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## Crafting rat genomes with zinc fingers

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Mammalian oocytes and zygotes are overtly and deceptively simple cells poised to elaborate a foundation to support the development of highly complex multi-cellular embryos. Yet these cells have turned out to be extraordinary resilient receptacles which provide a conduit for designs etched in laboratory notebooks to living animals and beyond.

The generation of the first transgenic mammals was achieved by injection of naked DNA into pro-nuclei of mouse zygotes three decades ago. This success provided the first indication that these cells were endowed with the machinery to support experimental genetic manipulation of their genomes. This remarkable feature was soon recognized to be a general property of other mammalian zygotes with the generation of transgenic rats, rabbits and farm animal species.

The technical simplicity of transgenic technology has led to its widespread application in pursuit of scientific questions as well as industrial applications such as protein production. However, the technology has, until now, only supported the insertion of exogenous DNA at random sites in the genome. It has proven to be extremely difficult to manipulate the genome in a site-specific manner by this route. Cui and colleagues, writing on page xxx of this issue, have finally overcome this barrier, making use of zinc finger nucleases (ZFNs) to stimulate targeted integration of transgenes by homologous recombination<sup>1</sup>. This technology will dramatically alter the speed and repertoire of genetic alterations that can be generated in a variety of mammalian species.

Evidence that mouse pro-nuclei are endowed with the machinery to support homologous recombination emerged more than 20 years ago, but the efficiencies were too low to be practically useful<sup>2</sup>. Instead, the technology to manipulate the mouse genome has relied on embryonic stem (ES) cells, which have been so extraordinarily receptive to homologous recombination. Up to now, mouse ES cells with targeted mutations are available for more than 12,000 genes<sup>3</sup>, thus they have become the genetic repository for the mouse. Unfortunately, the link between cultured ES cells and production of mutant animals could not be readily established in other species. ES cell lines isolated from species other than the mouse, rarely exhibit germ line colonization, although recent success has been reported in the rat<sup>4, 5</sup>.

The lack of authentic ES cells held back attempts to manipulate endogenous genes in most mammalian species for many years. This barrier was eventually overcome by utilizing a remarkable feature of the mammalian oocyte to re-programme a somatic cell nucleus, effectively converting it to a zygotic genome<sup>6</sup>. By performing gene targeting in cultured somatic cells and then using these for nuclear transfer, it became possible to manipulate endogenous genes in several mammalian species<sup>7</sup>. Despite these successes, the technical difficulties have been substantial because of the low efficiencies of both gene targeting in somatic cells and the subsequent re-programming of their nuclei.

The research reported by Cui *et al.* provides for the first time a route to directly manipulate the rat genome, an approach which bypasses the requirement for germ-line competent ES cells or somatic nuclear transfer. This approach also offers an avenue towards targeted modification of other mammalian genomes. The major difficulty in achieving gene targeting

with naked DNA injected into pro-nuclei is the very low efficiency of targeted rather than random integration<sup>2</sup>. Gene targeting is stimulated by several orders of magnitude in somatic cells by provision of a double strand break in the host genome<sup>8</sup>. Cleaving the mammalian genome at any defined site was not possible until ZFNs were developed. ZFNs are modular proteins which couple sequence-specific binding of zinc finger DNA binding domains to the nuclease domain of the restriction endonuclease *FokI*<sup>9</sup>. ZFNs function as homo- or hetero-dimers, cleaving DNA between two sequences recognized by the zinc-finger arrays (Figure, ZFN technology). The large number of different zinc fingers, their diversity of recognition sequences and their modularity will in principle enable them to be developed to target any DNA sequence<sup>10</sup>.

Following the initial demonstration of sequence-specific cleavage of the *Drosophila* genome<sup>11</sup>, ZFNs have been shown to stimulate targeted integration of a template sequence *via* homologous recombination in fruit flies, plants and human cells<sup>10</sup>. Cui *et al.* in this issue<sup>1</sup> and Meyer *et al.* in a recent report<sup>12</sup>, both demonstrate that co-injection of a pair of ZFN mRNAs with a targeting vector into pro-nuclei stimulates the frequency of gene targeting by workable levels, 2-20% (Figure, pronucleus injection route). Interestingly, live-born founder animals obtained from these experiments are mosaics which carry several different mutant alleles with deletions at the target locus, as well as correctly targeted alleles and unmodified wild-type alleles. Deletions are expected products following non-homologous end-joining (NHEJ) of cleaved DNA in the absence of targeting and have been described previously following expression of ZFNs in zygotes<sup>13</sup>. The transmission of multiple different mutant alleles from the same founder reflects germline mosaicism caused by expression and cleavage activity of ZFNs after DNA replication in maternal and/or paternal pro-nuclei. The ability to simultaneously generate a spectrum of mutations can be advantageous for genetic purposes.

Despite these advances, several questions remain to be addressed about ZFN stimulated pronuclear targeting. Molecular biologists are familiar with unwanted off-target activity of restriction enzymes. To what extent do ZFNs cleave other sites in the genome? The comparatively small size of deletions generated at illegitimate sites suggests that off-target cleavages will be hard to trace. Does the physical damage of the host genome observed in many transgenic animals generated by pronuclear injection occur in this setting too? The importance of the answers to these questions will undoubtedly depend on the frequency and type of unwanted events, their linkages to the desired genomic alterations and the context in which the technology is applied.

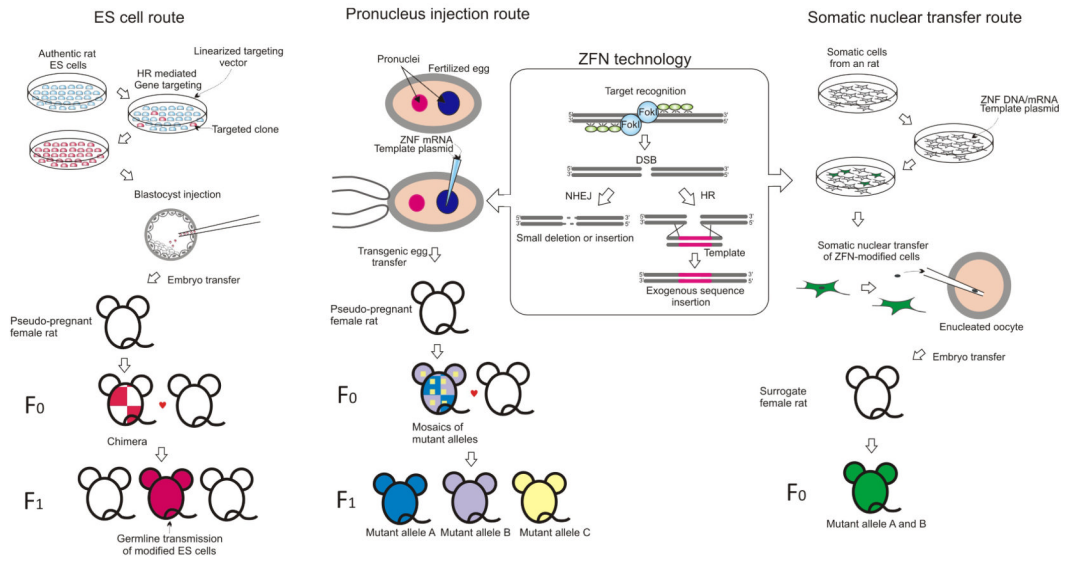
The mutant alleles generated using conventional gene targeting technology in mouse ES cells have steadily embodied greater degrees of sophistication over the last two decades. The repertoire of genetic alterations that can be achieved by ZFN-stimulated pronuclear targeting is fertile ground for exploration. Although mutant rats have recently been established using rat ES cell technology<sup>14</sup> (Figure, ES cell route), ZFN targeting applied directly to the zygote (Figure, pronucleus injection route) presents considerable advantages. Pro-nuclear injection of nucleic acids is well established, widely practiced and applicable to any strain. Moreover transmission of the engineered allele from founder rats is readily achieved. Provided ZFNs with the appropriate specificity can be generated, the community of rat researchers can look forward to rats with a myriad of defined genome modifications.

This technology will also find applications in a multitude of other species, which hitherto have required somatic cell re-programming to achieve directed modifications of their genomes. While vector-chromosome gene targeting has yet to be demonstrated in the pro-nuclei of farm animal zygotes, this is likely to be possible. ZFNs have also been shown to stimulate gene targeting in a variety of species, thus this technology can also be used in

combination with somatic nuclear transfer, removing a bottle neck in achieving directed modification by this route (Figure, somatic nuclear transfer route). While the promise of this technology will stimulate numerous applications, the terms, conditions and costs associated with commercially provided ZFNs can be prohibitive and may limit their potential.

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**Figure.**