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COMMENTARY

Pluripotent stem cells and other technologies will eventually open the door for straightforward gene targeting in the rat

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Although the rat is a preferred model in many fields of biomedical sciences, the inability to generate germline competent embryonic stem (ES) cells was a major drawback for research activities that aimed to elucidate gene functions. Several alternative strategies like *N*-ethyl-*N*-nitrosourea (ENU) or transposon-mediated mutagenesis were developed successfully for this species. Countless experiments in many laboratories around the world were undertaken to overcome this problem. Eventually, the successful establishment of rat ES cells and rat-induced pluripotent stem (iPS) cells was reported, 27 years after the first reported generation of mouse ES cells. Furthermore, the application of zinc-finger nucleases (ZFNs) to early-stage rat embryos demonstrated effectively that another way existed for generating knockout rats. ZFNs require only the standard techniques that are used to produce transgenic animals and are expected to comprise a major tool for the gene-targeted generation of knockout animals. These newly developed tools, in conjunction with the solid basis of the rat in the area of physiological and behavioral experiments, will not only close the gap between the rat and the mouse as the mammalian genetic model of choice, but also boost the significance of the rat as a model animal in research laboratories around the globe.

The rat, *Rattus norvegicus*, began its career as a laboratory animal in Europe in the 1850s and became the first mammalian species to be domesticated for scientific research. Its suitable size and easy handling might have been major selective points in early animal experiments that were part of studies on breeding, behavior, psychology, nutrition, endocrinology and genetics, among others topics.

The history of the rat as a model organism

The rat is now well known as being the most important animal for safety or toxicological testing of chemical compounds, including drug candidates. Since the development of inbred rat strains by King in 1909 (Lindsey, 1979), over 700 rat strains that comprise inbred and outbred strains of various natures (spontaneous mutant, congenic, recombinant inbred, consomic, transgenic, etc.) have been developed and are accessible through rat resource centers such as The National Bio Resource Project for the Rat in Japan (NBRP-Rat; http://www.anim.med.kyoto-u.ac.jp/nbr) and the Rat Resource and Research Center (RRRC; http://www.nrrrc.missouri.edu/). The development of these strains was based on a broad genetic background, which is very helpful for the exploration of gene functions in heterogeneous populations. This genetic variability that is made available by numerous rat strains gives an enormous advantage to the laboratory rat. When the research community began to move away from mammalian species such as dogs or cats around 40 years ago, the accessibility of physiological, behavioral and learning parameters was the decision criteria that lead to the rat being the model of choice. Studies on the cardiovascular system and drug response tests required a variety of administration methods, invasive operational strategies and repetitive sampling for which a minimal body size and moderate life span were compulsory. Also, learning and behavioral phenotypes can only be accessed in animal models that show at least a minimal ability to respond to the test parameter in question. Then, the rat turned out to be the most appropriate model for scientific reasons, as well as for economic reasons (cost/performance). Accordingly, the physiology of the rat, describing all major organs and their interactions, has been studied extensively and has been instrumental in creating a foundation for systems biology.

However, the most significant tool in the field of functional genomics during the last two decades is the discovery of ES cells. These cells were derived first from mouse blastocysts in 1981 (Evans and Kaufman, 1981) and this work allowed subsequently for the generation of various kinds of knockout and knock-in mice that were used to understand gene functions. Numerous attempts have been made to derive ES cells from the rat but, until recently, all had failed. Instead, sophisticated approaches such as the yeast-based screening assay of *N*-ethyl-*N*-nitrosourea (ENU)-mutated G1 offspring (Zan et al., 2003; Chen and Gould, 2004), transposon-tagged mutagenesis in the rat (Kitada et al., 2007), or unique archives of mutagenized rat sperm (Mashimo et al., 2008) were used to induce genetic mutations. Although the ENU-based mutants offer the advantage that serial allelic variations of targeted genes, in addition to knockout mutants, can be obtained, targeted modifications of specific DNA regions are difficult to achieve. ES cells are still the tool of choice for manipulating genomes in the context of functional genomics. Because ES cells did not exist in the rat, the utilization of this species in genetic experiments has been restricted for many years, despite the advantages conferred

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by the size and physiological accessibility of this animal. The utility of the rat as a model organism has now changed.

Rat ES cells

In the Rat Genomics and Models meeting at Hinxton, UK in December 2008, two major breakthroughs were presented that will lead to a paradigm shift in genetic research. Austin Smith reported on the successful generation of germline-competent rat ES cells and Aron Geurts demonstrated the simple generation of knockout rats using zinc-finger nucleases (ZFNs). Although the latter discovery describes a relatively new technology that was applied to mammalian species, the story on rat ES cell development was rather long and troublesome (Brenin et al., 1997; Prelle et al., 1999; Vassilieva et al., 2000; Buehr et al., 2003). One reason for this long journey was the genetic background of the teratoma developing mouse strain 129, from which the first mouse ES cells were derived. These cells required an empirically determined culture medium that is efficient for only ES cells that are derived from a few mice strains. However, strain 129 is considered an exceptional strain in terms of ES cell development. Fetal calf serum, a key component of this original culture medium used for strain 129-derived ES cells, stimulates various differentiation programs and initiates cell commitment. Unfortunately, these serum-based conditions served as the basis for the majority of the experiments that aimed to derive rat ES cells from the inner cell mass (ICM) of rat blastocysts. The reasoning for this was that extrinsic factors are required to sustain the pluripotent status of ES cells. However, it became more and more evident that these conditions only describe the particular situation for strain 129-derived ES cells and cannot be transposed on to the rat or other mammalian species. Growing knowledge on early-stage differentiation processes, as well as a paradigm shift on how pluripotency is defined (Silva and Smith, 2008; Ying et al., 2008), eventually allowed less empirical but more systematic approaches to derive rat ES cells from the ICM of rat blastocysts. The assumption that pluripotency exist for some cells at a particular stage in blastocysts led to the hypothesis that the suppression of signals that trigger cell differentiation is key for the successful derivation and propagation of ES cells, rather than stimulation by 'pluripotent factors'. As a first step, extrinsic factors like serum and serum substitutes were removed from the ES medium. This was not sufficient to prevent differentiation since the autocrine production of fibroblast growth factor 4 (FGF4) and the associated induction of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) pathway drive the cells into committed lineages (Kunath et al., 2007). The blockade of this MEK-ERK pathway and the removal of extrinsic factors were helpful for sustaining mouse ES cell self-renewal (Ying et al., 2008), but were not sufficient for the derivation of rat ES cells. Thus, it was reasoned that interference in the activity of another pathway became necessary. Glycogen synthase kinase-3 (GSK-3) plays a crucial role in various anabolic pathways and is active upstream of β-catenin as part of the Wnt signaling cascade. Limiting the activity of GSK-3 mimics canonical signaling of Wnt through β-catenin accumulation; together with MEK-ERK inhibition and serum-free culture conditions, the stability of pluripotent cells then improves dramatically. Under these conditions, ES cells could be generated from mice strains that were found formerly to be resistant to the production of ES cell clones from their ICMs. These conditions proved to be sufficient for several ES cells derived from various mice strains, but the addition of leukemia inhibitory factor (LIF) seemed to be helpful for the initial generation and long-term passage of such lines (Nichols and Ying, 2006; Silva and Smith, 2008; Ying et al., 2008). The proof of concept of 'the ground state of pluripotency' was shown subsequently by applying these conditions to the rat blastocyst. Either of the so-called 'three inhibitors' (3i) or 'two inhibitors' (2i) conditions (Fig. 1) resulted in the successful generation of germline-competent rat ES cells when combined with LIF-producing feeder cells (mouse or rat embryonic fibroblasts, MEF or REF, respectively) (Buehr et al., 2008; Li et al., 2008). Similarly successful results were obtained for a close relative of the ES cell, the induced pluripotent stem (iPS) cell (Li et al., 2009). Slightly modified 3i conditions were used to successfully sustain the pluripotency of reprogrammed rat liver cells as stable iPS clones that could produce rat chimeras. However, germline competence has not been demonstrated yet, but this can be expected in the near future, probably by means of 2i conditions. What is still missing are knockout rats that have been derived using stem cells. This is already

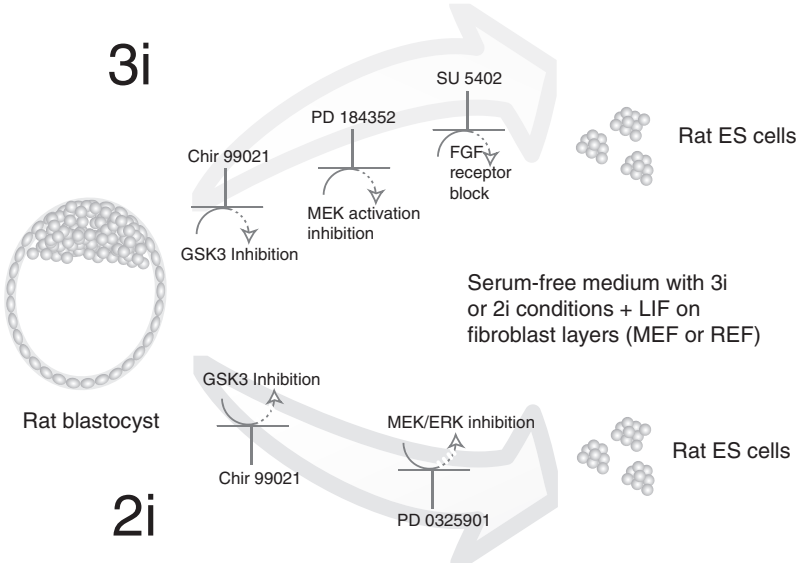

Fig. 1. Conditions used to generate rat ES cells. The ICM of rat blastocysts is plated on fibroblast feeder cells (mouse or rat embryonic fibroblasts, MEF or REF) and treated with 2i or 3i conditions in serum-free medium, typically N2B27. The addition of LIF appears to be mandatory under the above conditions. Chir99021, PD184352 and SU5402 are three chemicals that encompass 3i conditions, whereas medium that contains Chir99021 and PD0325901 describes the 2i conditions. Similar conditions can be applied to iPS cell cultures a few days after reprogramming has occurred.

Table 1. Rat models and knockout technologies

Preferred research areas for gene-modified rat models
Knockout rats will enable research in areas where they are the proven model organism of choice. This includes many aspects of:
Metabolic and cardiovascular diseases
Toxicology screening and drug tests
Behavioral and learning studies
Knockout technologies for the rat
ES cells are the tool of choice for creating knockout or knock-in animals and have now been derived for the rat
ZFNs use zinc-finger proteins associated with the DNA-cleaving domain of a restriction endonuclease to target a specific locus in the genome, in order to cause a double-strand break. Currently, this is the most tractable method for creating knockout rats
Genetic mutation in SSCs may eventually allow for 100% germline transmission, but these cells have not yet been used successfully to create knockouts
Transposon-tagged mutagenesis and ENU sperm archives facilitate cost-efficient generation of knockout rats using a non gene-targeted approach; the latter technique also produces interesting allelic variants that often resemble human alleles

in the pipeline for several laboratories that are dealing with the newly developed rat ES/iPS cells and we can expect to see these rats soon.

Other cell types and technologies are also being explored as avenues to create genetically modified rats (Table 1). Spermatogonial stem cell (SSC) cultures have been developed already for the rat but have proved resistant to attempts at producing successful homologous recombination events (Shinohara et al., 2006). The nature of the SSCs is different from ES cells, but the advantage of SSC-derived knockout rats would be guaranteed germline transmission. In any case, the next step will be to produce the first rat ES/iPS/SSC cell-derived knockout rat. As mentioned above, another gene-targeting technology has proven successful for generating knockout rats: ZFNs. Briefly, artificial customized zinc fingers, 3-6 fingers that can bind to up to 20 base pairs of DNA, are bound to the cleavage domain of the endonuclease *Fok*I. The RNA of this construct is injected into the pronucleus of fertilized oocytes. The RNA is then translated and the resulting ZFN is specific at targeting the genomic region, as determined by the artificially constructed zinc-finger sequence. Two of these molecules form a dimer and cut the genomic DNA. Following this, the internal repair machinery of the cell has several courses of action that it can pursue. However, a process called non-homologous end joining takes place frequently. As the name alludes to, the repaired double strand often results in the creation of a nonsense mutation and, therefore, a knockout animal. Although this technology is still in its infancy for use in mammals, it can be expected to become a method of choice for producing 'simple' knockout animals in the near future, whereas more complex strategies that include sophisticated, multi-step genetic modifications will be the preferable area in which ES/iPS/SSC cells are being used.

A bright future for rats

The use of both ES/iPS/SSC cells and ZFNs will become core applications in the biomedical sciences, which will use the rat as a model organism to elucidate gene functions and for the generation of animal models of human diseases. These methods will allow the development of rat models to address scientific questions where mouse physiology and morphology have presented challenges when addressing human disease. In particular, cardiovascular, neurological and behavioral studies, or experiments that require repetitive sampling, should make the rat a preferred model organism for many human diseases. The accessibility of phenotypes will eventually guide scientists to the most informative mammalian knockout, be it mouse or rat.

COMPETING INTERESTS

The authors declare no competing financial interests.

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