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Editorial

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The Arrival of 'knockout' Rats

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'Knockout' technology is a valuable tool in identifying causative genes resulting in disease states. Over the last three decades, it has only been possible to use the mouse for this purpose, owing to the non-responsiveness of rat embryonic stem cells (ESCs) to the culture conditions that were successfully used to construct 'knockout' mice. But during the last few years, this situation has changed and successful novel approaches for the construction of 'knockout' rats have brought the rat back centre stage.

There are clear advantages to using the rat for scientific purposes, notably its size. Its extensive use in pre-clinical toxicology has generated a robust database of normal biochemical and physiological values.

Molecular techniques used in the construction of 'knockout' mice have evolved over the past four decades, with initial progress in organised gene targeting work picking up pace in the late 1970s, when the ability to produce specific mutations in the target genes was demonstrated in cultured mammalian cells [1]. In the early 1980s, three related concepts brought the possibility of extending this work to the whole organism a step closer:

- Success in the efficient transfer of exogenous DNA sequences, linked to short viral sequences, into mammalian somatic cells
- Integration of these plasmids into the host genome
- Selection protocols for the desired homologous recombination products [1].

However, the frequency of targeted homologous recombination in these somatic cells was very low and, in order to use these techniques to target 1-cell zygotes, it was imperative to achieve a much higher frequency. At the time, this proved to be a major problem.

In the early-1980s, mouse ESCs were successfully isolated and cultured [2]. The use of these ESCs offered an alternative to the problem of low frequency homologous recombination. Gene targeting, selection and purification of ESCs proved to be a superior option compared with the existing experimental model of somatic cells. The introduction of these purified ESCs into a pre-implantation embryo and subsequent implantation into a foster mother produced chimeric offspring that were bred further and screened for 'knockout' individuals.

Protocols to isolate, culture and process mouse ESCs that resulted in the successful construction of a 'knockout' mouse model worked only for a limited number of inbred mouse strains, but the rat ESCs were completely non-responsive to these culture conditions and this situation proved to be a major obstacle in the development of 'knockout' rats.

This problem was eventually solved in 2008 when a team of scientists at the University of Cambridge, UK, developed culture conditions to which rat ESCs responded in the desired manner and were shown to transmit their genome successfully [3]. Using these culture conditions, the first 'knockout' rat model was constructed in 2010. In order to construct this 'knockout' rat, a vector targeting the p53 tumour suppressor gene was transfected into rat ESCs. This vector disrupted the p53 gene via homologous recombination. Successfully targeted cells showed one out of their two p53 genes to be disrupted. These cells were injected into rat blastocytes and transferred into pseudo-pregnant female rats to produce chimeric offspring. Regardless of the low germline transmission efficiency of the p53-targeted ESCs, this experiment proved to be a major advance in constructing a 'knockout' rat model [4].

Although long-term culture conditions were optimised for rat ESCs to which they responded in the required manner, there were still critical issues that needed to be addressed. In long-term cultures, rat ESCs accumulate changes in their karyotype. They also exhibit changed patterns of DNA methylation. These changes often result in the loss of germline competence, a pivotal factor in the construction of 'knockout' animals. Clearly, the non-availability of germline-competent ESCs in a large number of animal species warranted the need to look for alternatives. The use of spermatogonial stem cells (SSCs) showed potential that could be exploited to overcome most of the problems associated with the use of ESCs.

SSCs are found in very small quantities in the testes and produce sperm throughout the lifecycle of animals. As unipotent cells, they do this through self-renewal and differentiation processes. In the early 2000s, successful isolation and long-term culture conditions for mouse SSCs were developed and SSCs were shown to retain their proliferative potential, normal karyotype and stable germline potential for up to two years following culture. Genetic manipulation potential and HR frequency of these mouse SSCs was comparable to ESCs. In the mid-2000s, a 'knockout' mouse was constructed by transplanting genetically manipulated SSCs into the testicular seminiferous tubule of an infertile mouse where these cells underwent spermatogenesis. This male was crossed with a wild-type female mouse that resulted in the production of fertile heterozygous offspring [5]. Homozygous 'knockout' individuals were obtained by intercrossing these heterozygous mice.

This achievement opened up the option of constructing a 'knockout' rat by circumventing the need to use ESCs. In 2005, rat SSCs were isolated and successfully cultured for weeks without losing any of their vital characteristics and, in 2010, a 'knockout' rat model was successfully constructed using genetically manipulated SSCs [6].

To construct this 'knockout' model, the target gene was disrupted by a forward genetic approach. 'Sleeping Beauty' transposons were used to produce site-specific mutations in SSCs. The successfully

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targeted cells were screened and expanded before being transplanted into recipient founders who were allowed to breed naturally; their offspring were screened for 'knockout' individuals.

SSC-based technology has been successfully used to produce a range of rat 'knockout' models. Customized rat models are also commercially available. A significant advantage of this technology is the use of fewer animals (less than 25 compared with over 300) to create a 'knockout' rat model. This is an important aspect in terms of the 3Rs. In addition, the timescale required to develop a 'knockout' rat model using SSC-based technology is roughly half that of other available technologies.

These achievements in terms of isolating, culturing and genetic targeting of germline-competent cells of different epigenetic states, ESCs and SSCs, to construct 'knockout' rat models have opened up a range of options to manipulate genes of interest almost anywhere in the rat genome.

Recent advances in molecular technologies - Zinc-finger nuclease (ZFNs) and Transcription activator-like effector nuclease (TALENs) - have been successfully used to construct 'knockout' rat models by injecting gene targeting molecular complexes directly into an embryo that is transferred into a pseudo-pregnant female for chimeric offspring production, avoiding the need to use any type of stem cells.

ZFNs are synthesized by fusing the non-specific catalytic domain of the FokI restriction endonuclease and a modular array of individual zinc finger motifs that serve as a DNA- binding domain. The injection of ZFNs into an embryo generates sequence-specific double strand (DSB) breaks. Cellular response to these DSBs initiates repair mechanisms that take place through one of two distinct processes: non-homologous end joining (NHEJ), an error- prone mechanism, or homologous recombination (HR), a high-fidelity precise process. However, the balance between NHEJ and HR can be shifted in favour of the HR pathway by providing a donor repair template with ZFNs. 'Knockout' rat and mouse models have been successfully constructed by exploiting both NHEJ-mediated and HR-mediated repair processes following embryonic injection of ZFNs.

NHEJ-mediated repair processes summoned in response to ZFN-mediated DSBs in the recipient embryo result in unpredictable mutations, which clearly limit the use of this system. On the other hand, HR-mediated repair mechanisms can potentially be extended much further than for 'knockout' work. Conditional 'knockouts' and 'knockins', gene replacement and point mutations are all possibilities that may be exploited in the near future.

Transcription activator-like effector nuclease (TALEN) is produced by the fusion of TAL effector proteins to the FoKl nuclease domain. A very simple and highly efficient sequence- specific DNA recognition mechanism used by TALENs is a useful attribute. In 2011, an *IgM* 'knockout' rat model was successfully constructed by injecting TALENs into single-cell rat embryos [7]. All these technologies, embryonic stem cell based, spermatogonial stem cell based, ZFN- based and TALEN-based, come with specific advantages and limitations, with continuous efforts being made to overcome the latter.

By using these techniques, a number of 'knockout' rat models have already been produced and many more are in the pipeline. These advances have finally opened up the model organism of choice for the kind of experiments that have only previously been possible with mice.

References

1. Capecchi MR (2005) Gene targeting in mice: Functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet 6: 507-512.
2. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154-156.
3. Li P, Tong C, Mehrian-Shai R, Jia L, Wu N, et al. (2008) Germline Competent Embryonic Stem Cells Derived from Rat Blastocysts. Cell 135: 1299-310.
4. Tong C, Huang G, Ashton C, Li P, Ying QL (2011) Generating gene knockout rats by homologous recombination in embryonic stem cells. Nat Protoc 6: 827-844.
5. Kanatsu-Shinohara M, Ikawa M, Takehashi M, Ogonuki N, Miki H, et al. (2006) Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. Proc Natl Acad Sci 103: 8018-8023.
6. Ivics Z, Izsvák Z, Medrano G, Chapman KM, Hamra FK (2011) Sleeping Beauty transposon mutagenesis in rat spermatogonial stem cells. Nat Protoc 6: 1521-1535.
7. Tesson L, Usal C, Ménoret S, Leung E, Niles BJ, et al. (2011) Knockout rats generated by embryo microinjection of TALENs. Nat Biotechnol 29: 695-696.

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