continuously ongoing, no one has succeeded over the decades in isolating embryonic stem cells that can contribute to the germline of recipient animals. Cultured rat blastocysts and derivative cell lines rapidly lose pluripotency due to loss of Oct-4 transcription factor [78].

Nuclear transfer can be used as an alternative route to germline modification. Cloning of fertile rats through somatic cell nuclear transfer (SCNT; Box 4) has recently been shown to produce viable and fertile offspring [79]. In theory, this should enable the generation of targeted mutants by homologous recombination in somatic cells followed by nuclear transfer in oocytes, similar to what has been done for the pig [80,81]. This approach could have major advantages, because the strain of the donor nucleus essentially determines the strain of the cloned animal. Unlike potential ES-cell technology and ENU mutagenesis, which are strongly strain-dependent, this technology can produce knockouts, knockins or conditional knockouts for any strain of interest with similar efforts, without having to incross the mutation or transgene into the desired genetic background. However, homologous recombination in rat somatic cells and the use of these manipulated cells as the somatic donor nucleus needs to be demonstrated before this technique can be implemented for the production of gene knockouts [82]. In addition, vast optimization and improvements in the efficiency of the SCNT procedure are required, because the current efficiency of only two fertile progeny after implantation of 129 cloned embryos is far too low for routine application.

Recently, an alternative approach, circumventing the need for the laborious and inefficient SCNT, has come in sight, using rat spermatogonial stem cells (SSCs). These cells can be cultured for up to seven months and, after transplantation to recipient rats, donor stem cell-derived progeny can be obtained [83]. When homologous recombination can be achieved in these cultured SSCs, this approach would allow for the generation of knockout rats. However, these stem cells are relatively slow growing (doubling every five to six days) and selection for the occurrence of homologous recombination can have a devastating effect on the survival rate of the SSCs.

Future developments and conclusions

Rat biomedical research has moved over the years from phenotypic characterization to the identification of genetic traits by means of high-resolution QTL mapping, and is currently arriving at the next stage of coupling functional genetic components to the observed pathological states. Further expansion of the genetic toolbox for the rat and improvement of newly developed methods will be important to succeed.

The establishment of knockout technology for the rat now enables novel genetic approaches, such as quantitative complementation or QTL-knockout interaction for testing the candidacy of a gene at a QTL [5], conditional transgene expression in a null background [84], and sensitised genetic screens in knockout background [85]. These approaches together with the lentiviral delivery of transgenes, the use of siRNAs or shRNAs for gene knockdowns and the prospective availability of homologous recombination in the near future, enable us to conclude that the genetic toolbox for interference with endogenous gene expression in a targeted fashion is rapidly filling up.

Over the next few years, we expect to witness the identification of novel genes and polymorphisms underlying complex human diseases, such as hypertension and depression. Once causally verified, these genes become potential drug targets providing novel prospects for treatment. The elegant advantage of using genetic rat model systems in the initial discovery phase is that these well-characterized vertebrate model animals are directly available for drug screening, testing and validation.

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