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Use of genetically modified rat models for translational medicine

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Because of its relevance to human physiology, the rat may provide highly predictable models for the pharmaceutical industry. Until recently, the lack of efficient tools to manipulate the rat genome has drastically limited the use of this research model. Recent advances in gene expression and transgenic systems have provided new possibilities for the generation of informative rat models. This review presents a state-of-the-art transgenic technologies in the rat and their application to biomedical research. Novel technologies enabling the faithful expression of human genes in rats are focussed on specifically.

Introduction

The sequencing of the human genome has led to the identification of several target genes that could be putatively 'druggable'. Nevertheless, it is important to further analyse these targets *in vivo* in suitable animal models to enable a better control of target prioritisation and better management of R&D expenses. Characteristics of suitable animal models should include physiological parameters closely matching those of humans as well as proper model design to reproduce disease, enable target validation and compound evaluation. The retrospective analysis of data from mouse knockouts of the targets of the 100 best-selling drugs has highlighted the importance of the transgenic mouse model in this process [1]. Although an animal model can exhibit some aspects of a complex human disease, it rarely mimics it completely. Experts in the pharmaceutical industry, as well as at the U.S. Food and Drug Administration (FDA), have identified inadequate animal models as being one of the major hurdles to drug discovery and development. Therefore, the development of new animal models that have greater relevance to human biology is of crucial importance to optimise the discovery process. In this respect, the rat may constitute a model of choice in many areas of biomedical research. The rat exhibits physiological characteristics similar to those of humans and has for over a century been a key experimental model in biomedicine. Originally, the rat was used because it allowed experimental procedures, which could not be easily carried out in smaller rodents such as mice. This reason is still valid even though instruments have been miniaturised and surgical procedures improved in mice. The size of the rat facilitates sampling procedures, pharmacological development, stereotaxic neurological studies, neuroimaging and cardiovascular monitoring. Over the past few decades, rats have been shown to be superior to mice as *in vivo* models for several important human diseases such as hypertension, aging, infectious diseases, cancer and neurological disorders [2]. Since the development of the first transgenic rat lines in 1990 [3,4] more than 2000 publications indexed in PubMed report the use of transgenic rats in research. A regularly updated list of all original transgenic rat lines published from 1990 is available at http://www.ifr26.nantes.inserm.fr/img/pf/upload/Table%20-transgenic_jan2007.pdf. The collective experience indicates that genetically engineered rats play a crucial role in research programmes investigating mammalian physiology and the molecular basis of human genetic disorders [5,6]. The lack of tools available to manipulate the rat genome has dramatically slowed the use of this research model in several laboratories. The process of genome engineering in the mouse is mainly based on the ability of embryonic stem cells (ES cells) to colonise the blastocyst and contribute to germline transmission. These ES cells are then used as a tool to specifically alter, modify or add genes of interest before the injection into recipient blastocysts followed by reimplantation in foster mothers. The resulting chimeras will then transmit the genetic modification to the progeny resulting in the establishment of a stable heterozygous, then homozygous, genetically

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engineered mouse model. Unfortunately, all attempts to isolate pluripotent rat ES cells have failed despite intensive efforts in many laboratories worldwide. Meanwhile, other approaches enabling the development of genetically engineered rats have been successful and provide new opportunities for the development of relevant models.

This review presents the current state-of-the-art transgenic technologies in the rat and their application to target discovery and generation of disease models. We will address new approaches for the design and development of *in vivo* rat models, which have greater relevance to human physiology. These approaches are mostly based on novel technologies enabling the expression of human genes in rats while preserving their original genomic environment. As a result, the expression of the transgene is more physiological and better reproduces key parameters such as expression patterns, levels of expression and gene regulation.

Discussion

One of the simplest ways to study gene function and regulation *in vivo* is to alter the expression level of the target gene in some or all tissues. This can be achieved at the genome level by the introduction of additional copies of a gene (overexpression models) or by abolishing the gene's expression (loss-of-function models). Presently, the majority of rat lines produced correspond to the first category of transgenic models. Only a few lines have been produced with the aim of achieving partial or total loss-of-function [6].

Target validation and generation of disease models via gene overexpression

The first success reported in the rat was the generation of a transgenic hypertensive rat [3]. The establishment of transgenic rats has been crucial for the investigation of this pathology because hypertension is not well defined in the mouse. The development of a transgenic rat strain carrying the mouse REN-2 gene, and exhibiting fulminant hypertension, has provided evidence for a monogenic form of hypertension [3]. The data showed that the REN-2 transgenic rats display all the typical characteristics of hypertensive pathology, making them a relevant model for therapeutic interventions and studies on gene-related hypertensive processes. Transgenic rats harbouring the human components of the renin–angiotensin system (RAS), expressing the human renin and angiotensinogen proteins, were developed subsequently [7] to allow the study of specific interactions with the human transgene products *in vivo*. This double transgenic model has enabled specific testing of both human genes in rats. Recently, the rat has been shown to be a pertinent model for the evaluation of the efficiency and toxicity of antiviral compounds directed against HIV-1. The inability of the cells from transgenic rabbit or mouse models to support a robust productive HIV-1 infection had previously been a major limitation to the development of an alternative to non-human primate animal models for the study of host–pathogen interactions, virus transmission and dissemination, pathogenicity, and the evaluation of prophylaxic and preventive strategies. Rats transgenic for human CD4 (hCD4) and the human chemokine receptor CCR5 (hCCR5) were generated [8,9]. Co-expression of these human transgenes rendered primary rat cells permissive to infection by R5 viruses providing with rats developing clinical signs similar to those of AIDS in humans. The relevance of the hCD4/CCR5 transgenic rat as a suitable model for testing anti-HIV compounds was also demonstrated [8,9]. Other transgenic rat disease models have been generated through overexpression of the human gene of interest. In many cases, transgenic rat models have been proven to give a more accurate representation of the human disease than their mouse model counterparts. The relevance of rat physiology when compared to that of humans was further demonstrated in therapeutic areas such as autoimmune inflammatory disease, atherosclerosis and Huntington's disease [4,10,11]. Genetically modified rat models not only provide information on the physiological function of the target but can also illustrate the role of the gene product in disease states.

Conventional transgenic technologies

Most of rat transgenic models were generated via injection of a transgene into the pronucleus of a single-cell stage embryo. The transgene usually includes the coding sequence of a gene (cDNA) and an efficient gene promoter/enhancer. The promoter confers widespread or tissue-specific expression of the cDNA. Injected embryos are reimplanted into foster mothers to obtain offspring harbouring one or several copies of the transgene. Transgenic founders are most likely to have germ cells with the integrated transgene allowing stable transmission of the transgene to the progeny. Pronuclear injection has been widely used in mouse species since the early 1980s [12] but successfully achieved in rats only in the 1990s [3]. Specific features of the rat embryo have influenced the late shift of the technology to the rat. Superovulation regimens used conventionally in the mouse to generate embryos are less efficient in the rat [13]. In addition, single-cell rat embryos exhibit highly flexible pronuclear and plasma membranes that render transgene injection more difficult and increase cell lysis [14]. The conditions of *in vitro* incubation for the rat preimplantation embryos are also less well characterised than for their mouse counterparts.

Alternatively, rat overexpression models can be generated using recombinant retroviral vectors, particularly lentiviruses [15–17]. A distinguishing property of lentiviruses is their ability to infect both dividing and non-dividing cells. Retroviral vectors are usually delivered by injecting into the perivitelline space of the embryos or by simply incubating zona-free embryos in a viral solution. Very high efficiencies of transgene insertion have been reported after embryo infection with lentiviral vectors in mouse and rat species with 80–100% of the pups being transgenic [16,18,19]. The major drawback of these types of vector is their relatively reduced capacity (up to around 10 kb) for the insertion of expression cassettes. Nevertheless, it is important not to underestimate the pitfalls that may arise from the limitations of the technology. There is no control over the integration site and the copy number of the transgene. The transgene insertion site may not only impact upon transgene expression levels but may also modify the expression of genes at and around the site of integration. These alterations, referred to as 'position effects', can result either from altered transgene expression because of the neighbouring chromatin state or from the disruption of endogenous gene expression by sequences introduced along with the transgene such as promoters, enhancers, sequences regulating splicing and polyadenylation sequences. Some transgenic lines show phenotypic abnormalities

clearly associated with the 'position effect' [20]. Consequently, transgenesis by the random insertion method requires the creation of multiple transgenic lines with the objective of identifying one suitable for further research. This involves a time-consuming validation procedure to ensure that the phenotypes observed are because of transgene expression and not because of position effects.

Artificial chromosome vectors

Unpredictable or unfaithful expression patterns often reported in transgenic mouse and rat models may be overcome using artificial chromosome-type vectors. Thanks to their size, these vectors have the capacity to harbour genomic regions comprising genes and their regulatory sequences. Moreover, the size of the surrounding flanking regions can act as insulators protecting the gene of interest from potential interference from the host genome [21]. Artificial chromosome-type vectors present several advantages that make them very attractive for transgenesis in rats and mice. Introduction of these gene constructs into the germline of animals makes possible the study of the function and regulation of genes in conditions approximating their natural genomic context. Their cloning capacity, ranging from less than 100 kb to more than 1 Mb, allows the inclusion of most regulatory sequences required for the faithful regulation of a gene, allowing appropriate spatial and temporal expression of the inserted DNA. These vectors have been successfully used in the mouse using both pronuclear injection and transfection into ES cells to achieve random integration of large exogenous DNA fragments [22]. In the majority of transgenic mice generated in this manner, the transgene expression was found comparable to the endogenous levels. Furthermore, contrary to transgenic animals generated using conventional vectors, most transgenic animals generated with artificial chromosome-type vectors carry single or few (<5) copies of the integrated transgene, in agreement with the limited number of DNA molecules that are microinjected. The choice of vector is mainly driven by the size of the expression domain to be inserted and the expertise of the lab in manipulating the respective vector types. Transgenes up to 100 kb can be hosted by phage artificial chromosomes (PACs) whereas bacterial artificial chromosomes (BACs) are capable of mobilizing genomic fragments 100–300 kb in length. Bigger fragments (up to 1 Mb) can be cloned into yeast artificial chromosomes (YACs). Nevertheless, BACs have some advantages compared to YACs and PACs. BACs are circular plasmid DNA molecules. BACs are hosted in *Escherichia coli* and are therefore more convenient to propagate and purify because they do not require specific methods other than adaptations of existing protocols commonly applied to routine work with plasmids in bacterial cells. Gene overexpression using artificial chromosome vectors has been used successfully in the rat to secure faithful transgene expression patterns [23–25]. The expression of the human α-lactalbumin in the milk of transgenic rats was achieved after the microinjection of a YAC vector [24]. The 210 kb YAC DNA vector harbouring the entire human α-lactalbumin gene allowed position-independent and specific transgene expression. This contrasts with the highly variable expression of the bovine α-lactalbumin achieved in mice and rats after pronuclear injection of a conventional vector harbouring only partial 5′ regulating sequences of the α-lactalbumin gene [25–27]. The overall transgenesis efficiency achieved following pronuclear microinjection of large DNA molecules such as artificial chromosome vectors is usually low and the generation of several transgenic lines is labour-intensive [28]. These bottlenecks are associated with the difficulties of purifying and injecting large DNA fragments into single-cell embryos [23,29,30].

Sperm-mediated transgenesis

The development of alternative technologies based on sperm as a DNA carrier has alleviated the technical constraints associated with pronuclear injection of large DNA constructs. The most efficient strategy is the co-injection of DNA and a sperm head into an unfertilised metaphase II oocyte. This technology known as ICSI-mediated transgenesis achieves higher efficiency than pronuclear injection allowing the generation of more transgenic founders per injection session [28]. On the contrary, the manipulation of oocytes instead of embryos may facilitate the generation of transgenic lines in inbred strains [31]. Indeed, single-cell embryos derived from inbred strains are more prone to lysis after pronuclear injection than are oocytes after sperm injection (unpublished data). The recent development of microinsemination techniques based on ICSI in rats [32] has allowed the production of transgenic lines by means of injection of DNA-coated sperm into MII oocytes [33,34]. Two BAC constructs of 186 and 208 kb were successfully inserted into an outbred (Sprague–Dawley) genome with an efficiency of about 1–2% of transgenic offspring per reimplanted embryo [34] demonstrating the feasibility of this approach in rats. Until now, most of transgenic rats generated by ICSI have been obtained in outbred strains. By consequence, the efficiency of ICSI for the generation of transgenic rats in inbred strains remains yet to be demonstrated.

Conditional transgenic technologies

Being able to obtain the expression of a desired gene in a spatiotemporal manner is of paramount interest for the generation of animal models mimicking the development of certain physiopathological conditions. In addition, wide expression of a given transgene in all the tissues or even in restricted cell types may be associated with embryonic lethality or with complex phenotypes affecting multiple tissues. These limitations can be overcome using conditional transgenic systems. Cre/loxP systems including a termination 'Stop' cassette have been widely used in the mouse to obtain conditional gene expression since the pioneer work of Lakso *et al.* [35] who first described the Cre-mediated activation of an SV40 T antigen transgene in lens cells. When positioned between the promoter and coding sequence of the transgene, the Stop element suppresses transgene expression (Figure 1). The Stop element is flanked by recognition sequences for site-specific recombinases (e.g. loxP sites for Cre recombinase). Deletion of the transcriptional Stop cassette is catalysed by the Cre recombinase, thereby allowing the expression of the transgene within the tissue where Cre is expressed. Cre recombinase is often delivered through breeding with Cre-expressing animals (deleter line) or by injection of recombinant viral vectors encoding Cre. The Cre/loxP-based approach has been described only recently in the rat [36,37]. Ajiki *et al.* [38] used a DsRed2/GFP double-reporter

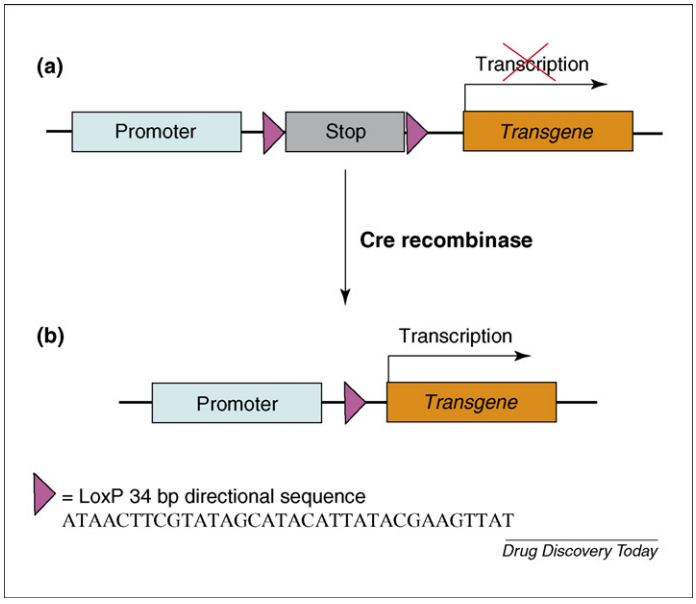

FIGURE 1

Conditional transgene expression using the Cre/Lox excision system. **(a)** The STOP sequence flanked with recognition sequences for site-specific recombinases (e.g. LoxP sites for Cre recombinase) blocks transgene transcription. **(b)** Cre recombinase catalyses the excision of the STOP cassette allowing transcription of the transgene within the tissue where Cre recombinase is expressed.

transgenic rat and an NCre transgenic rat to study the healing process of muscular tissue following composite limb transplantation. These rats exhibited the unique characteristic of changing from red fluorescence to green fluorescence by Cre/loxP recombination when cell-to-cell fusion occurred between the two transgenic lines. The Cre/loxP strategy was also successfully applied to the generation of a pertinent rat model for pancreatic cancer [37]. Most of Cre lines are generated by DNA pronuclear microinjection of Cre-expressing plasmid. Even though tissue-specific promoters are often used, the resulting expression pattern is highly dependent upon position effects associated with the integration site. This unreliable Cre expression pattern may lead to artifacts because of, for example, induction of transgene expression in unexpected tissues or at the wrong time during development [39]. Recently, several studies have reported the use of Cre-expressing lines generated using recombinant BAC vectors for the efficient, targeted expression of recombinase in specific tissues [40,41]. As ICSI increases the efficiency of large DNA fragment transgenesis in the rat, one would anticipate that reliable rat Cre-expressing lines could be generated using BAC transgenesis. A major drawback of the Cre/loxP system remains the irreversibility of the process. This limitation can be alleviated through the use of inducible expression systems that provide reversible temporal control of gene target expression.

The most documented inducible system makes use of the operator/repressor properties of the bacterial tetracycline (Tet) operon (Figure 2). Tetracycline-inducible transgenic systems allow for reversible temporal regulation of transgene expression [42]. The Tet system consists of two components. The first consists of a tetracycline-dependent transactivator (tTA) driven by a specific promoter. The second contains tet operator sequences next to a minimal promoter that control the transcription of the cDNA or gene of interest. The use of these two systems *in vivo* requires the generation of two sets of transgenic animals: one so-called transactivator animal, expressing the activator (tTA, rtTA) under the control of a given promoter and another set of transgenic animals, referred as to responder line, in which the expression of the transgene of interest is under the control of the target sequence for the tTA/rtTA transactivators. Breeding between the two strains allows the spatio-temporal control of transgene expression. Two versions of the Tet-inducible system exist. The expression of the transgene is activated or repressed by the presence (Tet-On system) or absence (Tet-Off system) of tetracycline or its derivatives, such as doxycycline, which can be easily administered *in vivo* through the drinking water. Numerous conditional transgenic mouse models using the tetracycline system have been developed for targets involved in all major therapeutic areas. In the rat, the application of the technology has been restricted to systems including single transgene under the control of ubiquitous promoters and tetracycline regulation, thus allowing temporal control of expression only [43–45]. The functionality of such systems *in vivo* has been hampered by insufficient levels of induction, leakiness and lack of tissue specificity. These problems are partly related to the insertion of the tet-responsive element in an inappropriate genomic environment (position effect). The tightness and range of regulation of the Tet system have been improved by the integration of the Tet regulatory sequences into a BAC vector [46]. This strategy should be applicable to the rat thanks to the recent advances achieved in the generation of transgenic animals using artificial chromosome vectors and ICSI.

Target validation and generation of disease models via gene invalidation

Gene expression inhibition is routinely achieved in the mouse by homologous recombination in ES cells. In the absence of a robust ES cell technology in the rat, alternative technologies have been adopted to generate loss-of-function models. Two main approaches have been used depending on whether the endogenous targeted gene is preserved or altered.

Dominant negative mutants

Gene expression knockdown may be achieved while preserving the endogenous genetic information. One possible approach is to generate dominant negative mutants. The basic principle of the dominant negative strategy is to introduce a gene to produce a mutant or heterologous protein that prevents the endogenous protein from performing the wild-type function. Efficient gene expression knockdown effect was obtained in the rat for the human growth hormone in the CNS [47], the oestrogen receptors (ERs) [48] in several tissues as well as the natriuretic peptide receptor at the cardiac level [49]. However, dominant negative constructs may lead to misleading results because of the interactions between the introduced gene product and parallel or intersecting signalling pathways.

RNA interference

An alternative to protein function inhibition is to target the RNA using anti-sense nucleic-acid-based inhibitors of gene expression. Among the developed strategies RNAi has become the technology

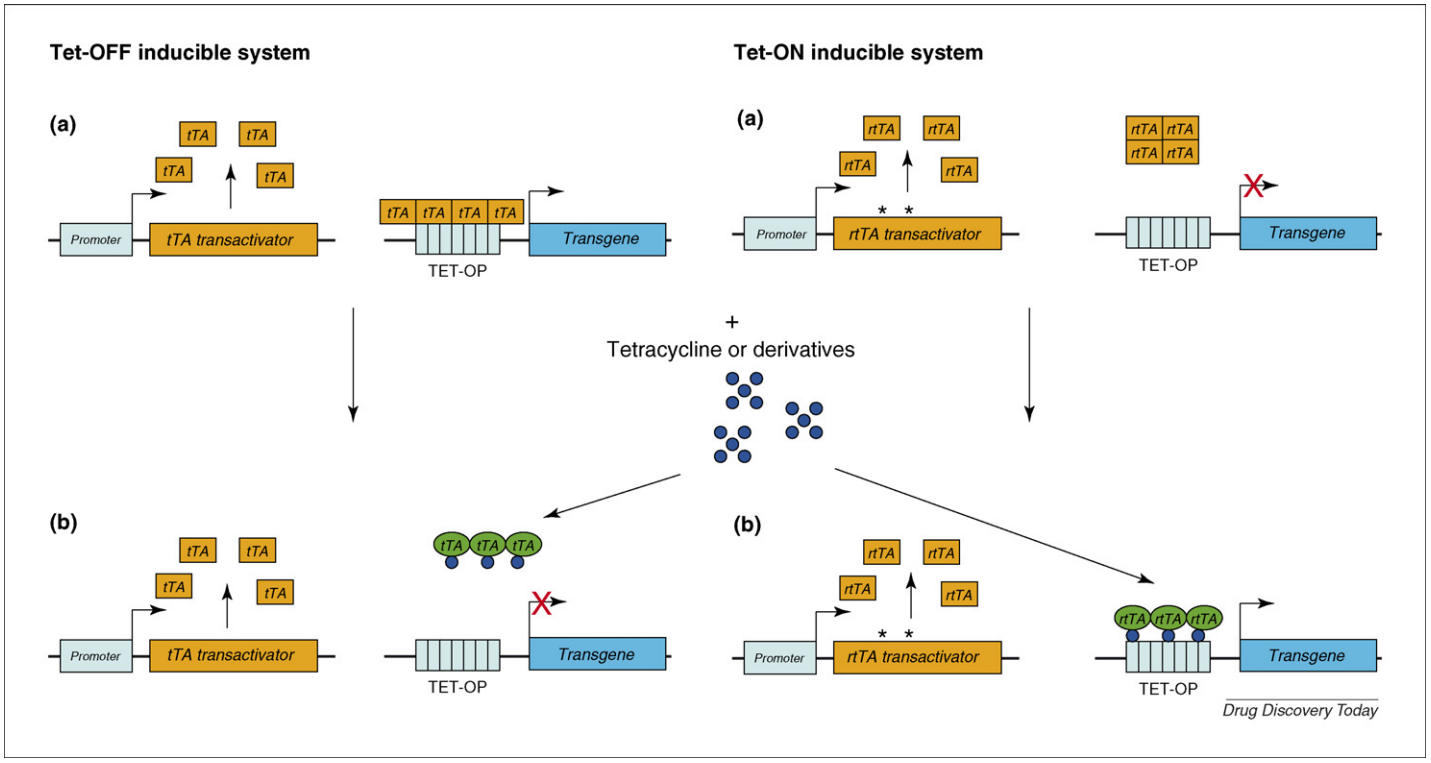

FIGURE 2

Conditional transgene expression using the tetracycline-inducible system. Tet-On system – **(a)** The tetracycline-controlled transactivator (tTA) is a fusion protein containing the *Escherichia coli* Tet repressor and the carboxy-terminal domain of the VP16 transcription factor from herpes simplex virus. In the absence of tetracycline or its derivates (such as doxycycline (Dox)), tTA binds to an array of seven cognate operator sequences (tetO) and activates transcription of a minimal human cytomegalovirus promoter. **(b)** In the presence of Dox, a conformational change occurs in the tTA inhibiting operon binding and thus transcription from the promoter is silenced. Tet-Off system – **(a)** The reverse tetracycline-controlled transactivator (rtTA) is a fusion protein containing a mutated form of the *E. coli* Tet repressor and the carboxy-terminal domain of the VP16 transcription factor from herpes simplex virus. In the absence of tetracycline or its derivates (such as doxycycline (Dox)), rtTA cannot bind its target and the system is inactive. **(b)** In the presence of Dox, rtTA binds to an array of seven cognate operator sequences (tetO) and activates transcription of a minimal human cytomegalovirus promoter.

of choice for generating transgenic animal models. RNAi is a sequence-specific post-transcriptional regulatory mechanism widely conserved in eukaryotes by which double-stranded RNA (dsRNA) causes sequence-specific degradation of mRNA sequence [50]. This approach has several advantages for obtaining gene silencing *in vivo* [51]. The feasibility of the modulation of gene expression by RNAi in rats and mice was first demonstrated by Hasuwa *et al.* [52]. In this study, an siRNA driven by a Pol III human H1 promoter allowed the complete or partial silencing of a reporter EGFP construct in a wide range of organs. The efficiency of RNA interference in depleting a specific endogenous gene product in the rat was recently demonstrated by Dann *et al.* [53], who targeted the expression of the germ cell-specific mRNA, dazl, to create a transgenic rat model with male sterility [53]. In all cases gene silencing was shown to be heritable, the progeny showing the same knockdown phenotype as the founders. *In vivo* RNAi has been achieved in the rat by DNA pronuclear injection [52] and transfection of embryos by lentiviruses [53]. These techniques do not enable control over the number of integrated copies or site of insertion of the transgene. Consequently, the expression levels of interfering RNA molecules can vary greatly within the transgenic founders and their progeny. This effect will be particularly important in the case of multiple transgene insertions with the segregation of the mutant alleles in successive generations. The generation of several lines is thus required to validate the siRNA effect and/or target specificity.

Chemical mutagenesis

Gene knockdown may be achieved by the alteration of the endogenous genetic information. Constitutive gene knockdown can be achieved in rodents by chemical mutagenesis of spermatogonial stem cells followed by gene-specific detection of mutations in the subsequent generation. This approach relies on the use of *N*-ethyl-*n*-nitrosourea (ENU) to generate heritable phenotypes. ENU causes random single base-pair mutations by direct alkylation of nucleic acids. The availability of high-throughput sequencing and mutation detection technologies has lead to the generation of screens for induced mutations in genes of interest. Gene-based screens have proven successful for a variety of species, including the mouse and more recently for the rat [54,55]. However, the technology suffers from some intrinsic limitations and drawbacks. ENU requires the generation of a large number of animals and, therefore, the allocation of considerable resources to animal facilities. Moreover, labour-intensive segregation screening analyses are required to ascertain that the observed phenotype is associated with the mutation in the target gene and not to random mutations introduced elsewhere in the genome. Finally, by creating point mutations, ENU mostly generates loss-of-function mutations restricting the technology to the production of knockout models.

Conclusion

Recent advances in genome engineering, gene expression and transgenic systems have provided new tools for the generation of informative transgenic rat models. In the absence of pluripotent ES cells, rat transgenesis relied mostly on DNA random insertion strategies. The inability to control transgene expression patterns has long been a major limitation of such approaches. Artificial chromosome vectors, allowing the inclusion of most of the regulatory sequences of a gene, constitute an efficient strategy for securing and achieving appropriate ubiquitous or tissue-specific transgene expression. Interest in such types of vector has been further underlined by the development of ICSI-mediated transgenesis in the rat, which facilitates the insertion of large DNA fragments. The ability to regulate gene expression in a spatio-temporal manner using the tetracycline-inducible Cre/loxP system in mouse and rat species now brings further improvements to the regulation of transgene expression. On the contrary, the recent validation of RNAi in *in vivo* models promises the development of efficient gene silencing procedures in the rat. Finally, the combination of procedures based on chemical mutagenesis of the germinal line by ENU and high-throughput screening methods has allowed the generation of constitutive KO rats.

The creation of 'humanised rats' in which endogenous genes have been replaced by their human counterparts through gene targeting is the next major step to be accomplished. Such an advance may be achieved in the coming years through technologies allowing the generation of animals from gene-targeted somatic or germ cells after nuclear transfer [56] or cell transplantation [57,58].

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