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ABSTRACTS

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Oral Abstracts

A method to convert mRNA into a gRNA library for CRISPR/Cas9 editing of any organism

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The clustered regularly interspersed palindromic repeats (CRISPR)/Cas9 (CRISPR-associated protein 9) system is a powerful tool for genome editing that can be used to construct a guide RNA (gRNA) library for genetic screening. For gRNA design, one must know the sequence of the 20-mer flanking the protospacer adjacent motif (PAM), which seriously impedes experimentally making gRNA. I developed a method to construct a gRNA library via molecular biology techniques without relying on bioinformatics. Briefly, one synthesizes complementary DNA from the mRNA sequence using a semi-random primer containing a PAM complementary sequence and then cuts out the 20-mer adjacent to the PAM using type IIS and type III restriction enzymes to create a gRNA library. This approach does not require prior knowledge about the target DNA sequences, making it applicable to any species.

Targeted insertion of polyA tracks with CRISPR-Cas9 allows titratable control of gene expression

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Embryonic lethality following global knockout is a major obstacle in the study of essential genes. Embryonic lethality may be overcome with heterozygous knockout, however haploinsufficiency and related phenotypes may not necessarily result. The most common solution tends to be tissue-specific knockout via the cre-lox system, however this method is dependent upon the availability of tissue-specific promoters as well as cre-driver mice. Additionally, only gene function in the targeted tissue may be studied.

The observation that insertion of polyA tracks into the coding region of target genes results in predictable reduction of gene expression presents an opportunity to study hypomorphic mutations in essential genes. Furthermore, length of polyA tracks is inversely correlated with gene expression, allowing for the generation of multiple animal models with defined levels of gene knockdown. Animal models with defined, diverse, heritable levels of gene knockdown represent novel tools to study essential genes, model small molecule inhibition of a target, examine diverse expression of a biomarker representative of human patient populations, among many other potential applications.

Here we will give an overview of the polyA track system, demonstrate its function in in vitro systems and conservation amongst diverse model systems. We will demonstrate its use with CRISPR-Cas9 to target endogenous genes, and lastly will present our progress in the generation of mouse models.

Single strand DNA-mediated knock-in for large genomic regions in rodents

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Emergence of genome editing technologies enabled us for producing genetically modified models in a variety of species. Development of efficient knock-in (KI) methods will facilitate easy and flexible genome engineering to introduce precise gene modifications at any target sites in any strains.

Recently we have developed the two KI strategies with CRISPR/Cas9 for the large genomic regions in rodents. One is the long single strand DNA (lssDNA)-mediated KI method. Microinjection and electroporation of originally synthesized lssDNAs with gRNA and Cas9 mRNA could produce several types of KI mice and rats with a good efficiency such as GFP-tagging, floxed and repeat sequence replacement.

The other strategy is a 2 Hit 2 Oligo with a plasmid (2H2OP) KI method. In the method, we could generate plasmid KI mice and rats by using CRISPR/Cas9 as "scissors" to cut at targeted sites in genome DNA and the plasmid DNA, and single stranded oligo DNAs as "paste" to ligate the ends of the cut sites. The methods could generate 200 kb BAC-KI rat without homology arms.

These gene KI technologies are essentially applicable to any targeted site, which can provide the generation of genetically humanized models for understanding the mechanisms of human disease and physiological function.

Employing single-stranded DNA oligonucleotides for the high throughput production of conditional knockout alleles in mice

Gaspero A., Wang, Y., Lorenzo, I., Liao L., Seavitt J., Dickensen M., Beaudet A., DeMayo F., Xu J., Heany J.

CRISPR/Cas9 genome modification technology has proven to be a rapid and effective means to generate genetically modified mice. The expedited production and economization offered through the advent of CRISPR/Cas9 technology has prompted the Knockout Mouse Project (KOMP) and larger International Mouse Phenotyping Consortium (IMPC) to transition from using existing ES cell-based resources to in vivo CRISPR/Cas9-mediated gene editing for the production of null alleles. However, the majority of IMPC ESC designs used for mouse production employed a flexible allele, which through breeding to different recombinase strains, could be changed into a variety of useful sub-alleles of interest to the scientific community, including conditional knockouts. To test the feasibility of using CRISPR/Cas9 gene editing to generate a large scale resource of mouse strains with conditional potential, we targeted 30 genes using CRISPR/Cas9 to introduce two loxP sites around a critical

region using pairs of single stranded oligonucleotide donor DNA molecules (ssODNs) as templates for homology-driven repair (HDR). For the 30 genes targeted, we successfully obtained putative founders harboring 2 loxP sites for 23 genes on the first microinjection attempt. Additionally, for 29 of 30 genes a null allele founder was generated from deletion of the critical region and repair of the double strand breaks by non-homologous end joining. We tested two different donor DNA designs (symmetric and asymmetric homology arms); however we observed no significant differences in the HDR efficiencies between the two approaches. The ability to introduce a loxP site at a specific target site was directly correlated to the efficiency of the sgRNA to mediate a double strand break. At least one founder with the 2 loxP sites in cis was obtained for 18 of 22 lines harboring 2 loxP. We did observe mutagenesis in the loxP sequences in 4 lines with 2 loxP in cis, reducing the total number of successful conditional alleles to 14 lines. Therefore, our study exemplifies the feasibility to obtain a conditional allele through CRISPR/Cas9-mediated HDR with pairs of ssODNs in roughly 50% of first microinjection attempts. Curiously, a pilot of conditional allele targeting using a long single stranded oligonucleotide donor DNA molecule (lssODN) for repair resulted in conditional founder production for all 3 genes targeted. This new donor may overcome the pitfall of ssODNs which often result in mutated loxP sites and targeting in trans. Our extensive study will be informative for future generation of more sophisticated alleles for IMPC and KOMP production.

Genomic rearrangements generated by CRISPR and characterization pitfalls

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The CRISPR/Cas9 system is now widely established as a tool for genome engineering in mouse zygotes. It has been used to generate mutants containing small insertions/deletions (indels) as well as single nucleotide polymorphisms (SNPs), loxP sites and, less efficiently and to varying degrees of success, for the introduction of targeting constructs.

The Wellcome Trust Sanger Institute has always been at the forefront of generating mouse models and we are routinely producing mice carrying critical exon deletions using the CRISPR/Cas9 system. As part of our R&D work we are working on improving the efficiencies of introducing point mutations and floxed alleles. We also investigate the pitfalls of using CRISPR/Cas9 for homologous recombination in zygotes.

Another part of our work focuses on generating mouse models to study human diseases. Deletions, duplications and inversions of large genomic regions covering several genes are an important class of disease causing variants in humans. Modelling these structural variants in mice so far required multistep processes in ES cells, which has limited their availability. We demonstrated before that it is possible to directly generate deletions, duplications and inversions of up to one million base pairs by injection of Cas9 into mouse zygotes[1]. We followed up on this study and are now able to show that thorough analysis of these mice (for both small critical exon deletions as well as larger rearrangements) is required in order to fully understand which alleles are present within each F0 founder animal and resulting mouse line.

1. Boroviak, K., Doe, B., Banerjee, R., Yang, F. & Bradley, A. Chromosome engineering in zygotes with CRISPR/Cas9. genesis n/a-n/a (2016). doi:10.1002/dvg.22915.

In-depth analysis of CRISPR off-targets in genetically engineered rodents

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CRISPR has quickly become a powerful tool for engineering of the mouse (and rat) genome. Several tools and algorithms exist that facilitate the design of sgRNAs, and CRISPR is routinely used for the generation of knock-out, knock-in and even conditional knock-out rodent models. Although most investigators are aware of the potential for off-target effects with CRISPR, it is safe to say that our field is still far from reaching a consensus on what to look for, what to worry about, and how to deal with concerns about animals created using CRISPR.

We routinely analyze all of our G0 founders for a list of potential off-targets using deep sequencing. From the analysis of more than 80 in-house projects we find that about 25% of the projects have evidence of off-target cutting by Cas9. While we do observe some correlation between predicted scores and off-target profile, from our data-set it is also evident that existing algorithms are still lacking in their predictive power. Based on our comprehensive data-set we have generated a list of recommendations for CRISPR project design and subsequent analysis of the resulting animals.

Cas9 RNA-guided nuclease—efficient model generation by microinjection and electroporation

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The Model Production Core at The Centre for Phenogenomics (TCP) is a national facility serving both academia and industry in Canada and internationally. TCP is also part of large-scale production co-ordinated by the International Mouse Phenotyping Consortium (IMPC). The use of Cas9 RNA-guided nuclease (RGN, aka CRISPR/Cas9) has revolutionized mammalian genome editing. Targeted mutations can now be generated by co-injecting zygotes with guide RNAs (gRNAs) specific to the target site, Cas9 endonulcease, and a homologous repair template. More recently electroporation has been

used to efficiently edit mouse zygotes. Here we show that we have successfully produced exon deletion and point mutant alleles with electroporation. For null allele production of 21 genes, we achieved an average 40% birth rate from embryos transferred and with 11% of embryos born as mutant pups. This represents ~fourfold improvement over microinjection mutagenesis rates, where we have observed ~3% of embryos born as mutant pups over >60 genes tested. Coupled with the improved birth rate (40% cf. 20%), the efficiency of electroporation has enabled us to replace microinjection with electroporation for both customer-specific and large-scale production of deletion alleles. We have also used electroporation to produce knock-in and conditional alleles and have preliminary data demonstrating success with a small number of loci. We are continuing to optimize electroporation parameters and will present data for different allele types comparing microinjection and various electroporation parameters.

Targeting human *SerpinA1* by Cas9 therapeutic gene editing reverts liver damage phenotypes in a humanized α1-antitrypsin disease mouse model

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Alpha-1-Antitrypsin (AAT) is a circulating serine protease inhibitor primarily secreted by hepatocytes and has protective functions in the lungs by targeting neutrophil elastase, which when unchecked causes protease related connective tissue damage. Point mutations in *SerpinA1*, the gene which encodes AAT, can cause protein miss-folding and aggregation in the ER of hepatocytes and subsequently liver damage. We have expressed Cas9 and a sgRNA molecule to target a human *SerpinA1* mutated allele expressed in the liver of a humanized transgenic model (PiZ mice). Our therapeutic gene editing approach was confirmed in disrupting the *hSerpinA1* gene and we saw a reversal of the disease phenotype associated with mutated *hSerpinA1*, including reduced circulating transaminases and hAAT levels, liver fibrosis and protein aggregation and a reduction in the expression of fibrosis markers. Furthermore, liver histology was significantly improved in terms of inflammation and overall morphology in *hSerpinA1* gene edited PiZ mice.

We have shown that CRISPR/Cas9 based therapeutic gene editing in an Alpha-1-Antitrypsin (AAT) mutated mouse model reverts the liver disease phenotypes associated with the AAT PiZ mutation and shows the utility and efficacy of this approach in a disease model relevant to human Alpha-1-Antitrypsin Deficiency.

Knocking out recombineering: an innovative and rapid approach for the targeted generation of complex allelic modifications

Kucera G., Bock C., Flores M., Flowers M., Kent J., Kuddar O., Soderling, S.

Gene targeting in embryonic stem cells is traditionally achieved by the use of homologous recombination between a targeting vector and the ES cell genome. These targeting vectors require subcloning of large homology arms from both upstream and downstream regions of the targeted allele. These targeting vectors are typically constructed in E.coli using Recombination-mediated Genetic Engineering (Recombineering). However, this technique is time consuming and inefficient, requiring Bacterial Artificial Chromosomes (BACs), the construction of several mini-targeting vectors, and multiple rounds of recombineering in specialized strains of bacteria. These steps require expertise and are significant limitations to the higher-throughput needs of academic and commercial transgenic facilities. We have developed a new approach for introducing complex modifications to alleles within ES cells that obviates recombineering techniques. We have designed custom donor plasmids for this novel approach that can be modified for targeted gene manipulation in greatly shorter time frames than with standard targeting vectors. This new strategy also greatly streamlines PCR-based screening of targeted ES cells and replaces the need for Southern analysis with more robust sequencing-based validation. We demonstrate the method works using standard ES cell selection strategies for gene manipulations, including conditional knockout alleles. In summary, these advances greatly simplify and reduce the effort required for the generation of custom mouse models.

CRISPR/Cas9 gene editing in mice

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Our facility has finished more than 200 genome editing projects in mice using CRISPR/Cas9 technology. All projects involved microinjection of reagents into zygotes, followed by implantation into pseudopregnant females, through surgical procedures developed previously for creation of conventional transgenic mice. Approximately 65% of the projects were site-specific transgene insertions and ~35% were targeted gene disruptions or deletions. Here, we describe our work, and share our experiences from working with investigators on project planning, designing and validating guide RNAs (gRNA), preparing the reagents, and microinjecting zygotes for the generation of genetically engineered mouse models.

We injected into mouse zygotes, (1) Cas9 mRNA or protein, (2) single guide RNA (sgRNA) or dual RNAs of synthetic crRNA: tracrRNA, and (3) DNA donor of either oligo nucleotide or plasmid DNA to generate gene insertions, or deletions in the absence of DNA donor. The DNA donors that we used were oligo nucleotides of 80–220 bases in size or plasmid DNA constructs ranged from 2 to 13.4 kb. We will

show the data on the comparison of gene editing efficiencies between using Cas9 mRNA vs Cas9 protein, sgRNA vs synthetic dual RNA of crRNA:tracrRNA, and pronuclear injection vs cytoplasm injection of CRISPR reagents.

With these methods, we generated mice with gene deletions or insertions ranging from a few bp to several kb. Based on analysis of the current data, the efficiency for gene deletion through NHEJ appears to be significantly higher (~66%) than gene insertion through HDR or HR (~18%). Our results demonstrate that CRISPR/Cas9 technology is a robust and rapid method for the generation of new genome edited mouse lines.

Posters

Maximizing certainty of ES cell germline transmission rates—a cascade of efficiencies using genetically sterile "goGermline" embryos

Roger Askew, Ozgene

A major challenge in the generation of germline transmitting chimeras with gene targeted embryonic stem (ES) cells, by conventional ES cell injection into a wild type (wt) host embryo, is the competition between sperm derived from ES cells and that from the wt host. A solution to this challenge is to replace the wt host embryo with an embryo deficient in development of sperm (Koentgen et al., Genesis 2016). To this end, we developed Tsc22d3-null embryos as sperm deficient hosts for the generation of chimeras. We refer to these Tsc22d3-null embryos as "goGermline" embryos. We have found that the ES cell genome is transmitted to 100% of the offspring of goGermline based chimeras. Eliminating competition from host-derived sperm resulted in increased efficiency in terms of animals numbers required to develop new mouse strains and also provided a means of developing mouse strains using previously recalcitrant ES cells. The resulting cascade of efficiency includes reductions in: colony size required for host embryo production; numbers of host embryos required for chimera generation; numbers of chimeras required to achieve ES cell germline transmission and numbers of F1 offspring produced to identify germline transmission of the targeted mutation. The goGermline technology refines the production of ES cell germline chimeras by replacing transmission of the host genome with that of the ES cell genome with a significant reduction of animal numbers required to generate new genetically engineered models.

Comparative gene expression analysis for identification of appropriate markers of pluripotency and germline competency

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Pluripotency is a defining characteristic of embryonic stem (ES) cells and a key factor in germline competency. The factors associated with germline competency in murine ES cells are not well understood. However, the potential to obtain chimeras and germline transmission decreases over time upon initiation of ES cell differentiation. Changes in the expression patterns of *Ankrd1*, *Hck*, *Oct4*, and *Nanog* have been previously detected during ES cell differentiation. Relative mRNA expression levels of *Ankrd1* and *Hck* in unmanipulated C2 ES cells were examined between 0 to 72 h after stress induction to determine whether there was a correlation between the expression patterns and the potential for germline transmission. The results showed changes in relative mRNA expression patterns of *Ankrd1* and *Hck*. In addition to the time course analysis, a direct comparison of the relative mRNA expression levels of *Ankrd1*, *Hck*, *Oct4*, and *Nanog* was performed on nine germline and six non-germline genetically modified cell lines to determine whether there was a correlation between gene expression and germline transmission. Germline competency of ES cells was confirmed by screening progeny from chimeric breeding sets for the targeted gene. A correlation was not detected between the expression levels of *Ankrd1*, *Hck*, *Oct4*, and *Nanog*, and germline competency.

Abbreviations: ES, embryonic stem; *Gapdh*, glyceraldehyde-3-phospahte dehydrogenase; LIF, leukocyte inhibitory factor; PCR, polymerase chain reaction.

CRISPR/Cas9 mutant mouse model creation through in vitro fertilization and zygote electroporation

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CRISPR/Cas9 has been widely adapted for animal model creation, while the standard protocol for CRISPR-based genome editing in mice still relies on microinjection into one-cell stage zygotes, a process that remains laborious, costly, and low throughput with considerable technical barriers. By using electroporation of CRISPR components into zygotes with intact zona pellucida (Kaneko et al., 2014) in combination with in vitro fertilization (IVF) technique, we succeeded in introducing non-homologous end joining (NHEJ)-mediated indels, exon deletions, and point mutations in the C57BL/6 N mouse genome. Commercially available Cas9 protein effectively introduced mutations while decreasing the need of quality control of in-house Cas9 mRNA. Knockout founder mice were obtained in some of the embryonic lethal genes of which we could not obtain founders via standard microinjection, showing that zygote electroporation with two-guide RNA strategy is applicable to generation of critical exon deletion alleles which meet the criteria needed for high-throughput production of the International Mouse Phenotyping Consortium (IMPC) knockout mouse resource. We extended our approach to frozen/thawed zygotes which were obtained by the administration of inhibin antiserum (IAS) and equine chorionic gonadotropin (eCG, known as IASe superovulation) followed by IVF,

resulting in decrease in the number of oocyte donors. The introduction of mutations into mouse zygotes by electroporation can be a convenient and efficient alternative to microinjection for mouse model creation.

Targeted gene replacement of 42 kb using asymmetric donors in mes cells

Oliver Baker et al.

Designer nucleases like CRISPR/Cas9 enable fluent site-directed damage or small mutations in many genomes. Strategies for their use to achieve more complex tasks like regional exchanges for gene humanization or the establishment of conditional alleles are still emerging. To humanize the Kmt2d gene, we built a hybrid mouse/human targeting construct in a BAC by recombineering. To simplify the possible outcomes, we employed a single Cas9 cleavage strategy and best achieved the intended 42 kb regional exchange with a targeting construct including a very long homology arm to recombine $\sim$42 kb away from the cleavage site. We recommend the use of long homology arm targeting constructs for accurate and efficient complex genome engineering, particularly when combined with the simplifying advantages of using just one Cas9 cleavage at the genome target site.

1. Baker et.al., "RAC-tagging: Recombineering And Cas9-assisted targeting for protein tagging and conditional analyses" *Scientific reports* (2016).
2. Baker et.al., "The contribution of homology arms to nuclease-assisted genome engineering" (*Accepted with NAR*).

Rat Resource and Research Center

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The Rat Resource and Research Center (RRRC) was established in 2001 with funding from the National Institutes of Health (NIH). The goals of the RRRC are to (1) shift the burden for maintaining and distributing rat models from individual investigators to a centralized repository, and (2) provide the biomedical community with ready access to valuable rat strains/stocks and other related services that enhance the use of rats in research. Currently, the RRRC has over 435 rat lines received through active recruitment of important rat models and donations from investigators. Upon importation of strains/stocks into the RRRC, sperm and embryos are cryopreserved to ensure against future loss of the model. The RRRC distributes live animals, cryopreserved sperm and embryos as well as rat embryonic stem (ES) cell lines. Quality control measures for all materials include extensive genetic validation and health monitoring. Due to high success rates with intra-cytoplasmic sperm injection, the RRRC uses sperm cryopreservation as a cost-effective method for banking large collections of single gene mutations and ensuring reliable recovery when models are requested. The RRRC has expertise in rat reproductive biology, colony management, health monitoring, genetic assay development/optimization, and isolation of germline competent ES cell lines from transgenic rats; our staff and researchers are readily available for consultation and collaborations. The RRRC has a number of fee-for-service capabilities such as a wide variety of genetic analyses, cytogenetic characterization including spectral karyotype analysis, strain rederivation, spermatozoa cryopreservation, isolation of specific rat tissues and microbiota characterization. Our website (www.rrrc.us) allows user-friendly navigation and provides information about all strains/stocks, cell lines, model donation procedures, on-line ordering, lists of services, and protocols. Current research efforts include refinement of models, characterization of the rat microbiota and its influence on model phenotypes, and generation of new rat models using CRISPR/Cas9 technology. In addition to the RRRC, the University of Missouri is home to two other NIH-funded animal resources: the MU Mutant Mouse Resource and Research Center (MMRRC) and the National Swine Resource and Research Center (NSRRC) as well as the MU Metagenomics Center (MUMC) and MU Animal Modeling Core (AMC). Together, these highly collaborative groups provide a variety of animal model-related services across species to facilitate biomedical research.

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"Replications, ridicule and a recluse" NgAgo and beyond

Khin, Nay Chin, Lowe Jenna, Starrs Lora and Burgio Gaetan

Genome editing and Genetics of Host-Pathogens interaction: Department of Immunology and Infectious diseases. The John Curtin School of Medical Research, the Australian National University, Canberra, Australia

Novel precision genetic technologies such as CRISPR/Cas9 genome editing technology offer novel avenues to a better understanding the mechanisms of diseases. Using CRISPR/Cas9 we are able to precisely modify the mouse or the human genome by creating knockout or a specific single nucleotide change to enable the study of the function of the gene of interest. The generation of these models lies on the ability of Cas9 to create a double strand break in the DNA and the repair to occur via the error prone Non-Homologous End Joining (NHEJ) or the precise Homology direct Repair (HDR) mechanisms. A large body of work have been recently dedicated to either improve the technology to generate efficiently knockout or knock-in mouse models (point mutations, tags or floxed alleles) or to explore novel alternatives to Cas9 enzymes such as orthologs of Cas9 or others prokaryotes defence systems against viruses such as *Natronobacterium Argonaute* (NgAgo). This rapid pace in the technology development has generated a lot of excitement but also some disappointment over the lack of reproducibility of the experiments. This led to a substantial loss of research time and funding. This short presentation will give an overview of the

initiatives undertaken to tackle irreproducible research in the field of genome editing technology and will discuss how to address these problems in the transgenesis technology community.

A new, easy and reliable cryopreservation protocol for zebrafish sperm

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Zebrafish is by now the second most used animal model in biomedical research and the increasingly fast generation of new transgenic and mutant fish lines in recent years urges for simple and efficient cryopreservation programs. The development of more effective, reproducible, easier and cheaper methods of sperm cryopreservation not only guarantees safe preservation of the genotypes but also addresses the inevitable space limitation to maintain live strains in fish facilities thus limiting research.

The most commonly used cryopreservation method relies in sperm being slowly frozen and then stored in liquid nitrogen at –180 °C. However, as in humans and rodents, this technique has drawbacks, including loss of motility and vitality, and membrane damage. For zebrafish, published protocols are ponderous, with multiple steps rendering them error-prone. More recent studies have demonstrated that the use of french straws can improve this method, however the cost of a programmable freezer is very high.

Vitrification, on the other hand, is a fast freezing method with several advantages over the existing method, including a significant increase in sperm motility. However, in zebrafish vitrification of primordial germ cells had very low success rates and entailed quite laborious steps. Simultaneously, there were suggestions that cryopreservation of these cells can have negative consequences in gametogenesis due to hypermethylation.

As part of the technological development program of the Champalimaud Fish Platform, a new, cheaper and faster cryopreservation protocol with high fertilization rates will be presented.

CRISPR/Cas9 for in vivo gene editing: the hidden face of paradise

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The efficiency and simplicity of CRISPR/Cas9 technology to make precise changes to the genome of living cells has led to new revolution in genetics.

This approach is now driving innovative applications from basic biology to biotechnology and medicine. To date, the *Streptococcus pyogenes* Cas9 (SpCas9) has been used broadly to achieve efficient genome editing in a large variety of species and cell types, including human cell lines, bacteria, zebrafish, yeast, mouse, fruit fly, roundworm, rat, common crops, pig, and monkeys.

The molecular mechanisms at the origin of the correction of CRISPR/Cas9 target double-strand breaks are mediated by the cell DNA repair machinery. However, those DNA repair mechanisms are highly complex and poorly amenable to control. As a result, targeted alleles with additional modifications, such as deletions, partial or multiple integrations of the targeting vector, duplications and more drastic rearrangements can be observed. Moreover, mosaicism taking place at preimplantation development stage renders the identification of unwanted genomic modifications at targeted locus very challenging in F0 generation animals. Finally, dsODN and ssODN used for the generation of Kin models can integrate randomly in the genome thus bringing additional complexity to resulting genotypes.

Many articles have emphasized the easy to use nature of the CRISPR/Cas9 system but few have explained the complexity of generated genotypes and the impact of CRISPR/Cas9 double strand breaks and downstream repair mechanism on the cell genome. Here, our aim is to illustrate the complexity of genotypes resulting from application of CRISPR/Cas9 through presentation of in vivo and in vitro gene editing data in mouse and/or rats. The data will be discussed in light of application of CRISPR/Cas9 to in vivo gene editing in mouse and rats and how it can impact safety of genetic therapy in humans.

Induced-pluripotent stem cells from transgenic mice NF-κB-RE-Luc: First steps in the generation of new reporter cell models for the study of inflammation

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The nuclear factor kappa B (NFκB) transcription factors is a key regulator of immune development, immune responses, inflammation, and cancer. Taking this into consideration, it would be of great value to have availability of new cellular models as biological tools to evaluate the contribution of NF-kB in inflammatory processes and to advance in the development of new, more efficient and selective drugs. In the last decade, induced pluripotent stem cells (iPSC) have become a promising tool for the study of pathologies and the screening of drugs. The present work aim to generate and characterize iPSC from murine embryonic fibroblasts (MEF) isolated from the reporter transgenic mice BALB/c NF-kB-RE-luc. To accomplish that, MEF were transduced using a lentiviral system carrying the STEMMCA vector (kindly provided by Dr. Mostoslavsky, Genetics Department, Harvard Medical School, Boston, USA) with the four Yamanaka factors (Oct-4, Sox2, Klf4 and c-myc). After two weeks, colonies were picked up, expanded and cryopreserved. Characterization of the iPSCs was performed by

determining the mRNA levels of the pluripotent genes Oct-4, Sox2, Nanog and GAPDH (as housekeeping gene). Furthermore, the expression of Oct-4, Nanog and SSEA-1 proteins were evaluated by immunofluorescence and in addition the SSEA-1 by flow cytometry. The iPSCs derived from the transgenic MEF showed consistent results in the expression of Oct-4, Nanog and Sox2 genes that are characteristic for pluripotent stem cells. In addition, the immunofluorescence images showed positive staining for Oct-4, Sox2 and SSEA-1 proteins in all analyzed colonies. Finally, flow cytometry results of the iPSC clones displayed more than 80% positive cells for the SSEA-1 marker. Ongoing work is focused on evaluating the reporter function of these clones by performing proof-of-concept experiments with known pro and anti-inflammatory compounds that modulate the activation of NF-κB. The generation of the iPSC reporter lines and their validation as new biological tools will be of fundamental relevance for a deeper understanding of NF-κB pathway in the development of different diseases and as a platform for drug discovery purposes.

Efficient CRISPR reagent generation and genome engineering

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To date at the GEiC, we have created genetic modifications in over 300 cancer and iPSC lines, ranging from simple KOs, ssODN-mediated point mutations, as well as large deletions to insertions using plasmid donors. We have also designed and generated over 1000 CRISPR reagents for cell line, mouse, zebrafish and worm model creation. We will share our process, which allows us to deliver reagents in the same conditions and format as when we measured nuclease activity and/or KI efficiency when applicable. For mouse models, we work closely with mouse cores on campus to optimize delivery of RNPs to zygotes, via microinjection and electroporation, and genotype pups.

Next gen sequencing (NGS)-based genotyping is essential in our workflow for high throughput nuclease activity validation and clone and founder identification. We will discuss our NGS genotyping data to demonstrate its usefulness on determining allele copy number and clonal purity as well as level of mosaicism in founder animals.

In short, we aim to utilize our experience in both cell lines and animal models and help researchers with their own genome editing effort.

Sperm cryopreservation of genetically engineered mouse models: why quality control by in vitro fertilization is important?

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The simplicity of the procedure, and its ability to generate hundreds of offspring from frozen sperm (Re-derivation by FRapid Expansion™ at Taconic Biosciences, Inc.) make sperm cryopreservation in mouse a robust, reliable, and effective method for the low-cost maintenance, and economical distribution of genetically engineered models (GEMs). However, the efficiency of re-derivation of the GEM line(s) from frozen sperm is critically dependent on the quality of frozen thawed sperm, which is often measured by fertilization rate (%FR) of frozen sperm in IVF. Here we discuss the effect of frozen sperm %FR of 225 GEM lines on the full-term development potential (% Live Birth; %LB) of the 2-cell embryos generated in IVF by the frozen sperm.

Sperm cryopreservation and IVF with frozen sperm was performed following the original protocol (Takeo et al., Biol Reprod 2008, 78(3), 546–551) using FERTIUP media (Cosmobio, USA). Based on the FR % in IVF (standard quality control at Taconic Biosciences for sperm cryopreservation), GEM lines were classified into 3 groups—Low %FR (15–30%), Medium %FR (31–60%), and High %FR (>61%). High ($n = 89$), Medium ($n = 90$), and Low ($n = 46$) %FR groups contributed 40, 40, and 20% of total GEM lines cryopreserved, respectively. While the average %FR varied significantly among the 3 groups, no difference was observed in the average %LB among the Low (28 ± 9%, range 15–46%), Medium (29 ± 11%, range 15–60%), and High (29 ± 11%, range 15–53%) %FR groups. However, 28% of GEM lines cryopreserved in Low %FR group required a second trial recovery attempt to achieve and/or confirm a %LB of 15% or higher (minimum benchmark at Taconic Biosciences, for sperm cryopreservation projects opted for trial live birth recovery), compared to 17 and 11% of GEM lines in High and Medium %FR groups, respectively.

We conclude that (1) cryopreservation of unique GEM lines may vary in the fertilization rate and the yield of viable 2-cell embryos for implantation into pseudopregnant recipients for full-term development, but the quality of those embryos as measured by % live birth rate is not influenced by the % fertilization rates. (2) In the absence of IVF metrics, sperm cryopreservation can still serve as an insurance policy to safeguard a line. However, the absence of such quality control data may lead to difficult in realizing the exceptional potential of this technology when attempting large scale cryorecoveries from the stock at a later point in time (often critical for certain research projects after cryopreservation of a GEM model).

Generation and characterization of a stable transgenic ZsGreen reporter rat line

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The rat is an increasingly popular animal model species used in biomedical research. However, due to previous limitations in available technologies that allowed efficient genetic engineering in the rat, there are relatively few genetically modified rat models. Here, we created a transgenic reporter rat carrying a

floxed-STOP-ZsGreen cassette under expression of the CAG promoter. ZsGreen was chosen as the fluorescent reporter for this particular model due to the fact that it has been shown to have up to 10-fold higher intensity than the standard eGFP. This is especially beneficial when tracking low levels of expression and it eliminates the need for secondary antibody staining. We have characterized two independent lines: F344-CAG.loxP.-STOP.ZsGreen Line 551 (RRRC#795) and F344-CAG.loxP.STOP.ZsGreen Line 561 (RRRC#797). Integration sites of the transgenes were determined by target locus amplification (TLA) technology and transgene copy number was measured via droplet digital PCR (ddPCR). Functional assessment for ZsGreen expression was accomplished by crossing the two reporter rat lines with two Cre recombinase-expressing strains: a CAG-driven Cre recombinase line (RRRC#301) and a mChAT-driven Cre recombinase line (RRRC#658). Double hemizygous animals from these crosses demonstrated intense ZsGreen expression in the expected tissues and cell types. While some evidence of ZsGreen transgene silencing was found in Line 551, ZsGreen expression continues to be strong in the double hemizygous offspring from crosses between Generation 4 Line 561 rats and Cre-expressing rats. This new CAG-loxP-STOP-loxP-ZsGreen genetically engineered rat model represents an important reporter rat that will be of great value to the research community across many different disciplines. All of the models used in this study are available through the Rat Resource and Research Center (RRRC) at the University of Missouri (www.rrrc.us).

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CRISPR/Cas9 mediated genome editing by electroporation of mouse oocytes

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Animal models are a powerful tool to understand the mechanisms underlying physiological and pathological processes in vivo. To date, mice remain the species most commonly used for genetic manipulation. The recent advent of CRISPR/Cas9 for gene editing has revolutionized the field of mouse transgenesis, thanks to unprecedented advantages: seamless synthesis, ease of use and superior efficiency. Meanwhile, gene transfer methods have also been improved with the recent report of direct electroporation of oocytes. Compared to microinjection, this method is much faster and does not require highly technical skills.

Yet, one limitation to oocyte electroporation is the use of large quantities of CRISPR reagents. Here, we present an optimized method to achieve high levels of editing with minimal amount of CRISPR reagents.

Genetics meets 3 R′s: genetic background of transgenic mouse strains

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Introduction: A defined genetic background and knowledge about the exact genetic background of inbred laboratory animals are critical for the validity and reproducibility of experimental studies. Substrains of a particular inbred strain of mice differ genetically and have developed a number of mutations, such as *Nnt*, *Snca*, *Dock*2, *Crb*1^{rd8} defects in substrains of C57BL/6, which can strongly impact the experimental outcome.
Methods: Our studies are based on highly informative STR Markers covering the 19 autosomes as well as X and Y chromosomes, which provide a unique fingerprint for any mouse strain or substrain. Additionally, presence or absence of mutations *Nnt*, *Snca*, *Dock*2, *Crb*1^{rd8} were tested. 69 different transgenic strains obtained from 17 scientific institutions in Germany and Austria were analysed.
Results: Our data have shown that 37% of transgenic models have a mixed genetic background with genetic contributions from various BL6 substrains and often show large remains from the donor strain. 19% carry a mixed wt/mut background for *Nnt*, 23% a mixed *Crb*1^{rd8} background. In particular, due to a lack of suitable genotyping platforms the characterisation of the Y chromosome was not in the focus of the scientific community so far. As it is accessible through our STR-marker system we have discovered that about 45% of transgenic lines still carry the Y-chromosome of the donor strain and, therefore, their genetic background should be regarded as Y-consomic. It has been proven that mismatching Y-chromosomes can show dramatic effects on the phenotype even for female descendants—through epigenetics.
Conclusion: In terms of the 3 Rs we recommend to put special focus onto the genetic background of transgenic animals and, if in doubt about the genetic history of a model, to carry out background analysis (including Y chromosome) because well defined genetic backgrounds increase the validity and reproducibility of experimental studies and therefore help reducing the size of experimental cohorts.

In addition, we have developed a method for refining genotyping methods, often carried out on DNA extracted from biopsy materials. Our genotyping kit for extracting DNA from swab samples includes lysis buffer and highly sensitive PCR chemistry and does not require biopsy materials. The kit can be applied on swabs from buccal mucosa of rodents and also on swabs from skin of Zebrafish.

C57BL/6NCrl mouse models generated via CRISPR/Cas9-mediated gene-editing

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With the development of CRISPR/Cas9 gene-editing tools, generation of novel knock-out and knock-in mouse models can be done more quickly and with greater precision compared to conventional methods. We created three new C57BL/6NCrl mouse lines via CRISPR/Cas9 technology to compare the efficiency of different CRISPR and genetic transfer methods.

For Proof-of-Concept purposes three mouse genes, Leptin, Leptin Receptor and CRB1, were identified for genetic modification due to the ease of measuring phenotype on a C57BL/6NCrl background. We used non-homologous end joining (NHEJ) or homology directed repair (HDR) CRISPR/Cas9 techniques combined with ES cell engineering, direct-pronuclear/cytoplasmic injection and electroporation genetic transfer. All putative founder mice were analyzed by an efficient method for screening CRISPR/Cas9 generated mutations. Our sensitive platform detects distinct heteroduplexes generated by each unique variant. These models were created as a part of our ongoing R&D efforts involving CRISPR/Cas9 technology licensed from the Broad Institute.

First, a mouse embryonic stem (ES) cell line was generated from C57BL/6NCrl mice. *In vitro* transfection of this line with a Charles River two-vector CRISPR/Cas9 system was optimized to obtain 73% gene-editing efficiency using NHEJ targeting in the Leptin gene and verified by sequencing. Despite the high frequency of gene-editing, the NHEJ approach was relatively inefficient at introducing the required frameshift. Therefore, we opted for a HDR approach for Leptin Receptor gene target. A 120-nt single-stranded repair template was designed to insert a premature stop codon in every reading frame, thereby also introducing a null mutation. Straightforward PCR screening identified up to 44% of the clones positive for the introduced sequence.

Mutated clonal ES cells were then injected into NMRI blastocysts. Chimerism >75% was observed in 72% of Leptin knock-out and 64% of Leptin Receptor mutation offspring (F0). These animals were backcrossed with C57BL/6NCrl, upon which successful germ-line transmission was observed. Direct embryo injection, using the same constructs as ES cell editing, was also successful in generating Leptin and Leptin Receptor knock-out. Over 80% of injected embryos that developed to full term pups contained deletions. Finally, electroporation was used to generate Leptin knock-outs and 90% of full term pups contained indels.

Thus, generation of C57BL/6NCrl knock-out mice was achieved via all methods, ES cell editing, direct embryo injection and electroporation. The phenotype of these new knock-out lines is currently under study. Moreover, this proof-of-principle study paves the road for development of additional novel C57BL6/NCrl mouse models by CRISPR/Cas9-mediated gene editing.

A comparison of pseudopregnant F1c (CBA/CaJ × C57Bl/10J) and Swiss Webster female mice as suitable recipients for embryo transfers

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In the creation of genetically modified mice, microinjected embryos are transferred into the reproductive tract of pseudopregnant recipient female mice to carry the embryos to term and serve as surrogate mothers until the pups are weaned. Pseudopregnancy is induced in the female by mating with a vasectomized male thereby preparing the female's reproductive tract to receive the surgically transferred embryos. Microinjected one or two-cell embryos are transferred to the pseudopregnant recipient female by exteriorizing the ovary and oviduct, locating the infundibulum, or small opening leading into the oviduct tube, and subsequently expelling the embryos into the oviduct. While several different strains are suitable as recipient females, the ideal recipient strain should be within the suggested weight limits, relatively docile, easy to work with, have relatively large litters, and demonstrate good maternal characteristics. At the Stowers Institute, we use the hybrid mouse strain F1c (CBA/CaJ × C57Bl/10 J) as pseudopregnant recipients. However, many transgenic facilities utilize outbred mouse strains such as Swiss Webster (SW), CD1, and ICR. The purpose of this study was to examine if the outbred strain of Swiss Webster mice would be a better recipient as compared to the hybrid F1c mice in terms of ease of surgical procedure and reproductive performance. To test this, we transferred an equal number of two-cell embryos (14–16) into 0.5dpc SW ($n = 16$) and F1c ($n = 17$) pseudopregnant females. To compare the ease of the surgical procedure between the two strains, various observations including vascularization of the surrounding bursa, presence of blood in the surgical field, length and orientation of the infundibulum, and relative ease of surgery were recorded. Experimental parameters analyzed were female's weight, pregnancy rate (number pregnant/number recipients), pups per female, and birth rate (number pups born/number embryos transferred). All data was analyzed by one-way ANOVA.

Replacing a transgene with another one in transgenic mice by CRISPR/Cas9 technology

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Interneurons play an important role in neuronal network regulations. Classification of this neuron-type is based on the presences of different calcium binding proteins, and peptides such as somatostatin. Identification of these cells are important for anatomical and electrophysiological studies, which can be achieved by direct expression of fluorescent marker proteins or using cell type specific recombinases to activate marker gene expression in transgenic mice. Labelling of specific interneuron subpopulations can be carried with combination of different recombinases driven by distinct cell-type specific promoters. Parvalbumin is a Ca binding protein present in muscles and a subpopulation of interneurons. Since neuronal specific enhancer region is localized far downstream from the parvalbumin gene, modified BAC construct is needed to drive marker gene expression to Pvalb interneurons in transgenic mice.

BAC-Pvalb-GFP mice were generated using PNI. Line #2, the brightest expressing line contains two copies of the transgene, it shows gfp expression in Pvalb specific interneurons without ectopic expression.

Parvalbumin expressing interneurons are functionally divergent often contain additional markers, such as somatostatin. Somatostatin free subpopulation could be labelled by crossing Pvalb-specific floxed Flpo recombinase transgenic mice to somatostatin specific Cre mice and flpo-reporter mice.

Pvalb-floxed-flpo transgenic mice could be generated by a unique approach that is to replace gfp with floxed-flpo in BAC-Pvalb-GFP mice by using CRISPR/Cas9 technology. Donor DNA construct contains homologous regions to the BAC-Pva-egfp sequence and loxPflpo-loxP at the Pvalb–egfp border.

Embryos were produced by IVF from homozygous FVB/AntTgBAC-Pvalb-gfp mice and preincubated in SCR7 solution. Cas9 mRNA, gRNA and ds donor plasmid were microinjected into the pronuclei of embryos and incubated in SCR7 containing G1 solution for overnight. Two cell stage embryos were transferred into recipient females. Six founders were identified out of 38 born pups, each containing flpo gene inserted into the gfp gene.

Flpo expression was characterized in two ways. AAV-fDIO-EYFP recombinant virus was injected into the forebrain and amygdala of Pvalb-flpo transgenic mice, and labelled cells were compared to Pvalb immunostaining. Pvalb-flpo transgenic mice were also crossed to CAG-frt-flanked STOP-tdTomato transgenic mice. In both cases strong reporter gene expression were found in the thalamus and globus pallidus, but flpo expression are not present in all Pvalb expressing cells.

Here we demonstrated how to replace a Pvalb interneuron specific gfp marker by floxed flpo using CRISPR/Ca9 technology.

This approach can be used to replace one transgene with another in a well-characterized transgenic line by using CRISPR/Cas9 technology.

Generation of interferon α/β receptor knockout sheep using CRISPR/Cas9 and SCNT techniques

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Zika virus (ZIKV) infection during pregnancy in humans leads to fetal infection in utero and contributes to microcephaly and other serious congenital neurological symptoms. Interferon (IFN) α/β receptor knockout mice have been used for the investigation of ZIKV infection. However, large animals, such as sheep, have the advantage that their size would enable them to better mimic the fetal syndrome in humans. Here, we report successful generation of IFN α/β receptor knockout (IFNAR-/-) sheep using CRISPR/Cas9 and SCNT techniques. A functional sheep IFNAR consists of two subunits, IFNAR1 and IFNAR2. We designed 3 single-guide RNAs (sgRNAs) specific for exons of sheep IFNAR1 (Gene ID: 443126) and 4 sgRNAs for IFNAR2 (Gene ID: 443363). The targeting vectors were constructed by using pX330 plasmid (Addgene) and each of them was transfected into 2×10^5 sheep fetal fibroblasts. Our results showed that 2/3 (66.7%) of sgRNAs for IFNAR1 and 4/4 (100%) of sgRNAs for IFNAR2 were efficient in directing Cas9 to generate targeted cleavages, with mutation efficiencies of 1–20%. We isolated single cell-derived IFNAR-mutated fetal fibroblast colonies by limiting dilution of cells transfected with the targeting vectors. Two female colonies, IFNARKO-13 and -54, harboring 28-nt and 1-nt biallelic deletions in exon 2 of IFNAR1, respectively, and one male colony, IFNARKO-57, harboring a 5-nt biallelic deletion in exon 1 of IFNAR2, were established and used as cell donors for SCNT. A total of 130 cloned 1-cell stage embryos were generated and surgically transferred into 9 synchronized recipients. Six of them (6/9, 66.7%) were confirmed pregnant by ultrasonography on day 40–45 of gestation. Five pregnancies (5/8, 62.5%; one pregnancy was terminated for IFNAR$^{-/-}$ fetal fibroblast isolation) developed to term, resulting in 4 live lambs. PCR/RFLP assays and sequence analysis showed that the one female lamb carried the mutation in exon 2 of IFNAR1, and the 3 male lambs in exon 1 of IFNAR2, which were identical to the donor colonies they originated from (IFNARKO-54 and -57). In conclusion, we have demonstrated that CRISPR/Cas9 combined with SCNT is a highly efficient system for generating IFNAR$^{-/-}$ sheep.

RHEB1 insufficiency and stress-induced seizures in aged male mice

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The mechanistic target of rapamycin (mTOR) is a central regulator of mammalian metabolism and physiology. Protein mTOR complex 1 (mTORC1) functions as a major sensor for the nutrient, energy, and redox state of a cell and is activated by ras homolog enriched in brain (RHEB1). Increased activation of mTORC1 pathway has been associated with developmental abnormalities, certain form of epilepsy (tuberous sclerosis), and cancer. Clinically, those mTOR related disorders are treated with the mTOR inhibitor rapamycin and its rapalogs. Because the effects of chronic interference with mTOR signaling in the aged brain are yet unknown, we used a genetic strategy to interfere with mTORC1 signaling selectively by introducing mutations of *Rheb1* into the mouse. We created conventional knockout (*Rheb1*$^{+/-}$) and a gene trap (*Rheb1*$^{\Delta/+}$) mutant mouse lines. *Rheb1* insufficient mice with different combinations of mutant alleles were monitored over a time span of 2 years. The mice did not show any behavioral/neurological changes during the first 18 months of age. However, after aging (>18 months of age) both the *Rheb1*$^{+/-}$ and *Rheb1*$^{\Delta/-}$ hybrid males developed rare stress-induced seizures, whereas *Rheb1*$^{+/-}$ and *Rheb1*$^{\Delta/-}$ females and *Rheb1*$^{\Delta/+}$ and *Rheb1*$^{\Delta/\Delta}$ mice of both genders did not show any abnormality. Our findings suggest that chronic intervention with mTORC1 signaling in the aged brain might be associated with major adverse events.

Optimized co-transfection of murine ES cells

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Programmable nucleases like CRISPR/Cas9 enable direct gene targeting in zygotes however, complex and therefore rare events like knock ins and precise gene editing in some cases still require the use of embryonic stem (ES) cells. Such methods usually rely on co-transfection of different plasmids or synthetic DNA molecules. While others have tested a range of transfection methods on ES cells (1), to our knowledge a systematic comparison of co-transfection efficiencies is missing.

We therefor compared the transfection, co-transfection and cost efficiency as well as the mean expression level achieved by a number of frequently used transfection methods.

In short, we found Lipofectamin 2000 (Life Technologies) to be the most effective co-transfection method also resulting in the highest expression levels. Xfect-mESC (Clontech) and use of the Nucleofector with mouse ES cells buffer and program A-023 (Lonza) have lower total transfection efficiencies but co-transfection ratio is comparable to Lipofectamin. However, Xfect-mESC and Nucleofection result in more moderately transfected cells, which might be an advantage if random integration or off-target activity is a risk. Cell viability was highest after transfection with Lipofectamin 2000, followed by Xfect-mESC whereas viability after Nucleofection was much lower. Our results give guidelines for the choice of the optimal transfection method, which however will always depend on the exact experimental conditions e.g. the possibility of selection, sorting or alike.

1. Tamm et al. Stem Cell Rev and Rep DOI 10.1007/s12015-016-9673-5.

Inducible regulation of RORγt in mice with a modified reverse tetracycline-controlled transcriptional silencer 'rtTS3'

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Inducible and tunable regulation system of gene expression in vivo is a powerful tool for identifying functions of a specific gene. However lots of such systems have been developed, they have some limitations in point of efficiency, specificity, or convenience. We challenged these issues with a modified reverse tetracycline-controlled transcriptional silencer 'rtTS3'.

We investigated the feasibility of our rtTS3-based inducible expression system targeting retinoid-related orphan receptor gamma t (RORγt) gene. We fused Krüppel-associated box (KRAB) domain and reverse tetracycline repressor of 'TET-On 3G' to improve doxycycline (dox)-dependent binding affinity for tetracycline responding elements (TRE). We first compared the rtTS3 and the former type of 'rtTS-2SM2' in vitro. The efficiency of transcriptional repression of luciferase driven by CAG promoter was up to ~90% with rtTS3 compared to ~80% with rtTS-2SM2. The leaky repression in the absence of dox was only ~10% with rtTS3 compared to ~50% with rtTS-2SM2.

To regulate the RORγt expression in vivo, TRE sequence was inserted into the RORγt promoter region. The rtTS3 transgenic mice with ubiquitous rtTS3 expression driven by CAG promoter were generated and crossed with RORγt-TRE mice. Administration of dox by feeding resulted in >99.5% repression of the RORγt expression in thymus, spleen, and lymph node. We also examined our system in other tissues. Because the expression of RORγt was limited in above tissues, we focused on 'RORγ' which was splice isoform of RORγt. The promoter of each splice form was only ~3 kb away from each other. Dox-dependent repression of RORγ was observed in skin and muscle with an efficiency of >99.5%, in heart and lung with that of >80%. These results demonstrate that the rtTS3-based inducible expression system provides a powerful strategy for temporal and efficient inhibition of endogenous genes.

Can the RNA-guided AsCpf1 nuclease be useful for generation of knock-in mice?

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Highly efficient CRISPR-Cas9 systems have revolutionized vertebrate genome editing. Target sites within the genome for the widely used Cas9 protein from *streptococcus pyogens* are limited by the G-rich PAM (NGG) preference. The set of target sites can be expanded by using Cas9 orthologs or Cas9 variants which have been engineered to recognize other PAM sequences. Recently, however, a new CRISPR/Cpf1 class of endonuclease has been identified that utilizes a 5′ T-rich PAM site (TTTV, B. Zetsche, 2nd Programmable Nucleases Course 2017). This unique PAM site provides an advantage over Cas9 for targeting regulatory regions, particularly for organisms with an AT-rich genome. Similar to Cas9, Cpf1 is a single RNA-guided endonuclease but unlike spCas9, it does not use a tracrRNA and generates a double stranded break with 5′ overhangs.

We have previously demonstrated that the *Acidaminococcus* Cpf1 RNA-guided nuclease (AsCpf1) has in vivo activity in mouse and can be used to effectively inactivate genes following pronuclear injection at a high concentration (Watkins-Chow et al. *G3* 2017). We observed an efficient targeting rate (41–100%) and germline transmission despite a high degree of mosaicism in founder mice. Cpf1-mediated mutations were mostly small deletions of less than 20 bp. This demonstration of AsCpf1 function greatly expands the available CRISPR systems and DNA target sites for in vivo mammalian genome editing.

The utility of Cpf1 for knock-in of cargo DNA for genome editing is unknown. We are currently testing several types of cargo for insertion. Single stranded oligonucleotides with small homologous arms are being tested using an approach similar to methods widely used for Cas9. Also, double stranded cargo with compatible overhangs are being used to test if DNA ligation can occur independent of homologous recombination. Initial results are promising and awaiting further characterization and sequence confirmation. Full results with several targets and types of cargo following pronuclear injection into mouse embryos will be reported.

Genome editing at TIGM: production of various mutant alleles via CRISPR/Cas9

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Texas A&M Institute for Genomic Medicine (TIGM) has been housing the world's largest library of knockout C57BL/6 N ES cells and provided transgenic mice for researchers worldwide. As interest in the gene trap lines declined, TIGM repositioned itself as a provider of basic transgenic core services to researchers within the Texas A&M system and external customers as well. Among the most recent additions to our array of services was the successfully adopted production of mutant conditional and nonconditional knock in and knock out mouse lines by CRISPR/Cas9 including indels, point mutations, deletions and conditional-ready (floxed) mutants. Will present technical details on the most recent successful editing projects many of which have been produced with the help of Sigma-Aldrich CRISPR Core Partnership.

Keywords: CRISPR/Cas9, indels, point mutation, deletion, conditional-ready, floxed

Generation of ES cell-derived mouse kidneys in anephric rats by blastocyst complementation

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Generation of transplantable human organs from pluripotent stem cells (PSC) is the ultimate goal of regenerative medicine. Xenogeneic blastocyst complementation is attractive approach to generate functional organs from PSCs, as previously shown effective in xenogeneic apancreatic rodent models (Kobayashi et al., Cell, 2010; Yamaguchi et al., Nature, 2017). We have already presented to generate *Sall1* KO rats, from which 2nd and 3rd exons encoding DNA binding-domain of *Sall1* locus are eliminated (Goto et al., 108th SRD, 2015; Goto et al., 63rd JALAS, 2016). Here, we report the first successful generation of three-dimensional kidneys in anephric rats by blastocyst complementation with Venus-positive rat ES cells or GFP-positive mouse ES cells. In Experiment 1, blastocysts derived from sib-mating of heterozygous *Sall1* KO rats were injected with rat ES cells. Transfer of 80 injected blastocysts resulted in the harvest of 52 live pups, including 24 allogeneic chimeric rats, on postnatal Day-0. Genotyping showed that these allogeneic chimeric pups composed from 8 *Sall1*-wildtype pups, 11 heterozygous *Sall1*-KO pups, 4 homozygous *Sall1*-KO pups, and 1 undetermined pup. Kidneys in 3 out of the 4 homozygous *Sall1*-KO pups had strong Venus fluorescence, while those in heterozygous *Sall1*-KO pups exhibited mosaic-like fluorescence. In Experiment 2, blastocysts derived from sib-mating of heterozygous *Sall1* KO rats were injected with mouse ES cells. Transfer of 252 injected blastocysts resulted in the harvest of 110 live fetuses, including 76 xenogeneic chimeric rats, on E20.5 to 22.5. Genotyping showed that these xenogeneic chimeric fetuses composed from 27 *Sall1*-wildtype fetuses, 31 heterozygous *Sall1*-KO fetuses, and 18 homozygous *Sall1*-KO fetuses. Kidneys in 12 out of the 18 homozygous *Sall1*-KO fetuses had strong GFP fluorescence, while those in heterozygous *Sall1*-KO fetuses exhibited mosaic-like fluorescence. Based on flowcytometric quantification, mouse lymphocytes were detected at a maximum proportion of 12.1, 10.4, and 5.7% in spleen cells of the *Sall1*-wildtype, heterozygous *Sall1*-KO, and homozygous *Sall1*-KO xenogeneic chimeric rats, respectively. These data indicate that mouse kidneys were successfully generated from ES cells in the developmental niches for kidneys of homozygous *Sall1*-KO rats. Functional integrity of the regenerated xenogeneic kidneys remained to be demonstrated.

Generation of knockout, knock-in, and humanized mouse models using the CRISPR/Cas9 technology: lessons learned and open questions

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The CRISPR/Cas9 genome editing system has established itself as a versatile technology for inducing precise genetic alterations in a number of different species, and has proved to have the potential to increase the efficiency and speed of producing genetically engineered models of human disease. At Taconic Biosciences, we use both an in vivo strategy utilizing one-cell embryos and a complementary in vitro strategy utilizing ES cells to generate both mouse and rat models with CRISPR/Cas9. We will provide a broad data-set to illustrate our experiences using these two strategies within our production pipeline.

The major advantages of in vivo gene editing using CRISPR/Cas-based methods are the significantly reduced time frame and effort involved in establishing new mouse or rat models and the ability to utilize almost any available mouse and rat strain. The relative simplicity of this method compared to ES cell-mediated approaches facilitates the generation of founder animals with reduced costs and effort, providing an attractive alternative to homologous recombination-based approaches. We have implemented CRISPR/Cas9 technology in vivo for the accelerated generation of knockout and simple knock-in (e.g. point mutations and small tags) mice and rats and successfully generated more than 200 models in the past three years.

However, the genetic modifications that can be introduced in the genome by the in vivo approach are currently limited to relatively simple allelic configuration, such as single base substitutions, gene deletion, and insertion of short sequences.

To overcome this limitation, we have combined the use of CRISPR/Cas9 technology with the advantages of utilizing large targeting constructs in ES cells. The combination of the two technologies allows for the generation of large and complex alleles with the precision and efficiency provided by the CRISPR/Cas system and, importantly, the humanization of specific loci in the mouse genome by gene replacement to create relevant model for preclinical drug testing.

The embryo cradle: a novel and versatile tool for manipulating mouse embryos

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The traditional strategy for penetration of early-stage embryos requires the presence of an intact early zona pellucida and involves compression of the embryo between a holding pipette and a sharp glass injection pipette, presented from opposite directions. The Embryo Cradle is an innovative tool that combines the holding and injection pipettes into one easy co-axial microtool: the manipulating pipette is contained within the holding pipette. The major advantage of the Cradle is that the manipulating pipette contacts the embryo in a region that is constrained under the regulated forces imparted by the holding pipette. The original Cradle was developed to manipulate llama blastocysts, which are $\sim$1 mm in diameter. The objectives of the present project were to successfully redesign the tool to handle smaller embryos, including preimplantation mouse embryos, and to test its ability to inject embryonic stem cells (ESC) into 8-cell through blastocyst-stage mouse embryos and to biopsy 8-cell through hatched blastocyst-staged embryos. Embryo survival and viability following manipulation was close to 100%, and live pups were born from every type of procedure. Embryos did not need to be chilled prior to ESC injections; in fact, successful injections were performed on 8-cell through blastocyst-stage embryos using the heated stage. Another unique feature of the Cradle is that it can be used to remove cells from 8-cell embryos prior to injection of ESC. Our long-term goal is to develop a versatile, easy-to-use and affordable tool that can be used to perform any type of manipulation on preimplantation mouse embryos, including ICSI, nuclear transfer, biopsy, and injection of cells, DNA, RNA or proteins. Since the Embryo Cradle reduces embryo stress, which may increase survival, and allows for manipulation of embryos at a wide range of developmental stages, it could reduce the number of embryos that need to be collected and manipulated to obtain desired experimental results.

Complexity of the animals generated by Crispr

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Crispr is a highly efficient genome editing tool while analysis and breeding of the animals generated by Crispr may take much longer time.

We have used Crispr to generate animals by zygote microinjection or ES cell approach. We learned that most F0 animals are mosaic of multiple genetic modifications. When crossed with a wildtype mouse, the progenies will inherit some of these modifications.

We'll show some of the examples to reveal the complexity of the animals generated by Crispr. For example, we used Crispr and oligo to generate a point mutation and detected 58 positive F0 mice by PCR with a primer outside the oligo arm and a primer with point mutation. We then sequence 33 of them and found 3 homozygotes, 1 heterozygote, 16 with mutation + insertion or deletionl, 3 with homozygotes + deletion, 8 with point mutation + unspecified mutation, 1 homozygotes for deletion and 1 homozygote for insertion.

Another example is a triple genes knockout project. The ear piece genotyping of F0 mice showed 1–2 deletions but most of the F1 progenies carried triple gene knockouts.

Beware of these modifications would help us to analyze the genotype and select correct allele for further breeding.

Electroporation of Rat Embryonic Fibroblasts to Assess Function of CRISPR-Cas9 Plasmids

Elizabeth D. Hughes, Wanda Filipiak, Michael Zeidler, Thomas L. Saunders

CRISPR-Cas9 genome editing technology has greatly enhanced the ability and speed with which genome edited animal models can be generated for use in research, including in species for which no genome editing technology was previously available. Gene targeted rat models are now produced as efficiently as for mice. In any project involving use of animals it is vital to minimize the number of animals used to maximize animal welfare. For CRISPR-Cas9 technology this is addressed by first confirming the CRISPR-Cas9 reagents are active in cultured mouse cells or cultured rat cells. This process removes the need to use mice and rats to produce fertilized eggs for microinjection and testing of Cas9 activity in animals. This also reduces the number animals used because only proven Cas9 reagents are used in the production of animal models. The replacement of testing in mouse and rat eggs by cell culture systems has the potential to significantly reduce the numbers of animals used in research. In particular, this approach eliminates the need for superovulated rat egg donor females and eliminates the need for the technically demanding five-day rat blastocyst culture in two-phase medium systems for CRISPR-Cas9 reagent validation.

In the generation of genome edited rats the University of Michigan Transgenic Animal Model Core co-electroporates a puromycin resistance marker with CRISPR-Cas9 plasmids into primary rat embryonic fibroblasts to test for the presence of Cas9 induced insertion or deletion (indel) mutations. The use of cells in culture obviates the need to use rats to produce zygotes for Cas9 activity testing. Cas9-induced double strand DNA breaks repaired by non-homologous endjoining often result in the presence of indels. Genomic DNA is prepared from surviving electroporated cells and tested for Cas9-induced indel mutations. Indel mutations are detected as superimposed sequences (peaks-on-peaks) in sequencing chromatograms of PCR-amplified DNA fragments spanning the expected Cas9

cut site. Indel mutations can also be detected by T7 endonuclease I assay for mismatched DNA. The Core uses on-line tools to design the CRISPR-Cas9 construct (http://crispor.tefor.net) and builds plasmids with protocols described by Ran et al. (2013. Nat Protoc. 8:2281–2308). Only those plasmids with measurable activity are used to produce gene targeted rat models for use in research. The University of Michigan Transgenic Animal Model Core has successfully generated many genome edited rats using this approach. The CRISPR/Cas9 Pipeline for genome editing in rats will be presented with representative results and analysis of gene editing outcomes.

Implementing novel technologies in a transgenic facility: creating conditional alleles with CRISPR/Cas9 in mouse zygotes and embryonic stem cells

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Recently, several technologies emerged that had a great impact on the transgenic field. Techniques such as genome editing using CRISPR/Cas9, transposons as vectors for transgenesis and improved "2i" culture conditions for embryonic stem cells (ESCs), expanded the transgenic toolbox considerably. Now genetically modified mouse strains can be generated in a shorter time-frame on almost any strain or genetically modified mouse model (GEMM) background. With all these new possibilities, for many it has become unclear which approach is the most suitable to create a new mouse strain. Based on our experience, >80 new strains in the last 3 years, we have created a decision guide that helps to identify the optimal approach to obtain the desired mouse model taking into account the type of genetic modification, the method to introduce this modification in the genome and the injection route, i.e. zygote vs. blastocyst. This decision guide considers the reliability of each approach, the time required to introduce the genetic modification in the mouse germline and the additional time required for cross-breeding to obtain the final experimental mice. An example of how a novel technology alters the choice of approach is seen for the generation of conditional knockout mice. Previously, we used conditional targeting vectors in wildtype ESCs. This approach was reliable, but technically demanding and time-consuming. In the last three years, we shifted towards a one-step CRISPR/Cas9 approach based on the methods of the Jaenisch group. Now, two gRNAs are designed flanking a gene or gene cluster together with two homology-directed repair oligos encoding LoxP sites. These are introduced simultaneously with Cas9 in either zygotes or (GEMM-)ESCs, depending whether the conditional allele is required on a wildtype or GEMM background, respectively. Via the zygote route, we have succeeded in 9 out of 11 projects, resulting in "floxed" mice on either FVB/N or C57Bl/6 background. On average, we found 5.1% of the founder pups correctly targeted. In the 2 non-successful projects, the customer was satisfied with the full knockout allele that was created as a by-product. Via the ESCs route, we created in one step a hemizygous allele (Flox/Del) in ESCs from a GEMM of small cell lung cancer. The resulting chimeras were germline competent, highly chimeric and directly used in tumor induction experiments using Cre recombinase. In conclusion, careful implementation of novel technologies in a transgenic facility can lead to improved mouse models that are generated reliably within a short time-frame.

Validating custom and imported rodent lines during colony creation with PCR, qPCR and ddPCR

Emily Hunley, Deborah Siler, Carol Cain-Hom, Anna Pham, Maria Martinez, Sanjeet Aujla, Rhonda Wiler, and J. Colin Cox

As a high-throughput genetic analysis laboratory in one of the largest rodent vivaria used for drug discovery, it becomes crucial that the hundreds of thousands of mammalian genotypes we return to several hundred researchers each year are scientifically robust. The need to genetically inspect and validate lines during colony creation is of critical importance. Prior to the development of this standard operating procedure, we would notice that at low frequency, new colonies would be found to contain hidden, undisclosed, or confounding genetic elements (e.g., recombinases driven under various promoters, antibiotic resistance genes, etc.). When this occurs, it can have a significant impact on both research integrity and animal welfare. To mitigate these risks, we developed SOPs and present them here.

In short, first generation animals are screened using a capillary electrophoresis system (ABI 3730XL) to confirm expected amplicon length while detecting unexpected amplicons of similar sizes. If necessary, copy number variation analysis is performed via ddPCR (BioRad QX200), and is used to further characterize abnormalities, such as off-target construct integrations. If animals are imported from another institution, they are additionally screened for CRE, iCRE, FLP, FLPo, DRE, puromycin res., and neomycin res., using qPCR(TaqMan) to detect any undisclosed genes that may be present. In addition, ESC constructs are confirmed at the time of injection, giving the added benefit of ensuring our in-house assays detect the mutation of interest before mice are born. Finally, CRISPR G1 animals are monitored for any additional mutations that may have occurred. By following these protocols we catch unwanted genetic modifications early, and are able to prevent waste of animal life as well as protecting the integrity of our investigator's research.

Efficient homology-directed repair using single-stranded DNA templates

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Homology-directed repair (HDR) is a valuable tool for genome editing when combined with the CRISPR/Cas9 system. HDR

with a targeted repair template after a double-stranded DNA (dsDNA) break enables the generation of knock-in strains or animal models, including insertion of reporter genes or the introduction of specific mutations. Double-stranded DNA can be used as a repair template, however knock-in efficiency can be low. Further, linear double-stranded templates can insert at any break present in the genome, including off-target sites. Several recent studies have demonstrated that single-stranded DNAs (ssDNAs) are superior HDR templates. ssDNAs can be obtained as synthetic oligonucleotides or made from longer dsDNA templates. Here, we report optimal conditions to make point mutations and epitope tag insertions using IDT Ultramer® Oligonucleotide ssDNA HDR templates delivered with a Cas9 ribonucleoprotein complex. Investigations of strand choice (targeting vs. non-targeting strand), homology arm length, strand symmetry, Cas9 variants (wildtype, nickase, HiFi), chemical modification, and Ultramer purification conditions are shown and a rule set for HDR using oligonucleotide-based templates of 200 bases or less is presented. IDT Megamer™ ssDNA fragments are used for insertions of 200–2000 bases. Initial experiments suggest that these long ssDNA HDR templates show similar advantages and behavior as their shorter oligonucleotide counterparts. Furthermore, Megamer ssDNA fragments injected with Cas9 ribonucleoprotein complexes into mouse zygotes successfully produce correctly targeted alleles in live offspring.

Efficient generation of gene knockout rats using CRISPR/Cas9

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Since its development as genome editing tool, the CRISPR/Cas9 system has been widely adapted as an efficient tool to generate knockout or transgenic animals. The CRISPR/Cas9 system harbors two essential components; single-guide RNA which directs Cas9 endonuclease to cleave specific DNA sequence. In general, microinjection technique, which involves direct injection of CRISPR/Cas9 into one-cell embryos were utilized for generating knockout rodents. However, this technique has several disadvantages: 1. User has to be well-trained to manipulate single zygotes; 2. User can only inject one cell at a time (long and laborious); 3. Low viability after microinjection is observed. To overcome these, we adapted electroporation system which is relatively easy to handle and up to 100 zygotes can be electroporated at once. Using this method, we established a protocol to generate knockout rats with high efficiency. With this procedure, we generated *slc16a2* knockout rat (up to 83% homozygous pups) which has a potential to be utilized as a model for Allen-Herndon-Dudley syndrome, a leukodystrophy with thyroid hormone transport deficit. Electroporation-based genome editing technology provides a simple and efficient methods to produce knockout rat models.

Keywords: CRISPR/Cas9, Electroporation, Embryos, Knockout, Rat

Transfection of mouse pluripotent stem cells for gene-editing with CRISPR/Cas9 in a feeder-free culture system

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The introduction of readily accessible reprogramming and gene-editing platforms enable researchers to interrogate the conserved roles of genes in fundamental development and disease processes. While numerous recent studies have demonstrated that functional human genetic experiments can be performed using human induced pluripotent stem cell (iPSC) models, often the cellular phenotypes observed in in vitro assays are not predictive of the pleiotropic functions that a gene can have across a range of organ systems. Appropriate animal models continue to be needed to confirm that complex genotype–phenotype associations observed in patient cells in a dish translate into impacts on whole organismal biology and health. Targeting conserved loci in mouse iPSCs or embryonic stem cells (mESC) can provide comparative in vitro results with the added benefit that gene-edited pluripotent stem cells (PSCs) can also be used to generate new in vivo mouse models. Two impediments to making traditional transgenic or knockout mouse models have been the inefficient introduction of gene-editing tools through electroporation and the cumbersome isolation and screening of PSC colonies surviving selection. Over the decades since their discovery, most mouse pluripotent stem cell culture systems continue to require co-culture with a supporting mouse embryonic fibroblast (MEF) layer that can confound subsequent genetic analyses. Here we present a new mouse ESC culture medium that was optimized to support (1) spheroid colony morphology in feeder-free conditions (2) maintenance of pluripotency markers and (3) the ability to support downstream differentiation. In addition, we have optimized chemical transfection of mouse ES cells to introduce CRISPR/Cas9 gene-editing tools by co-delivery of DNA, mRNA and Ribonucleoprotein complexes. This new medium enables continued mouse PSC cell proliferation after gene-editing in a feeder-free workflow, while maintaining pluripotency. Taken together this work highlights two solutions for streamlining the targeting of mouse PSCs cells for new model creation.

Generation of novel neurofibromatosis type i rodent models

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Neurofibromatosis Type I (NF1) is an autosomal dominant single gene disorder with over 1700 different documented patient mutations in the 57 exon gene, affecting approximately 1 in 3000 individuals. Neurofibromas, a benign peripheral nerve sheath tumor type, are the cardinal feature affecting

>90% of patients and can manifest in numerous tissues, with no effective treatments available. Animal models to study NF1 disease in vivo have been previously limited to the use of null alleles, or conditional knockout alleles in the mouse. To establish more relevant pre-clinical animal models, the Transgenic and Genetically Engineered Models (TGEMs) Core at the University of Alabama at Birmingham (UAB), in conjunction with the UAB Neurofibromatosis Program, is creating mouse and rat models of patient mutations using both traditional ES cell targeting and CRISPR/Cas9 technology. Mouse models developed to date include the nonsense mutation c.2041 C>T; p. Arg681X, and missense mutations c.2542G>C; p.Gly848Arg and c.2393_2408del16. Additional model development is currently underway in the mouse including c.2919_2920insTT, c.5425C>T; p.Arg1809Cys, c.1466A>G; p.Tyr489Cys, c.2970-2971delAAT, and c.499_502delTGTT. The c.3827G>A, p.R1276Q mutation is being created in both mice and rats, with novel null alleles in the rat confirmed. The CRISPR based projects have been successful both with in vitro transcribed and commercially available synthetic guides and were confirmed to have on-target activity in blastocyst assays before animals were created to reduce the numbers of live animals required for each project. To further reduce animal use, CRISPR guides are now being validated in vitro on PCR products. Design of CRISPR guides, repair templates, and ES cell targeting vectors as well as injection methods and results for these projects are presented.

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Application of oocyte-cytoplasmic injection for CRISPR-Cas9 gene-editing in mouse

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The CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeats/associated protein 9) offers a facile method for genome editing in many model organisms, including human embryos. However, the low efficiencies of current genome editing protocols require a time-consuming, laborious, and costly screening workflow that entails animal breeding, often resulting in repeated microinjections. An optimized workflow should aim to reduce failures early in development to preclude filling procedural pipelines with poorly chosen late-stage products likely to fail. Current protocols target gene editing in zygotes by pronuclear or cytoplasmic injection of editing components. However, studies suggest that mouse zygotes interrupt their normal developmental program soon after fertilization and undergo cell division. If gene targeting is not fully efficient or cells divide prior to gene corrections, genetic mosaicism will result, compromising the efficiency of editing and complicating the evaluation of success. We set out to improve the overall process of mammalian gene editing, with the aim of producing a CRISPR/Cas9 kit effective at the egg cell/fertilized zygote stage. To accomplish this goal, we need to improve the understanding of cell cycle processes related to gene insertion by homology directly repair (HDR) that affect the action of CRISPR/Cas9 delivery between egg cells and the fertilized zygotes. To that end, we are developing a new method for a powerful prediction system for CRISPR/Cas9 editing in egg cells in advance and prior to in vitro fertilization (IVF), allowing in vitro cultured blastocyst embryos to be diagnosed prior to animal production. Our preliminary results document the ability of this new method to rescue previously failed gene insertion procedures, providing a significant improvement of model production of up to 100%. The results will help efficient application of gene targeting that will accellerate the identification and validation of successfully targeted cells or embryos that will enable creation of animal models of human diseases.

Harnessing the one-step generation of genetically engineered mice with CRISPR/Cas9 in zygotes

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The CRISPR/Cas9 system allows for one-step generation of genetically engineered mice by microinjection into zygotes, and a number of approaches to enhance the efficiency of genome modifications have been reported recently. However, there appears to be a room for improvements on the technologies to insert specific sequences and gene cassettes in targeted manners. Here we report our current approaches for the generation of designed-null alleles with insertion of termination codons or conditional (floxed) alleles with ssODN, as well as cassette knock-in alleles. For generating knock-in mice efficiently, we attempted to optimize three previously described methods: (1) homologous recombination (HR), (2) that using a long single strand DNA as a donor (lssDNA), and 3) the PITCh system employing microhomology-mediated end joining. The *ROSA26* locus was used as a model site for the insertion of a GFP expression cassette. Initially, Cas9 mRNA was used for microinjection of about 200 zygotes per condition, yielding GFP knock-in embryos only by the lssDNA method. On the other hand, GFP knock-in embryos were obtained more efficiently with a combination of Cas9 mRNA and protein in all methods. The knock-in efficiencies (KI out of pups) are 11.3% by HR, 9.0% by lssDNA, and 44.1% by PITCh, respectively. We will discuss potential pros and cons of these methods, and present several examples of establishment of designed-null and floxed mice, as well as knock-in mice.

Genome engineering using the Cre/loxP system

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The Cre/loxP system is one of the most valuable tools for genome engineering. In this system, Cre recombinase efficiently catalyzes recombination between two consensus loxP sequences, enabling the conditional transgenesis or knockout of genes to study gene functions in specific tissues or at a specific time. Within the last ten years, the high-throughput generation of conditional alleles for mouse genes in embryonic stem (ES) cells has been achieved by the International Knockout Mouse Consortium. Unfortunately, the rat has lagged far behind the mouse for various technological reasons, the availability of rat ES cells available for complex genome editing and technology to efficiently perform HDR via in vivo genome editing. With the advances in recent genome editing, in vivo modifications using the Crispr/Cas system as well as the availability of multiple Rat ES cell strains, it is crucial to create a library of Cre driver strains to have available to the research community. Therefore we are in the process of creating multiple lines by utilizing the Sleeping Beauty Transposon system to create multiple tissue specific and inducible Cre drivers. So far we have created 8 Cre strains covering various tissues (Brain, Kidney, adrenal gland, epithelial cells and ubiquitous expression. We have assessed the tissue specificity as well as the inducibility by crossing the Cre driver strain with a Rosa26-Lox-Stop-Lox-tdTomato strain and then look for tdTomato expressing cells via epifluorescence and confocal imaging. Further characterization to determine tissue specificity as well as co staining for cell specific markers via IHC is underway.

Comparison of standardized nomenclature for CRISPR, ZFN, TALEN mutations across species

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The widespread use of CRISPR/Cas and other targeted endonuclease technologies in many species has led to an explosion in the generation of new mutations and alleles. The ability to generate many different mutations from the same target sequence either by homology-directed repair with a donor sequence or non-homologous end joining-induced insertions and deletions necessitates a means for representing these mutations in literature and databases.

Standardized nomenclature can be used to generate unambiguous, concise and specific symbols to represent mutations and alleles. The research communities of a variety of species using targeted endonuclease technologies (CRISPR/Cas, ZFN and TALEN) have developed different approaches to naming and identifying such alleles and mutations. The International Committee on Standardized Genetic Nomenclature for Mice and Rat in conjunction with MGI (Mouse Genome Informatics, www.informatics.jax.org) and RGD (Rat Genome Database) has developed allele and strain nomenclature guidelines for endonuclease-mediated (em) mutations that provide basic recognizable information about the mouse and rat at a glance. Other organism-specific research communities (Arabidopsis, worm, fly, maize, frog, yeast and zebrafish) have developed allele nomenclature that incorporates the method of generation within the official allele/mutant symbol or use metadata tags that include method of generation or mutagen.

Use of standard nomenclature and data object IDs ensures unique identity of allele and strain names in scientific discourse, publications, data knowledgebases and in data analyses. MGI serves as the authoritative source for these mouse gene, allele and strain nomenclatures. In addition, assignment of the Mammalian Phenotype Ontology terms for describing phenotypes and Disease Ontology (DO) to describe human diseases/syndromes to genotypes. As of May 2017, over 50,785 distinct mutant alleles in mice (including 561 em alleles), more than 311,921 mammalian phenotype terms are annotated to these mutants, and more than 1534 human hereditary diseases modeled in mice are currently integrated into MGI. Using unique nomenclature symbols enables unambiguous association of these terms to the mice for which they serve as a model.

A mouse model of MeCP2 overexpression

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MeCP2 is mainly expressed in brain, nuclear localized and binds to methylated DNA. Whereas loss-of-function mutations cause Rett Syndrome, overexpression of wildtype MeCP2 leads to MeCP2 Duplication Syndrome, demonstrating that an excess of MeCP2 is detrimental. Using a previously established mouse model for MeCP2 Duplication Syndrome, we have knocked in either wildtype or mutant MeCP2 into the Tau locus. Expression from this locus mimics expression of MeCP2 knock-in alleles from the endogenous locus, and homozygous expression of wildtype MeCP2 from the Tau locus is lethal.

Two important domains have been mapped within MeCP2: The MBD (methyl-DNA binding domain) and the NID (NCoR/SMRT interaction domain). Missense mutations which cause Rett Syndrome are clustered almost exclusively within these 2 domains, whereas neutral mutations found in healthy humans are distributed throughout the protein, with the exception of these two domains. We are testing if the same 2 domains of MeCP2 involved in Rett Syndrome, namely the MBD and the NID, also are crucial for MeCP2 Duplication Syndrome. We do this by testing if overexpression of Rett mutants in a MeCP2 wildtype background causes lethality similar to overexpression of wildtype MeCP2, or if we can observe other toxic effects causing a detectable phenotype.

It has been demonstrated in the past that in mice MeCP2 deficiency is reversible. For therapeutic purposes it is important to restore enough MeCP2 function, but to avoid an MeCP2 overdose. Overexpression of hypomorphic MeCP2, such as

Rett-mutants, could achieve this. By crossing our Tau-MeCP2-knock-in alleles into MeCP2 null animals we are able to test wether overexpression of mutated MeCP2 can ameliorate the symptoms of MeCP2 knock-out mice.

Streamlining the generation of knockout and knockin mice using the CRISPR-Cas system

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There have been many reports of gene editing in mice using the CRISPR-Cas system but although knockouts with indels are now relatively straightforward to produce, the efficiency for knockins is variable and depends on several factors. We wished to develop a higher-throughput platform for the generation of mouse knockouts and knockins that was easy, rapid, and required neither high amounts of technical skill nor expensive microinjection equipment. Consequently, we adopted a raft of published methods including use of electroporation to replace microinjection, multiplexing of projects, and use of homologous recombination with long single-stranded oligos to generate precise mice with null mutations or knockin alleles containing fluorescent reporters, loxP sites, the SNAP-tag, or cre recombinase. Additionally, we utilised the recent hyperovulation method with an in-house generated inhibin antiserum to halve the number of mice that are required for embryo generation while substantially reducing the cost of this method. Taken together, these techniques have significantly reduced the time to generate and genotypically characterise the mutant mouse lines we create as well as reduce the number of mice used per project, in line with the principles of the 3Rs.

Streamlining the genetics of the laboratory rat through genome editing and assisted reproductive technologies

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Recent genomic accessibility of the rat now permits complementary genetic and molecular approaches to utilize rats for biomedical studies. CRISPR/Cas9 technology has revolutionized rat biology by allowing rapid, reliable, and cost-effective generation of genome-edited rat models. In order to enhance access to these state-of-the-art research models, the UW Genome Editing and Animal Models Core (GEAM) offers a comprehensive suite of CRISPR genome engineering services of mice, rats and swine for both on-campus and off-campus users. To date, UW GEAM has reliably designed and generated a wide variety of gene knockouts, small knock-ins, and precise deletions in outbred SD and inbred ACI and F344 rats. Conditional gene knockout projects in inbred Lewis rats are on-going. Our complete start-to-finish design and synthesis of injection-ready reagents in combination with our unique rat embryo transfer methods enhance our production results.

In vitro fertilization (IVF) procedures are utilized to create a cohort of aged matched animals and to rescue difficult breeding lines. UW GEAM used IVF to rescue valuable lines of mice and rats that exhibited limb paralysis due to genetic mutations they were harboring. Using fresh sperm from animals with limited mobility, we obtained two-cell embryos through IVF procedures, successfully generated offspring and rescued these lines.

Cryopreservation of embryos or sperm is necessary to protect against catastrophic loss or genetic drift as well as decrease animal husbandry costs. We have stored thousands of frozen rat embryos on unique background strains including Copenhagen and recovered live offspring after 13 years in liquid nitrogen storage. CRISPR/Cas9 microinjected embryos can also be cryopreserved as two-cell embryos using slow-freezing methods until suitable recipients are obtained.

Rederivations are routinely performed to eliminate undesirable pathogens. Aseptic embryo transfer rederivations can easily be done on rat strains that respond to exogenous hormones. Unique rat strains such as Wistar Furth, Wistar Kyoto, Brown Norway and ACI are low responders to exogenous hormones. Therefore, we used timed matings and caesarean sections to successfully rederive over 15 valuable rat lines carrying genetic variants mapped to specific cancers. This large project was previously attempted and mismanaged by a commercial vendor who genetically contaminated many of the rat lines submitted for rederivation.

We are currently utilizing developing CRISPR approaches such as long single stranded DNA donors and electroporation to enhance our gene editing services, and are eager to stay on the cutting edge of all assisted reproductive technologies.

Improved oviduct transfer surgery for genetically modified rat production

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Rat embryo transfer surgeries are becoming more common with targeted nucleases increasing the demand for rat models. This protocol details pre-surgical preparation, techniques for placing embryos into the oviduct, and post-surgical care of rats to parturition. Direct application of mouse oviduct transfer protocols results in limited success in the rat. By combining techniques from several widely used protocols, the number of live pups born to healthy dams was increased. This protocol is distinct from previously published protocols by modification of anesthesia administration, analgesia, peritoneal sutures, incision location and number of transfers per animal.

The ability to reliably produce healthy pups after microinjection and embryo transfer surgery is critical to model creation and the increased likelihood of creating multiple founder animals gives confidence in observed phenotypes. Therefore, as birth rates were low relative to reported rates even with varied concentrations of injection solution, modifications were

systematically made to the existing mouse embryo transfer protocol to better suit the rat.

Multiple publications describe transferring embryos to the oviducts of both horns of the bipartite uterus; however, this increases the length of time the animal is under anesthesia and requires either a midline incision and traversing the peritoneal cavity to reach the lateral reproductive tract, or creating two separate incisions. These options are less than ideal since either will increase stress of the animal and thereby the likelihood that the pregnancy will be aborted. By creating a single lateral incision and administering analgesia both preoperatively and postoperatively the stress of the animal is minimized. The use of isoflurane over injectable anesthetic agents minimizes risk of toxicity (such as with tribromoethanol), injury from IP injection, and repeated dosing, all of which are associated with higher mortality rates following rodent surgery.

The greatest improvements in litter number and size followed the addition of ampicillin and epinephrine to the procedure [62 born/298 transferred (20.8%) versus 91 born/248 transferred (36.7%) post addition of ampicillin and epinephrine; all projects]. Although the surgery is performed aseptically, ampicillin was shown to increase the number of pups born to rats and use of epinephrine on the ovarian bursa reduced bleeding and thereby trauma to the animal, as well as reduced the length of time required to find the infundibulum. These modifications have been used individually in multiple reports; however, this is the first protocol to combine the most advantageous aspects of each protocol while refining procedures that may be detrimental.

Towards the role of the epigenetic factor PRDM9 in meiosis of *Rattus norvegicus*

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PRDM9 (PR/SET-domain 9) is a histone-3-lysine-4-trimethyl(H3K4me3)transferase that determines the double-stranded breaks (DSBs) initiating meiotic recombination in the mouse, human, and cattle. The rapidly evolving DNA-binding domain of the PRDM9-encoding gene can set off the meiotic paradox (the most often used recombination sites—hotspots—disappear most quickly from the population). Deletion of the *Prdm9* gene from the laboratory mouse induces a shift of the recombination initiation hotspots to other H3K4me3 sites including promoters and a complete arrest of meiotic prophase I. On the other side, dogs, birds, and bakeŕs yeast that lack PRDM9 have recombination sites conserved in related species and often located near promoters. Moreover, a human female that could produce offspring without PRDM9 function was identified. We therefore investigated if PRDM9 is essential for finishing up meiosis in another rodent species, *Rattus norvegicus*. Mutants carrying deletions in one of the exons encoding the PR/SET domain were produced by injecting mRNAs of programmed endonucleases into rat zygotes. Four resulting founder animals were bred to homozygosity. We will present the results of their phenotyping. Nevertheless, the rat could be utilized to model human meiotic development.

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Development of KCNQ1 knockout golden Syrian hamsters as a model of Jervell and Lange-Nielsen syndrome

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The *KCNQ1* gene encodes the pore-forming subunits of voltage-gated potassium (K^+) channels that form heteromers with different regulatory subunits to dynamically regulate cellular repolarization by facilitating K^+ efflux during an action potential. Mutations in the *KCNQ1* gene have been found to be associated with a plethora of human diseases, with the most frequently observed disease as long QT syndrome (LQTS), a life-threatening cardiac arrhythmia manifested as prolonged QT interval and increased risk for torsade de pointes which can trigger syncope, seizures and sudden cardiac death (SCD) in young individuals. In general, heterozygous mutations in the *KNCQ1* gene are manifested as autosomal dominant LQTS (Romano-Ward Syndrome; RWS) and homozygous mutations as autosomal recessive LQTS (Jervell-Lange Nielsen syndrome; JLNS). In addition to the symptoms observed in RWS, JLNS patients are also characterized by congenital total bilateral sensorineural hearing loss.

We recently successfully established a CRISPR/Cas9-mediated gene targeting technology in the hamster and have produced *KCNQ1* knockout (KO) hamsters as a model of JLNS. From gene therapy point of view, hamsters are more permissive than mice are for the replication of adenovirus-based gene therapy vectors, thus offering great advantages in testing gene therapies for JLNS. Other potential advantages of the hamster over the mouse in modeling JLNS, or cardiac arrhythmia in general, may include: (1) similar lipid metabolism profiles between hamsters and humans, a physiological feature that is very often related to the development of heart diseases, (2) hamsters are much more readily to develop obesity under Western diet than mice are and that obesity is closely associated with LQTS, and (3) the much lower heart rate in the hamster than in the mouse (310–480 in hamsters vs. 400–700 in mice), which is more closer to human heart rate. Considering the facts that certain *KCNQ1* KO mice do not display electrocardiogram (ECG) abnormalities at all, a

hamster model of JLNS which may better mimic the human conditions is needed.

In this report, we have conducted preliminary characterizations on *KCNQ1* KO hamsters and demonstrated that homozygous KO hamsters display some of the salient features of JLNS, including a total loss of auditory brainstem responses (ABR) and prolonged QT intervals. Consistent with the ABR results, detailed histopathological analysis by H&E staining on the cochlea isolated from $KCNQ1^{-/-}$ hamsters shows: (1) collapsed Reissner's membrane, indicating the disappearance of endolymph, (2) severe degeneration of sensory cells (inner hair cells and outer hair cells) and supporting cells in the organ of Corti, (3) thinning of the stria vascularis of cochlear duct, and 4) some (secondary) degeneration of spiral ganglion neurons. Very interestingly, two $KCNQ1^{-/-}$ hamsters suddenly dropped dead while being handled during an experiment. We are conducting more observations to confirm that the animal deaths are indeed stress-induced SCD and not by other unknown causes. We also observed that compared to their wild type and heterozygous KO ($KCNQ1^{+/-}$) siblings, the $KCNQ1^{-/-}$ hamsters also show slower growth. Our preliminary data indicate that the *KCNQ1* KO hamster is a promising model of JLNS.

Highly efficient screening method for founders of CRISPR/Cas9-generated conditional knockout mice using full-length PCR

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The CRISPR/Cas9 system is widely used to modify the mouse genome for generation of conventional and conditional gene knockout mice. Because the two loxP cassettes used to flank the regions to be deleted to generate conditional KO mice are recombined individually upstream and downstream of the genomic region to be deleted, the generated founder mice are born with the two loxP sites of cis-(integrated in the same allele) and trans-(integrated in different alleles) configurations. It is necessary to breed the founder mice to get offspring for identification of the cis- and trans-loxP sites. Breeding is sometimes time consuming. To circumvent this problem, we tested the possibility of genotyping the founder mice for the configuration of the two loxP sites. The genomic DNA of founders was directly amplified by full-length PCR following TA cloning and sequencing. We tested 9 cKO cases of different targeting gene loci with the loxP-flox region from 1.7 to 5.5 kb. Successful identification of the cis/trans-loxP configuration was achieved in 8 of the 9 founder mice and was subsequently confirmed by breeding of the founder mice with Sox2-Cre mice. One founder mouse diagnosed to be trans-loxP configuration gave an offspring of cis-configuration. The offspring was confirmed to be a conditional KO mouse carrying the correct loxP-floxed allele by breeding with Sox2-Cre mice. This unexpected finding although needing further investigation, raises concern about the accuracy of the full-length PCR method. In conclusion, the full-length PCR method is useful for efficient identification of the cis/trans-configuration of the loxP cassettes in cKO founder mice generated by the CRISPR/Cas9 system. It is advisable to breed the trans-loxP mice for confirmation of their cis/trans loxP status in cases when no cis-loxP founder mice have been generated.

Four-dimensional phenotypic analyses of mouse and human kidney development

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Advances in the ability to generate double-stranded DNA breaks have led to a plethora of enabling approaches by which genetic modifications can be generated for the study of novel phenotypes. To scrutinize phenotypes, parallel progress in complementary microscopy technologies, are required.

We have developed broadly applicable imaging tools to resolve in high-resolution the 3D and 4D development of the human and mouse kidney in both the normal and mutant setting. Using whole-mount imaging of intact kidneys we generate comparative 3D morphological maps of the nephron-producing progenitor niche and reveal systemic differences between human and mouse development. Through long-term 3D time-lapse imaging of transgenic mouse reporter strains we capture whole kidneys at both a macro-anatomical and subcellular level and demonstrate new cellular behaviors as well as the challenges associated with describing highly dynamic phenotypes.

Collectively, we present a suit of imaging tools for four-dimensional phenotypic analyses that can be implemented to examine wide-ranging developmental and adult phenotypes.

A sensitive method for detecting multiple mutations generated by CRISPR/Cas9 genome editing

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Charles River Laboratories

CRISPR/Cas9 is an efficient genome editing tool to introduce targeted small INDELs, therefore both alleles could be mutated simultaneously. In addition, Cas9 could remain active after cell division, resulting in founders so called mosaics that carry multiple mutations. It is essential to have a sensitive and reliable method to identify various mutations.

The most commonly used methods are E7 endonuclease 1 (E7D1), and Surveyor mutation assay, both of which use endonucleases that cleave single stranded DNA due to mismatch between the wildtype and mutant allele. Obviously, mutations that are homozygous could be missed, and many times small deletion or insertion such as SNPs may also be undetected.

At Charles River, we have developed a sensitive platform, which could reliably identify small INDELs with high resolution which may also allow for a SNP detection. The identification is possible due to high resolution and sensitive detection of laser induced fluorescent signals. This technique is especially useful for detection of heteroduplex formation, which is less abundant than homoduplex, but migrates differently. Multiple INDELs can be detected simultaneously, and signal intensity may correlate with an abundance of the mutation. This platform is superior to the two commonly used methods.

The creation and characterization of a knock-in rat optogenetics toolbox

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The rapid progress of programmable nuclease-based gene editing technologies (zinc finger nuclease and CRISPR/Cas9) has made genetic modification relative straightforward in rats, which could serve as better models for neuro-behavioral studies but are not yet widely available. We therefore initiated the efforts to create an optogenetic toolbox, consisting of multiple knock-in rats including neuron-specific Cre driver lines, Cre-activity-dependent excitatory and inhibitory opsin expression lines as well as a fluorescent protein-based Cre-activity reporter line. We performed mRNA in situ hybridization and antibody staining-based immunohistochemistry analysis on brain sections and validated the expected labeling/activity pattern of different Cre lines. We envision that the commercial availability of these lines will offer powerful tools for neuroscience research with genetically modified rat models, particularly in the optogenetics field.

CRISPR/Cas9 genome editing, from plasmid DNA microinjection to dual guide/RNP electroporation: evolution of a small core facility for transgenic mice

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This work aimed at simplifying each step of CRISPR/Cas9 genome editing to improve the efficiency of our small core facility, and to facilitate access to this technology for users.

In 2014, in order to simplify reagent preparation by users unfamiliar with RNA synthesis and handling, we tested the microinjection of a plasmid allowing Cas9 nuclease and sgRNA expression into mouse fertilized oocytes. Unfortunately, the plasmid approach was not very efficient in generating NHEJ driven mutations and unsuccessful for the one attempt of HDR editing.

We switched to the more efficient RNA injection approach. Despite difficulties for some users to obtain good quality RNA, different knock-out and single point mutation projects were successfully completed.

We then tested the "cloning free approach" described by Aida et al., combining it with chemical modifications described by Hendel et al. This consisted in using RNP (ribonucleoprotein) complexes between Cas9 nuclease and guide RNA, where classic sgRNA was replaced by chemically synthetized and modified tracrRNA and crRNA (dual guide). One comparative experiment showed that dual guide/RNP was at least as efficient as RNA injections to generate single point mutation through HDR. This much simpler approach was adopted, as users only need to purchase their specific crRNA (±ssODN), while tracrRNA and Cas9 protein are provided by the core facility. Knock-out, deletion and single point mutation projects were achieved with this approach.

Our last goal was to facilitate component delivery into zygotes, usually dependent on microinjection, a time consuming step requiring experienced technicians. We tested electroporation using the NEPA21 electroporator. Electroporation has already been described using sgRNA/RNP, but not with dual guide/RNP. Conditions were optimized to use embryos with intact zona pellucida. So far, we have achieved 2 knock-out, 1 double knock-out and 2 single point mutation projects in C57BL/6 J or FVBxB6D2F1 embryos and compared efficiencies between dual guide/RNP injection and electroporation. Our results showed that electroporation significantly increases embryo viability (81–97%) compared to microinjections (33–75%). Mutation efficiency through NHEJ mechanism reached 100%, and up to 69% HDR efficiency was achieved using asymmetric ssODN as described by Richardson et al. Overall, electroporation reduces the number of embryos used and saves time.

In summary, we succeeded in simplifying preparation of high-quality checked CRISPR/Cas9 components and largely improved their embryo delivery by using electroporation. This facilitates users' work and increases the efficiency of our small core facility in providing genome edited mouse models.

Derivation and profiling of mouse epiblast stem cell lines (mEpiSCs)

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While mouse embryonic stem cells (mESCs) derive from the inner cell mass of the embryo at blastocyst stage (Evans and Kauffman, 1981), mouse epiblast stem cells (mEpiSCs) derive from the epiblast of the early post-implantation embryo (Brons et al. 2007). mEpiSCs are pluripotent cells that can proliferate indefinitely in culture and differentiate to derivatives of all the three germ layers either in vitro or in teratomas, similarly to mESCs. However, other characteristics of mEpiSCs such as flat-colony morphology, culture conditions, X chromosome

inactivation status, specific marker expression, low clonogenicity and poor contribution to chimeras when injected into blastocysts make them closer to the human embryonic stem cells (hESCs) state than the mESCs (Brons et al. 2007; Nichols and Smith, 2009). Both hESCs and mEpiSCs are considered primed pluripotent stem cells (PSCs), in contrast to naïve PSCs such as mESCs (Nichols and Smith, 2009). Therefore, studing mEpiSC would help to better understand the nature of hESCs and their potential applications in stem cell therapy, as well as the mechanisms involved in the initial differentiation into the three germ layers.

Here, we present the derivation of several mEpiSC lines from different strains of mutant mice. We follow a previously established protocol (Sugimoto et al., 2015) and confirm that inhibition of the WNT secretion leads to a high derivation efficiency. Moreover, we simplify the culture conditions showing that mEpiSCs can be established in vitro in a feeder-independent manner. The genetic profile of the newly derived mEpiSC lines will be shown as well as their performance in further characterization assays, such as in vitro and in vivo differentiation experiments.

Novel rodent models of renal cystic disease

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Cystic kidney disease can be caused by genetic, developmental or environmental factors and animal models have played an important role in the study of this disease. We are generating and characterizing several new rodent models for two such cystic disorders: Polycystic kidney disease (PKD) and Nephronophthisis (NPH). PKD is one of the most common genetic diseases in humans and it is characterized by grossly enlarged kidneys due to fluid-filled cysts. Mutations in the *PKD1* gene are responsible for the majority (85%) of autosomal dominant cases. Nephronophthisis is an autosomal recessive disorder that is the most frequent cause of end-stage renal disease in the first three decades of life and is characterized by normal or slightly enlarged kidneys with cysts in the corticomedullary junction. We used an ES-cell based targeting strategy to create a *Pkd1* conditional knockout rat carrying a floxed allele that when knocked out will completely abolish the production of polycystin 1. A targeting vector containing exons 2–6 of the rat *Pkd1* gene flanked by *lox*P sites was inserted into rat ES cell line F344.Tg.EC4011 using a CRISPR/Cas9-assisted knock-in strategy. Chimeric animals were produced by injecting the targeted ES cells into F344 blastocysts. Founders were assessed for germ line transmission. To evaluate whether two unique human point mutations in the *Bicaudal C homologue* 1 (*BICC1*) gene are responsible for NPH in two unrelated individuals, mouse models with the same point mutations within the *Bicc1* locus were created using a CRISPR/Cas9-assisted knock-in strategy. While several founders had cystic kidney disease, genetic analysis identified multiple alleles with not only the expected knock-in but also a number of indels indicating a high degree of mosaicism at the *Bicc1* locus. The surviving mutant animals are currently being bred to generate lines with only the knock-in allele. Heterozygotes can then be intercrossed to generate homozygous animals which would be predicted to have cystic kidney disease. This study exemplifies the potential power of using CRISPR/Cas9 technology to generate animal models to validate putative human disease alleles but also illustrates how the high degree of mosaicism often seen with CRISPR/Cas9 genome editing can confound the analysis.

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Preliminary characterization of p53 knockout golden Syrian hamster created by CRISPR/Cas9 system

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The tumor suppressor gene p53 is the guardian of the genome and mutations in the p53 gene are frequently presented in human cancers. Inactivation of p53 in a variety of animal models results in early-onset tumorigenesis. In this study, using CRISPR/Cas9 system, we created a p53 knockout (KO) golden Syrian hamster that carries a 1-nucleotide insertion in exon 5, resulting in a reading frame-shift and premature stop codons in the p53 gene. Both homozygous and heterozygous p53 KO golden hamsters developed a wide spectrum of tumors, such as tumors in the bowel, prostate, lung and liver. Homozygous p53 KO golden Syrian hamsters survived for an average of 121 days before succumbed to tumors, while about 50% heterozygous p53 KO golden Syrian hamsters died from tumors between 84 and 320 days of age. The tumor types developed were the same between homozygous KO and heterozygous KO hamsters. Interestingly, defects in neural tube closure were observed in female homozygous p53 KO hamsters resulting in exposed brains and perinatal lethality. Our data suggest that the p53 KO golden Syrian hamsters developed by us will provide an alternative model for the research in understanding carcinogenesis and other diseases, developing diagnostic biomarkers, as well as evaluating therapeutics.

Development of a mouse model suitable for in vivo genome editing efficiency studies

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The CRISPR-Cas9 system has been widely used as a popular tool for in vitro and in vivo genome editing applications. This system has a potential to apply to the gene therapy for correcting the human disease causing mutations. There are already a proof of concept reports that showing the feasibility of in vivo gene therapy applications. Nevertheless, this area needs several improvements including assessing gene correction efficiencies of the strategies that are investigated. A mouse model to precisely and rapidly assess the gene correction efficiency would serve as a valuable tool for expediting the research in this area.

In this study, we generated transgenic (Tg) reporter mouse (ΔeGFP Tg), containing a defective eGFP coding sequence and then used the model for gene correction in CRISPR gene editing experiments. TheΔeGFP Tg mice were generated by injecting Cas9 mRNA and sgRNA target to the eGFP gene into the fertilized eggs derived from the single-copy eGFP Tg mouse previously generated in our laboratory. Six pups were recovered and all showed no eGFP fluorescence. We also confirmed that each pup has mutation within the eGFP gene. The two lines of ΔeGFP Tg mice were established and maintained as a homozygous colony. We next designed new gRNAs targets to the mutated eGFP genes and sgRNAs were synthesized. CRISPR components (Cas9 mRNA, sgRNA, and repair DNA template) were then injected into fertilized eggs obtained from the ΔeGFP Tg mice to examine if ΔeGFP gene is repaired. Aboiut 25% of eggs exhibited eGFP fluorescence at the blastocyst stage, and the precise repair was confirmed by sequencing. We next used this system for in vivo gene correction in the adult mice. We performed injection of a plasmid expressing Cas9 and sgRNA together with the repair DNA template into the skeletal muscle of ΔeGFP Tg mice, followed by electroporation. After one week, the muscle tissue of the mice treated showed eGFP fluorescence, indicating that ΔeGFP gene was repaired in the area. Taken together, these results indicate that the ΔeGFP Tg mice developed can be used for fluorescence-based evaluation of efficacy and delivery of genome editing components for gene repair.

Keywords: CRISPR/Cas9, gene therapy, in vivo

Overall evaluation of gene modified mouse production by embryo-based genome editing

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The Laboratory Animal Resource Center (LARC) in University of Tsukuba performs gene modified animal production service publicly. Not only Knock-out strains, also Knock-in, point mutation, and flox strains have been produced by embryo-based genome editing in LARC. Here, we introduce the production processes and results of each kind of mutation.

In almost all cases, we use CRISPR expression DNA plasmid vectors and micro-inject them into pronucleus of C57BL/6 fresh embryos from natural mating. In Knock-out mouse production, two CRISPR target sites per gene are designed for excising the critical exon(s) of target gene. We succeeded in inducing 13 bp to 2.2 Mb excision and the average efficiency of excision mutation induction was 31.0% in 44 projects. In Knock-in mouse production, DNA plasmid donor including 5′ and 3′ homology arms (approximately 1.5 kb each) and insert fragment (0.2 to 7.8 kb) are co-microinjected with CRISPR expression DNA plasmid. The average efficiency of Knock-in induction was 7.8% in 44 projects. Interestingly, there was no correlation between lengths of insert fragments and Knock-in efficiencies. In contrast, the Knock-in efficiencies were correlated with CRISPR cleavage activities. In point mutant mouse production, we used single strand oligo DNA donors. The average efficiency of point mutation induction was 6.5% in 43 projects. There is very little CRISPR target candidates for specific point mutation induction. When there is no CRISPR target, we use the donor plasmid DNA which carried target point mutation in central arm and two synonymous substitutions in borders of each 5′ and 3′ arm. In flox mouse production, two CRISPR expression DNA plasmids and one flox donor DNA plasmid are co-microinjected. The flox donor contains 5′ homology arm-loxP-central arm-loxP-3′ homology arm. In this way, both flox and excised Knock-out mutations were induced. The average efficiency of flox mutation induction was 3.9% in 18 projects. The longest flox region which we induced was 5.1 kb.

These data conclusively demonstrate that embryo-based genome editing with CRISPR expression DNA vector is very useful for inducing every kind of mutations in almost all genetic loci in mouse.

Generation of new CRISPR-edited mouse models for investigating non-syndromic types of albinism

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Albinism is a rare human genetic condition characterized by severe visual abnormalities (foveal hypoplasia, abnormal ganglion cells chiasmatic projections, nystagmus, photophobia and iris transillumination) that may be often, but not always, presented with associated skin, hair and eye hypopigmentation, and affects approximately 1:17,000 newborns. Albinism is genetically heterogeneous. Mutations in at least twenty

genes are associated with the corresponding types of albinism (Montoliu et al. PCMR 2014; Montoliu and Marks, PCMR 2017). Syndromic and non-syndromic forms exit. Syndromic forms impact many different cell types in the body, beyond pigment cells, are less frequent and more severe. Syndromic forms include Hermansky-Pudlak syndrome (HPS, 10 subtypes known, HPS1 to HPS10) and Chediak-Higashi syndrome (CHS, 1 type known). Non-syndromic forms have been traditionally characterized according to pigmentation defects impacting hair, skin and eyes (oculocutaneous albinism, OCA) or only the eyes (ocular albinism, OA). Currently, seven types of OCA are known (OCA1 to OCA7) whereas only one type of OA (OA1) has been reported. Additional forms are being characterized and studied within the albinism spectrum of cases, such as FHONDA (Montoliu and Kelsh, PCMR 2014) and other gene candidates. Historically, we have been using genetically modified mouse models to investigate OCA1 variants (Giraldo and Montoliu, PCMR 2002; Lavado and Montoliu, Front. Biosci. 2006; Murillo-Cuesta, PCMR 2010) associated to mutations in coding and non-coding genomic sequences, using artificial chromosome-type transgenes. More recently, we have been using CRISPR-Cas gene-editing approaches (Seruggia and Montoliu, Transgenic Res. 2014; Mojica and Montoliu, Trends in Microbiology 2016) to functionally assess the relevance of DNA regulatory elements in the tyrosinase gene (Seruggia et al. Nucleic Acids Res. 2015). Moreover, we are now extending the use of CRISPR technology to generate multiple new mouse mutants carrying patient-specific mutations (avatar mice) in which we will be able to investigate the molecular underlying mechanism causing the phenotypic alterations observed in all these cases of albinism. These CRISPR mice will be instrumental to devise adapted therapeutic approaches by which we could possibly treat or alleviate the visual abnormalities found in this human rare disease. In this presentation we will summarize our current efforts and status of development of these numerous new mouse mutants for the different types of albinism generated with the help of CRISPR technology. Small and large deletions, inversions, base-pair substitutions, INDELs have been reproduced in mice after being first genetically diagnosed in patients.

Use of vitrified/warmed zygotes by spatula-MVD to generate CRISPR/Cas9 conditional knockout mouse

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Small transgenic facilities have the great challenge to perform efficient and costeffective services relying on research demand. Genetically modified (GM) mice generation by CRISPR/Cas9 system have been increasing steadily since it allows easy and accurate gene edition for specific locus targeting in the zygote genome. This revolutionary tool have led to a widespread use for biomedical and drug discovery research. In order to optimize the whole GM generation process (from microinjection to cryopreservation of the produced lines), we developed the spatula-MVD system, modified from Tsang and Chow, to vitrify both ovine and murine embryos (Tsang and Chow 2009; Schlapp et al., 2014; Dos Santos-Neto et al. 2015). This adaptation allow us to easily cryopreserve hundreds of GM lines, improving the efficiency of the system. The aim of this study was to produce GM mice using microinjected spatula-MVD vitrified embryos. For that, F2 zygotes from 8-week B6D2 superovulated females were harvested from natural mating. Zygotes were vitrified using spatula-MVD method as described before (Dos Santos Neto PC et al. 2015) and stored in a LN2 tank until use. Eight months later, they were thawed and immediately used for CRISPR/Cas9 microinjection into the cytoplasm. Embryos were injected with Cas9, two RNA guides, and two ssODN carrying a loxP site for a conditional knockout mouse generation. After microinjection, embryos were incubated overnight in 50 μM SCR7 in M16. The following day, two-cell embryos were transferred to 0.5 dpc B6D2 surrogates mothers. Zygote recovery rate after warming was 89.8% (97/108), with a survival rate of 92.8% (90/97). Zygote survival rate immediately after microinjection was 77.7% (56/72). Twenty-four h later, surviving 2-cell embryos (19/56) were transferred to a single surrogate mother, who delivered eight pups. One out of eight carried the complete 3′ loxP site, and another one carried a truncated form of the 3′ construction. The insertion efficiency of 3′ loxP site was 12.5%. In our hands, these results support the use of spatula-MVD zygote vitrification to generate GM mice. These data allows to generate a huge zygote bank ready to use on every microinjection day independently of the superovulation outcome, optimizing thus the whole process.

Improved GONAD (*i*-GONAD) (I) as *ex vivo* manipulation-free genome-editing system allowing efficient knock-out, large deletion, and knock-in

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Abstract: Genome-editing via Oviductal Nucleic Acids Delivery (GONAD) is a novel technique enabling genome editing in mice through intraoviductal instillation of genome editing nucleic acids (e.g. Cas9 mRNA and sgRNA) followed by an in vivo electroporation. It does not require ex vivo manipulation of zygotes, either microinjection or in vitro electroporation of isolated embryos. The genome-editing frequencies in our previous reports (Takahashi et al., *Sci Rep* 5: 11406, 2015; Gurumurthy et al., *Curr Prot Hum Genet* 15.8.1–15.8.12, 2016) was relatively low and the results were limited to only generating only *indel* mutations. In addition, the previous GONAD was carried out at the gestation stage of 2-cell embryos (corresponding to Day 1.5–1.7), which also typically produces higher mosaicism.

In this study, we tested CRISPR ribonucleoprotein (Cas9 protein + crRNA/tracrRNA) on mice at Day 0.7 of pregnancy, which contain fertilized eggs at late one-cell stage. We

observed that frequency of *indel* mutations was increased, reaching up to 100%. The funders also resulted in germline-transmitted genome-*edited* mouse lines. In this *i*mproved GONAD (*i*-GONAD) protocol we observed lower mosaicism than the previous protocol. We next tested if *i*-GONAD can introduce precise single nucleic acid change via homology directed repair (HDR) by rescuing the defect in the *tyrosinase* (*Tyr*) locus in ICR mice. *i*-GONAD procedure that included an ssODN repair template successfully generated founder mice with repaired allele with an efficiency of about 50% and the resultant mice exhibited agouti coat color. Further, we showed that *i*-GONAD can be applicable to generate large deletion ($\sim$16 kb) in the mouse genome and reporter-gene knock-in mice. Since the GONAD does not require ex vivo manipulation of zygotes, it is possible to produce these genome-edited mice even by the researchers who do not possess special skills for ex vivo handling and micromanipulation of embryos.

A "gene editing journey" from gene targeting to CRISPR: impact of CRISPR/Cas in the generation of genetically modified mice

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The Transgenic Mice Unit at the CNIO has been dedicated to the generation of cancer mouse models for almost twenty years. For most of this time, the technology we used to introduce targeted mutations in the mouse germ line was gene targeting by homologous recombination in embryonic stem cells. This way we have engineered hundreds of genetically modified mice carrying knockout, knockin and sophisticated conditional or inducible alleles. The first targeted nucleases available, such as ZFNs and TALENs, increased the repertoire of gene editing strategies although they did not replace gene targeting for general mouse genome manipulation. In the last three or four years, however, the use of the CRISPR/Cas-based gene editing tools have revolutionized not only the way we generate targeted mice but also the way we approach the use of mouse models to answer biological questions. Constitutive knockout alleles were rarely made by gene targeting, since similar effort, time and economic cost was involved in generating the corresponding conditional alleles. Conversely, making constitutive knockouts or introducing constitutive point mutations with CRISPR in the mouse is now even faster and cheaper than importing a mouse strain by rederivation. This makes it worthwhile to generate CRISPR-based constitutive models for quickly answering some relevant biological questions, requiring the use of an in vivo approach directly in F0 mice. Introducing this type of mutations, even multiple ones at a time, in a mouse strain already carrying multiple modified alleles by CRISPR injection in zygotes, is also an efficient, fast and easy. However, generation of conditional alleles (floxed alleles) or large and sophisticated knockin constructs, as they are routinely created by gene targeting in ES cells, requires further optimization with CRISPR. We will present data to illustrate these aspects of the use of the CRISPR/Cas system in mice and its impact in our work engineering mutant mice.

Opportunity Knock's

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This talk will discuss how developments in transgenic technologies could impact on how the scientific community and Society as a whole assess ethical issues arising from the application of such technologies to animal models used in research.

Public concern regarding the use of animals for research purposes is just one aspect of Societal opinions that the UK and other governments monitor. By identifying trends in public opinion both governments and the scientific community can choose to proactively engage with the public on specific topics, to acknowledge concerns and encourage responsible research and innovation (RRI). The Concordat on Openness on Animal Research and other equivalent national initiatives are a great example of proactive engagement of this kind. The genetic modification of living organisms through the application of transgenic technologies is another research area that gives rise to Societal concerns, and has been consider by the Nuffield Council on Bioethics who published a report of their discussions *Genome Editing: an ethical review* (2016). The Nuffield Council on Bioethics are updating their report on *Genome Editing* and so it is perhaps timely for ISTT members to discuss how developments in transgenic technologies might contribute to greater implementation of the 3Rs given that these principles are now firmly embedded as central to the practice of responsible animal research. There may also be benefits from reflecting on whether current levels of public engagement with respect to the development of transgenic technologies is appropriate, or indeed whether broader concerns regarding the reproducibility, reliability and translatability of research are applicable to and/or need to be addressed by the transgenic community.

Effects of voltage strength on development and quality of electroporated porcine embryos

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Introduction: Recently, we established the GEEP (gene editing by electroporation of Cas9 protein) method, in which the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system is introduced into porcine zygotes by electroporation, leading to high-efficiency disruption of the targeted gene. To further determine the suitable conditions for electroporation in the GEEP method, we investigated the effects of pulse polarity and voltage on the development and quality of porcine embryos.

Methods: In the first experiment, we examined the effect of pulse polarity with unipolar and bipolar pulses on the

development of porcine in vitro fertilized (IVF) zygotes. The zygotes were electroporated by either unipolar or bipolar pulse, keeping the voltage, pulse duration, and pulse number fixed at 30 V, 1 ms, and five repeats, respectively. In the second experiment, we tested the effect of electroporation voltages on development and quality of porcine embryos. The zygotes were electroporated by a range of electroporation voltages at 20–40 V, using five 1-ms unipolar pulses. In the third experiment, we compared the effect of two electroporation **voltages (25 and 30 V) on the efficiency of genome editing in the porcine zygotes. The zygotes were electroporated with 400 ng μl^{-1} of Cas9 mRNA and 200 ng μl^{-1} of single-guide RNA (sgRNA), targeting the *FGF10* gene by electroporation at 25 and 30 V.

Results: We observed that the rates of blastocyst formation in zygotes electroporated by bipolar pulses decreased as compared with the rate in those electroporated by unipolar pulses. The rates of cleavage and blastocyst formation of zygotes electroporated at the voltages of 35 and 40 V were significantly lower ($p < 0.05$) than those electroporated at the lower voltages. Moreover, the apoptotic nucleus indices of embryos derived from zygotes electroporated at voltages more than 30 V were significantly higher ($p < 0.05$). When the zygotes were electroporated with Cas9 mRNA and sgRNAs targeting sites in *FGF10* exon 3 at voltages of either 25 V or 30 V, a higher efficiency genome editing in the resulting blastocysts was obtained at 30 V.

Conclusions: The results demonstrate that the bipolar pulses had a detrimental effect on the development of electroporated zygotes. Moreover, when using five 1-ms unipolar pulses, the electroporation voltage of 30 V may be suitable for introducing CRISPR/Cas9 system into pig IVF zygotes.

Analysis of mouse blastocysts to assess in vivo activity of sgRNAs

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A major challenge for transgenic facilities has been to identify the optimal sgRNA for use in vivo. There are online tools used to predict which sgRNA will produce the best cutting with the fewest off target sites and in vitro analyses (Surveyor/T7E1) used to test the ability of the sgRNA to direct Cas9 cutting. However, online tools and in vitro tests are not always predictive of in vivo capabilities. *In vivo* detection of the desired mutation can be impaired both by the degree to which the mutation is present and the adjacent "noise" in the system caused by additional mutations. We have devised a simple method to test sgRNAs in vivo. The injection components are divided into a small (10 µl) testing sample and a large (30 µl) injection sample (frozen for later use). A small number of embryos (10–20) are injected with the test sample and then cultured 2.5–3.5 days to verify that the injection components will support embryonic development. DNA is then obtained from the embryos that develop further in order to examine how well the sgRNA cut and, if supplied, donor DNA inserted. Nested PCR is performed to amplify the region in which cutting and/or homology driven repair (hdr) should occur and the PCR products are sequenced. The efficiency of cutting and insertion is determined; both indels and point mutations can be simultaneously detected. Since the Surveyor/T7E1 methods utilize gel shifts based on fragment size, and because the assayed mutation is often within 30 bp of the PAM, this in vitro method will lack the precision to identify small insertions and deletions, especially if they exist in only a few cells due to mosaicism. Upon analysis of the injected blastocysts, we were able to identify both indels and point mutations that occurred at a level of approximately 5% or more. As well, we could clearly identify both bi-allelic and homozygous mutations. We conclude that the use of this simple, in vivo analysis will allow us to easily identify the most effective sgRNA/donor DNA pairs for generating mutations via pronuclear injection.

Lmx1b-Dre, a CRISPR generated knock-in mouse for Dre mediated labelling of excitatory dorsal horn neurons

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The Cas9/CRISPR technology has revolutionized the generation of transgenic animals allowing precise targeted modification such as site specific deletions (knock-out) and integrations (knock-in) by directly manipulating mouse zygotes. We have used Cas9/CRISPR to generate a mouse line specifically expressing Dre recombinase in excitatory dorsal horn neurons. The P2A-Dre sequence was inserted just upstream of the termination codon of the mouse Lmx1b gene by microinjection of C57BL/6 J zygotes. Our results demonstrate the feasibility of rapidly generating novel recombinase driver lines using Cas9/CRISPR technology directly in mouse embryos. These driver lines can be used with newly developed Dre reporter lines or in combination with Cre/Dre tandem recombinase systems to achieve unprecedented resolution in lineage tracing experiments.

Using CRISPR to investigate the role of a placental-specific long non-coding RNA in mouse

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The placenta is a distinguishing feature of mammals and is essential for normal embryonic development. During pregnancy, the placenta serves as the interface between the fetus and the mother. Many unsuccessful pregnancies are the result of abnormal placental development, therefore it is important to identify the genes that control the normal state of development. Our hypothesis is that long non-coding RNAs (lncRNAs) that are expressed uniquely in the placenta play an essential role in

the development of this organ, and when dysregulated will perturb implantation and normal embryonic development. Extensive transcriptomic analysis has now confirmed that 70–90% of the genome is transcribed, yet only 1–2% codes for proteins, therefore a large portion of the genome is non-coding. These non-coding regions were once thought of as junk, but non-coding transcripts such as lncRNAs are now known to be involved in important regulatory networks that drive developmental programs. It is the goal of this study to identify lncRNAs that are important for placental development. In this study, transcriptomic analysis of E7.5 mouse extra-embryonic tissues has identified several placental-specific lncRNAs. From this data one lncRNA in particular, *483Rik*, was selected for further study. RT-PCR analysis has revealed specific expression of *483Rik* in the placenta at multiple time points during development. Furthermore, in situ *hybridization* has revealed expression in the labyrinthine region of the placenta, where exchange between the maternal and fetal circulatory systems occurs. To understand the function of this lncRNA, the CRISPR system has been utilized to delete a large portion of the gene. Three heterozygous *483Rik* knock-out mice have been generated and are now being bred to analyze resulting phenotypes which will reveal any functional role of *483Rik* in the placenta. Additionally, CRISPR is being used to produce a reporter cassette knock-in at this locus to leverage this gene for future placental-specific transgene expression. The placenta is arguably the most important organ for early mammalian growth, yet is the least understood. Identifying genes that are essential for normal developmental is the first step in understanding the causes for abnormal development. This study is expected to expand our current understanding of the role of lncRNAs in reproductive health and early pregnancy loss as it relates to placental development.

Multiple transgenic rabbit models of human diseases

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Successful generation of the first transgenic rabbits was reported in 1985. Since then, transgenic rabbits have been generated and used as animal models for a variety of human diseases and as bioreactors for production of pharmaceutical proteins. Although transgenic mice are still dominantly used in biomedical research, it is known that the causative mutations resulting in human diseases do not cause corresponding pathological changes in mice in some instances. Additionally, a number of techniques are difficult to apply to mouse models due to their small size and phylogenetical features. Alternatively, the rabbit (*Oryctolagus cuniculus*) with an intermediate size between rodents and large farm animals and closer phylogenetical features to primates has attracted more attention in recent years in biomedical and pharmaceutical research. Transgenic rabbits have been most commonly used as models for studies of cardiovascular diseases, such as atherosclerosis, hypertrophic cardiomyopathy and long QT syndrome, in addition to their use in studies of certain infectious diseases. Our lab was one of a few labs in the world which successfully generated several of the earliest transgenic rabbits. Since then, more transgenic rabbit lines have been produced in this lab, including the expression of papillomaviral genes, EJras gene, HLA-A2.1 gene, LQT1 and LQT2 genes, etc. These transgenic rabbits have been used as models for studying the tissue specific expression of papillomavirus infection and cancer development, development and regression of keratoacanthomas, host immune reaction to human papillomaviral antigens and long QT syndrome.

V2G locus editing for production of GLP1Ser8-M3R transgenic pig islets

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Xenotransplantation of modified pig islets could be a possible medical treatment for T1/T2 diabetic patients. Programmable nucleases (ZFNs, Talens and CRISPR/Cas9) allow precise genetic engineering into a selected genomic locus. We characterised a genomic sequence identified as a new porcine safe-harbour genomic V2G locus (EP2921048) for the safe and efficient expression of transgenes. Recently we reported that islets treated in vitro with adenoviral vector, expressing a dipeptidyl peptidase-resistant form of GLP1 (GLP1Ser8), and a constitutively activated muscarinic receptor (M3R) genes, demonstrated an increased glucose-stimulated insulin secretion (Mourad et al., 2017). The aim of the present work was the creation of targeted modified-V2G pigs, expressing the Insulin-GLP1Ser8M3R transgene in islet beta-cells. Primary porcine adult fibroblasts ($2x10^6$ cells) of low PERV A/B and PERV C negative boar were co-transfected (Nucleofector, V-024 program) using the V2G-Talens set (4 μg) and the V2G-InsGLP1Ser8M3R targeting vector (4 μg; LeftHomologyArm = 568 bp; RightHomologyArm = 1187 bp; Insert = 11.2 Kb; floxed-$Puro^R$;2x MARs from chicken beta-actin gene). Two days post transfection (DPT) cells were plated (200 cells/150 mm dish) and selected with Puromycin. Resistant colonies were picked up, expanded for PCR and cryopreserved for Somatic Cell Nuclear Transfer (SCNT). All colonies were PCR-analysed (3 assays) for the targeting event occurred into the V2G locus and the presence of the intact coding sequence. Selected colonies were used for SCNT (Lagutina et al., 2006) and resulting cloned pigs were analysed.

Eighty-eight well growing colonies were analysed by PCR and 5 (5,7%) KI at the V2G locus colonies were sequenced. In SCNT experiments 654 morulae/blastocysts (MC/BL, 49%) were transferred into 7 synchronised sows, all became pregnant and delivered 42 alive, 8 stillborn and 5 mummified piglets with developmental efficiency 8,4% from MC/BL. The analysis of piglets confirmed the monoallelic-targeted transgene insertion in the V2G locus. However during extensive molecular characterisation of the piglets we identified also random integrations of targeting vector.

In conclusion, Talens-mediated gene targeting of pig genome using short homology arms to KI a large transgenic cassette and production of healthy piglets was confirmed at V2G-locus. Transgenic Ins-GLP1Ser8-M3R piglets are currently studied for the in vivo and in vitro characterization of their islet's glucose-stimulated insulin secretion. However screening for random integrations should be always included if this has to be avoided during the genomic characterization of targeted cells/animals to establish a fully characterized pig founder line meeting the regulatory requirements for future clinical trials.

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Management of mouse sperm freezing and quality control

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Generally, in research an increasing set of transgenic and gene-deficient mouse lines and intercrosses thereof is maintained in the animal facility. Due to a changing research focus some mouse lines are not currently under investigation. These lines are then routinely bred in a "maintaince mode" in order to keep the genetic modification. This is especially the case if the gene modification will not be considered by repositories. However, even with a very limited set of breeders, offspring is constantly arising and animal numbers increase, ultimately limiting animal capacity for "active lines" and increasing costs an animal numbers. The management of transgenic and gene-deficient mouse lines therefore requires management strategies to reduce the number of breeding lines, to reduce cost and to diminish typing work.

In order to do that, systematic sperm freezing was introduced in the laboratory, based on the protocol of Takeo & Nakagata[1] for mouse lines on a C57BL/6 N background. A set of report and data forms was developed for documentation and quality control of cryopreservation and in vitro fertilization (IVF). Up to now, 10 lines have been successfully cryopreserved and successfully rederived by IVF of mouse oocytes (C57BL/6 N). In order to test sperm viability and gene modification PCR conditions for different alleles were optimized based on the method of Scavizzi et al.[2], allowing typing of fertilized embryos already at the blastocyst stage. This approach reduces the numbers of foster mice and born offspring required for proof of sperm viability and freezing of the correct gene modified mouse line.

Frozen sperm also was used to transfer mouse lines to other laboratories, reducing animal stress during transport and reducing quarantine procedures at the receiving animal facility.

Routine sperm freezing therefore was introduced into the management of our transgenic and gene-deficient mouse lines in the laboratory which keeps all generated alleles available and provides a simultaneous reduction of animal numbers required in research.

1. Takeo T, Nakagata N.; Biol Reprod. 2011; 85:1066-72.
2. Scavizzi et al.; Transgenic Res. 2015; 24:921-7.

Screening for off-target CRISPR mutations: it's never too late

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The CRISPR-Cas9 system is a quickly growing tool to simply and effectively manipulate the genome of an organism. In our case of genetically engineered rodent models, this system helps to heavily reduce timeframes of custom animal production. CRISPR is quickly becoming popular though off-target mutations are still a concern. While WGS-NGS can be performed to address this, it is costly and time consuming. Instead, a straightforward PCR validation can be used.

When founders are identified within a germline colony, they can be genotyped by qPCR (TaqMan), a quick and highly efficient way to interrogate loci though allelic discrimination. However, this process prevents discovery of off-target mutations. Thus, we perform an initial PCR validation followed by fragment analysis using the ABI 3730XL Capillary Electrophoresis system. With resolution of a single nucleotide, this genetic analyzer can reveal peaks that don't fall into expected bins, demonstrating off-target mutations.

These off-target mutations can be further analyzed using ddPCR (such as BioRad's QX200 device) which verifies how many copies of each allele an animal carries, another important validation step. It is crucial to prevent breeding of these animals to maintain colonies with expected/proper mutations for investigators' studies. Here, we present a case scenario where we identified an off-target mutation from CE platform and validate on ddPCR platform. This is one lesson where the oldest technology happens to be an essential tool in this emerging field.

RNAi and CRISPR/Cas9 based *in vivo* models for drug discovery

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With the advent of CRISPR/Cas9 technology, the speed and precision in which genetically engineered mouse models can be created is unprecedented. We now have at our disposal a genetic toolbox that will enable the rapid generation of sophisticated mouse models of human disease. We have a powerful new SplashE algorithm for reproducible RNA interference (RNAi) technology, which can be exploited experimentally to effectively and reversibly silence nearly any gene or gene combinations not only in vitro but also in live mice. Here, we take advantage of these powerful technologies and combine both CRISPR/Cas9

and inducible RNAi-mediated gene silencing to not only model disease, but also mimic drug therapy via RNAi in the same mice, giving us advanced capabilities to perform preclinical s tudies in vivo. Furthermore, we have harnessed the power of CRISPR/Cas9to create a novel platform for more rapid and cost-effective production of RNAi models that will serve as entry into the generation of higher organisms such as rats and guinea pigs with reversible gene silencing capabilities in the near future. Undoubtedly, extending our RNAi technology into other species will transform cancer research by providing model systems in which disease and therapeutic outcomes more closely resemble their human counterparts. The ability to better model clinical disorders and evaluate genetic and environmental stimuli in advanced mammalian species will increase our confidence in predicting drug responses in humans and push drug discovery research into a new era.

Genome editing in rats: generation of a target point mutation by pronuclear or cytoplasmic injection or by electroporation of CRISPR/Cas9

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CRISPR/Cas9 system has become a powerful and efficient tool to edit genomes. It can be applied on biomedicine for the development of new gene therapy approaches, and in biotechnology for the generation of new genetically modified organisms. It allows to produce knockout and knockin models avoiding need of embryonic stem cells (ESC) for gene targeting by homologous recombination and opening the possibility of genome edition for all animal species.

Rats are physiologically, genetically and morphologically closer to humans, have a well-characterized and rich behavioral display for neurological studies, and have larger size than mice. But the difficulty of working with rat ESC has limited the generation of genetically modified rat models. CRISPR system has overcome these troubles and allows genome editing by direct injection of one cell embryos.

Here we show and compare the results obtained in the generation of a rat model carrying a point mutation by pronuclear or cytoplasmic microinjection or by electroporation of CRISPR system in one cell embryos.

Generating murine models in NZBWF1/J background for systemic lupus erythematosus

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The hybrid between New Zealand Black (NZB) and New Zealand White (NZW) mice, NZBWF1/J, is a classic model for systemic lupus erythematosus (SLE), an autoimmune disease. Generating mutant models in the NZBWF1/J background is technically challenging, requiring time-consuming backcrossing of mice to generate SLE models in this background. To reduce the timeline, BAC DNA was injected via conventional pronuclear injection into NZW zygotes, and positive mice expanded from the resultant G0 founder mice (those expressing the transgene) were crossed to wild type NZB mice to generate transgenic (TG) models in the NZBWF1/J background. C57BL/6 N and NZW strains were compared using two BAC constructs. Relative to NZW mice, C57BL/6 N mice exhibited a significantly higher mean embryo yield (30.1 vs. 9.0), birth rate (18.7% vs. 1.4%), and mutation rate (1.3% vs. 0.1%). For knockout (KO) models, CRISPR reagents were injected into the cytoplasm of NZB and NZW zygotes, and homozygous KO mice produced from NZB and NZW G0 founders were intercrossed to generate the KO mice in the NZBWF1/J background. The birth rate after cytoplasmic injection was higher in the NZW strain than in the NZB strain (18.3% vs. 6.0%). However, mutation rates were similar between NZW (2.3%) and NZB (1.8%) strains. To increase the oocyte yield, an ultra-superovulation technique using inhibin antiserum (IAS) and hCG was implemented, which increased embryo yield for the NZW strain from 9.0 per donor to 25.5 per donor by natural mating. To reduce the number of stud males used for zygote production, IVF was performed. The average fertilization rate was 65.9 and 0.4% for NZB and NZW, respectively, which was unexpected given that the NZB strain is the challenging breeder of the two. Our approach demonstrates that first generating TG and KO models in NZW and/or NZB strains, followed by intercrossing, is a feasible alternative to generate SLE models in the NZBWF1/J background.

SMPD1 knockout golden Syrian hamsters develop spontaneous hyperlipidemia with elevated apolipoprotein B cholesterol

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Atherosclerosis is the most common cause of heart disease, the leading cause of death worldwide. Development of atherosclerosis is caused by accumulation of modified lipoproteins in the vascular wall and subsequent inflammation. Acid sphingomyelinase (ASM) is an enzyme coded by the *SMPD1* gene that is commonly proposed as a therapeutic target for atherosclerosis. ASM directly affects endogenous cholesterol levels through the metabolism of sphingomyelin, which sequesters cholesterol in cell membranes and lipoproteins. Metabolism of sphingomyelin reduces endogenous cholesterol production, promotes cholesterol efflux, and reduces cholesterol absorption from the intestines. Humans with mutations causing ASM deficiency develop cholesterol abnormalities that lead to atherosclerosis at a young age. Unfortunately, also ASM exhibits an apparent contradictory role in the vascular wall where metabolism of sphingomyelin causes lipoprotein aggregation, a modification that promotes their retention and lesion development. To develop appropriate therapeutics and accurately predict their systemic effects, it is necessary to

employ animal models that demonstrate both the positive and negative effects of ASM on atherosclerotic lesion development. The only animal models available to investigate ASM regulation are mice, which differ significantly from humans in cholesterol metabolism. *SMPD1* knockout (*SMPD1*$^{-/-}$) mice develop hypercholesterolemia with elevated high density lipoprotein (HDL) cholesterol and low apolipoprotein B (ApoB) cholesterol, while humans with ASM deficiency exhibit elevated ApoB cholesterol and low HDL cholesterol. The lipid profile of *SMPD1*$^{-/-}$ mice protects them from atherosclerosis, opposite ASM-deficient humans. Thus, the *SMPD1*$^{-/-}$ mouse cannot be used to study the effects of ASM on blood lipids or to predict the systemic effects of therapeutics regulating ASM. Golden Syrian hamsters more closely resemble humans in cholesterol metabolism, thus we suspected that ASM deficiency in hamsters would yield blood lipid changes similar to ASM-deficient humans. We have successfully employed the CRISPR/Cas9 system to create a strain of *SMPD1* knockout (*SMPD1*$^{-/-}$) golden Syrian hamsters. We collected blood for analysis from an 11 week-old female *SMPD1*$^{-/-}$ hamster carrying a 17 base pair deletion in both alleles of exon 1. Results demonstrated that this individual developed spontaneous hyperlipidemia with elevated ApoB cholesterol (314 mg/dL; normal 21.02 ± 3.9 mg/dL) and reduced HDL cholesterol (75 mg/dL; normal 104.08 ± 10.56 mg/dL). We expect that this model can be applied to future studies investigating the contributions of ASM to atherosclerosis and as a model to test therapeutics that regulate ASM activity.

CRISPR and the 3Rs—reducing animal numbers in high-throughput mouse production

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The CRISPR/Cas9 system has revolutionised the way in which researchers study genetics and biological processes. Due its ease in disrupting gene function it is now a major part of the continuing IMPC project to knock out all protein-coding genes in the mouse genome. One of the main attractions of CRISPR is the greatly-reduced time needed to create mutant mouse models, as experiments can be performed directly in mouse zygotes without the need for gene-targeting in ES cells.

By comparing our existing ES cell-based high-throughput mouse production to our new CRISPR pipeline, we show that the germ line transmission rate is much more predictable using CRISPR. This has allowed us to optimise many aspects of our mutant founder production, including PCR screening, embryo transfer numbers and founder mating numbers. F0 mosaics transmit the mutation over a much wider range of percentage mutagenesis than their ESC-chimera counterparts.

We demonstrate that in addition to efficiency gains in cost and time, there is also a significant reduction in animals required to achieve GLT. When applied to the scale of projects such as the IMPC, the benefit from a 3Rs-perpective is enormous.

Improved GONAD (*i*-GONAD) (II): usefulness of rhodamine-dextran for monitoring the success of the GONAD and of gonadotrophin-based regulation of the timing for the GONAD

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We previuosly developed a method (called "genome-editing via oviductal nucleic acids delivery, GONAD") that does not reqiure ex vivo handling of preimplantation embryos for producing genome-edited mice (Takahashi et al., *Sci Rep* 5: 11406, 2015; Gurumurthy et al., *Curr Prot Hum Genet* 15.8.1–15.8.12, 2016). As demonstrated in our first report, GONAD is performed by simple instillation of CRISPR/Cas9-genome editimg components into the lumen of the oviduct corresponsing to 2-cell stage (day 1.5 of pregnancy) and applying in vivo electroporation (EP). Here we assessed some parameters of the GONAD procedure in order to improve it further. First, we tested if day 0.7 of pregancny, the time that correspondes zygotes stage, is suitable for GONAD. This addresses the high rate of mosaicism that can likely occur if the procedure is performed at day 1,5 of pregancny (2) Our results show that GONAD at Day 0.7 of pregnancy (16:00–19:00), corresponding to the late zygotic stages, was more efficient which could be because almost cumulus cells that potentially hamper uptake of nucleic acids instilled intraoviductal are thought to be detached at this stage. Second, to address the inconvenience of superovulation timing to prepare the animals for the procedure (evening in the day when vaginal plug is found), we thought that the timing of GONAD may be shifted forward by administering low dose of gonadotrophins that are used to superovulate at an early time. To verify this strategy, B6C3F1 hybrid female mice were administrated intraperitoneally with 1 to 2 IU of PMSG on 11:00, followed by hCG of the same dose 48 h later. Then, the females were mated to males. In the next morning, the GONAD was performed towards the pregnant females on 11:00 to 13:00 (which corresponds to Day 0.7). Third, we tested if adding 1 μg/μl of rhodamine-dexran 3 kDa to the instillation solution can serve as a visible flouresecent marker for succesfullness of the GONAD procedure. The instillation mixes also contained 0.5 μg/μl of EGFP mRNA in order to test whether the rhodamine-dexran can be used as a fluorescent marker alternative to the EGFP. The oviducts dissected on 11:00, 24 h after hCG administration, had cumulus cell-free zygotes. GONAD trials at this stage led to production of ~60% of embryos showing both red and green fluorescence, suggesting usefulness of

rhodamine-dexran 3 kDa to monitor the successful uptake of substances by an embryo. We are now testing whether genome edited live born pups can be obtained by these improved GONAD parameters.

CRISPR/Cas9 genome editing pipeline for mice and rats

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CRISPR/Cas9 is a RNA guided nuclease that produces double strand breaks in DNA. Mouse and rat zygotes repair the chromosome breaks with non-homologous endjoining (NHEJ) or homology directed repair (HDR). NHEJ repair often produces deletions in critical regions that knockout gene expression. HDR repair can introduce new information into the genome that causes the expression of proteins with mutant amino acid codons or cDNAs that code for fluorescent reporter proteins such as EGFP or recombinases such as iCre. sgRNA targeting critical regions in Pipeline genes are identified by selecting sgRNAs predicted to be active. sgRNAs are prepared in plasmid, synthetic sgRNA, or as synthetics crRNA and tracRNA. sgRNA is validated in vitro: 1) cleavage of PCR templates in vitro after mixture with Cas9 protein and in vivo: 2) microinjection into mouse zygotes and testing blastocysts for NHEJ repair. sgRNA used to target genes in mouse ES cells are prepared in plasmids and tested in ES cells.

CRSIPR/Cas9 technology was used to produce 955 gene-edited founders for 106 new genetic mouse and rat models. Examples of models produced include large and small deletions of the genome for gene knockouts or truncations; point mutations; conditional alleles (floxed genes); reporter knockins (fluorescent proteins, Cre recombinase, FLP recombinase, cDNA, etc.); and transgenes targeted to the ROSA26 locus. Results will presented on 1) producing gene knockouts in mouse and rat strains, 2) introducing coding SNPs knockins with oligonucleotide in mice and rats, 3) producing reporter gene knockins in mice and rats, 4) producing floxed genes with a novel one-cut strategy in mice. Analysis shows that mosaic founders occur frequently. Mutations observed in founders vary from deletion/insertion of a few nucleotides to the deletion of several hundred base pairs. These patterns are observed in gene knockouts in both mouse and rat models. The efficiency of CRISPR/Cas9 targeting is lower in inbred C57BL/6 J mice than in hybrid mice. Experiments are underway to evaluate the activity of recombinant enhanced specificity Cas9 protein (eSpCas91.1).

Production of mouse and rat gene knockout models with reagents designed and produced in the Transgenic Core are guaranteed. The efficiency of oligonucleotide knockins is lower, the majority of SNPs succeed. ROSA26 knockins have good efficiency. The introduction of complex alleles such as multi-reporter knockins (e.g. iCre-P2A-mCherry) are less efficient and cannot always be guaranteed. Compared to preceding technologies, CRISPR/Cas9 significantly increases access to mouse and rat genomes for the generation of biomedical research models.

Introduction of targeted point mutations in mice using CRISPR-Cas9

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Creation of point mutations by conventional gene targeting in mouse embryonic stem cells is a slow and laborious process. We report here our findings using CRISPR-Cas9 to create point mutations in three genes, *Eef2 k*, *Cic*, and *Kcnma1*, in C57BL/6 J, B6/CD-1, and BALB/cJ genetic backgrounds. Injections were performed using oligo concentrations at either 50 or 100 ng/ul, Cas9 mRNA concentration between 25–100 ng/ul, and gRNA concentrations between 12.5–50 ng/ul. Overall, we found that from 913 embryos transferred, 49 pups were born ($\sim$5%) with 36 present at weaning. As the number of *Cic* pups born was particularly low, we performed Caesarian sections on several *Cic* embryo-recipient female mice just prior to delivery. We found many small "white spots" in the uterine horns of the recipient females suggesting that the injected embryos had implanted but that they had died early in development. Following genotyping of the pups for the three different mutations, at least 10 of the 36 mice showed evidence for gene editing by gel analysis and or by sequencing. Four mice were identified that had the correct mutation (*Eef2 k*—1 mouse, *Cic*—1 mouse and *Kcnma1*—2 mice). Genotype analysis performed by our core on *Kcnma1* mice revealed that 3 of the 4 mice born contained the introduced MvaI restriction site although mouse #1 had other events suggesting it might be mosaic for multiple events. Mouse #2 and mouse #3 appeared homozygous for the targeted mutation as no wild-type allele was observed. This was confirmed by breeding analysis. Sequence analysis revealed that mouse #2 had the introduced CT to TC mutation and no other change nearby. Analysis of four potential off-target genes detected no other alterations in mouse #2. Sequence analysis of progeny from mouse #3 revealed that approximately half inherited the correct CT to TC mutation, while the other half inherited the CT to TC mutation plus a 1 base pair deletion 2 nucleotides upstream of the targeted nucleotides. Together our results suggest that the number of pups born from oligo injections are reduced, but that a significant number of the pups that are born carry the introduced mutation and that mice with the correct point mutation, including homozygous founders, can be identified as soon as 6 weeks after injection.

Precision medicine in action: using CRISPR/Cas9 to generate a human disease SNP rat model by electroporation of a single stranded DNA oligo

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The aim of precision medicine is to create customizable prevention and treatment options in populations with specific diseases and disorders. Coupling genetic and molecular profiling with development of precision animal models is a critical first step in precision medicine. Desmin-Related Myofibrillar Myopathy (DRM) or desminopathy is a heritable mypothic disease caused by mutation in the gene that codes for the Desmin protein, which regulates muscle structure/function. Mutations to Desmin result in contraction-induced skeletal muscle injury, idiopathic cardiomyopathy, arrhythmogenic right ventricular cardiomyophathy (ARVC) and premature death. Although Desmin knockout mice are currently available, rats are a superior model species for neuromuscular disease, and therefore, ideal for studying desminopathy. We set out to replicate the exact two-point mutation in the Desmin gene, originally identified in a family of patients, using a precision SNP knock in by single stranded DNA oligo assisted homology directed repair (HDR). Specifically, we sought to replace nucleotides AG with CC in the 1036–1037th coding region within the 246th amino acid on exon 6. To generate this model, we tested both electroporation and microinjection of complexed Cas9 protein with single stranded (sg) RNA guides and a single stranded DNA oligo (ssODN) engineered to replace the two base pairs within the Desmin gene by HDR. In our first experiment, we transferred a mix of microinjected and electroporated embryos into a 9-week old Spraque-Dawley rat. One of the three offspring successfully carried the two precision SNPs, another possessed an indel and the third was not edited. At maturity, the HDR Desmin mutant male was bred to a wild-type female, yielding 3 HDR offspring of a litter of 5, thus confirming the heritability of the mutation. The second experiment set out to determine whether electroporation would be an effective gene modification technique compared to traditional microinjection. Three 9-week old Spraque-Dawley females were implanted with electroporated or microinjected (two and one rats respectively) zygotes. One pup from the electroporated embryo group carried the two precision SNPs in the pathomimetic Desmin gene. All other pups ($n = 4$) across both groups contained frame-shift indels. Despite small animal numbers, we successfully reproduced the specific familial Desmin mutation using electroporation in Sprague–Dawley rats. We have additionally demonstrated the ability to establish a line of Desmin rats containing the precision SNP knockin using ssODN assisted HDR by electroporation and subsequent breeding.

Non-surgical embryo transfer in rats: a 3Rs refinement for assisted reproductive technology

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A 3Rs concern for rat research models is the requirement for surgical transfer of embryos after targeted mutagenesis or as an assisted reproduction technique. In order to eliminate the need for surgical embryo transfer, rat non-surgical embryo transfer (rNSET) technology has been developed. NSET technology supports humane production, maintenance, preservation, and transport of existing and newly developed rat strains.

Anatomical measurements of the rat reproductive tract were compared in two rat strains with significant differences in body size (>50 g); Sprague–Dawley (SD) and Fischer 344 rats. No statistical difference was observed between the two strains of rats for various aspects of reproductive tract measurements. The similarity in the essential parameters for rNSET device and speculum design enabled development of a single device. Prototypes were tested for ease of use, depth of insertion, and safety. One device was then chosen for embryo transfer testing.

Comparison transfer studies were performed with blastocysts (4.5dpc) from SD females transferred by surgical or rNSET technique to 3.5dpc pseudopregnant SD recipients. For the rNSET procedure, the cervix of the female was dilated with 1–2 IU oxytocin. The rat speculum was inserted vaginally. The rNSET device catheter was inserted through the speculum, past the cervix, and into the uterine horn where the embryos were deposited. The rNSET device and speculum were then removed. For surgical embryo transfer, a dorsal midline incision was made, through which the ovary and uterine horn were exposed. A small incision in the uterine horn was created and embryos were delivered by pipette. The ovary and uterine horn were returned to the abdominal cavity and the incision closed with suture and wound clips. The efficiency of transfer (live births/embryos transferred) was similar for rNSET (40%) and surgery (37%).

The use of a non-surgical procedure as a replacement for a surgical procedure provides clear advantages to the welfare of animals in research. However, to determine if a non-surgical procedure produced a more stressful situation than a surgical procedure (with appropriate anesthesia and analgesia), the stress response for two noninvasive stress biomarkers was measured. For these studies, weight loss and fecal corticosterone levels were compared for rats having surgical (isoflurane, 4 mg/kg meloxicam, 0.05 mg/kg buprenorphine) and non-surgical (no anesthesia/analgesia) embryo transfer. No increase in stress was observed over control animals for either embryo transfer method. Therefore, this non-surgical embryo transfer technique provides an efficient 3Rs alternative to surgical embryo transfer for the rat research model.

The phenotype of Taz null mutation in mouse closely resembles Barth syndrome

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An accurate mouse model of Barth Syndrome (BTHS) would greatly facilitate an understanding of the molecular bases of how mutation of the *Tafazzin* (*Taz*) gene causes the pleiotropic effects observed in the clinic. We have generated

a mouse line carrying a conditional knockout mutation of the Taz gene.

Analysis of RNA and protein confirmed the KO mice no longer express Taz. As observed in patients with Barth Syndrome, Taz^{KO} mice show decreases in levels of the mitochondrial phospholipid cardiolipin and increases in monolysocardiolipin levels, in heart, liver and skeletal muscle.

The Taz^{KO} mice are around 30% smaller than their wild-type littermates at 6–8 weeks. KO males are born at less than 50% of the Mendelian ratio. Preliminary and on-going phenotyping of Taz-KO mice using echocardiography and ECG indicate that Taz-KO mice exhibit cardiac left ventricular wall thinning, reduced cardiac systolic and diastolic function and prolongation of the QT interval.

Assessment of mitochondrial morphology by electron microscopy shows substantial alterations in the Taz^{KO} mice, particularly in the heart. Expression profiling of heart tissue and metabolomic analyses of heart, plasma and urine confirmed significant alteration of mitochondrial function in the Taz^{KO} mice.

Taken together these results suggest these mice represent a good model of the clinical consequences of Barth Syndrome, which will be useful for understanding the processes underlying the disease and for testing the efficiency of possible therapies, to reverse the changes observed in the Taz^{KO} mice.

Efficient use of vitrified or refrigerated pronuclear zygotes for gene-modification in rats with CRISPR/Cas9 system

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New genome-editing tools, such as ZFNs, TALEN and CRISPR/Cas9, have enabled the generatation of gene modified models effectively, not only small experimental rodents but also large domestic animal species. The MCW Gene Editing Rat Resource Center (http://rgd.mcw.edu/wg/gerrc, GERRC) is in the process of generating ~200 genetically modified rat strains using these technologies. Cryopreservation or cooled storage of pronuclear (PN)-stage zygotes makes routine generation of transgenic rats more convenient. Rat embryos can be cryopreserved by 2-step freezing (Hirabayashi et al., Exp Anim 1997) or vitrification procedure (Eto et al., Cryobiology 2014). More than 58% of the cryopreserved embryos are capable of developing to full-term when transferred to pseudopregnant females. However, with regard to cryopreserveation of pronuclear-stage zygotes, previous reported that 95% of vitrified zygotes appeared morphologically normal and only 22% of them developed into fetuses following oviductal transfer (Takahashi et al., Transgenic Res 1999). In this study, pronuclear stage rat zygotes were cryopreserved by vitrification or 1-day stored at 4 °C, and then used for generation of knocked-out (KO) rats with CRISPR/Cas9 system. Transfer of fresh, 4 °C-stored, and vitrified zygotes retrieved from SS rats (SS/JrHsdMcwi, RGD: 61499) gave birth to viable offspring at 81, 62, and 76%, respectively. Three different sets of guide RNA/Cas9 dual expression plasmid, designed for inducing mutation in 3 targeted genes, were microinjected into the PN zygotes, resulting in comparable survival rates (fresh, 90–97%; 4 °C-stored, 88–91%; vitrified, 86–92%) and offspring rates (fresh, 6–39%; 4 °C-stored, 9–38%; vitrified, 8–38%). Efficiencies of generating KO rats were not different among the fresh group (8%, 23/300), 4 °C-stored group (9%, 10/115), and vitrified group (4%, 12/268) when data for targeting different genes were pooled. These results indicate the successful generation of KO rats using vitrified and refrigerated PN zygotes for the first time.

Keywords: Cooled storage, CRISPR/Cas9, Pronuclear zygotes, Rat, Vitrification

Optimization of CRISPR reagent delivery using the zygote electroporation of nucleases (ZEN) method

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The traditional approach to the delivery of CRISPR/Cas9 reagents (sgRNA, Cas9, donor molecules) into mouse embryos for genome modification is via direct microinjection. This necessitates having expensive microinjection equipment, significant microinjection expertise, and multiple rounds of injection over several days. Recently Sean Chen, et al.[1] describe zygote electroporation of nucleases, or ZEN, as an alternative approach for delivery of CRISPR reagents. ZEN entails weakening of the embryo zona pellucida to allow delivery of CRISPR reagents by electroporation using a standard electroporator. The minimal methodological expertise required and standard molecular biology equipment used allow for very high numbers of embryos to be prepared in a short amount of time (40–50 embryos/ZEN). Even though ZEN has proven to be efficient, there still remain questions regarding the most optimal parameters for achieving the highest mutation rate with subsequent low mortality when performing ZEN. We have tried to address these questions by investigating several parameters to optimize efficiency, including: 1) Cas9 protein vs. Cas9 mice (mixed background and pure C57Bl6 background) 2) number of pulses 3) concentration of reagents (gRNA, Cas9 protein, donor) 4) background strain of mice (F1 or pure C57Bl6) 5) zona softening with tyrodes vs. no tyrodes 6) donor size and 7) efficiency rates of NHEJ, point mutations, donor insertion. We present here data to address each of these areas either at the blastocyst stage or in live mice using High Resolution Melt Analysis (HRMA), Restriction Enzyme PCR Analysis, or PCR Size Shift Analysis.

1. Highly Efficient Mouse Genome Editing by CRISPR Ribonucleoprotein Electroporation of Zygotes. THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291,

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Multi-gene combined intervention of metastasis in nasopharyngeal carcinoma mediated by gene targeting with high efficiency

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The project aims to establish a multi-gene combined intervention technique based on multi-locus gene targeting and transcription activator-like effector nuclease(TALEN).

The multi-locus gene targeting vectors containing E-cadherin(E-cad) gene, Endostatin gene, the shRNA expression element of Bmi-1 gene and the homologous recombination leader sequences were constructed by the multi-locus gene targeting technique established by the authors. TALEN was designed, screened and identified for specific recognition and cleavage of human rDNA. The cleavage efficiency was as high as 78.5%. The multi-locus gene targeting vector containing each target gene and TALEN eukaryotic expression vector were transfected into Nasopharyngeal Carcinoma CNE2 cells with different combinations.

The results showed that intervention of E-cad or Bmi-1 mediated by multi-locus gene targeting significantly inhibited the migration ability, invasive ability and epithelial-mesenchymal transition (EMT) of nasopharyngeal carcinoma cells. Furthermore, double gene intervention significantly reduced the migratory ability of nasopharyngeal carcinoma cells and significantly decreased the expression of vimentin protein. The subcutaneously transplanted tumor model of human nasopharyngeal carcinoma in nude mice was established. adenovirus containing Endostatin and Bmi-1 shRNA could inhibit the growth of tumor cells, but there was no significant difference in tumor inhibition rate between these two groups, which showed that intervention of endostatin significantly inhibited the growth of tumors in nude mice.

In this study, the multi-locus gene targeting technique combined with TALEN was applied to tumor multi-gene intervention, which holds the promise of overcoming the limitations of single-gene intervention and revealing more subtle interactions of multiple target genes in tumor metastasis. The technique can been applied to other tumor types, or combined with other biological treatments such as cell therapy and oncolytic virus therapy, thereby contribute to the development of comprehensive cancer therapies with broad application prospect.

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Generation of *TP53*-modified pigs by GEEP method: CRISPR/Cas9-mediated gene modification introduced into porcine zygotes by electroporation

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Introduction: Recently, we established gene editing by electroporation of Cas9 protein (GEEP); a simple method for CRISPR/Cas9 gene editing, that involves the introduction of Cas9 protein and single-guide RNA (sgRNA) into in vitro-fertilized (IVF) zygotes by electroporation. In this study, we applied the GEEP method to target the *TP53* gene, one of the most frequently mutated genes in cancers, to generate *TP53* gene-edited pigs as a cancer model.

Methods: First, we designed eight sgRNAs targeting *TP53* and each sgRNA with Cas9 protein was introduced into IVF porcine zygotes by the GEEP method. After in vitro culture of zygotes, the efficiency of genome editing in the resulting blastocysts was evaluated. As a control, zygotes were cultured without electroporation treatment. Next, we generated *TP53*-modified piglets by the GEEP method. Two sgRNAs (sgRNA1 and sgRNA2), which induced high-efficiency genome editing in the first experiment, were chosen for the GEEP method. Three groups of zygotes were genetically edited using different combinations of these sgRNAs. Groups 1, 2, and 3 were electroporated with sgRNA1, sgRNA2, and both sgRNA1 and sgRNA2, respectively. Same numbers of zygotes from each group were transferred together into the oviducts of two estrous synchronized-recipient gilts. Group 1 and 2 were intended to cause short insertion or deletion, while group 3 was intended to cause long deletion of *TP53* gene. Finally, genomic DNA of piglets was analyzed to determine whether mutations were introduced into the *TP53* gene.

Results: The rates of blastocyst formation in the edited zygotes were similar to those in control, irrespective of the introduced sgRNA. Two recipients that received edited zygotes became pregnant and gave birth to total 11 piglets. Sequencing of the *TP53* genomic regions flanking the target sites revealed that 8 of the 11 piglets carried mutations in the *TP53* gene. Among the 8 mutant piglets, two piglets did not have wild-type sequences, indicating that they carried biallelic mutations in the *TP53* gene. The other piglets exhibited mosaic genotypes, and three piglets had a long deletion ($\sim$142 bp) genotype caused by the combination of sgRNA1 and sgRNA2.

Conclusions: We generated *TP53*-modified pigs by the GEEP method with high efficiency. Our result indicates that the introduction of two kinds of sgRNA by the GEEP method can induce long deletion in a gene in zygotes. The GEEP method appears to be a powerful tool to achieve gene modification in pigs.

Key words: CRISPR/Cas9, Disease model, Pig, Electroporation, GEEP method, TP53 gene

Extending the range of alleles obtained using CRISPR/Cas9 technology in mouse embryos: Generation and validation of mutations

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Mouse models are valuable tools to understand genes functions, genetic diseases and to develop and test new therapeutic treatments in vivo. The ability to introduce tailored modifications within the mouse genome is essential to generate them. The CRISPR/Cas system has brought new perspectives for the generation of mouse models in a more efficient and precise fashion, at reduced price, all within a shorter time scale.

Here we report the use of the CRISPR/Cas9 technology at the Mary Lyon Centre, MRC Harwell Institute, to introduce a wide range of modifications within the mouse genome through different methods.

We first present our high throughput mouse production pipeline that generates alleles containing indels, tailored deletions or point mutations through direct injection into zygotes. We report the use of the CRISPR/Cas9 technology to engineer and enhance the genetic background of the C57BL/6 N mouse strain by correcting mutations in the *Cdh23* and *Crb1* genes.

Finally, we present data obtained for enhancing the homologous recombination rate in one-cell embryos through the use of long single stranded-DNA and the strategy for validation of such mutations.

Developing these methods and tools for genome engineering will enable the generation of a range of increasingly complex alleles in mice.

Oxidative stress-induced tumorigenesis: lesson from the experiments with DNA repair-deficient mice

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Reactive oxygen species (ROS) are generated in vivo through normal metabolisms, and the generation of ROS is further enhanced by the exposures of chemicals or radiation. ROS induce oxidative DNA damages that cause mutagenesis and tumorigenesis in mammals. Thus, the anti-mutagenic activity against oxidative DNA damages could be a factor that determines the susceptibility for tumorigenesis among individuals. MUTYH is an adenine DNA glycosylase that can remove adenine incorporated opposite to a most abundant oxidative damaged base, 8-oxoguanine, and thus suppresses the spontaneously occurring mutagenesis caused by ROS. We established *Mutyh*-deficient mice, and confirmed the association between MUTYH deficiency and the recessive form of hereditary colorectal cancer in humans. Furthermore, our findings suggested that the abnormality in Wnt signaling pathway is causatively associated with oxidative stress-induced tumorigenesis in the intestine of *Mutyh*-deficient mice (Sakamoto K. et al., Cancer Res., 2007, Isoda T. et al., Int. J. Biol. Sci., 2014). Thus, *Mutyh*-deficient mice provides an ideal model system to investigate the process of intestinal tumorigenesis.

To assess the dose-dependent relationship between the levels of oxidative stress and the degree of tumor incidence, we performed oxidative stress-induced intestinal tumorigenesis experiments using *Mutyh*-deficient and wild-type mice. Mice were divided into five groups, and were administrated the different doses of potassium bromate; 0, 0.05, 0.1, 0.15, 0.2%, in drinking water for sixteen weeks. No tumor was developed in *Mutyh*-deficient mice untreated or treated at the dose of 0.05%, whereas a number of tumors were observed in small intestine of all the mice treated with the dose of 0.1% or higher. The average number of tumor per mouse was 8.8, 41.6, 61.8 at the dose of 0.1%, 0.15%, 0.2%, respectively. In contrast, the average number of tumor per wild-type mouse was only 0.9 at the dose of 0.2%. These results indicated that the intestinal tumorigenesis correlates to the level of oxidative stress in *Mutyh*-deficient mice, and suggested that DNA repair system is a constituent of the mechanism underlying practical threshold of oxidative stress-induced tumorigenesis. In addition, we will present our data about oxidative stress-induced mutagenesis after treatment of potassium bromate at different doses for 4 weeks.

The naturally short-lived turquoise killifish sheds light on the basis of vertebrate lifespan

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The turquoise killifish (*Nothobranchius furzeri*) is an annual freshwater fish that inhabits savanna habitats in southeast Africa, and is adapted to survive in an environment characterized by short rainy seasons and long dry seasons. Turquoise killifish are adapted to hatch, rapidly achieve sexual maturation and reproduce during the brief rainy season, and can survive as diapausing embryos encased in the mud during the dry season. In captivity, these fish live between 4 to 8 months, depending on the strain and on the housing conditions, and can survive in diapause from several months up to at least two years. We recently sequenced and assembled the turquoise killifish genome and developed methods to efficiently achieve transgenesis and genome editing via CRISPR/Cas9. We identified the regions of the genome of this species associated with a strong signature for positive selection, compared to longer-lived species. We found that several genes involved in DNA repair mechanisms, genomic stability, nutrient sensing and ageing-related pathways, were under strong selection in this species. Additionally, performing genetic crosses between short-lived

and long-lived populations of this species, derived from more or less dry regions throughout the habitat of this species, we mapped the genomic regions associated with longevity in this species. A single genomic locus, present in the sex chromosome in a region characterized by suppressed recombination, was responsible for ~30% survival difference in different genotypes. Ongoing work in our lab is aimed at identifying the genomic regions associated with the traits that are under stronger selection in harsh annual environments and those that are mostly divergent between annual and non-annual African killifish species. Additionally, we strive to develop turquoise killifish as a short-lived experimental vertebrate model to identify novel mechanisms underlying the vertebrate aging process. We recently discovered that the gut microbiota plays a causal role in modulating host life span and in ongoing work we are dissecting the molecular mechanisms underlying this process.

Off-target analysis of CFTR-knockout sheep produced by CRISPR/Cas9 and somatic cell nuclear transfer

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Cystic Fibrosis (CF) is an autosomal recessive genetic disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR)* gene that affects over 30,000 people in the U.S. The CFTR is a cAMP-regulated Cl^- channel responsible for regulation of the anions transport primarily in the epithelial cells. We have recently generated CFTR± and $CFTR^{-/-}$ lambs using CRISPR/Cas9 (targeting the exons 2 or 11 of the CFTR gene) and Somatic Cell Nuclear Transfer. All homozygous animals exhibited small intestine obstruction (meconium ileus). Additionally, the majority of the $CFTR^{-/-}$ animals had pancreatic hypoplasia or aplasia, gallbladder hypoplasia, urethral obstruction, and hydronephrosis. Most of the symptoms are commonly observed in human but are less severe. However, hydronephrosis and severe pancreas hypoplasia observed in the homozygous and some of the heterozygous animals are not common CF characteristics and might have been caused by off-target mutations created by CRISPR/Cas9. Therefore, we conducted an evaluation to identify any mutations in possible off-target sites in the genome of the cloned CFTR-knockout sheep produced by the CRISPR/Cas9 approach. Based on the two on-target sgRNA sequences used for the gene targeting, we searched the whole sheep genome and predicted all the possible off-target sequences using an online software (Benchling). We selected all off-targets within genes and those in unknown genome regions that have at least 0.9% probability to be targeted. The animals presenting non-specific CF phenotypes were used for analysis. The DNA fragments spanning the off-target regions were amplified by PCR and subsequently sequenced by Sanger method. The sequences were aligned against those of the original cell line used for gene editing to identify possible mutations. A total of 19 off-targets were analyzed, 9 selected based on the on-target sequence at exon 2, and 10 at exon 11 of the CFTR gene. The sequence analysis results showed that none of the animals had mutations in the analyzed off-target sites. Given the severe phenotypes (e.g., hydronephrosis) we observed even in the heterozygous animals, we hypothesize that either the disruption of CFTR may lead to more severe symptoms in sheep than in humans or they could be a result of incomplete reprogramming during cloning.

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Generation of hhypoxanthine–guanine phosphoribosyltransferase gene knockout pigs by somatic cells nuclear transfer

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Abstract: The hypoxanthine-guanine phosphoribosyltransferase (HPRT) catalyses the purine bases hypoxanthine and guanine to synthesize inosine. HPRT gene is on the long arm of the X chromosome. In human, complete or severe deficiency of HPRT activity leads to Lesch-Nyhan disease (LND) which is one kind of inherited neurogenetic disorder of purine metabolism. Features of LND include hyperuricemia, intellectual impairment, and selfinjurious behavior. The HPRT knockout mouse had been established as an model for LNS, However, the mouse model did not have behavioral phenotype like selfinjurious. Pigs have been utilized for build many kinds of disease models because of the physical similarity with people. Recently, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system has been successfully used to produce many gene targeted animals. Gene targeted somatic cells can be used as donor for somatic cell nuclear transfer (SCNT) to produce gene modified animals. To establish the pigs model of LND which is closer to human, we applied Cas9/sgRNAs and homologous recombinant system to effectively direct gene editing in pigs fetal fibroblasts and then mutant cell colonies were used as donor to generate HPRT knockout pigs through SCNT. As a result, we constructed the Cas9 and sgRNA vectors targeted to exon2 of HPRT, in the meanwhile, the homologous recombinant vector contained markerfree element targeted to exon2 was constructed also. Then the Cas9 and homologous recombinant vectors cotransfected pigs fetal fibroblasts, followed G418 screen were performed and the positive cell strains which were HPRT deficiency by Cas9 mediated homologous recombination were obtained.

To produce HPRT knockout pigs, male and female HPRT-null cell lines were used as nuclear donors for SCNT. Untill now, 1735 cloned embryos were transferred into 5 recipients. We are looking forward to generating the HPRT knockout pigs as an ideal model that could mimic Lesch-Nyhan disease.

Keywords: HPRT, CRISPR/Cas9, SCNT, Pigs

Modifying the opossum genome using CRISPR

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Mammals are comprised of three subclasses, etymologically defined in Greek by their evolutionary ranking and, inevitably, by the natural arrogance of man. Man falls into the group Eutheria meaning 'true beasts'; marsupials belong to the Metatheria (meta—'changed'); and egg-laying mammals belong to the Prototheria (proto—'first'). Marsupials are therefore defined by their 'otherness', their name literally translated as 'pouch-like', a reference to the post-partum care of their young. The body plan of marsupials is however a lot more varied than their name suggests: some provide refuge for their young in the form of a teat pad, some provide a full pouch, and others are physically intermediate between the two.

The laboratory opossum *Monodelphis domestica* is a marsupial which has long been used as a research tool. It has responded well to laboratory domestication and has contributed to the study and treatment of human pathology, early mammalian development and evolutionary biology. The sequencing of the laboratory opossum genome in 2007 highlighted important differences in metatherian and eutherian gene regulation mechanisms, and shed light on the path of mammalian evolution. As the opossum's scientific repertoire expands so must the toolbox available to fully exploit the 'otherness' of this fascinating species.

The ability to genetically modify the opossum genome not only informs the state of man. It has repercussions for the future of countless species hanging over the precipice of extinction at the hands, (or rather paws) of marsupial species. As we come to respect the laboratory opossum for its contributions to human knowledge, we must also censure its close relative the common brushtail possum (*Trichosurus vulpecula)* for its devastating effect on the ecology of New Zealand. While it enjoys differing degrees of protection on mainland Australia, in New Zealand the government has announced a USD$6 billion drive to eradicate mammalian pest species by 2050. CRISPR/Cas9 technology therefore steps to the fore, and shows its promise in another developing field—genetic biocontrol. Conversely, in areas where they are endangered, our work has the potential to assist in marsupial conservation. In the process of creating GA opossums we have developed a method of embryo transfer to live births, a world first.

We wish therefore to present to our colleagues our journey in modifying the opossum genome via zygote microinjection of CRISPR/Cas9 genetic material. As far as we know we are the first to harvest, microinject, culture, transfer and deliver to term live marsupial pups. The main focus of our poster presentation will be the technical achievements and obstacles that we faced and eventually overcame, as well as those we did not, and those we still hope to.

Efficient generation of genetically edited rodent models using CRISPR-Cas9 by high throughput electroporation of zygotes

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The emergence of CRISPR-based endonuclease technology represents a paradigm shift in our approach to generating precision disease animal models. This rapidity has also led to several methods for employing CRISPR-Cas9 with varying levels of success. The CRISPR-Cas9 technique uses Cas9 nuclease and a guide RNA (gRNA) that can be delivered into zygotes by either microinjection or electroporation to cleave specific genomic sites. Our hypothesis is that optimizing the CRISPR-Cas9 preparation and delivery method will increase efficiency in generating mutant rodent models leading to reduction and refinement of animal use. With this aim in mind we compared the effects of continuous strand (single guide, sgRNA) vs two separate strands (paired guide, pgRNA), Cas9 mRNA vs Cas9 protein, microinjection vs electroporation, and multiplex vs single gene embryo transfer into pseudopregnant recipients to generate ExDels (exon deleted mutants). Our ex vivo results demonstrate that Cas9 nuclease preparation and electroporation had the largest impact on generation of ExDels. To compare gRNA and Cas9 preparations, we tested 12 randomly selected genes (from the KOMP2 project) and found that pgRNA with Cas9 protein was the superior microinjection mixture. Further we found that electroporation was overall significantly more efficient for generating ExDel mutants and SNPs via oligo insertion (ssODN). We then compared Cas9 protein by electroporation at both 8uM and 16uM across 63 and 33 genes respectively and found 16uM protein to be 1.6 fold more efficient. Additionally, we investigated the impact of transferring zygotes from different gene editing experiments into a single recipient and failed to find a significant variation in the survival rate, litter rate, litter size, or mutant rate compared to transfer of zygotes from one gene editing experiment. Overall, we have tested 23 genes by pgRNA with 16uM Cas9 by electroporation and have an average ExDel per live pup born rate of 31% of 187 live pup births. Similarly we have tested 10 genes using sgRNA with 16uM Cas9 by electroporation and have an average ExDel per live pup born rate of 28% of 79 live pup births. These are both significantly higher than microinjection of sgRNA with Cas9 mRNA which has an average ExDel per live pup born rate of 11% of 2008 live pup births across 226 unique gene targets. In summary, electroporation has increased successful germline editing efficiency by nearly threefold compared microinjection, reducing animal use by more than 3500 mice annually, or> 40%.

Assessing the effects of filtering CRISPR reagents through a centrifuge tube filter prior to microinjection

Michael Woods, Brendan Doe, Caroline Sinclair, Ellen Brown, Katharina Boroviak, Graham Duddy, Ed Ryder

WTSI Mouse Pipelines

We have assessed using a centrifuge tube filter on CRISPR reagents that have proven difficult to microinject using our standard protocol. The hope is that we can achieve an equal or better rate of F0 mosaic production whilst making the microinjection component easier. For the most part, reagents that we receive for CRISPR injection can be easily injected without filtering. Occasionally we find that, when attempting injection, there is no flow or debris can be seen in the injection needle preventing successful injection. The way that the CRISPR reagent handles in microinjection may depend on the allele type; deletion, point mutation or loxP for example. The reviewed data is from microinjection sessions where reagents are injected pre and post filter and also sessions where it was only possible to microinject after filtering. A centrifuge tube with a 0.22 μm cellulose acetate filter was used and reagents spun for 1 min at 11–13000 rpm. F0 mosaics are produced at a favourable rate from filtered reagents compared to non-filtered and we have generated a number of F1 hets from several injections. There have been occasions where F1 hets have been produced from non-filtered reagent and not the filtered reagent. In conclusion, where a reagent cannot be injected easily there is value in filtering and microinjecting, though reasonable attempts should be made to inject a reagent without filtering.

Ca_v 1.2 sequence and mutually exclusive splicing study to develop pig models of cardiac and neuropsychiatric disorders

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The cardiac L-type calcium channel (Ca_v 1.2) is involved in Ca^{2+} currents regulation in the cells. Mutations in its gene (CACNA1C) are the cause of multiple cardiac and neuropsychiatric disorders and some of them can not be adequately reproduced by *in vivo* mouse models. Human Ca_v 1.2 is ubiquitously expressed and it undergoes to mutually exclusive splicing of its exons 8 and 8A leading to isoform 14 and isoform 17 respectively. The two isoforms ratio differs from tissues and isoform 17 is more expressed in heart and brain.

The aim of the study is to analyse the porcine CACNA1C gene focusing our interest on the genomic sequences of exon 8 and 8A and on their alternative splicing. Three different boars biopsies were taken establishing their primary fibroblast cell lines. For each line, DNA and total RNA were extracted retro-transcribing their mRNAs.

As in human (Abernethy et al., 2002), following PCR sequencing analysis of three different pig cells lines confirmed the intron-exons organisation and sequences without any polymorphisms. Whereas, isoform 17 splicing was correctly confirmed by RT-PCR and subsequently sequencing analysis, during the electrophoretic run of isoform 14-specific RT-PCR, we found otherwise a noticeable extra band, ~100 bp upper than the expected that suggested the inclusion of exon 8A (104 bp). In fact, exon-8 and 8A splicing is not so strictly mutually exclusive but in cells could be present also both the double exon-included or exon-skipped mRNA products (Tang et al., 2010). To confirm this hypothesis, RT-PCR was TOPO-TA cloned to isolate the fragments and sequencing analysis also demonstrated the presence of the exon 8-8A product. However, the inclusion of both exons produces a frame shift in the mRNA upstream of many exon–exon junctions so these transcripts undergo to degradation by the nonsense-mediated RNA decay pathway (Lejeune et al. 2005; Boutz et al. 2007; Moon et al. 2016).

Together these data confirm the Ca_v1.2 switch into exon8 and exon8A expression in pig and their sequences are conserved in the species. The next steps will be the confirmation of these results into the brain and cardiac tissues performing real-time PCR to analyse the two isoforms ratio in pig. In conclusion, pig is similar to human and genome edited swine models may be obtained using the CRISPR/Cas9 system and the Somatic Cell Nuclear Transfer to study diseases associated with Ca_v1.2 mutations and to test new therapies.

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Recombineering bacterial artificial chromosome transgenes

Michael G. Zeidler, Thomas L. Saunders

Bacterial artificial chromosomes (BACs) contain large segments of chromosomal DNA (average size is 197,000 bp). Transgenic mouse technology provides scientists with a powerful tool to investigate gene function in a small mammal whose physiology resembles that of humans in many ways. The use of large BAC DNA transgenes provides more information to cells and tissues in the mouse so that gene expression occurs at physiological levels in the appropriate cell types while recapitulating normal developmental expression timing. Genomic libraries prepared in BACs for the mouse and human genome sequencing projects are a ready source of large DNA transgenes. BAC recombineering technology is used to modify BAC genomic clones to (1) express proteins resulting from point mutations, (2) mark specific cell populations with fluorescent protein reporters, or (3) express exogenous proteins in a cell specific fashion expression.

The extensive DNA sequence contained in BACs confers significant advantages in understanding gene expression. Shorter DNA fragments often do not contain enough gene expression information to completely reproduce normal gene expression patterns. The large size of BACs precludes their facile manipulation and mutagenesis by standard molecular

biology techniques. Recombineering methods permit the precise insertion of reporter cassettes in BACs by homologous recombination. Point mutations and other cassettes can also be introduced into genes in the genomic context provided by the BAC by recombineering. The consequences of these changes are studied by transfecting BAC DNA into cultured cells or by preparing BAC transgenic mice. Human disease genes caused by point mutations can be introduced to develop relevant in vivo disease models. Easily detected reporter genes allowing for straightforward detection of gene expression in cells and transgenic mice.

A series of specialized plasmids were developed to prepare transgenes and targeting vectors exclusively homologous recombination with PCR amplified DNA fragments or plasmids and BACs. Simple procedures, such as reporter knockins can be prepared and verified by pulsed field gel restriction mapping and insert sequences in a few weeks. Recombineering was used to knockin eGFP or DsRed-MST reporters in-frame in genes of interest, insert single point mutations at user-defined locations, build targeting vectors for the manipulation of the mouse genome. A series of genetic modifications and subcloning of genomic fragments of various sizes were prepared to analysis genetic control elements in animal models. BAC transgenic mice prepared from these BACs are used for lineage tracing in developmental models, to inactivate conditional alleles of genes in a tissue specific manner, and to model human disease.

Towards an optimized workflow for CRISPR/Cas mediated mouse transgenesis

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In contrast to conventional embryonic stem cell transgenesis the use of site-directed DNA endonucleases in mouse zygotes has enabled fast generation of mouse models with scarless introduction of specific mutations, allowing to precisely recapitulate the genetic background of human diseases. Due to its simplicity in design, economic advantage and exceptional efficiency, the CRISPR/Cas9 system has become the method of choice for genetic editing in mice and other species. We have established a standardized workflow for cloning-free CRISPR/Cas9-mediated mouse transgenesis in strain C57Bl/6 mice via pronuclear injection in our facility. By now we have successfully generated more than 100 mutant mouse lines with genetic modifications of various complexity ranging from simple knock-outs and introduction of point mutations to multiplex knock-outs and introduction of entire transgenes. Nevertheless, pronuclear injection is not only technically demanding but also invasive and restricted to zygotes with visible pronuclei. To overcome these limitations, we have recently explored electroporation as an alternative route to deliver CRISPR/Cas9 components into mouse zygotes. In order to maintain an easily adaptable workflow we employed a standard electroporation system (Bio-Rad Gene Pulser XCell^{TM}) and used intact zygotes to omit delicate zona pellucida weakening with acidic Tyrode's solution.

To determine differences in embryo viability, we compared the competence of C57Bl/6 zygotes to form blastocysts upon electroporation or pronuclear injection. Embryo viability significantly improved. In fact, electroporation had no effect on development and is even comparable to the development of non-treated zygotes, proving that electroporation is less harmful than pronuclear injection. We also investigated the ability to modify zygotes without visible pronuclei via electroporation, whose presence is a requirement for pronuclear injection. Indeed, electroporation resulted in similar ratios of transgenic blastocysts compared to pronuclear injection demonstrating that this technique is less selective regarding the embryonic stage while retaining full efficiency of transgenesis.

Hence, our results highlight electroporation as a promising route for less harmful and selective CRISPR/Cas9 mediated mouse transgenesis and demonstrate its potential to profoundly decrease animal numbers.

Analysis of mouse genome changes after CRISPR/Cas9 editing in vivo

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The CRISPR/Cas9 system is currently considered as the most powerful technology for genome editing in vitro and in vivo. The CRISPR/cas9 technology is developing and modifying very quickly. Presently several different modifications are used to edit the genome. We performed more than 20 knock-out (KO) and knock-in (KI) projects of different genes in mouse models and analyzed genome changes at the targeted loci.

We found that around 90% of indels resulting from DNA cleavage by CRISPR/cas9 are deletions (1–20 bp) whereas the other 10% are insertions (1–6 bp) that occur via nonhomologous end-joining (NHEJ) pathway repairs.

Analysis of founders showed that the origin of indels in regions of PAMs is variable in different regions of DNA. Approximately half of the targeted regions have a frequency of 15–20% (indels/# of newborn mice), 40% of the targeted regions have 5–14% frequency of indels/# of newborn mice and 10% of PAMs are somehow resistant to gRNA/Cas9. Such variability apparently depended on the binding efficiency of individual gRNAs and the cleavage efficiency of Cas9.

Comparison of CRISPR KO generation resulting from injection of 2 pX330 (Cas9/gRNA) plasmids and (Cas9 mRNA + 2 gRNAs) into pronuclei *versus* injection of (Cas9 mRNA +2 gRNAs) into the cytoplasm of zygotes, demonstrated that injection of (Cas9 mRNA +2 gRNAs) into the cytoplasm has an efficiency of 16% whereas the two first methods of injection into pronuclei have only 5–6% success rate.

Analysis of generation of CRISPR KO and CRISPR KI projects showed that the efficiency of generation of CRISPR KI is in three- to fourfold less than CRISPR KO projects. To improve the CRISPR KI success rate we used the SCR7 inhibitor of NHEJ. Addition of SCR7 inhibitor of NHEJ

(0.7–1 mM) in CRISPR cocktail for injection into cytoplasm increases CRISPR KI efficiency approximately twofold.

In summary, CRISPR/Cas9 system already has widespread success for genome engineering and its further optimization will improve and accelerate a production of new animal models.

Whole genome analysis of a germline transmitted calf by next generation sequencing

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Recently, we published a microinjection method for generating transgenic cattle using the DNA transposon system and their analysis by next-generation sequencing (*Yum* et al. Sci Rep. 2016 Jun 21;6:27185). In that study, we generated transgenic cattle using two different types of DNA transposon system, sleeping beauty (SB) and piggybac (PB), carrying Yellow fluorescent protein with SB (SB-YFP, female) and green fluorescent protein with PB (PB-GFP, male) under the control of the ubiquitous CAG promoter, respectively. The female and male founder cattle have been grown up to date (the female age: 40 months old, the male age: 33 months old) without any health issues. In genomic instability and blood analysis, there was no significant differences between wild type and founder cattle. In the present study, we confirmed germ-line transmission of the transposon-mediated transgene integrations and ubiquitous and persistent expression of transgene in second generation of offspring (F1). The F1 was born without any assistance and expressed GFP in the eyes without UV light. The ubiquitous expression of GFP was detected in skin fibroblast from the ear tissue and confirmed by genomic DNA PCR, which suggest that the transgene from the PB-GFP was successfully transmitted. Unfortunately, no transgene from SB-YFP were identified. To confirm the transgene integration site, the genomic DNA from blood was extracted and performed next-generation sequencing (NGS). The GFP gene was integrated in chromosome 4 (two copies), and 6. All the integrated position was not related with coding region and there was no significant difference in genomic variants between transgenic and non-transgenic cattle. To our knowledge, this is the first report of germ-line transmission through non-viral transgenic founder cattle. Those transgenic cattle will be valuable resource to many fields of biomedical research and agricultural science.

Keywords: Bovine embryos, Microinjection, Transposon, Next-generation sequencing, Germ-line transmission

Joint intervention of E-cadherin and Bmi-1 genes mediated by TALEN transgene in nasopharyngeal carcinoma cell

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The aim of this study was to establish transcription activator-like effector nuclease(TALEN) -mediated E-cadherin(E-cad) and Bmi-1 intervention of the metastasis of nasopharyngeal carcinoma, providing experimental basis for gene therapy of nasopharyngeal carcinoma.

On the basis of the common multi-locus gene targeting vector pUC-DS1-DS2 constructed by our laboratory, the E-cad and Bmi-1 shRNA expression element were inserted by In-fusion technique to construct multi-locus gene targeting vectors pUC-DS1-CMV-E-cad-2A-Neo-DS2,pUC-DS1-Bmi shRNA-Zeo-DS2. The E-cad and Bmi shRNA gene targeting vectors were transferred with TALENs to CNE-2 cells individually or simultaneously. The integration of target genes were detected by PCR, the expression of E-cad,Bmi-1 and marker protein (Vimentin,α-1-Catenin,Fibronectin) of epithelial-mesenchymal transition(EMT) were detected by Western Blot. The effects of E-cad and Bmi-1 intervention on CNE-2 were measured by Transwell chamber, CCK-8 and flow cytometry.

The E-cad and Bmi shRNA expression elements were successfully integrated into the genome of CNE-2 cells;the protein expression levels of E-cad was up-regulated, the protein expression levels of Bmi-1 was down-regulated. Compared with the control group,the migration and invasion ability of single gene or double gene transfection group were decreased significantly, the late apoptosis was promoted and the expression maker protein Vimentin, Fibronectin and α-1-Catenin were down-regulated and up-regulated respectively of EMT. Furthermore, double gene intervention could inhibit the migration ability and EMT process of nasopharyngeal carcinoma cells significantly compared with single gene intervention.

The study combined TALEN technique with gene targeting technique to intervent the metastasis of nasopharyngeal carcinoma in vitro, which will establish a key technique for gene therapy and animal genome editing.

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Production of immunodeficient rabbits by multiplex embryo transfer and multiplex gene targeting

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Immunodeficient mice have been used predominantly in biomedical research. Realizing that large animal species may have an enhanced ability to predict clinical outcome relative to mice, we worked to develop immunodeficient rabbits by CRISPR/Cas9. Given high efficiency of CRISPR/Cas9 mediated knock out, we reasoned that it is possible to use fewer animals and embryos for production of KO founders. One feasible approach is to pool embryos targeted for different genes for embryo transfer, referred to as multiplex embryo transfer, to reduce the number of recipient animals. We also note that a major advantage of Cas9 mediated gene targeting, the multiplex targeting capacity, has not been fully exploited in gene targeting rabbit production. We first demonstrated that multiplex embryo transfer efficiently produced multiple lines of single-gene knockout (SKO) founders. Embryos microinjected with single sgRNA targeting *Foxn1, Rag2, IL2rg* or *Prkdc* were pooled for embryo transfer. As few as three recipients were used to produce twenty SKO founders for four genes. We then demonstrated the powerful multiplex targeting capacity of CRISPR/Cas9. First, two genes on the same chromosome were targeted simultaneously, resulting in three *Rag1/Rag2* double knockout (DKO) founders. Next we microinjected forty-five embryos each with five sgRNAs targeting *Foxn1, Rag1, Rag2, IL2rg and Prkdc*, and transferred them to two recipients. Five founders were produced: one SKO, two DKO, one triple-gene KO and one quadruple-gene KO. The present work demonstrates that multiplex embryo transfer and multiplex gene targeting can be used to quickly and efficiently generate knockout rabbit founders with less recipients. Four lines of SKO (e.g. *Foxn1, Rag2, Il2rg, and Prkdc*) immunodeficient rabbits, as well as multigenic KO immunodeficient rabbits have been produced. These animals may prove useful for biomedical research.