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Gene Targeting in the Rat: Advances and Opportunities

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Abstract

The rat has long been a model favored by physiologists, pharmacologists, and neuroscientists. However, over the last two decades, many investigators in these fields have turned to the mouse because of its gene modification technologies and extensive genomic resources. While the genomic resources of the rat have nearly caught-up, gene targeting has lagged far behind, limiting the value of the rat for many investigators. In the last two years, advances in transposon- and zinc finger nuclease-mediated gene knockout as well as the establishment and culturing of embryonic and inducible pluripotent stem cells have created new opportunities for rat genetic research. Here, we provide a high-level description and potential uses of these new technologies for investigators using the rat for biomedical research.

Use of the rat in biomedical research

The rat was the first mammalian species domesticated for scientific research, with work dating back to before 1850 and some of the first genetic studies in animals demonstrated that rat coat color is a Mendelian trait ¹. The prevalence of the rat in biomedical research is second only to human; there are more scientific publications using rat than any other model system based on PubMed searches [JS1]. As a model system, the rat genomic tool box is rich ² and new sequencing technologies are moving the community toward improvements in the draft rat genome sequence ³ by adding new strain assemblies ⁴. Ever improving repositories for storing, integrating, and mining genomic information such as the Rat Genome Database (<http://rgd.mcw.edu>) and for collecting and distributing the more than 500 existing rat model strains by the Rat Resource & Research Center (RRRC, <http://www.nrrc.missouri.edu>) and the National BioResource Project (NBRP-Rat <http://www.anim.med.kyoto-u.ac.jp/NBR/>) provide a resource platform for scientific discovery in the rat. Despite this, the mouse is the preferred model for genetic dissection of mammalian biology and disease because of the longstanding existence of core technologies for targeted manipulation of its genome.

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Disclosure Statement

The authors currently collaborate in a funded effort to knock out genes using zinc-finger nuclease technology and provide them as a resource to the research community (Howard Jacob 1RC2HL101681) and Howard Jacob is the Principal Investigator of the Rat Genome Database (2R01HL064541). Sigma-Aldrich, Inc and MCW, Inc have a joint license agreement that could result in MCW receiving royalties for animal sales for commercially successful strains.

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In the preceding years, different strategies had been employed to manipulate the rat genome using transgenic, siRNA knockdown and ethyl-nitrosyl urea (ENU) methodologies⁵. We build on the prior state of the art by outlining the new techniques that add key tools to the rat genetic tool box. From the manipulation of rat genes within gametes and their precursors, pluripotent stem cells, or directly within embryos, the majority of these new technologies has appeared within the last 2 years and creates many new opportunities for the use of the rat as a biomedical research model. With the existing rat genomic tool box and the new technologies outlined here, it is reasonable to anticipate significant growth in genetic research using rat over the next few years.

Mutagenesis via sperm manipulation

One potential access point to manipulate the rat genome is the male gamete. Investigators have made significant strides in determining the conditions for isolating, culturing and utilizing rat spermatogonial stem cells (SSC)^{6–8}. Using a transgenic rat that expressed enhanced green fluorescent protein (eGFP) exclusively in the germ line, SSCs can be separated from other somatic cells and cultured for up to 12 passages^{6, 7}. These cells can be transfected with a selectable plasmid (a precursor step to genetic manipulation by gene targeting), while retaining competency to colonize and develop into spermatids upon transplantation into testes⁶; and lentivirus transduction and transplantation of SSCs leads to highly efficient generation of transgenic rats^{7, 8}. The initial SSC cultures have been derived from outbred rat strains. It will be interesting to see if SSCs from inbred strains, which would allow for genetic mapping approaches, can be similarly derived and manipulated. The potential to introduce foreign DNA and apply selection during culture of SSCs holds real promise for achieving gene-targeting by homologous recombination. While the advent of other technologies outlined below may reduce the need for this strategy for individual genes, the genetic manipulation of SSCs, which can then be maintained in cryopreservation, has potential for saturation mutagenesis of the entire rat gene set.

The Kyoto University Mutant Rat Archive (KURMA) has already made significant strides toward mutagenizing the entire rat genome by combining efficient methods for screening, preserving, and reanimating mutant strains. The potent mutagen ENU, which primarily causes single-base point mutations when applied to the gonads of male rats, has been used to produce several mutant rat models⁵. By cryopreserving sperm from thousands of first generation (G1) offspring of ENU-mutagenized males, a frozen library of mutant rat sperm can harbor mutations in a significant fraction of the genes⁹. DNA from these G1 males can be screened in pools using a novel approach which takes advantage of the preference of the Mu transposon element for single-nucleotide mismatches¹⁰. Intra-cytoplasmic sperm injection (ICSI) can then be used to generate a live rat from the frozen sperm of the identified G1 animal. Because the resource can be maintained as a frozen repository, it is a cost-effective approach which could allow for the indefinite preservation of the tens of thousands of samples which would be required for saturation mutagenesis⁹.

Transposon-mediated insertional mutagenesis

The generation of mutant resources by chemical mutagenesis has been complemented by the adaptation of transposon-mediated gene-trap insertional mutagenesis using the *Sleeping Beauty* (SB) transposon system in rats. This strategy was developed and implemented for random saturation mutagenesis in mouse^{11, 12} and has now been adopted in the rat^{13, 14}. The strategy is based on the assumption that the random insertion of a gene-trap transposon into a gene is likely to cause a null mutation by disrupting the transcribed mRNA at the site of insertion (Box 1).

Box 1**Transposon-mediated knockout**

Random gene knockout in the rat can be accomplished using gene-trap mutagenesis in transgenic strains. Transposons can facilitate the random insertion of the gene trap cassette into genes. In order for the transposon to jump, the presence of an active transposase enzyme is required. As a general strategy, two transgenic animals are generated, one carrying the gene-trap transposon and the other expressing the transposase (Figure I). These two strains are bred together to create so-called 'seed' male rats which carry both elements. In their spermatogonia, the transposase catalyzes the excision of one or more transposons from the donor site and inserts it into the genome at a new site ¹⁴. As with many transgenics ⁵, the transposon transgene is incorporated as a concatamer of tandem copies of the transposon, increasing the number of substrates for the transposase. Sometimes the insertion lands in a gene and disrupts it, creating a transposon knockout (TKO) mutation. The insertion events are captured in the seed male's offspring and segregated away from the transposase gene so they no longer jump.

The major limitation of the transposon mediated mutagenesis is that, like ENU-mutagenesis, it is essentially a random process with respect to knocking out genes. However, the transposon can be modified to have specific attributes which can make them useful tools. For example, the BART3 gene-trap transposon ¹⁴ interferes with expression and splicing of endogenous gene pre-mRNAs upon insertion while reporter gene-based traps can allow for easy tracking of animals with transposon insertions in genes and revealing their expression patterns ¹³. Another limitation of transposons as gene transfer tools, as with virtually all vectors is that there is a limit to the cargo size.

Figure I.

(a) The simplest gene-trap cassette consists of a splice acceptor (SA), reporter gene, and polyadenylation signal (pA). Insertion of the gene-trap cassette into an endogenous gene disrupts the normal splicing of the gene and result in the incorporation of the reporter gene and pA into the nascent messenger RNA (mRNA) which is then translated into a fusion peptide between the endogenous gene and the reporter protein. (b) The gene-trap cassette is cloned into a transposon vector (red arrowheads). Transgenic rats harboring this gene-trap transposon are bred to transposase-transgenic rats to create doubly transgenic 'seed' males and the transposons are mobilized in developing sperm. These events are captured by breeding the seed male to generate G1 offspring and screening for gene trap-induced mutations (m). Putative TKO mutations can then be bred to homozygosity for phenotyping.

One advantage of gene-trap insertional mutagenesis is that the investigator can modify both the transposon (mutator) and delivery of the transposase (helper) to generate a variety of model types. For example the transposon can be modified to carry a marker (e.g. *Tyrosinase*, *eGFP*, or *β -Galactosidase*) to simplify the tracking of mutant animals. A second advantage of the transposon method is that only a few transposon insertions are created per G1 animal, which can be easily identified, tracked and segregated using PCR methods and breeding^{13, 14}. This is in contrast to ENU mutagenesis, where thousands of mutations are created in each G1 animal, some of which can potentially be transmitted along with the mutation of interest to subsequent generations.

The PhysGen Program for Genomic Applications (<http://pga.mcw.edu>) used both the ENU and insertional mutagenesis strategies to develop 117 mutant rat strains (91 transposon-mediated knockout (TKO) and 26 ENU-induced), all of which are cryopreserved and are available through the RRRC and the Knockout Rat Consortium website (<http://www.knockoutrat.org>). Intriguingly, the use of SB-based gene trapping with the aforementioned advances in culturing and genetic selection in SSC has now been demonstrated to be a potentially powerful avenue to saturation mutagenesis *in vitro*¹⁵. Like the KURMA archive, a frozen repository of mutagenized SSCs could provide a valuable resource to the research community.

Although in the TKO approach the expression of transposase component is required only in the germ cells to mobilize the gene-trap transposon vector, variations in how the transposase is delivered offers other opportunities to generate novel models. Placing the transposase under a tissue specific, inducible promoter can be used to generate a somatic mutation in a tissue of interest. This strategy has been used in mice to screen for cancer genes and modifiers within a specific tissue¹⁶. Another use of transposons is as a transgene-harboring vector to catalyze the integration of the transgene to generate transgenic rats after pronuclear microinjection along with a source of SB transposase. In our hands, this system markedly increases the efficiency of developing transgenic rats with single-copy transgene integrations¹⁷.

Zinc-finger nucleases

Zinc-finger nucleases (ZFNs) are engineered proteins which combine the highly sequence-specific DNA binding ability of multimeric zinc-finger protein domain, where individual zinc-finger motifs capable of binding triplets of DNA sequence are linked together, with the nuclease activity of the restriction endonuclease *FokI*¹⁸. The plasticity of the zinc-finger domain allows for the design of ZFNs which can bind specifically to a broad range of sequences^{19, 20}. Libraries of zinc-finger motifs have been engineered with sufficient complexity that ZFN reagents can be designed to target most user-defined sequences. The nuclease domain functions to cleave the target sequence. Because the nuclease domain must form a dimer to cleave double stranded DNA, two ZFNs are needed to target a specified sequence. When introduced into a cell, the two ZFNs bind to their respective binding sites, positioning their nuclease domains to interact and introduce a site-specific double-strand break (DSB) in the chromosome. This break is subsequently repaired by the cell using either the highly-conserved homology-dependent repair (HDR), or non-homologous end joining (NHEJ) DNA repair pathways¹⁸. In the case of HDR, the sequence is repaired precisely and the ZFN heterodimer can reform on the repaired target sequence and re-cut. Since NHEJ-mediated repair is less accurate than HDR, it occasionally results in the loss or addition of nucleotides, resulting in a mutation²¹. This mutation, in turn, often results in a frameshift in the gene coding sequence, leading to a truncated and/or nonsense peptide. Beginning with pioneering work in the fruit fly²², engineered ZFNs have been used to generate site-specific mutations in a variety of cells and embryos from several species^{23, 24}.

Initial experiments applying ZFNs to the rat produced site-specific mutations in genes with a surprisingly high efficiency^{25, 26}. ZFNs can be used to produce heritable, site-specific targeted mutations in the rat by combining *in vitro* transcribed ZFN-encoding nucleic acids to the one-cell embryo via standard transgenic microinjection techniques (Box 2). Action of the ZFNs during the earliest cell divisions leads to a high percentage of modified chromosomes in the resulting offspring. Modified alleles are transmitted through the germ-line and can be backcrossed to establish multiple strains with unique mutant alleles.

Box 2**Gene knockout via ZFNs**

Zinc-finger nucleases are engineered proteins which are designed to target and disrupt genes when introduced into a cell by inducing a double strand break in the chromosome. Innate cellular DNA repair mechanisms repair the break, although frequently deleting small bits of sequence. When the ZFNs induce a break in the coding sequence of a gene, the deletion created during repair disrupts the function of that gene. ZFNs can be injected into the rat embryo in either plasmid DNA or mRNA form to disrupt a target gene. Thus far, commercially developed reagents have been successful in knocking out genes in the rat ^{25, 26}, although academic sources of reagents are demonstrating improved utility ^{57, 58} and may be equally applicable to the rat. ZFN mRNA is injected into the rat embryos derived from superovulated females using standard pronuclear injection techniques, although cytoplasmic injection can also work ^{25, 27} (Figure I). Injected embryos are transferred to pseudopregnant females and the resulting offspring are screened for ZFN activity using a CEL I nuclease assay, which is based on the affinity of this enzyme for heteroduplex (mismatched) DNA ²⁷. ZFN-modified founder animals are bred to assay for inheritance of the germline modification and to create uniformly modified F1 rats. About one-third of the mutations will result in an in-frame deletion or insertion that may or may not disable the gene. For this reason, characterization of ZFN-induced alleles by sequencing to identify desirable alleles is essential prior to attempting germline transmission.

Figure I.

ZFNs can be applied to the rat embryo to knock out genes. The engineered reagents are designed, assembled and tested *in vitro*, either commercially or by the user. Active ZFN pairs are introduced by pronuclear microinjection and transferred to pseudopregnant females which have been mated with vasectomized males. The resulting offspring are

screened for mutations in the target gene and confirmed by sequencing before breeding to establish a colony for phenotyping.

There are three major advantages of this strategy. First, it is very rapid, in the order of months. Second, it has worked in all strains tested to date^{26–28}. Third, it does not result in the incorporation of exogenous DNA. One current limitation of ZFNs relates to design for small genes or closely related gene families due to the requirement of unique sequences for which ZFNs can be assembled from the existing libraries, both of which could hamper selective design. A second limitation is a potential, albeit rare, off-target effect where ZFNs cause DSBs and mutations at undesired loci. Such effects were noted in the application of ZFNs to the zebrafish^{29, 30}, but were not found to be prevalent in either rat study^{25, 26}. Nevertheless, the potential effects of rare off-target events would be easily mitigated by backcrossing, resulting in the loss of any potential undesired mutations.

Since it was first reported, two other groups have reported rat gene knockouts using this approach^(26; <http://www.sageresearchmodels.com>) and we have knocked out 54 genes ourselves in a span of 10 months. The protocol has been highly efficient, requiring injection of an average of 297 embryos per target gene (range 71–1283), screening an average of 29 live born pups (range 3–208) and has resulted in an average of 3.5 knockout founders per gene (range 1–14) (PhysGen Knockout Team, unpublished). The knockout of this many target genes demonstrates the reproducibility of the approach and also that the current commercially owned libraries of ZFN reagents are sufficient to target and knockout most, if not all genes in the rat genome. The approach is also very rapid, as demonstrated by the publication of an X-linked severe combined immunodeficiency (X-SCID) rat model by knocking out the interleukin 2 receptor gamma with very high efficiency²⁶. From target site selection to published homozygous knockout phenotype, this study took approximately 12 months (personal communication). This speed and ease is unprecedented and opens up entirely new research opportunities for investigators interested in using rats. The Sigma Advanced Genetic Engineering (SAGE) Lab also recently applied the same approach to mice³¹. Other potential hurdles to ZFN engineering in the rat include the cost (\$25,000 USD) and associated licensing agreement (\$7,500 USD) associated with the commercial ZFNs used in the rat studies. We have recently discussed these issues and alternative ZFN sources in another recent review²³, but add here that engineering a knockout mouse can cost as much as \$100,000 USD and take a whole year to generate a single animal³². Thus, considering the speed and reproducibility, the ZFN method is a cost effective way to generate novel knockout rat models.

Finally, as HDR is a cellular repair mechanism for a DSB which is active in most cells, it is possible to provide a template in the form of an exogenous DNA fragment so that the HDR mechanism results in precise incorporation, or 'knockin' of the template. Several groups have now used ZFNs to facilitate targeted integration of new sequences into genomes by stimulating the HDR mechanism. To date, ZFNs have been used for knockin of a few base pairs up to 9-kilobase expression constructs into *Drosophila* embryos and cultured human cells^{22, 33–39} and, recently, ZFNs were used to target expression cassettes into human embryonic- and induced pluripotent stem cells^{40, 41}. It will be very interesting to see if similar approaches can be routinely applied to knock new sequences into the rat embryo genome by microinjection. Gene knockin in the rat embryo would potentially allow for many exciting new approaches including the generation of conditional gene alleles to allow for temporal and/or spatial ablation of gene function in different cells and tissues as has been done routinely in the mouse⁴².

Embryonic stem cells

In mice, targeted knockout and knockin genetic engineering is most often done in cultured embryonic stem cells and has yielded thousands of genetically modified strains. Rat ESCs have been sought for these same purposes and claimed by many, yet never meeting the three criteria that define authentic ESCs: unlimited symmetrical self-renewal *in vitro*, comprehensive contribution to primary chimeras, and generation of functional gametes for genome transmission. Today, the major need for rat ESCs is for knocking in genes and sequences, not knocking them out, as there are now many alternative strategies available⁵. The reasons for failure to develop ESCs in rats related to the lack of maintained pluripotency in culture. Numerous strategies were tried without success^{43–48} until it was found that using cell culture media that works for mouse was not sufficient for maintaining rat ESCs and has likely led to years of failed attempts^{44, 49}.

In a previous mouse study, it was demonstrated that the use of three inhibitors (3i: FGF receptor inhibitor SU5402; inhibitor of MEK activation PD184352; GSK3 inhibitor CHIR99021) and a ‘defined’ basal culture media containing no fetal bovine serum (FBS) successfully maintained the ground state of pluripotency of mouse ESCs cultured on feeder layers of mouse embryonic fibroblasts (MEFs) and did not require activation signals from the LIF/STAT3 and BMP/SMAD pathways, which were normally required for maintaining pluripotency⁵⁰. This 3i protocol was also successful in generating rat ESCs from the inner cell mass (ICM) of cultured blastocyst (Box 3). However, although the cells displayed many pluripotent characteristics and yielded chimeric rats, they failed to yield genetic transmission due to chromosomal aberrations arising during culture⁴⁴. The substitution of SU5402 with a more potent MEK inhibitor PD0325901, along with CHIR99021 (2i) led to more stable culturing conditions for deriving rat ESC when leukemia inhibitory factor (LIF) was added to enable STAT3 signaling^{44, 49}. The use of DIA-M cells⁴⁴ or a mixture of mouse embryonic fibroblasts (MEFs) and L-cells as feeder layers⁴⁹ were found to be optimal for isolating rat ESCs from the SD (Sprague Dawley) and DA (Dark Agouti) strains, respectively. Roughly, 30–60% of ICM explants will outgrow and propagate into cell lines under these conditions, with little indication of an impact caused by strain differences^{44, 49}. Finally, two of nine cell lines, one SD-derived and one DA-derived, produced chimeric rats which transmitted the ESC genome to its offspring.

Box 3

Development of authentic rat embryonic stem cells

Germline competent ESCs from widely used rat strains hold tremendous potential for manipulating the genome through gene targeting by homologous recombination, as preclinical tools for modeling cell therapies, for tissue engineering, and for understanding stem cell biology. ESCs are derived from the inner cell mass (ICM) of blastocyst stage embryos isolated from pregnant female rats (Figure I). Culturing media containing small molecule inhibitors with or without the addition of leukemia inhibitory factor (LIF) allow the ICM to propagate and maintain a pluripotent state^{44, 49, 51}. Characterization of stem cell markers such as early transcription factor, alkaline phosphatase and surface marker expression, as well as gene methylation patterns, is followed up with pluripotency assays under *in vitro* differentiation protocols and teratoma formation after subcutaneous injection into immunocompromised mice. Teratomas are tumors which spontaneously derive from the injected stem cells, consist of cell types and tissues derived from the three embryonic germ layers: ectoderm, endoderm and mesoderm, and are considered a definitive measure of pluripotency. Finally, a test of germline competency by injection of cells into a host blastocyst to create a chimeric animal distinguishes truly pluripotent cell lines. Only cell lines whose genomes are reproducibly transmitted to offspring of the

chimeras will ultimately constitute authentic rat ESCs lines useful for downstream applications.

Figure 1.

Rat ESCs are derived from the inner cell mass (ICM) of blastocyst-stage embryos and cultured under conditions which allow them to maintain their pluripotent state. Media formulas containing cocktails of small molecule inhibitors with or without LIF (described in the main text) allow the pluripotent embryonic cells to propagate. Several stages of characterization are required to validate a good ESC line including marker gene expression, pluripotency assays, and karyotyping before attempting to produce chimeric animals by injecting the donor ESCs back into a host blastocyst. Chimeras are then backcrossed to the donor strain to test for germline transmission of the ESC genome. Germline competent ESCs can be genetically manipulated to produce knockout rats, are useful for understanding stem cell biology, and are potentially applicable to tissue engineering. ³Combined results reported by Refs [44] and [49]. Nine cell lines generated under 2i conditions from 3 rat strains were injected into host blastocysts and two cell lines demonstrated germline competency.

A similar approach using an inhibitor cocktail media including the additional inhibitors of Rho-associated kinase (Y-27632) and TGF- β signaling (A-83-01) to prevent apoptosis and enhance proliferation was used to derive Wistar, LEA (Long Evans Agouti) and hybrid Wistar/LEA ESCs with⁵¹. A mouse ESC-like basal culture media containing FBS and MEFs was used as feeders in this study, but LIF was not necessary. The majority of cell lines demonstrated chimerism and germline transmission and could be stably transfected with a reporter transgene to produce genetically modified rats ⁵¹.

In the ultimate demonstration of the utility of the rat ESC, the DA ESCs derived by the 2i + LIF method described above were recently used to create the first targeted gene knockout rat by gene targeting using homologous recombination ⁵². In this report many properly targeted cell lines developed chromosome abnormalities as in the earlier studies, but after careful evaluation, cell lines were found (two out of 20 examined) which had normal chromosome complements and led to the production of the viable knockout ⁵². This is an historic achievement as the *p53* knockout rat validates the culminated effort of many to enable targeted genetic engineering in the rat ESC.

Clearly, the generation of authentic rat ESCs, and successful transgenesis by transfection and gene knockout by homologous recombination, marks the beginning of a new era for rat genetics. However, numerous challenges remain including the development of culture conditions for long-term stable passage, the development of authentic ESCs from additional widely used strains, and the identification of donor/host strain combinations for efficient germline transmission of the chimera. Certainly, putative ESCs for a variety of strains are now being developed. It will be interesting to see how many of these new cell lines, and the existing DA ESCs, can be used widely and reproducibly for gene knockout and knockin approaches. Importantly, however, key milestones have been achieved in the establishment of the 2i culturing method and demonstration of gene targeting, thus opening up numerous research opportunities.

Induced pluripotent stem cells

Induced Pluripotent Stem Cells (iPSCs) are ESC-like cells derived from humans, mice, and rats which are generated by the genetic reprogramming of differentiated cells into to a ground state of pluripotency⁵³. The development of useful iPSCs is principally similar to ESCs - the goal is to develop a pluripotent cell type capable of differentiating into every cell type of the adult animal and which can be potentially genetically manipulated (Box 4). However, rather than taking the precursor cell from the inner cell mass of a blastocyst, a somatic cell is reprogrammed to the earliest developmental stage by introducing key transcription factor genes.

Box 4

Development of iPS cells in the rat

An exciting prospect for generating pluripotent rat stem cells involves genetic reprogramming of differentiated cells such as fibroblasts using forced expression of key regulators of stem cell gene expression. Viruses are used to deliver genes coding for the stem cell factors *Oct3/4*, *Klf4*, *Sox2*, *c-Myc*, and in one report, *Nanog*^{54–56} (Figure I). Forced expression of these factors can lead to the rare de-differentiation of a small number of somatic cells into pluripotent cells called induced pluripotent stem cells (iPSCs). These cells share many features with ESCs including morphology, gene expression profiles and the ability to differentiate into the primary three germ layers. *c-Myc* is typically dispensable, but can improve the efficiency of establishing iPS clones. Various protocols and medias, with^{54, 56} or without⁵⁵ small molecule inhibitors (described in the main text), have been used to culture and propagate rat iPS cultures. Once individual cell lines are established, they are characterized using the same methods described for ESCs to identify clones which have strong characteristics of pluripotency. Although until now no rat iPSC line has been demonstrated to be germline competent, multiple iPSC lines from mice have achieved this final test of pluripotency. Presumably, iPSCs will have the same potential for genetic manipulation, tissue engineering and understanding of stem cell biology.

Figure I.

iPSCs are derived by the forced expression of specific transcription factor genes, typically by viral transduction of somatic cells such as fibroblasts. Rare cells are reprogrammed into a pluripotent state, subcloned and propagated. Once established, they are characterized like the ESCs (See Box 3).

Three strategies have thus far yielded putative iPSCs from rat. A combination of three mouse transcription factor genes (*Oct3/4*, *Sox2*, and *Klf4*) transfected by retrovirus, allowed for reprogramming of a hepatocyte progenitor cell line into iPSCs when cultured on a layer of MEF feeder cells⁴⁹. The addition of three chemical inhibitors (PD0325901, A-83-01, and CHIR99021) and LIF to a 'defined' culture media which contained no FBS allowed isolation of iPSCs in this study. Chimeric animals can be generated from these progenitor cell-derived iPSCs; however, none of these animals transmitted the donor genome to an offspring.

A similar approach tested the transduction of human reprogramming factor genes in combinations of three (*Oct3/4*, *Sox2*, and *Klf4*), four (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*), or five (*Oct3/4*, *Sox2*, *Klf4*, *c-Myc*, and *Nanog*) into neural precursor and embryonic fibroblast cells while culturing in media containing FBS, PD0325901 and CHIR99021, and LIF on feeder cells derived from rat embryonic fibroblasts⁵⁴. These culturing conditions are very similar to the 2i conditions used for ESCs except that the basal culturing media differed and would not be considered 'defined' because it contained FBS. In the third study, four transcription factor retroviral vectors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) were used to transfect and reprogram adult bone marrow and ear-tip fibroblasts under undefined culturing conditions using MEFs and no small molecule inhibitors⁵⁵. In this latter study, the addition of LIF was not necessary to maintain rat iPSCs in these conditions and could not maintain their pluripotency in the absence of feeder cells. At a glance, this seems to contradict the findings that inhibitors are necessary to maintain pluripotent rat stem cells^{44, 49, 54, 56} and that LIF enhances the culturing of rat ESCs as described earlier, but the culturing conditions used in all three studies are variable so a direct comparison is difficult.

Neither of the latter two studies using undefined culturing conditions reported testing their iPS cells via blastocyst injections; however, the gene expression studies and differentiation assays suggested they had pluripotent iPSCs^{54, 55}. It remains to be seen if culture conditions used in all three studies can be optimized to allow for long-term stable culturing and germline transmission of rat iPSCs. Another issue may be the relationship between the donor/host strains, as was found for ESCs^{44, 49}. With the apparent ability to produce and manipulate rat ESCs, is there any utility for rat iPSCs? We believe that rat iPSCs are likely to have tremendous utility for directed differentiation and phenotyping *in vitro*, as well as

preclinical modeling strategies for tissue generation and organ repair. With or without germline transmission, these reports suggest that rat iPSCs with the described levels of potency may be sufficient for looking at organ or tissue repair in the rat without additional work.

Concluding remarks

There are 594 strains and many more substrains of rats currently listed at RGD. The tools outlined here are now likely amenable to site-directed mutagenesis in the majority if not all of these strains. The journey from QTL mapping to gene validation techniques⁵ has evolved rapidly in the past two years. Now, not only can genes be tested in the context of QTL, but they can also be knocked out in a large number of accepted models to generate more appropriate and valuable representations of human diseases.

The ability to knockout genes in the strains of choice is critical and revolutionizes the field. The availability of embryonic and induced pluripotent stem cells offers the prospects of using homologous recombination to knockin genes of choice, including human genes. These new developments significantly enhance the rat genomic tool box and will enable many of the same experimental genetic approaches that have been enjoyed by mouse researchers and have driven the genetic study of mammalian biology and disease for more than two decades.

Given the role of the rat in the pharmaceutical industry, and the ability of these tools to impact drug discovery, should there be a project to knock-out every gene in the rat? Clearly one could make the argument that there is value since the mouse and rat, being separated by ~40 million years³, are quite distinct from each other. The sequence comparisons between the two rodents have proved useful, and would be more so if the rat had a finished sequence and more precise gene models. Consequently, having the ability to compare the same gene disrupted in three different species, mouse, rat and human, has tremendous power. The technologies make the prospects feasible in the rat; the biology makes it compelling; the ability to leverage rat and mouse data logical; and the ability to help patients sooner likely.

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