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ABSTRACTS

Abstracts of the 19th Transgenic Technology Meeting (TT2025)

Zurich, Switzerland, April 24–27, 2025

Oral presentations

O-1: IVF protocol and media modifications enables mouse embryo production to be conducted solely on the laboratory benchtop without the need for CO_2 incubation

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Session 2—New Frontiers in ARTs, Main Hall—G1, April 25, 2025, 11:30 AM—1:00 PM.

Mouse IVF technologies over the past decade has revolutionised the way we rederive and cryopreserve mouse strains. But this technology has its limitations. IVF experiments must be performed in a laboratory with a CO_2 or Tri-gas incubator. In Australia we have extraordinary strict quarantine rules for the importation of cryopreserved sperm. The aim of this project was to show proof of concept and to produce an IVF protocol that would enable the core facility to perform IVF and rederivation within a quarantine facility without the need of an incubator.

Variations to the standard CARD IVF protocol were performed comparing 3 IVF medias, 3 IVF containers and 2 heating sources. Frozen and fresh sperm was also used across all scenarios. After the post insemination washes, fertilization was determined by the presence of 2 pronuclei and embryos were: (1) Transferred into 0.5dpc pseudopregnant females or (2) cryopreserved and subsequently thawed for embryo transfer. Fertilized zygotes were also cultured over night on heating stage or incubator for subsequent embryo analysis, transfer or cryopreservation.

Proof of concept for live births following the IVF was successful in the variations of: (1) IVF in dishes on a bench heating stage; (2) the use of 2 different buffered culture media; (3) insemination with both fresh and frozen sperm and (4) the embryo transfer of fresh and frozen/thawed zygotes and 2 cells.

These results show that performing IVF is possible in the situations when an incubator is not available. This would allow technicians to conduct IVF experiments and embryo transfer within a quarantine facility or for a pathogen compromised facility -where oviducts/epididymis are not allowed to leave the facility- for IVF generated embryos to then be transported to an SPF for embryo transfer.

O-2: Efficient transgenic mouse modeling through CRISPR-RNP gene editing delivered via virus-like particles

Ms Da Eun Yoon[1,4,5], Tae Yeong Jeong[4,5], Sol Pin Kim[6,7], Jiyun Yang[4,5], Soo-Yeon Lim[6], Sungjin Ok[4], Sungjin Ju[4,5], Jeongeun Park[4], Su Bin Lee[6], Soo-Ji Park[4,5], Sanghun Kim[5], Dr Hyunji Lee[5,8], Dr Daekee Lee[9], Soo Kyung Kang[6,7], Seung Eun Lee[10], Annika Hummel[1], Natalie Fischer[2], Dr Soo Jin Min-Weissenhorn[3], Dr Je Kyung Seong[6,7,11], Dr Kyoungmi Kim[4,5]

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Session 5—Trends in Transgenic Technologies, Main Hall—G1, April 26, 2025, 9:00 AM—10:30 AM.

The production of diverse/accurate transgenic models for therapeutic or basic research into various diseases is crucial. While CRISPR technology has enabled precise gene editing, many researchers have delivered them to zygotes as plasmids, mRNA or RNPs using traditional methods (e.g. microinjection, electroporation, GONAD) to create mutant mice. However, these technologies require specialized skills and expensive equipment, limiting access to them for many labs. Moreover, they can cause physical damage to embryos and interfere with normal development.

We present a novel, non-viral approach using virus-like particles (VLPs) to address these issues and enable more research facilities to create the exact animal models they need. VLP can carry larger gene-editing cargo, do not contain viral DNA, and pose no risk of viral genome integration. Additionally, packaging CRISPR as RNPs in VLPs minimizes off-target problems.

We produced VLPs containing CRISPR-RNPs and then co-cultured them with fertilized zygotes or during IVF to introduce mutations into embryos without causing physical damage.

VLP-Cas9 showed 44.7% editing efficiency and had a heterozygous genotype for frameshift-induced knockout by deletion of 29-nucleotide in Plin1. The mutation was transmitted to the next generation and mice had smaller adipocytes. Editing by VLP-BEs were also successful and we could observe increases in VLP productivity and up to 6.5-fold higher editing, especially after codon-optimization of a part of gag. We were also demonstrated multi-target editing with different CRISPR systems simultaneously. For knock-in, we co-cultured AAVs containing donor DNA and VLPs with zygotes, and succeeded in replacing exon5 of mouse Kcnq4 and part of intron with human sequence. VLP-treatment during the IVF procedure, we obtained hetero Tyr-mutant mice with H420R (A-to-G) without off-target effects.

This method further simplifies and accelerates transgenic model generation without requiring special techniques or equipment and can be widely applied for customized mouse models in diverse research fields.

O-3: Detection of gene doping and genetic manipulation in Thoroughbred horses

Dr Edward Ryder[1], Ms Jillian Maniego[1], Dr June Swinburne[1], Ms Caitlin Harding[1], Ms Jocelyn Habershon-Butcher[2], Ms Pamela Hincks[1]

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Session 8—Alternative Animal Models, Main Hall—G1, April 26, 2025, 4:50 PM—5:55 PM.

Introduction and aims

The development of gene therapy and genome editing techniques in model organisms offers great promise for treating genetic disease. One unintended consequence of this technology is the potential misuse to influence performance in human and animal athletes, also referred to as 'gene doping'. This represents a serious threat to animal welfare if equine sport becomes a testing ground with little regard to dosing levels or toxicity. We have established a research programme, supported by the British Horseracing Authority, to investigate gene doping detection methods in horse sports and Thoroughbred racing. Research currently focuses on two main pillars: gene transfer via DNA vectors, and gene editing.

Methods and results

For gene transfer via viral and non-viral vectors, we have evaluated qPCR, dPCR, and next generation sequencing (NGS) for both screening and confirmatory work. Given the number of potential targets for screening, a platform has been developed utilising next-generation sequencing to test for multiple targets within a single assay, and tested on over 1200 samples. Administration studies have been performed in whole blood and plasma to compare methods, instrumentation, determine detection time, and inform stand-down times for future approved veterinary therapies. Both screening and confirmatory approaches have been validated for future forensic work.

Detection of genetic manipulation via methods where no foreign DNA is introduced, such as CRISPR, offers a unique challenge which will rely on a deep understanding of natural variation within the Thoroughbred population. To this end, we have created an NGS-based enrichment panel based on a candidate gene approach, and surveyed over 150 horses as part of a pilot project. In addition, long read sequencing has been used to characterize and haplotype the natural variation in over 1200 samples across the whole myostatin gene, which is directly linked to performance in flat races.

O-4: Innovative rodent models for controlling temporal and spatial activation of Cre using a split recombinase system with chemical-inducible dimerization domains

Dr. Daniel Davis[1], Dr. Andrew Kelleher[1]

[1]University of Missouri, Columbia, United States

Session 9—Innovations in Animal Model Design, Main Hall—G1, April 27, 2025, 9:00 AM—10:45 AM

Precise spatiotemporal control of gene expression is essential for understanding complex biological processes, tracing lineage relationships, and defining disease mechanisms. Among the most widely used tools in genetically engineered animal models are tamoxifen- and doxycycline-inducible Cre recombinase systems. However, while these systems allow for control of gene expression in a tissue- and time-specific manner, they have notable limitations. Tamoxifen-inducible Cre lines have significant drawbacks due to unintended physiological effects in endocrine-sensitive and other vulnerable tissues. Similarly, doxycycline-inducible Cre lines require drug doses sufficient for antibiotic activity, which can disrupt

mitochondrial protein production and induce dysbiosis. These systems are also known for the risk of leaky expression leading to experimental variability.

Advances in chemical-inducible dimerization (CID) systems offer a promising alternative for precise genetic control, as split Cre proteins tagged with CID domains remain non-functional until activated by small molecules. Here we have developed innovative split Cre mouse models engineered with gibberellin-CID linkers. Gibberellin (GIB) is a plant-based phytohormone with no known mammalian receptors. This system has the potential to provide high precision and rapid induction without notable off-target effects, addressing many limitations of current models. The first model we generated harbors a CMV-iCre N-half (1 to 229)-GID1 cassette knocked into the Rosa26 safe harbor locus as well as a CMV-GAI-iCre C-half (230 to STOP) cassette inserted into the H11 safe harbor locus. This model allows for ubiquitous activation of Cre upon GIB administration. The next model we generated contains a Foxa2-GAI-iCre C-half (230 to STOP) driving tissue-specific expression in an estrogen-sensitive tissue. Comparisons with tamoxifen-inducible CreERT2 models highlight the GIB-Cre system's ability to avoid estrogen receptor-mediated off-target effects, validating its utility for studies in hormone-responsive tissues. Notably, the GIB-induction system has the potential to substantially advance current Cre-loxP paradigms by offering a neutral, physiologically inert route to spatiotemporal genetic manipulation.

O-5: Microbiome characterization of the gastrointestinal contents of laboratory mice to improve rigor and replicability of disease models

Professor James Amos-Landgraf[1], Emeritus Professor Craig Franklin[1], Associate Professor Aaron Ericsson[1]

[1]Mutant Mouse Resource and Research Center, University of Missouri, Columbia, United States

Session 10—3Rs, Main Hall—G1, April 27, 2025, 11:05 AM—12:40 PM

Introduction: The Mutant Mouse Resource and Research Center (MMRRC) acts as a repository for genetically unique mutant mice and investigates factors that impact the replicability of mouse models. Our previous work revealed that various supplier-derived microbiomes could significantly impact the phenotypes of well-established mouse models of human disease.

Aims: To determine the extent of variability of microbiomes across colonies, we characterized the microbiomes of 351 mouse models submitted to the MMRRC from around the world for cryopreservation and archiving.

Methods: We performed 16S rDNA sequencing and analysis of fecal DNA isolated from mice upon arrival to our institution.

Results: In comparing these microbiomes to the four major US mouse suppliers, the vast majority showed investigator-specific microbial profiles that were distinct from either supplier origin microbiomes or other mouse colonies from the same institution. Some laboratories did overlap with a given supplier and those mice often could be traced back to the vendor where the mice were created. Several opportunistic microbial species including Helicobacter, which are not found in most supplier-derived microbiomes, were often present in investigator colonies.

Conclusions: Given this variability, the MMRRCs goals to optimize microbiome replicability to improve mouse model replicability are addressed with a two-pronged approach. First, mice can be rederived with one of the common supplier-origin microbiomes that most closely aligns to the submitted model. Second, detailed microbiome data from the submitted model is provided so that investigators can manipulate the microbiome at their discretion, for example by adding back targeted pathobionts at their institution. To improve rigor of mouse models we recommend that mouse colony microbiomes be routinely surveyed to identify possible factors involved in phenotypic changes. We also recommend that conversations between transgenic cores and investigators involve not only the specifics about alleles and genetics but also the microbiomes of the resulting mice.

P-021: 3R Award: Refined and accurate tamoxifen administration in mice via encouraged voluntary consumption of palatable formulations

Dr Dominique Vanhecke[1], Viola Bugada[1], Dr Regula Steiner[2], Prof. Bojan Polić[3], Prof. Thorsten Buch1[1]

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Session 10—3Rs, G1 Lecture Hall, HPH Building (Level G), April 27, 2025, 11:05—12:40

Drug administration in preclinical rodent models is essential for research and the development of novel therapies. Existing compassionate administration methods are mostly incompatible with water-insoluble drugs such as tamoxifen or do not allow for precise timing or dosing of the drugs. For more than two decades, tamoxifen has been administered by oral gavage or injection to CreERT2–loxP gene-modified mouse models to spatiotemporally control gene expression, with the numbers of such inducible models steadily increasing in recent years. Animal-friendly procedures for accurately administering water-insoluble drugs such as tamoxifen would, therefore, have an important impact on animal welfare. To encourage voluntary drug intake by mice, we formulated palatable solutions compatible with water-insoluble drugs, such as tamoxifen, that can be administered using a micro-pipette. We evaluated the acceptance of the new formulations by mice during training and treatment and assessed the efficacy of tamoxifen-mediated induction of CreERT2–loxP-dependent reporter genes. Both sweetened milk and syrup-based formulations encouraged mice to consume tamoxifen voluntarily, but only sweetened milk formulations were statistically non-inferior to oral gavage or intraperitoneal injections in inducing CreERT2-mediated gene expression. Serum concentrations of

tamoxifen metabolites, quantified using an in-house-developed cell assay, confirmed the lower efficacy of syrup- as compared to sweetened milk-based formulations. We found dosing with a micro-pipette to be more accurate than oral gavage or injection, with the added advantage that the method requires little training for the experimenter. The new palatable solutions encourage voluntary consumption of tamoxifen without loss of efficacy compared to oral gavage or injections and thus represent a refined administration method.

Tech expert presentations

TE-1: Efficient production of in vivo fertilized oocytes from IASe-treated female mice by synchronizing timings of ovulation and mating

Satohiro Nakao[1], Kotono Ito[1], Chihiro Sugahara[1], Yoshino Wagasugi[1], Naomi Nakagata[2], Toru Takeo[1]

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Session 3—Embryo Production & Cryopreservation, Main Hall – G1, April 25, 2025, 2:15 PM—3:35 PM

In vivo fertilized oocytes are useful for producing genetically modified animals. In mice, 8 to 10 oocytes are ovulated and almost all oocytes are fertilized by natural mating. Previously, we developed a high-yield superovulation technique using inhibin antiserum and equine chorionic gonadotropin (IASe, ultrasuperovulation) that produces 100 oocytes from a single female C57BL/6 mouse at 4 weeks old. However, after mating with the IASe-treated female mouse, only about 20 oocytes were fertilized. To overcome the limitation of in vivo fertilization, we examined the effect of synchronizing the timings of ovulation and mating, and we had success in improving the in vivo fertilization efficacy in the IASe-treated female mice (Nakao et al., PLoS One, 2023).

First, we confirmed the ovulation timing and classified it into three periods, pre-, during-, or post-ovulation. Then, female and male mice were mated at the pre-, during-, or post-ovulation timing, and fertilization rates were evaluated. Mated female mice at the during- and post-ovulation timing showed higher fertilization rates than that at pre-ovulation. The number of embryos obtained at the post-ovulation timing was three times higher than at the pre-ovulation timing. After embryo transfer, the fertilized oocytes were normally developed into live pups.

These results suggested that synchronizing the timing of ovulation and mating increased the efficacy of fertilization in female mice with ultrasuperovulation treatment. This technique will be applicable to efficiently produce in vivo fertilized oocytes used for producing genetically modified mice and achieve the reduction of animal numbers following the 3Rs principle.

TE-2: Improved workflow for embryo production, timing of gene editing, embryo transfer and founder mouse generation

Mrs Jade Desjardins[1], Mrs Marie-Pier Cloutier[2], Mrs Nobuko Honma-Yamanaka[2], Mrs Hedyeh Rahiman[2], Mrs Isabelle Gamache[2], Mrs Mary Pallet[2], Dr Yojiro Yamanaka[3], Mrs Mitra Cowan[2]

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Session 3—Embryo Production & Cryopreservation, Main Hall – G1, April 25, 2025, 2:15 PM—3:35 PM

Despite significant advancements in genome-editing technologies, generating genetically modified animals remains a complex and resource-intensive process. Given the time, effort, and resources required to produce founder mice, we present a set of standard operating procedures (SOPs) that can be implemented across various platforms to enhance pipeline efficiency, reduce costs, and minimize the number of animals needed for production. In this workflow, we will cover best practices in key areas, including superovulation for embryo production, in vitro fertilization (IVF), best timing for microinjection or electroporation of 1-cell and 2-cell embryos, and optimal animal husbandry techniques for managing small litters or founder mice with health complications. By implementing these best practices, we have reduced the number of animals required for embryo production and pseudo-pregnant females by over 41%, while also preventing losses due to small litters. Additionally, generating synchronized embryos through IVF has provided greater flexibility in scheduling and better staff time management. This approach has also helped maintain our homologous recombination (HR) and homology-directed repair (HDR) frequency across all model generation, with an average success rate of 20% for 35 projects completed in 2024.

TE-3: The development of a vitrification protocol for the fast and safe storage of mouse oocytes and zygotes

Ms Tina Hodgson[1], Hollie Lane[1], Fabio Delaqua[1], Laura Denti[1], Helen Horsler[1], Juliette Horwood[1], Ben Davies[1], Katharine Crawley[1]

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Session 3—Embryo Production & Cryopreservation, Main Hall – G1, April 25, 2025, 2:15 PM—3:35 PM

Introduction: Vitrification offers several advantages over conventional slow-rate freezing, but has limitations, particularly for sensitive development stages. Perhaps for these reasons, few facilities have developed robust protocols to enable the vitrification of oocytes and their subsequent thawing and efficient in vitro fertilization. The development of equilibrium vitrification techniques, which combine the benefits of both slow-rate freezing and conventional vitrification while mitigating their drawbacks, has significantly improved the

cryopreservation outcomes for delicate embryonic stages, including oocytes and zygotes.

Aim: Develop a robust vitrification protocol, utilising hyperovulation, with high viability and good fertilization rates for oocytes and good in vitro development for embryos. This would allow large-scale freezing of oocytes and zygotes, for use in rederivation and genetic modification pipelines, reducing the need for mice breeding on the shelf and increasing the flexibility of scheduling genetic modification and combining multiple alleles.

Method: Based on the Qui et al. 2021 paper which addressed 2-cell embryo vitrification, a method was developed using an equilibrium media with 5% Ethylene Glycol and 5% DMSO followed by a vitrification media containing 10% Ethylene Glycol, 10% DMSO, Ficoll and Sucrose. The embryos are stored on Cryolocks in LN2.

Results: We were able to freeze up to 50 oocytes per Cryolock with 100% viability on thawing, an average fertilisation rate of 88% using C57BL/6 J oocytes with B6CBAF1 sperm, and a 94% 2-cell to blastocyst development rate – comparable to fresh controls. Fertilisation rates with sperm from GM mice were also comparable. Whilst a decrease in birth rate was observed when compared to fresh controls, (31% vs 39%, n = 4), results were within an acceptable range.

We were also able to freeze up to 40 zygotes per Cryolock with 100% viability on thawing, an average 2-cell development rate of 97% for C57BL/6 J or B6CBAF1, and 85% 2-cell to blastocyst development rate.

TE-4: Spatula MVD: a simple, fast, economical and effective device for mammalian embryo vitrification

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Session 3—Embryo Production & Cryopreservation, Main Hall – G1, April 25, 2025, 2:15 PM—3:35 PM

Spatula MVD is a homemade carrier device validated for vitrification of mouse and sheep preimplantation embryos. It is prepared using a gel loader tip in which a petal-like plate (1 mm2) is created for loading the embryos, as the original version from Tsang & Chow (1). Embryos are rapidly equilibrated in two homemade solutions of increasing cryoprotectant concentrations, loaded into the spatula, and then directly immersed in liquid nitrogen. Up to 30-40 preimplantation stage embryos can be placed on the spatula, which is then sealed with a 0.5 mL straw. The use of spatulas instead of cryotubes, which are often used to store vitrified embryos, saves cost and space in liquid nitrogen dewars. In addition, the warming process requires only a homemade sucrose solution of decreasing concentration to rehydrate the embryos and restore their usual appearance. Our lab has been using this method successfully for over 10 years to vitrify mice and sheep embryos in different preimplantation states (2, 3, 4, 5).

In our experience, the Spatula MVD has demonstrated an average recovery rate (recovered/vitrified embryos) of 88% and a survival rate (viable/recovered embryos) of 93%. Vitrification of mouse embryos has yielded better results in both rates compared to traditional slow freezing, although no differences in reproductive outcomes (pregnancy, delivery and birth rates) have been observed.

This vitrification method is a straightforward cryopreservation technique that can be used to store surplus embryos, back up genetically modified mouse lines, and facilitate the exchange of mouse lines among researchers worldwide.

The Spatula MVD is an easy-to-assemble, low-cost, minimum-volume vitrification device with high embryo holding capacity and optimal nitrogen dewar occupancy. In our hands, this method has proven to be simple, fast and robust.

(1) Tsang, W. H., & Chow, K. L. (2010). Cryopreservation of mammalian embryos: Advancement of putting life on hold. Birth Defects Research. Part C, Embryo Today: Reviews, 90(3), 163–175. https://doi.org/10.1002/BDRC.20186

(2) Dos Santos Neto, P. C., Vilariño, M., Barrera, N., Cuadro, F., Crispo, M., & Menchaca, A. (2015). Cryotolerance of Day 2 or Day 6 in vitro produced ovine embryos after vitrification by Cryotop or Spatula methods. Cryobiology, 70(1), 17–22. https://doi.org/10.1016/J.CRYOBIOL.2014.11.001

(3) Dos Santos-Neto, P. C., Cuadro, F., Barrera, N., Crispo, M., & Menchaca, A. (2017). Embryo survival and birth rate after minimum volume vitrification or slow freezing of in vivo and in vitro produced ovine embryos. Cryobiology, 78, 8–14. https://doi.org/10.1016/j.cryobiol.2017.08.002

(4) Meikle, M. N., Schlapp, G., Menchaca, A., & Crispo, M. (2018). Minimum volume Spatula MVD vitrification method improves embryo survival compared to traditional slow freezing, both for in vivo and in vitro produced mice embryos. Cryobiology, 84, 77–81. https://doi.org/10.1016/j.cryobiol.2018.07.005

(5) Schlapp, G., Meikle, M. N., Pórfido, J. L., Menchaca, A., & Crispo, M. (2024). Zygote cryobanking applied to CRISPR/Cas9 microinjection in mice. PloS One, 19(7). https://doi.org/10.1371/JOURNAL.PONE.0306617

TE-5: das-CRISPR: A new method to achieve monoallelic gene editing in mouse embryos

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Session 7—Tech Expert Session 2 (Microinjection & EP and AAV), Main Hall – G1, April 26, 2025, 2:15 PM—3:35 PM

CRISPR-Cas9 technology is a powerful tool extensively used for genome editing in mouse and many other species. Cas9 efficiently, and often, cuts both alleles in mouse zygotes leaving many edited embryos without a functional protein that may be needed to sustain development, to survive postnatally or to reproduce, thus complicating its overwhelmingly advantageous use in making gene modifications. About 25% of

mouse genes are essential for embryonic development and another 7% are necessary for fertility, thus for these genes it is desirable to maintain a functional allele to establish viable lines from edited embryos.

Allele specific editing is difficult to accomplish in inbred mouse lines and it is not practical to rely on naturally occurring SNPs between different strains. Exclusive monoallelic editing is challenging with current CRISPR methods. Several strategies have been used to minimize the damage caused by biallelic editing, such as 2-cell embryo microinjection and pronuclear transplantation. Undesired biallelic editing that lead to inactivation of essential genes encountered in our facility necessitated the development of a simple, yet highly effective, method to sequester one allele from editing in a mouse zygote. Our method, amenable for both microinjection and electroporation of mouse embryos, combines different amounts of functional Cas9 and dCas9 separately complexed with the same sgRNA sequence. We have found that the greater amounts of dCas9 complexes can bind and protect target sites while the lower amount of functional Cas9 complexes access the unoccupied target sites and the result is a higher frequency of monoallelic gene editing, compared to using just Cas9 complexes alone. We named this method das-(dCas allelic sequestration) CRISPR.

TE-6: Efficient generation of founder mice carrying a point mutation of essential genes by microinjection of two-cell embryos with CRISPR-Cas9 system

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Session 7—Tech Expert Session 2 (Microinjection & EP and AAV), Main Hall – G1, April 26, 2025, 2:15 PM—3:35 PM

Microinjection of CRISPR-Cas9 reagents into a single blastomere of two-cell embryos was reported to overcome the obstacle of zygotic microinjection to generate viable founder mice with several lethal gene disruptions. Recently, we achieved similar outcomes in mouse models of a lethal point mutation of an essential gene (embryonically lethal, VclY100F, and postnatally lethal Gpt2R134C, Igf1rR1096C). After several failures with zygotic microinjections, we adopted single blastomere microinjection of the two-cell embryos with a mixture of Cas9 ribonucleoprotein (RNP) and homology-directed repair (HDR) donor of single-stranded oligodeoxynucleotides (ssODN). We successfully generated multiple founder mice containing 7% to 48% lethal mutations of interest. In these experiments, the injected blastomere survival rates are consistently high (about 75%) across projects. Therefore, we explored both blastomere cytoplasmic injections of two-cell embryos and successfully generated male founders containing relatively higher levels of lethal mutations (33 to 94%) than the single blastomere injection (InsrR1109C & Igf1rR1096C). Some of these founders survived to 8 weeks old and were used to collect functional spermatozoa for in vitro fertilization. Further, we introduced a rescuing oligo donor containing two to three nucleotide silent mutations in a one-to-one proportion to the donor oligo containing the target lethal mutation in the CRISPR reagents that were microinjected into both blastomeres of the two-cell embryos. Preliminary data modeling Gpt2R134C mutation suggested a combination of the included rescuing silent mutation donor in the CRISPR reagents and both blastomere cytoplasmic injection of the two-cell embryos increase the chance of generating founders carrying the lethal point mutation and create a unique targeting site in some mice after knocking in the silent mutation allele. This unique targeting site can be used for the next round of targeting and will eliminate recessive lethality in generating lethal point mutations in essential genes.

TE-7: An easy and efficient method to detect concatemers in rAAV-mediated knock-in projects using stuffer DNA

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Session 7—Tech Expert Session 2 (Microinjection & EP and AAV), Main Hall – G1, April 26, 2025, 2:15 PM—3:35 PM

Introduction

Genome editing with the CRISPR/Cas9 system is a powerful tool for creating animal models. However, achieving precise knock-ins often faces inefficiencies, particularly due to concatemerization of donor DNA during integration. Recombinant adeno-associated viruses (rAAV) have emerged as a promising solution for efficient donor DNA delivery, offering improvements in knock-in workflows.

Aims

This study aimed to optimize an rAAV-based knock-in protocol for rodents and establish a robust method to detect and characterize concatemers using PCR targeting "stuffer" DNA sequences.

Methods

We developed 10 new animal lines (4 rat and 6 mouse) using an optimized protocol inspired by Mizuno et al. (2018). Zygotes were electroporated with CRISPR/Cas9 ribonucleoproteins and incubated for 5-6 h in an rAAV donor DNA preparation. Embryos were then washed and re-implanted into foster females on the same day. To detect concatemers, we included a "stuffer" sequence -non-essential 'junk' DNA- at one end of the donor DNA, positioned between ITRs. Detection of this sequence via PCR indicates that homologous recombination did not occur correctly, suggesting the presence of concatemes at the target locus. Comprehensive allele characterization was performed using 5' and 3' PCR, Sanger sequencing, and droplet digital PCR copy counting (ddPCR).

Results

At least two positive founders per project were obtained with fewer than 80 embryos, demonstrating high efficiency. The inclusion of stuffer DNA enabled effective detection of

concatemerized donor DNA through PCR. In one example, a 3764 bp knock-in in Sprague Dawley rats achieved 100% knock-in efficiency, with concatemerization easily identified using this approach. Other examples will be shown to highlight the success and potential of this screening approach.

Conclusions

The use of stuffer DNA in rAAV constructs simplifies concatemer detection, improving the reliability of knock-in generation. This optimized workflow aligns with the 3Rs principles by reducing animal use and refining experimental methods.

TE-8: Design and delivery of rAAV donor templates for knock-in in mouse embryos

Dr Monica Sentmanat[1], Mr Zi Teng Wang[1], Ms Evguenia Kouranova[1], Mr Samuel Peters[1], Ms Wan Ching Chang[1], Mr Jed Lin[1], Mr Yong Miao[1], Ms Julie Ritchey[2], Ms. Julie O'Neal[2], Mr. John Dipersio[2], Mr Michael White[3], Ms Mia Wallace[4], Ms Xiaoxia Cui[1]

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Session 7—Tech Expert Session 2 (Microinjection & EP and AAV), Main Hall – G1, April 26, 2025, 2:15 PM—3:35 PM

Mouse models with multi-kilobase knock-in (KI) are necessary yet challenging to create. The combination of electroporation of Cas9/gRNA ribonucleoprotein complex (RNP) and rAAV donor delivery enables highly efficient KI. We report over 100 KI mice created with rAAV donors and variables affecting KI efficiency. To overcome the payload limit of the rAAV genome, we co-delivered two or three rAAV donors with CRISPR RNPs and achieved up to 6.6 kb KI via sequential insertion events. To thoroughly characterize the genome after editing, we developed a target-captured long-read sequencing assay (TCLR) to verify precise KIs as well as identify undesirable editing events genome-wide in founder animals that can impact model reproducibility, including partial insertions, concatenated donors, and random integrations. Additionally, we successfully applied the same approaches in cancer and stem cell lines, including those where KI was not possible previously due to low tolerance to exogenous DNA. In summary, we report two new strategies to significantly improve efficiency and precision in multi-kilobase KI models: using CRISPR and multiple rAAV for large KIs and a much-needed, comprehensive genome-wide analysis for both on-target precise insertion and unwanted, potentially phenotypic-skewing edits.

Poster presentations

P-001: Optimizing animal breeding strategies through advanced software solutions

Frank Brand[1], Mr Thorsten Buch[2], Philippe Bugnon[2]

[1]Berlin School of Economics and Law / Department of Business and Economics; Quantitative Methods, Berlin, Germany, [2]University of Zurich / Institute of Laboratory Animal Science, Schlieren, Switzerland

Reduction of surplus animals in breeding programs is an essential undertaking to fulfill 3R requirements during husbandry. Yet, animal breeding outcomes are inherently influenced by combinatorial and stochastic processes, making the decision of which animals to place into breeding for an optimal outcome (e.g., least number of surplus animals) a mathematical challenge. To address this, we present a user-friendly online software solution to support comprehensive breeding planning, incorporating factors such as available animals, Mendelian genetics, fertility, litter size, cage contingents, and experimental cohort dimensions. This tool consolidates and enhances existing breeding planning algorithms and calculates an optimal solution for the set target parameters. At every breeding generation, it will give the scientist, breeding manager, or caretaker instructions on which animals to place into breeding, keep, or euthanize.

Taken together, by facilitating optimal breeding strategies, our approach aims to significantly reduce surplus animal populations, with projections indicating a potential decrease of up to 59% in extreme breeding scenarios, and potentially hundreds of thousands of surplus animals prevented in Europe alone. It promises to improve breeding practices and thus promote ethical practices in husbandries.

P-002: Stochastic planning of cohort- and colony-based breedings

Mohammad Hussainy[1], Achim Tresch[1], Frank Brand[1], Mr Thorsten Buch[1], Philippe Bugnon[1]

[1]University of Zurich/Institute of Laboratory Animal Science, Schlieren, Switzerland

Breeding outcomes are governed by complex combinatorics and can be modeled by stochastic processes. This requires carefully selecting various parameters, such as the number of animals bred, maintained, and euthanized for each generation. To address these challenges, we have developed a cohort-based breeding planner that incorporates Mendelian genetics, fertility, and litter size, which is already available on our website (www.ltk.uzh.ch). We have now complemented this planner with an additional tool that facilitates stochastic planning for colony-based breeding. This new tool depends on the colony index (productivity) of specific animal strains or lines. It enables the calculation of the necessary number of breeders, taking into account the required confidence, colony index, shelf life, and weekly demand. This calculator is accessible a user-friendly web interface. The impact of optimal breeding planning is considerable; it has the potential to reduce the number of surplus animals generated during complex breeding

operations. Our calculator will make the planning of colony-based breedings more precise, helping to optimize the number of animals involved.

P-003: GV-SOLAS specialist information: breeding planning for laboratory mice

Johannes Schenkel[1], Stefan Nagel-Riedasch[2], Branko Zevnik[3], Mr Thorsten Buch[4]

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Efficient and effective breeding planning is pivotal in biomedical research to reduce the number of animals required for generating experimental cohorts and maintaining rodent colonies. The Committee for Genetics and Laboratory Animal Breeding of the GV-SOLAS has developed an in-depth guide (https://www.gv-solas.de/wp-content/uploads/2024/12/Breeding-planning_2024.pdf), catering to skilled professionals, facilitating the design of tailored breeding programs to meet specific research needs. The primary objective is to optimize breeding outcomes and minimize the number of experimental mice and surplus animals.

To accurately plan breeding procedures, a comprehensive understanding of genetic characteristics and other pertinent parameters influencing breeding outcomes is pivotal. By utilizing standardized breeding methods, various types of breeding can be categorized, allowing for the precise calculation of breeding animals and expected offspring within a well-defined confidence framework.

We outline the process of breeding planning, emphasizing the importance of addressing potential challenges while striving to generate suitable experimental cohorts. Additionally, we delve into the limitations of achieving cohort breeding without generating surplus animals. By raising awareness about the intricacies involved in breeding planning, we aim to encourage researchers in the biomedical field to engage in responsible and efficient animal utilization.

P-004: Effect of luteinizing hormone releasing hormone (LHRH), pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) on pregnancy, parturition, and litter size in superovulated mice

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Induction of superovulation by injection of exogenous gonadotropins is a common practice in several mammalian species. This is typically achieved by intraperitoneal (i.p.) injection of Pregnant Mare Serum Gonadotropin (PMSG) to promote follicular growth, followed by i.p. injection of human Chorionic Gonadotropin (hCG) to induce ovulation. Our lab has previously demonstrated that PMSG delivered subcutaneously (s.c.) instead of i.p. can significantly augment superovulation in younger females and that s.c. injection of LHRH can improve the response of older females to endogenous hormones.

Luteinizing Hormone Releasing Hormone (LHRH) administration is used in cycling mammals to desensitize the pituitary, thus suppressing endogenous gonadotropin release, leading to the induction of cycle synchronicity upon withdrawal. Moreover, when given to anestrous animals, it initiates pituitary gonadotropin release and resumption of the estrous cycle. While the PMSG/hCG superovulation protocol, with or without LHRH, increases the yield of oocytes and pre-implantation embryos, our objective was to utilize LHRH injection to control the estrous cycle and facilitate synchronization of injected gonadotropins with endogenous gonadotropins, thereby maximizing plug rate, pregnancy rate, and, most importantly, litter size.

We have found that administration of either standard or higher doses of PMSG and hCG, with or without prior LHRH injection, decreases pregnancy rate and litter size, and delays parturition. Reducing the doses of PMSG and hCG, in combination with prior LHRH injection, increased the pregnancy rate and restored litter size. Conversely, this regime negatively impacted lactation and had no impact on restoring timely parturition. Mice given LHRH followed by lower doses of PMSG/hCG gave birth to large, healthy litters, however most of the pups died within a day or two with no milk spot, indicating they had not been nursed. Ongoing experiments will determine whether inducing superovulation in females who have previously reared a litter will overcome this lactational disturbance.

P-005: Managing genetically engineered animals through the lens of the 3Rs

Dr Jean Cozzi[1], Engineer Jimmy Mancip[1], Mendy Queritet[1], Dr Miriam Hopfe[2]

[1]Charles River, Dardilly, France, [2]Charles River, Erkrath, Germany

The preclinical scientific community has long supported the principles of the 3Rs (Replacement, Reduction, and Refinement). For many years, Charles River has embraced these principles, taking responsibility for all aspects of our work and driving progress for both patients and animals that depend on our research. This presentation focuses on the management of genetically engineered rodent models through the lens of the 3Rs.

Key topics include the development and implementation of efficient embryology and genotyping refinements, highlighting how these techniques can reduce the number of animals used and improve the quality of research outcomes. Additionally, pioneering techniques for model generation are explored, showcasing how cutting-edge methods can replace more invasive procedures and refine the use of genetically

engineered models. Non-invasive sampling methods for genotyping, based on tears, feces, hair sampling, and oral swabbing, will also be presented. Finally, powerful digitalization solutions for animal identification and colony management will be discussed.

By integrating these principles into the management of rodent models, Charles River demonstrates a commitment to ethical research practices. This approach not only enhances animal welfare but also improves the reliability and reproducibility of scientific data, ultimately benefiting the broader scientific community and advancing biomedical research. This presentation aims to share these advancements and inspire continued adherence to the 3Rs within the research community.

P-006: Controlling genetic integrity in mouse research models: the genocheck service

Dr Jean Cozzi[1], Dr Guillaume Pavlovic

[1]Charles River, Dardilly, France, [2]Phenomin-Institut Clinique de la Souris, Illkirch-Graffenstaden, France

When reusing published genetically modified mouse models, researchers encounter complexities due to insufficient access to critical genetic information. Details on genetic background and mutations are often lacking, complicating genotyping and impacting the interpretation of scientific results.

For instance, studies have shown that the verified genetic background frequently does not match the indicated one (e.g., Dobrowolski et al., 2018, Transgenic Res. doi: 10.1007/s11248-018-0073-2). Similarly, many models exhibit actual mutation functions that differ from the expected ones (e.g., Lindner et al., 2021, Methods. doi: 10.1016/j.ymeth.2021.04.001). Inadequate control over these factors compromises research quality by hindering reproducibility and leading to unnecessary experiments and the use of animals, which could be avoided with adequate prior scientific information (aligning with the 3Rs principle).

In line with the ARRIVE guidelines for documenting laboratory animals, the Laboratory Animal Genetic-Reporting (LAG-R) framework was developed to standardize and improve genetic information (Teboul et al., 2024, Nat Commun. doi: 10.1038/s41467-024-49439-y). These recommendations have been co-signed by numerous experts, societies, and infrastructures. Their adoption should facilitate the use of genetically modified animals. However, critical genetic information is often missing when importing or using mouse models. To address this, we developed GenoCheck, a new service providing initial verification of this essential information.

Our presentation will highlight common genetic deviations in genetically modified mouse models and demonstrate how GenoCheck can mitigate risks in scientific projects.

P-007: Breaking the year barrier ethically: accelerated backcrossing in mice using IVF and SNP techniques

Dr Jean Cozzi[1], Engineer Jimmy Mancip, Mendy Queritet

[1]Charles River, Lyon, France

We introduce a novel method for accelerated backcrossing in mice, integrating in vitro fertilization (IVF) and single nucleotide polymorphism (SNP) analysis. This approach significantly shortens the backcrossing process to less than a year, resulting in a 40% reduction in the number of animals used and a decrease in cage occupancy.

IVF facilitates the rapid production of progeny, while SNP analysis allows for precise genetic monitoring and selection. By combining these techniques, we streamline the traditional backcrossing timeline without compromising genetic fidelity.

Backcrossing is an essential process in genetic research, used to transfer a specific mutation or genetic trait from one genetic background to another. It allows researchers to study the effects of specific genes in different genetic contexts, enhancing the reproducibility and robustness of experimental outcomes. This method ensures that any observed phenotypic changes are due to the introduced mutation, rather than other genetic variables.

The traditional approach, even when using SNP analysis to accelerate the process, remains too slow and requires a large number of animals. This results in extended timelines and higher resource utilization, impacting both efficiency and ethical considerations.

Our results demonstrate the method's efficiency in conserving animal resources, aligning with the 3Rs principle (Replacement, Reduction, Refinement). This method has substantial implications for genetic research, potentially setting a new standard for mouse model development in laboratory settings.

P-008: Enhancing mouse research model colony management and the 3rs through sperm and blastocyst genotyping

Dr Jean Cozzi[1], Engineer Jimmy Mancip[1], Dr Miriam Hopfe[2], Mendy Queritet[1]

[1]Charles River, Lyon, France, [2]Charles River, Erkrath, Germany

Genetically modified mice are vital in fundamental research and as models for human diseases. Traditionally, confirming their genotype involves generating live pups, requiring significant animal use, time, cage space, and resources. However, sperm and preimplantation embryo genotyping offer a promising and ethical alternative.

Our collaborative efforts between the genotyping and reproductive and genetic engineering platforms have developed robust automated PCR protocols for genetic analysis using fresh or frozen-thawed sperm and individual preimplantation embryos. A single straw of sperm provides sufficient DNA for PCR, producing fragments up to 1.5 kb. These samples can be stored for weeks without losing quality. Similarly, the crude extract from a single blastocyst suffices for genotyping, with amplification products up to 320 bp, and can be stored or shipped for days.

This collaboration significantly enhances cryostock validation, saving numerous animals per strain and reducing the need for cage space. The method is also beneficial for evaluating and optimizing gene editing technologies like CRISPR/Cas9, particularly for complex alleles with low efficiency rates.

Moreover, sperm genotyping enhances ES-based transgenesis by early identification of chimeric males capable of germline transmission (GLT). Using digital droplet PCR and automated analysis, we predict germ-line transmission, allowing IVF with only one male showing the best parameters, saving around 50 animals per model, and shortening project timelines by 3 to 6 months.

Our integrated approach not only aligns with ethical research principles by minimizing animal use but also streamlines processes and optimizes resource utilization, showcasing the strength of our collaborative platform.

P-009: IVF: The engine of evolution in the modern embryology lab

Dr Jean Cozzi[1], Engineer Jimmy Mancip, Mendy Verrier

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The production of mouse embryos at early developmental stages is crucial for the efficient management of transgenic mouse colonies and for maintaining research excellence. Therefore, it is necessary to have a robust and efficient embryo production pipeline. Approximately 10 years ago, the Charles River EU embryology platform refined its procedures for early development mouse embryo production. Previously, the pipeline relied on in vivo fertilization by mating males with hormone-primed females. However, this approach had several weaknesses. In contemporary embryology, in vitro fertilization (IVF) has emerged as a pivotal driver of key evolutionary advancements.

Through a retrospective analysis of 10 years of activity on our platform, we highlight the diverse applications and benefits of IVF and derived technologies in managing genetically modified mouse models. IVF allowed us to significantly reduce the time and resources required for generating, maintaining and securing genetically complex mouse colonies, adhering to the principles of the 3Rs (Replacement, Reduction, and Refinement). We also drastically reduced the timelines for generating congenic lines and generation of recombinase edited alleles. Moreover, the integration of advanced techniques, such as laser-assisted IVF, has expanded the capabilities of traditional IVF.

This approach has led to the saving of thousands of work hours and mice. By showcasing the latest advancements and methodologies, we demonstrate that relevant technologies and appropriate staff training can allow faster and more reliable research while dramatically reducing the resources and animal number needed.

P-010: Cervical stimulation as an alternative method to induce pseudopregnancy and generate genetically engineered mice

Ms Claudia Solbes-Godina Riera[1], Mr Albert Duarte Santana[1], Mr Hakam Jiroudy[1], Dr Fabien Delerue[1]

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Introduction: Genetically Engineered (GE) mice are essential to advance our understanding of gene functions, disease mechanisms and to develop treatment strategies. To generate GE mice, fertilized zygotes are implanted into pseudopregnant females. Typically, pseudopregnant females are selected when they present a vaginal plug after mating overnight with vasectomized males. The need for vasectomized males is costly, and alternative methods to induce pseudopregnancy in female rodents are needed to reduce the impact on the animals (ethical refinement). In the past, chemical and mechanical stimulation showed limited efficiency in female rats (Filipiak & Saunders, Transgenic Res. 2006). This may be due to the short reproductive cycle of rodents, which complicates its synchronization. Recently, cervical manipulation has been reported to successfully induce pseudopregnancy in rats (Kaneko et al., Sci Rep. 2020) and mice (Wake et al., Sci Rep. 2023) using sonic vibrations.

Aims: We assessed the suitability and the efficiency of cervical manipulation to induce pseudopregnancy in mice. The overarching goal was to induce pseudopregnancy on demand, with high efficiency and without the need for vasectomized males.

Methods: Vaginal smears collected from randomly selected CD-1 females, and staining with crystal violet allowed for estrus identification. Females in estrus, exhibiting predominantly cornified squamous epithelial cells, were then cervically stimulated in the morning using a specifically designed sonic vibrator, before embryos transfer (1-cell or 2-cell fertilized B6J zygotes).

Results: Following cervical stimulation and subsequent embryos transfer, we successfully achieved pregnancies and live pups without the need for vasectomized males. The pregnancy rate was relatively low, with a success rate of approximately 16–20%.

Conclusion: The induction of pseudopregnancy by cervical manipulation represents an important progress towards the 3Rs, bypassing the need for vasectomized males. Further optimization is required to increase the success rate and implement sonic vibrations as part of a workflow to generate GE mice.

P-011: Multistep allelic conversion in mouse pre-implantation embryos by AAV vectors

Msc. Pavla Dostálová[1], Petr Nickl[1], Irena Jeníčkova[1], Cyril Bařinka[2], Jana Kopkanová[1], Petr Kašpárek[1], Radislav Sedláček[1]

[1]Institute Of Molecular Genetics Of The Czech Academy Of Sciences, Prague, Czechia, [2]Institute of Biotechnology of the CAS, Prague, Czechia

Site-specific recombinase technology is a powerful tool with various applications, including mouse transgenesis. This study aimed to investigate the use of adeno-associated viruses (AAV) as a delivery system for Flp and Vika recombinases, which had previously shown inefficient in vitro protein synthesis. The Flp, Vika, Cre, and Dre recombinases were produced as AAV vectors and applied to mouse embryos carrying a transgenic cassette for Cre/Dre/Flp/Vika-dependent expression of GFP protein. The efficiency of gene conversion using AAV vectors was found to be comparable to the traditional methods of

protein electroporation or mRNA injection. The results demonstrated that Flp or Vika AAV-based delivery achieved high efficiency, similar to protein electroporation or mRNA injection, while offering the advantages of being non-invasive and reducing animal consumption. This study showcased the versatility of adeno-associated virus vectors and their potential application in transgenic practices. The findings contribute to simplifying the process of generating completely converted mice, thereby minimizing animal usage and advancing the field of mouse transgenesis.

P-012: Transgenic and archiving module: implementation and enforcement of the 3Rs principle

Mrs. Lucie Dufkova[1], Petr Nickl, Michaela Krupkova, Csilla Michalikova, Pavla Dostalova, Eliska Machalova, Farkas Kerenyi, Elena Vikhrova, Katerina Zelena, Marketa Joskova, Anna Kolihova, Radislav Sedlacek

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Transgenic and Archiving Module (TAM) is a key part of the Czech Centre for Phenogenomics (CCP). TAM provides complete service, from the initial gene-targeting design, generation of the rodent model, to the genotyping, breeding and archiving.

Implementation and enforcement of the 3R concept for each step of the whole process, from planning the experiment, selection of technology, harm-benefit analysis, husbandry conditions, colony management of genetically modified lines to actual procedures, are crucial for all three modules of CCP, except TAM also Animal Facility Module (AFM) and Phenotyping Module (PM).

TAM is responsible for the generation of novel genetically modified mice and rats using state-of-the-art technologies that allow the reduction of animal consumption in line with 3R principles. For example, CRE/FLP mediated allele conversions using AAV vectors enables the conversion from tm1a to tm1d in a single animal during IVF-based reanimation/rederivation from sperm, resulting in 80% of fully converted animals. Furthermore, mouse/rat model generation using programmable nucleases (TALEN, CRISPR/Cas9) using electroporation instead of microinjection reduces the number of donors animals, more than 90% of murine embryos continue their development.

In conclusion, before starting a new project a harm—benefit analysis is performed and also knowledge of veterinarians of AFM and scientists from TAM and PM of CCP is incorporated. The health and well-being of newly generated genetically modified lines are effectively monitored for clinical abnormalities, the number of animals used are continuously analyzed and measures that can enhance animal welfare are implemented.

P-013: The Midwest CRISPR animal modeling symposium (M-CAMS)—a novel meeting format to fulfill the need for a technician-focused forum in the field of animal transgenesis.

Judy E. Hallett[1], Kylee A. Burnside[1], Corinne E. Piotter[1]

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The first Midwest CRISPR Animal Modeling Symposium (M-CAMS) was hosted by the Transgenic and Genome Editing Facility (TGEF)—Purdue Institute for Cancer Research on Oct 11, 2024, in West Lafayette, IN. TGEF is a small animal model core facility in a large university in the US Midwest. M-CAMS was intended to be a small, local meeting with broad appeal to those in the field of animal modeling, having educational value to scientific investigators (PIs), companies, students and—most importantly—technicians. Our small meeting attracted participants from 38 institutes across 22 US states and three countries.

Our specific objectives were:

- To present the latest developments in the field of gene-edited animal model creation to researchers in the field and other interested scientists.
- To showcase the high caliber of research and world leaders in the field, based in the Midwest.
- To bring together Midwestern animal model facilities to share specialized knowledge; solidify and expand our support network; and encourage collaboration.
- To facilitate communication between key suppliers and core facilities to guide product development in line with requirements of new technologies.

Our intention was to create a financially accessible, in-person, intimate environment that would foster new and existing relationships with neighboring colleagues, particularly those involved in technical aspects of the field.

The central ideas behind the meeting format were:

- Free Registration
- Focus on Technology, Techniques, Technicians and Training (4Ts)
- Single day event
- Protocol-based presentations
- "No Tech Left Behind" Travel Awards
- Exclusively Plenary sessions

The success of M-CAMS highlights the need for a new format of meeting with a firm focus on technicians. The novel, 4Ts format that we adopted was enthusiastically received by participants at every level, clearly demonstrating that small, local, technician-centric meetings, such as M-CAMS, are a valuable complement to the ISTT-TT Meetings.

P-014: Pitfalls to 2-cell embryo collection

Mr Larry Johnson[1], Tama Taylor-Doyle[1], Emily Kuo[1], Ava Sutphin[1], Dr Teodora Georgieva[1]

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CRISPR-mediated transgenesis primarily targets the S-phase in developing embryos, making 2-cell embryos ideal for genetic manipulation due to their longer S-phase. However, oviduct dissection to obtain this stage of embryo carries an often under-appreciated risk of embryo loss. Collection of 2-cell embryos requires excising the entire oviduct, from the ostium to the utero-tubal junction (UTJ), due to their scattered distribution along the tube. Performing this dissection without inadvertently tearing or cutting any the oviducts, is difficult, and sometimes results in loss of valuable embryos. Any artificial opening along the oviduct can result in the loss of any embryos located between the artificial opening and the UTJ. Without close examination of the oviduct or careful attention to where the flushing fluid is expelled, it can be easy to misinterpret a low yield as stemming from other factors, such as hormone issues, leading to unnecessary troubleshooting and wasted time and resources. Additionally, holding the oviduct steady during flushing can be difficult, especially for less experienced technicians.

To address these challenges, we have optimized procedures and identified specific tools to address each of these concerns, maximizing embryo yield, improving training, and saving time and resources. These methods align with the 3Rs of animal research and, combined with other recent super-ovulation advances achieved by other researchers, have enabled us to achieve $\sim$ 100 2-cell embryos with just five donor females per session.

P-015: Microinjection needle "mushroom" storage solution

Mr Larry Johnson[1], Dr Teodora Georgieva[1]

[1]GEMM Core/University of Arizona, Tucson, United States

Microinjection specialists often use plasticine (a type of children's modeling clay) for storing injection needles because it can easily be shaped to fit the user's preferred dimensions and fits well in various plastic storage boxes, such as empty pipette tip boxes. However, over time, the oil from the clay can seep into the tips of the stored needles, rendering them unusable. To address this issue, we sought a material that would not leave a residue. The solution came in the form of a product known as Dual-Lock by 3 M, which resembles a hook-and-loop system like Velcro. This product features tiny mushroom-shaped projections that interlock when pressed together, creating a strong bond between two attached objects. We tested narrow strips of this tape and found it effective for securely holding glass microinjection needles while allowing for easy removal without breaking them. To implement this solution, strips of Dual-Lock material were attached to solid aluminum bars, which were then affixed to the bottom of a plastic storage box using double-sided tape. A critical design feature of this container is the series of rows of small plastic "mushrooms" projecting from the tape's surface. These mushrooms allow glass tubing to be securely nestled against their stems and held firmly in place by the mushroom caps. This system provides a means for the long-term storage of microinjection needles without the contamination of needle interiors from the oil exuded by plasticine, while also avoiding residue on surfaces such as the storage box and the hands of the microinjectionist.

P-016: A journey optimizing iGONAD for efficient gene editing in C57BL/6 J Mice

Abigail Kaija[1,2], Dr. Larisa Ryzhova[1], Anne Harrington[1], Dr. Lucy Liaw[1,3]

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The improved oviductal nucleic acid delivery (iGONAD) system offers a promising alternative to traditional microinjection techniques for creating genetically modified mice. However, its application in the widely used C57BL/6 J (JAX#000664) strain has been challenging due to lower pregnancy rates and smaller litter sizes (Kobayashi et al., 2020). Our aim was to optimize a functional iGONAD protocol for C57BL/6 J mice in our facility using the NEPA21 square wave electroporator, thus, reducing the number of animals used in genetic modification procedures while maintaining editing efficiency. Our journey began with trying to obtain viable offspring from C57BL/6 J mice subjected to ex vivo oviduct electroporation with only buffer. We began by testing various electroporation parameters, including voltage, pulse length, and pulse intervals. We evaluated the impact of female age and weight on success rates. Throughout the process, we monitored impedance values, pregnancy rates, and litter sizes. Initial experiments with C57BL/6 J mice demonstrated that the viability of eggs post-electroporation was increased in females heavier than 24 g or older than 16 weeks. We observed an association between lower impedance values and increased pregnancy rates. Our next step was to replicate a previously published experiment to verify targeting efficiency and repair (Kobayashi et al., 2020; Ohtsuka et al., 2018). To complete our journey, we decided to compare our optimized iGONAD procedure to modifications successfully targeted in our facility using conventional microinjection techniques. Our progress suggests that with further refinement, iGONAD can become a viable tool in our facility for gene editing in C57BL/6 J mice, aligning with the 3Rs principles.

P-017: BLAST-ddPCR: A novel method to simultaneously measure homology directed repair and AAV uptake in blastocyst

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Mouse Model creation has been significantly simplified by manipulating embryos with electroporation and AAV infection

to deliver donor DNA. Currently, there is no basis on how the AAV is applied to embryos. There are multiple variables to consider and there is no current standard across mouse model generation facilities.We have developed BLAST-ddPCR which allows dual measurement of both homology direct repair (HDR) sucess rate and AAV uptake per cell. We validated our method by varying AAV MOI, incubation peroid, embryo droplet size and number of embryos per droplet. We identify certain parameters that effect AAV uptake more. Simultaneously, we also HDR rates in those same cells.

P-018: The LAG-R guidelines, a framework for standardizing and improving laboratory animal genetic reporting

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Introduction

A major problem in biomedical research is poor reproducibility, with many scientific results not being successfully replicated. Many biological factors can explain this, from sampling effects to the influence of environmental factors such as microbiota. But other factors that are much easier to address, such as a lack of access to methodological details, raw data, and research materials, are also playing a major role in the reproducibility crisis. In particular, inadequate reporting of the genetics of laboratory animals in research papers is common.

Methods

ARRIVE has been a pioneer in demonstrating to researchers using in vivo animals the importance of structured documentation for animal research. In the same vein, we brought together a large number of leading international experts, consortia, and learned societies in the field of animal genetics to define the LAG-R recommendations for Laboratory Animal Genetic Reporting.

Results

Our recommendations have been published in Nature Communications (https://doi.org/10.1038/s41467-024-49439-y). This LAG-R framework defines a set of guidelines to support more complete documentation of the genetic make-up of animals that are used in research.

Conclusions

The purpose of LAG-R is to help authors better describe the information they should already have in scientific papers, but also to provide reviewers with a checklist of essential genetic information that needs to be included in a publication. LAG-R provides an easy-to-understand list of recommendations with the goal of improving reproducibility, reliability, and overall scientific rigor.

P-019: Right—left bias in uterine function of naturally mated mice and in ovulatory response following superovulation in mice and rats

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Previous research performed by our lab, transferring suboptimal numbers of embryos into pseudo-pregnant mouse surrogates, determined that the right uterine horn is more supportive of embryo implantation and survival after embryo transfer than is the left uterine horn when embryos are limiting. In this study we have examined whether the superiority of the right over the left side is also observed in the uterus when embryos are abundant, either from a normal or a superovulated cycle; and whether this bias also extends to the ovary. We have also examined whether the female rat reproductive system shows a similar right bias.

To induce superovulation, juvenile and adult female mice were administered Pregnant Mare Serum Gonadotropin (PMSG) and human Chorionic Gonadotropin (hCG), with adult mice also receiving Luteinizing Hormone Releasing Hormone (LHRH) prior to PMSG. Rats, regardless of age, were injected with LHRH, PMSG, and hCG. Right and left oviducts were collected from superovulated animals and number and quality of embryos were recorded. Implantation sites were observed in both uterine horns at various intervals after natural mating.

Observations of uteri from naturally mated mice showed a similar bias toward the right uterine horn as in our previous study with transferred embryos, with more implantation sites (pups and reabsorption sites) in the right uterine horn than the left uterine horn of inbred mice. This bias was not observed in outbred mice carrying hybrid embryos or in superovulated mice.

Right side bias was less pronounced with regard to ovarian function and appears to vary between strains. There is no significant difference in ovulation rates between right and left ovaries in the superovulated rats. On the other hand, in superovulated mice, right-left bias in ovaries appears to vary by strain, with some strains showing a higher total number of embryos originating from the right ovary.

P-020: The iMouse System—a visual method for standardized digital data acquisition reduces severity levels in animal-based studies

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In translational research, using experimental animals remains standard for assessing the effectiveness of potential therapeutics. At the same time, minimizing the impact on the well-being of the animals regarding the 3R is mandatory. To fulfil this goal and therefore evaluate the severity level, animals must be inspected several times a day. It is noted that these visual assessments disrupt the animals during their resting periods, resulting in elevated stress levels and consequently affect the results of scientific studies.

We examined the feasibility of implementing a digital monitoring system (iMouse) in a translational study conducted within home-cages. Our objective was to reduce or replace manual visual inspections during experiments and to examine whether digitally available data from this study can be used to train an algorithm capable of distinguishing between activities.

We successfully demonstrated the feasibility of integrate the system into the existing IVCs and established remote access to the overserved home cages.

Accordingly, digital surveillance of the experimental animal reduces their stress level. Furthermore, the digitally acquired data out of the home cages proved instrumental in training algorithms capable of analysing e.g. the long-term drinking behaviour of the animals.

In summary, our work has yielded an integrated, retrofittable, and modular system that serves two critical criteria for the 3R. Firstly, it reduces the severity level of the animal by executing visual inspections. Secondly, it refines the traceability and transparency of animal-based research studies. The standardized iMouse system enables the analysis of data sets and the generation of new digital biomarkers.

P-026: Emilin-1 Deficiency impairs cardiovascular development and function in Zebrafish

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Emilin-1 is an extracellular matrix (ECM) glycoprotein belonging to the EMILIN/Multimerin family, abundant in elastic tissues such as the heart, blood vessels, lungs, and dermis. It plays a crucial role in various cellular processes,

including elastogenesis, cell adhesion, maintenance of vascular cell morphology, and modulation of blood pressure homeostasis.

To better understand Emilin-1's role in cardiovascular health, we used Danio rerio as an in vivo model, chosen for its easy genetic manipulability and cardiovascular similarities to humans. Using CRISPR- Cas9 technology, we generated emilin-1a and emilin-1b knock-out zebrafish lines.

These single mutants were then bred to generate a double knock-out line, allowing us to examine the compound effects of both gene knockouts on cardiovascular development and function.

Initially, we confirmed the absence of Emilin-1 through immunofluorescence and Western Blot analysis. Subsequently, we observed reduced breeding success and survival rates in the emilin-1a/emilin-1b double knock-out, especially during early developmental stages.

We then assessed the impact of the absence of Emilin-1 on heart and blood vessel development by using transgenic fluorescence lines specifically marking endothelial and smooth muscle cells. We observed alterations in vessel morphology in the double knock-out, including an increased diameter of the dorsal aorta (DA), posterior cardinal vein (PCV), and an enlarged aortic bulb. The latter finding is particularly significant as it mirrors characteristics of aneurysmal conditions, suggesting a potential role for Emilin-1 in maintaining aortic integrity and preventing pathological dilation.

Furthermore, cardiac function measurements using pyHeart4Fish software indicated increased cardiac size and a general reduction in blood circulation efficiency in the double knock-out.

This research enhances our understanding of Emilin-1's role in cardiovascular health and its impact on vascular diseases. Our zebrafish model offers valuable insights that could lead to new therapies and improve early diagnosis and prevention of cardiovascular conditions.

P-027: Experimental follicle culture for in-ovo interference assays in primary chicken follicles

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Experimental follicle culture for in-ovo interference assays in primary chicken follicles

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In birds the germ plasm accumulation and organization into the Balbiani body during early folliculogenesis, and the segregation of germ plasm during the initial cleavage divisions in the early embryo are crucial for the specification of the germline manifested in primordial germ cells. In contrast to mammals, the germline is the first cell lineage specified during ontogenesis. The chicken Bucky ball protein (cBuc) has been recently identified as germ plasm organizer in birds1,2. To investigate the regulatory network of germline specification from germ plasm to primordial germ cells during early folliculogenesis, we established a follicle culture system (in ovo) on fertilized chicken eggs.

Therefore, separated ovarian follicles were experimentally stimulated by lipofection and the stimulated follicles were implanted in the nourishing environment of the chorioallantoic membrane of in-ovo cultured chicken embryos in surrogate shells; within 24 h the ovarian tissue fragments became vascularized and continued with folliculogenesis. First data provide information on inhibited transcription of Bucky ball after si-RNA transfection especially in primary follicles 1,2. The in-ovo culture system allows experimental access to critical phases of germ plasm formation and folliculogenesis in chicken to dissect the role of cBuc and other genes for germline specification.

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P-028: Engineering an organ donor pig for xenotransplantation

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It has been a dream for scientists to engineer a pig with lower immunogenicity, capable of serving as an organ donor for pig-to-human xenotransplantation. However, it faces physiological, immunological, and infectious barriers. This study aims to utilize gene-editing technologies and a recombinase-mediated cassette exchange (RMCE) technique to engineer a miniature Lee-Sung pig as a better organ donor. Lee-Sung breed is picked due to its human-like body size, well-adapted to the local environment, and its relatively homogeneous genetic background. So far, we are able to perform artificial ovulation induction on the sows, edit a gene in fertilized eggs, and culture the edited zygotes to a blastocyst stage in vitro. At the same time, we succeed in employing CRISPR/Cas9 technology to simultaneously knockout three glycosylation genes, namely

GGTA1P, CMAH, and B4GALNT2, which can effectively mitigate hyperacute rejection, in a Landrace-Yorkshire hybrid breed. Furthermore, we designed an exchangeable "PAD" cassette placed in the pig genome, which enables future integration of a CARGO (DNA) fragment carrying human immune-modulatory genes, such as CD47, HO1, and etc., via RMCE. Currently, we successfully obtain two triple-knockout (TKO) and a dual-knockout (DKO) founder sows. Genetic disruptions of the targeted glycosylation genes are confirmed by Sanger sequencing genomic PCR products. The feasibility of our RMCE design also shows promising results in our preliminary in vitro tests. In conclusion, we made significant progress on pig genetic engineering and we will use the technical foundation built to overcome barriers on the pig-to-human organ xenotransplantation.

P-029: High-yield induced ovulation in adult fat-tailed dunnarts by pmsg treatment combined with estrous cycle synchronization

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Mature oocyte production is crucial workflow for assisted reproductive technologies. We recently established the first protocol to reliably generate mature oocytes from juvenile fat-tailed dunnarts (Sminthopsis crassicaudata), a laboratory based marsupial animal model. However, when this method was applied to mature animals with established endogenous estrous cycles, it yielded inconsistent results. Therefore, the objective of this study was to improve methods to generate a high-yield induced-ovulation protocol to collect higher numbers of oocytes from adult female dunnarts. To achieve this, we first synchronized the estrous cycle in adult female dunnarts using luteinizing hormone-releasing hormone (LHRH). After four injections given every three days, 86% of the adult female dunnarts responded to the LHRH treatment, with their estrous cycles synchronized at the diestrous stage of reproductive cycle. We then induced ovarian follicle development through PMSG stimulation for 6 days followed by hCG administration 48 h after the last PMSG injection to trigger ovulation. By combining estrous cycle synchronization and PMSG stimulation, we obtained an average 12 oocytes per adult dunnart. Thus, estrous cycle synchronization followed by the PMSG-hCG treatment yielded consistent, highly efficient induced-ovulation in adult dunnarts. This protocol could be extrapolated for other valuable marsupial species to support conservation and captive breeding efforts.

P-030: A sheep model of sickle cell disease (SCD) generated by CRISPR/Cas9 and somatic cell nuclear transfer closely resemble human SCD

A Sheep Model of Sickle Cell Disease (SCD) generated by CRISPR/Cas9 and Somatic Cell Nuclear Transfer closely resemble human SCD Iuri Viotti Perisse[1], Caroline E. Kuczynski[2], Ying Liu[1], Tayler Patrick[1],

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Introduction

SCD is an inherited blood disorder caused by a point mutation at the sixth codon of the β-globin (HBB) gene, which results in the substitution of a glutamate for valine triggering polymerization of hemoglobin and erythrocytes sickling. Sheep and humans exhibit a high degree of homology at the HBB gene. Additionally, their anatomy, organ physiology, and immune system development closely parallel that of humans.

Aims

Our aims were to introduce SCD mutation in the sheep HBB gene, produce SCD lambs by Somatic Cell Nuclear Transfer (SCNT) and characterized their phenotype.

Methods

We used CRISPR/Cas9 to introduce the SCD mutation into exon 1 of the sheep HBB gene and a silent mutation (SM) in the fetal HBG gene. Single-stranded oligodeoxynucleotides (ssODNs) were designed for introduction of the mutations. CRISPR/Cas9 and ssODNs were transfected into sheep fetal fibroblasts (SFFs). Sanger sequencing was used for SFF genotyping and SFFs containing SCD and SM were used for SCNT to produce SCD cloned lambs.

Results

We isolated three SFF colonies with biallelic mutations (SCD and SM) in the HBB and HBG genes, respectively (3/30). These cells were used for SCNT and 104 SCNT embryos were transferred into 8 estrus synchronized recipients. Three of the recipients (3/8) delivered three SCD lambs. One lamb died within 24 h after birth and two lived for almost a year. The SCD genotype was confirmed by sequencing. These two sheep exhibited clinical and laboratory parameters that recapitulate human SCD. SCD sheep display a positive SickleDex test, sickled erythrocytes and polychromasia, abnormal hemoglobin with the same migration pattern as human hemoglobin S (HbS), and the presence of HbF at birth.

Conclusion

Our findings support the translational relevance of the SCD sheep model and highlight its potential use in the development of novel SCD therapies.

P-031: My catshark teacher. Establishment of gene editing tools to study the origin and evolution of the vertebrate brain

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Introduction

The evolution of brains from the earliest bilateral animals to the diverse forms seen today, is a central question in biology. Traditional model organisms like mice and flies may not be ideal for reconstructing brain evolution. There is a growing

need to study "slow-evolving" species that occupy key positions in the evolutionary tree, as they can provide insights into ancestral brain structures.

The catshark (Scyliorhinus canicula), is an interesting model for developmental studies: slow evolver, lays transparent eggs year-round, embryos can be cultured (allowing direct manipulation), and its reference genome is open and of high-quality.

Aims

1.Develop a reliable and reproducible gene editing protocol in catsharks.

2.knock-out (KO) and knock-in (KI) candidate genes.

3.Investigate evolutionary brain mechanisms in basal vertebrates

Methods

1.Design gene editing strategies and test different methods to deliver reagents, determining the conditions that produce the best edition efficiency and survival rate.

2.Deliver the construct into the telencephalic ventricle of stages 29-30 embryos. Euthanize at stage 31 and collect tissue. Characterize potential phenotypes and confirm edition events.

4.Generate reporter genes for candidates identified by transcriptomic data.

Results & Conclusions

This project represents a pioneering effort to implement gene editing tools in the catshark. Our initial results have yielded promising results while targeting the Foxg1a gene.

We have also gained invaluable experience working with a non-canonical model organism. This has posed unique challenges, but it has also opened new opportunities for innovation.

P-032: Development of GGTA1/CMAH knockout cattle via CRISPR/Cas9 for xenotransplantation and hypoallergenic meat production

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Xenotransplantation offers a promising solution to the global organ shortage, but immunological barriers caused by interspecies genetic differences remain a major challenge. To address this, we developed GGTA1 (produces the α-Gal epitope) and CMAH (converts Neu5Ac to Neu5Gc) knockout (KO) cattle using CRISPR/Cas9 technology to reduce immune rejection risks and explore the production of low-immunogenic meat. By targeting these genes, our goal was to create cattle optimized for xenotransplantation and hypoallergenic food applications.

We designed sgRNAs targeting GGTA1 and CMAH and introduced them into bovine embryos via electroporation of Cas9/sgRNA ribonucleoprotein. Genetic analysis revealed successful KO rates of 50% for GGTA1, 56.6% for CMAH, and 23.3% for double KO embryos. Implantation of 27 embryos yielded 14 calves (51.9% birth rate). Further analysis using mini-sequencing confirmed on-target mutations (GGTA1: 2.2–73.4%, CMAH: 7.3–100%) with no significant off-target effects observed.

Detailed gene and protein sequence analyses of calves with high mutation rates (#1, #2, #6, #13) provided critical insights into the functional consequences of the genome editing. In GGTA1, early stop codons caused by small indels were confirmed in #1 and #2, resulting in protein loss. For CMAH, 100% KO efficiency was observed in #1, #2, and #6. However, in-frame deletions in #1 and #6 maintained partial protein function, whereas #2 showed early termination. Flow cytometry analysis corroborated these findings, with α-Gal antigen deficiency identified in #1 and Neu5Gc deficiency in #2. Notably, in-frame deletions in GGTA1 or CMAH failed to abolish antigen expression entirely, underscoring the importance of KO pattern in achieving the desired phenotype.

Postnatal monitoring confirmed that the GGTA1/CMAH double KO cattle exhibited normal growth and health. This study establishes a strong foundation for the development of gene-edited livestock tailored for medical and dietary applications, addressing the challenges of immune rejection in xenotransplantation and providing hypoallergenic meat for sensitive populations.

P-033: Facilitating disease modeling using genetically modified rabbits

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The use of laboratory animals in biomedical research is essential for advancing our understanding of human biology and disease. Mice have been the primary choice for experimental models due to their genetic similarity to humans and practical advantages such as short life cycle, rapid breeding, and small size. However, it is known that mice cannot phenocopy some diseases of interest or at times pose technical challenges related to lack of translatable tools. In these circumstances, the use of alternative species may generate more accurate models that can bridge the translational gap between preclinical studies and clinical applications. However, there must be a clear biological justification for using species other than the mouse. Rodent models of human vision defects face significant challenges due to structural differences, organ size and accessibility, reproducibility of disease models, and vision tuned for nighttime. We identified the use of rabbits as valuable organisms offering distinct advantages for Ophthalmology models. With their larger size and more human-like physiological features, rabbits provide a platform with the potential for improved instrumentation and the availability of specialized tools and techniques. Here, we detail the successful generation of genetically modified rabbits through direct embryo modification. We demonstrate robust recapitulation of human phenotype using a precision ssODN-mediated point mutation as well as CRISPR KO to model Glaucoma-associated gene variants. Together, the anatomical and

physiological characteristics of rabbits can enable researchers to better model and characterize important human conditions.

P-035: Enhancing the efficiency of multi-gene modified cloned pig production using porcine endometrium organoid-derived intra-organoid fluid

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Introduction: Improving embryo production efficiency is critical for the generation of genetically modified cloned pigs.

Aims: To address this, porcine endometrium organoids were developed to replicate the uterine microenvironment, featuring apically polarized epithelium characterized using ZO-1, Vimentin, Ki67, and Pan-cytokeratin markers. Intra-organoid fluid (IOF), extracted from the organoids, was evaluated in an in vitro culture (IVC) system for its effects on parthenogenetic (PA) and somatic cell nuclear transfer (SCNT) embryos.

Methods: The SCNT embryos were derived using GGTA1/CMAH/iGb3 triple-knockout (TKO) donor cells to produce multi-gene modified pigs. Experimental groups included control (standard IVC), IOF-supplemented IVC, and uterine fluid (UF)-supplemented IVC.

Results: In PA embryos, IOF significantly enhanced cleavage (80.7% ± 1.4) and blastocyst formation rates (26.1% ± 1.4) compared to the Control group (76.4% ± 1.1, 20.7% ± 1.4, respectively) and demonstrated similar efficacy to the UF group (81.3% ± 1.1, 24.1% ± 1.2). IOF's ability to mimic uterine fluid highlights its potential to create in vivo-like conditions in IVC systems, crucial for supporting the development of SCNT embryos. The ongoing SCNT experiments aim to validate IOF's role in improving the developmental competence of embryos for producing genetically modified pigs.

Conclusions: These findings suggest IOF as a novel medium component, bridging the gap between in vivo and in vitro systems, and advancing reproductive biotechnology. This innovation holds promise for increasing the efficiency of multi-gene modified pig production, contributing to precision livestock engineering and regenerative medicine.

P-036: NMGN congenital anomalies cluster pipeline for identifying disease-causing mutations

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The MRC National Mouse Genetics Network (NMGN) is a UK-based initiative bringing together genetic experts to investigate a wide range of human diseases and intensify clinical translation using mouse models. Recent advances in sequencing technologies have significantly accelerated the identification of potential disease-causing variants in patients with congenital anomalies. However, establishing the pathogenicity and mechanistic relevance of Variants of Uncertain Significance (VUS) is challenging. As the Congenital Anomalies Cluster, we will generate and characterize over 25 clinically relevant mouse models, using the Mary Lyon Centre at MRC Harwell as a central hub for mouse production and phenotyping. To do this, we have created an online portal for the international clinical and scientific community to submit their VUS of interest (https://cagdb.nmgn.mrc.ukri.org). Each VUS is curated by the Cluster, cross-referenced to functional evidence from literature, bioinformatic metrics and model systems, and then scored for relevance by our external clinical and internal scientific advisory boards. Regardless of the outcome from advisory boards, comprehensive data are compiled for return to clinicians enabling improved clinical interpretation. We use a variety of genome editing strategies to create the most accurate models and are currently implementing base editing techniques. We use embryonic, peri- or post-natal F0 screening and/or establish mouse lines to characterize our models. Our cluster comprises 5 laboratories across the UK with different expertise and additional associates, allowing us to investigate multi-systemic diseases and complex syndromes. Our phenotyping pipeline uses various volumetric imaging modalities (μCT, HREM), histological and behavioural approaches. We aim to implement manual and automated systematic screening strategies and align human and mouse ontology for anomalies during embryonic and perinatal stages. To ensure diagnosis return and clinical translation, we constantly improve our pipeline focusing on high-quality results, reproducibility, collaborations and data sharing.

P-037: The swollen ampulla during embryo transfer in B6C3F1 recipient mice as a reduction and refinement factor in the 3R concept.

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Assisted reproductive technologies are used extensively for the maintenance of mouse models in animal research. Mice of different genetic backgrounds are being used as donor and as recipient animals for the cryopreservation and the embryo transfer. In vitro fertilization can be especially useful for breeding of transgenic mice with fertility problems. However,

mouse strains with different genetic backgrounds vary in their reproductive performance. The cryopreservation and thawing cycle affects the embryo survival and revitalization rate. The presence of structures like the vaginal plug of recipient mice can influence the embryo transfer result.

This retrospective study includes and analyzes the pregnancy- and birth-rates of the embryo transfers performed in our animal facility during a period of 6 years. The embryos of different genetic backgrounds were fertilized either in vivo or in vitro and were either freshly isolated or cryopreserved. All recipient females had a vaginal plug on the day of embryo transfer, and their ampulla was documented, if it was swollen or not.

The B6C3F1 recipient mice with swollen ampullae showed an almost threefold higher pregnancy rate compared to the recipients without swollen ampullae. The cryopreservation process reduced the birth efficacy of the embryos, regardless of whether they were fertilized in vivo or in vitro. Moreover, a significant effect of genetic background of donor mice on birth rate was found. C57BL/6 J mice and mice with a mixed genetic background had 34% higher birth rates than did C57BL/6N mice.

Although the transgenic mouse models provide important insights into the mechanisms and pathophysiology of disorders, their use is a controversial topic and undergoes a 3R make over. Thus, the swollen ampulla, as a strong indicator for a successful pregnancy, and a better understanding of the factors that affect the reproductive outcome, can lead to significant ET refinement and animal reduction.

P-039: In vitro generated or in vitro cultured mouse embryos lead to significantly reduced success rates after surgical and non-surgical embryo transfer using the NSET® or TCET® device.

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Introduction

Embryo transfer (ET) is pivotal in assisted reproductive technologies for laboratory mice, enabling the rederivation of mouse strains, revitalization of cryopreserved sperm and embryos, and establishment of transgenic lines. Surgical ET (SET) remains the standard procedure despite its invasiveness. Switching from the traditional surgical ET to non-surgical methods using transcervical devices would represent a significantly lower stress burden for the animals and therefore be in line with the 3Rs.

Aim

This study aimed to compare SET with non-surgical ET methods in terms of success rates and animal welfare. In addition, we investigated the effect of different embryo generation methods on ET success.

Methods:

We evaluated 217 recipient mice experiencing either SET, or non-surgical ETs with the NSET® or TCET® device (both commercially available). Blastocysts for ET were generated either in vitro with IVF and subsequent culture, or ex vivo, with subsets cultured from flushed zygotes or morulae. Success metrics included implantation and birth rates, and pup outcomes. Stress was assessed non-invasively by measuring fecal corticosterone metabolites (CMs).

Results

SET and non-surgical methods showed comparable implantation rates (76-85%) for ex vivo embryos, with NSET® offering the simplest, least invasive approach. Independent of the transfer method, birth rates were significantly higher for ex vivo (55-67%) compared to in vitro embryos (4-22%). In addition, flushed morulae yielded superior outcomes over flushed zygotes (birth rate: 73% vs. 19%), highlighting culture-related obstacles. Stress was highest for recipients with SET, as indicated by doubled CM levels, compared to non-surgical methods.

Conclusion

Non-surgical ET methods, particularly NSET®, demonstrate equivalent success rates to SET for ex vivo embryos while minimizing stress. However, in vitro embryo generation remains less efficient, with reduced viability linked to extended culture durations.

P-040: Cultivating a next-generation network of young experts in transgenic core and bioresource communities

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Transgenic technologies have created great value in basic science and biomedical industries. To advance science and technology related to transgenic technologies, many core facilities and bioresource centers are established worldwide. In these institutes, highly skilled technologists, researchers, administrators, managers, and directors work together to achieve their missions. In the post-corona era, international collaborations are getting easier based on online communication tools and data-sharing systems. On the other hand, the opportunities to build face-to-face networking and take hands-on training have been decreasing. In addition, nurturing the next generation through question-driven research and learning comprehensive viewpoints is important to continuously develop science, technologies, and the transgenic and bioresource community. Recently, we conducted some projects to promote international and multidisciplinary communications and collaborations. In this activity, our graduate students,

young researchers, and technicians visited highly established institutes, deepened their understanding of the system of animal experiments, and conducted collaborations related to reproductive technologies and bioresources. In addition, we organized the meetings to facilitate science communications beyond research fields. Furthermore, we opened science classes for teenagers to support their understanding of science and research. In conclusion, these activities help to cultivate a next-generation network of young experts for generating novel innovations in transgenic core and bioresource communities. In this presentation, I will share my research project on reproductive technologies, the realized values of visiting abroad institutions (France, Finland, Taiwan, and Brazil), and multidisciplinary activities from the view of a doctoral student.

P-041: The integrity and fertility of cryopreserved spermatozoa is impacted by different storage temperatures in a mouse strain-dependent manner

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Introduction

Liquid nitrogen (LN2, − 196 °C) is a reliable resource to cryopreserve and store spermatozoa. However, the alternative with dry ice would reduce the hazardous risks posed and the costs related to LN2.

Aim

We evaluated the effect of a LN2-free cryopreservation method on sperm integrity and fertility from commonly used mouse strains in biomedical research.

Methods

Spermatozoa from 3-month-old C57BL/6NTacCnrm (B6N), C57BL/6JCnrm (B6J), BALB/cByJCnrm (BALB/c), Crl:CD1(ICR) (CD-1), 129/SvImJCnrm (129) and FVBN/JCnrm (FVB) males were collected. For each strain, half of the sperm was cryopreserved and stored via LN2 or using dry ice and maintained at − 80 °C in an ultra deep freezer. Over one year, the fertility and the integrity of the spermatozoa via IVF, sperm viability, sperm kinetics, morphology, ultrastructure damages and DNA oxidation were evaluated.

Results

The IVF rate, vitality and morphology were stable up to 1 year except in B6J and BALB/c (significant decrease after 6 months and 3 months, respectively, at − 80 °C compared to LN2). In all strains, the total motility of spermatozoa decreased after 1 month at − 80 °C and more ultrastructure damages were found in the − 80 °C group compared to LN2. In B6J and BALB/c, only 17.4 and 4.2% of spermatozoa, respectively, remained intact after 12 months at − 80 °C whereas this value was 45% in B6N. The level of DNA oxidation was significantly increased in sperm maintained at − 80 °C in BALB/c only.

Conclusion

Results indicate that spermatozoa from B6N, CD-1, 129 and FVB can be cryopreserved and stored at − 80 °C up to at least 1 year without impacting their fertility even though a decrease in motility and ultrastructure damages was observed. Spermatozoa from B6J and BALB/c are most temperature-sensitive. A low level of cryodamages has no impact on IVF and the LN2-free method can be used for cryopreservation and storage of specific mouse strains.

P-042: Increased male fertility by ROS production after D-Aspartate treatment of B6N spermatozoa

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Introduction

We showed that D-Aspartate (D-Asp) treatment increases the fertility of spermatozoa from 9- and 16-week-old C57BL/6N (B6N) males. However, little is known about the mechanisms of action of D-Asp. Reactive oxygen species (ROS) plays a crucial role in sperm fertility, notably in capacitation and the acrosome reaction.

Aim.

We investigated the link between D-Asp, ROS and fertility of B6N spermatozoa.

Methods.

Cryopreserved-thawed B6N spermatozoa from 9- or 16-week-old males (n = 5) were treated for 1 h or 5 h with 4 mM D-Asp. The level of ROS was detected with dihydroethidium staining. Immunostaining was performed using a primary antibody against guanine oxidated to measure the level of DNA oxidation. Results are presented as mean grey intensity (MGI) that is proportional to the fluorescent signal. The capacitation and the acrosome reaction rates were determined after 1 h of co-incubation of D-Asp and the anti-oxidant N-acetyl-cysteine (NAC, 2.5 mM).

Results

In spermatozoa from 9-week-old and 16-week-old males treated with D-Asp, a significantly higher level of ROS was found for 1 h compared to the control (MGI: 383 vs. 287 and 387 vs. 298, respectively) and 5 h (MGI: 580 vs. 301 and 565 vs. 297, respectively) as well as for DNA oxidation after 1 h (MGI: 307 vs. 275 and 311 vs. 281, respectively) and 5 h (MGI: 378 vs. 257 and 375 vs. 271, respectively). In 9-week-old and 16-week-old mice, incubation with NAC and D-Asp reduced the capacitation rate by 42.2% and by 23.9%, respectively, and the acrosome reaction by 26.0 and 20.6%, respectively, compared to D-Asp alone.

Conclusion

These results demonstrate that in vitro D-Aspartate treatment increased the level of ROS. The addition of an anti-oxidant inhibited the beneficial effect of D-Asp on sperm maturation. This study gives new insights into the mechanisms of action of D-Aspartate and the beneficial role of ROS in male fertility.

P-043: Highly Multiplexed CRISPR/Cas9 Long Read Sequencing for Cost Effective Characterization of ROSA26 Insertions

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Identifying and characterizing genetically modified animal models using traditional PCR and sequencing methods often present significant challenges. Long-read sequencing (LRS) has revolutionized this process, enabling comprehensive characterization without PCR amplification; however, the high cost—typically thousands of US dollars per individual animal—remains a major drawback.

CRISPR/Cas9 LRS increases coverage by isolating the intended genomic target region with dual, flanking CRISPR/Cas9 cuts. In theory, the consequent increase in sequencing depth should allow multiplexing of multiple animals in a single sequencing run, substantially reducing costs. Oxford Nanopore Technologies previously offered a CRISPR/Cas9 LRS platform that has been discontinued and was challenging to run more than one sample at a time.

Recently, Pacific Biosciences (PacBio) introduced the PureTarget assay, designed to enable sequencing of up to 48 LRS samples per run. To assess the multiplexing ability with PacBio sequencing, we compared our previously-developed, in-house CRISPR/Cas9 assay with the PureTarget assay, both using gRNA sequences flanking 12 kb around the ROSA26 locus to characterize targeted insertions. These approaches allowed us to sequence 12 samples in a single flow cell, yielding comparable coverage ranging from 150X to 2000X reads per sample. Overall sequencing depth was sufficient to characterize the ROSA26 locus across all samples at a significantly reduced cost per animal.

Given the high coverage achieved in this trial experiment, we believe further optimization enabling sequencing of up to 48 samples per run is feasible and these experiments are in progress. Furthermore, CRISPR/Cas9 sequencing on the PacBio platform will allow for comprehensive characterization of a target sequence in founder animals, directly isolated from genomic DNA, at cost under $250 per sample.

P-044: The current activities of CARD mouse and rat bank

Professor Toru Takeo[1], Dr Satohiro Nakao[1], Mr Katsuma Yamaga[1], Ms Serina Kuroshima[1], Mr Ryusei Maeda[1], Mr Keisuke Masuda[1], Dr Nobuyuki Mikoda[1], Dr Yoshiko Nakagawa[1], Dr Jorge Sztein[1], Professor Naomi Nakagata.[1]

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Genetically engineered mice have contributed to advancing our understanding of gene functions and developing innovative approaches to treat human and animal diseases. Since 1998, the Center for Animal Resources and Development (CARD) at Kumamoto University has served as a hub research center to drive collaborative research projects using mouse resources. To establish a robust system of the CARD mouse bank, we have continuously developed various reproductive technologies and shared our advancements globally through online manuals and hands-on workshops. These improved reproductive technologies are helpful in efficiently managing and sharing mouse resources, while also reducing the number of animals required for research. In 2019, we expanded our scope by establishing the CARD Rat Bank, focusing on developing cutting-edge methods for rat sperm cryopreservation and in vitro fertilization using frozen-thawed or cold-stored sperm. These innovations have enabled the efficient archiving and distribution of genetically engineered rats, further supporting the global research community. In my presentation, I will introduce our advancements in reproductive technologies for mice and rats, explore their applications, and highlight the latest activities on the CARD Mouse and Rat Bank.

P-045: Dramatic Increase in zygote production after serial superovulation with hyperova in NBSGW mice—a case report

Mr Xiaojun xing[1], Dr. Suxia Bai[1], Dr. Lei Tian[1], Dr. Timothy Nottoli.[1]

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Inhibin antiserum (IAS) combined with eCG, known as IASe or commercially as HyperOva, can enhance ovulation in various mouse strains. However, for certain inbred and mutant strains, HyperOva has not been shown to be more effective than conventional superovulation.

NBSGW (Jax#026622) is an immunocompromised strain suitable for engraftment of human hematopoietic stem cells without irradiation. To generate NBSGW zygotes for microinjection for generation of a CRISPR-mediated knockin, 42 females in 3 rounds were initially superovulated conventionally and mated. 225 oocytes and 68 zygotes were collected from 9 plugged females ($\sim$ 25 oocytes/female, 7.6 zygotes/female; $\sim$ 5.4 oocytes and 1.6 zygotes/total females).

16 females from the initial cohort that did not plug in the initial sessions were retained for several weeks. 0.15 ml HyperOva was administered per female, followed 46 h later by 7.5 IU hCG and mating with the same males as before. With this one-round PMSG-hCG and one round Hyperova-hCG, a total of 119 oocytes and 104 zygotes were collected from 7 plugged females ($\sim$ 17 oocytes/female, 14.9 zygotes/female). From five remaining female mice that were not plugged and not pregnant, HyperOva-hCG was administered again several weeks later. With three serial rounds of superovulation, all 5 females plugged, and 240 oocytes and 197 zygotes were collected, yielding an 82.1% fertilization rate ($\sim$ 48 oocytes/female and 39.4 zygotes/female). From this batch, 118 zygotes were microinjected with CRISPR-Cas9 reagents and

transferred as 2-cell embryos the next day, resulting in the birth of 25 pups (21.1% birth rate).

Compared to the first round of PMSG-hCG, sequential superovulation increased plugging performance and the number of oocytes and zygotes per female 8.9-fold (48/5.4) and 24.6-fold (39.4/1.6), respectively. This case report indicates that ovulation performance may improve with age or multiple attempts, and serial superovulation is a worthy subject for further study in both genetically modified mice and wild-type mice.

P-047: Ex vivo survivability of two-cell mouse embryos within the reproductive tracts during extended incubation

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Our facility, like many others, provides an embryo cryopreservation service for labs located on different campuses in different cities. It is known that two-cell mouse embryos can maintain viability in cold storage for up to four days (PMC7677088). However, many labs may not be comfortable with isolating two-cell embryos. We therefore initiated a pilot study to examine ex vivo survivability of two-cell embryos within the female reproductive tract (ovary, oviduct, uterus) for easy delivery without shipping live animals to our facility.

To this end, we incubated reproductive tracts containing two-cell embryos in plain DMEM at either room temperature (RT) or 4C for 4 to 6, and 24 h, before collecting the embryos for vitrification. Embryos were thawed after 3 to 5 days in LN2, visually inspected to assess their quality, and either cultured to blastocysts or transferred directly into pseudopregnant females to determine their viability.

Our results showed that more than 80% of these embryos can survive inside dissected reproductive tracts at room temperature or 4C for 4 to 6 h. Furthermore, we were able to obtain viable two-cell mouse embryos after 24 h at 4C, albeit with a lower percentage of survivability as determined by development in culture and live born pups.

Our present study showed that it is possible for different vivaria to set up mating and harvest female reproductive tracts containing two-cell mouse embryos prior to delivering them to distal facilities for subsequent cryopreservation or other manipulation. This workflow allows us with more flexibilities and also lowers the cost to ship early mouse embryos without any live animals.

P-048: Stability of long-term cryopreserved human embryonic stem cells.

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Long-term cryopreservation of human embryonic stem cells (hESCs) presents significant challenges in maintaining cell viability and functionality over extended periods. Commonly used cryoprotectants, such as dimethyl sulfoxide (DMSO) and ethylene glycol, protect cells from damage during the freezing and thawing processes. The concentration and type of cryoprotectant can significantly impact cell viability and post-thaw functionality. Despite many hESC lines being cryopreserved for over ten years, their stability has not been fully elucidated.

In this study, we evaluated the stability of long-term cryopreserved (10 years or more) and thawed hESC cell lines, specifically SNUhES42 and SNUhES43. After thawing, the cells were replated on vitronectin-pre-coated dishes using Essential 8 medium. We assessed the expression of undifferentiated stem cell markers, including Oct4, SSEA-4, and Tra-1–61 through immunostaining. Additionally, the expression of markers representative of the three germ layers was analyzed using RT-qPCR, and karyotypic analysis was performed using metaphase-fixed chromosomes.

Our findings demonstrated that the thawed hESCs could attach stably and expand, though their growth rate was slower than non-thawed hESCs. Notably, the expression of specific undifferentiated stem cell markers remained high after expansion. Karyotypic analysis revealed that the cell lines maintained a normal karyotype after passages 1 and 5.

These results indicate that long-term cryopreserved hESCs can be effectively utilized for further research and clinical applications. However, maintaining chromosome stability is critical; therefore, we recommend regular short-interval checks to monitor genomic integrity.

P-049: Management of mouse sperm freezing and quality controls

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An increasing set of gene modified mouse lines is maintained for research in animal facilities. Due to changing research topics some lines are not in experiments. These lines are bred in a "maintenance mode" to secure the genetic modification, especially if the line is not considered by repositories. However, even with a minimal number of breeders, offspring is constantly arising, increasing costs and animal numbers.

The management of gene-modified mouse lines, considering three "Rs" therefore requires management strategies to reduce the number of breeding lines and typing work. To achieve this, systematic sperm freezing was introduced, based on the protocol of Takeo & Nakagata for C57BL/6N mouse lines. Moreover, different media and conditions were evaluated. Report forms were developed for documentation and quality control of cryopreservation and in vitro fertilization (IVF). More than 40 lines have been cryopreserved and successfully rederived by IVF of mouse oocytes (C57BL/6N).

To produce oocytes for fertility control by IVF, a significant reduction in the number of animals per IVF was achieved by replacing PMSG with HyperOvaCARD® for superovulation of BL6 females, while maintaining or even increasing oocyte yield. To verify sperm viability and gene modifications, PCR conditions were optimized based on the method of Scavizzi et al., allowing typing of fertilized embryos at the blastocyst stage. This approach reduces the numbers of foster mice and offspring for proof of sperm viability and correct genotype.

Frozen sperm was used to transfer mouse lines to other facilities, reducing transport animal stress and reducing quarantine procedures. Here, storage and shipping protocols using dry ice instead of liquid nitrogen were evaluated. Thus, we consider routine sperm freezing as a standard measure for the management of gene-modified mouse lines keeping all generated alleles, enabling simplified transfer of mouse lines, and providing a significant reduction of animals and costs.

P-054: Double FLEex: A leak-proof system for controlling recombinase-mediated expression of gene-targeted transgenes

Mr Roger Askew[1], Staff Scientist Jonathan Gauntlett[1], Project Coach Jacqui Watts[1], Ethics Officer & Senior Technician Nicole McLeod[1], Research Assistant/Mouse Embryologist Leah Mcgrady[1], Graduate Student MaKenna Browne[2], Professor David Kirsch[2], Radiation Oncologist Peter Hendrickson[3]

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Introduction

We previously reported that the commonly used StopFlox element, also known as Lox-Stop-Lox, is prone to a rare but potentially catastrophic leak by way of a Cre-independent deletion of the StopFlox. The example that inspired this work was a leaky gene-targeted StopFlox-regulated, CIC-DUX4 fusion oncogene model. In this case, all 104 chimeras carrying the StopFlox-regulated transgene died before sexual maturity from spontaneous tumors resulting from Cre-independent deletion of the StopFlox.

Aims and methods

As a potential solution, we developed a Double FLEx strategy in which two exons of the initially targeted fusion oncogene start out FLExed, with one FLEx being Cre-mediated and the other Flp-mediated and inverted relative to the transcript orientation. With this design, we hypothesized that we could completely prevent expression of the CIC-DUX4 fusion transcript by necessitating multiple precise recombination events.

Results and conclusions

As demonstrated in our TT2023 poster, we generated and tested this Double FLEx construct in ES cells and found that, indeed only Cre and Flp in combination effectively activated expression of the CIC-DUX4 transcript.

In this report, we demonstrate the function of the Double FLEx and Single FLEx CIC-DUX4 alleles of this transgene in mice. Double FLEx CIC-DUX4 mice are fully protected from spontaneous tumors driven by Cre- or Flp-independent recombination. However, mice with single FLEx alleles generated by crosses with either germline-Cre expressing or germline-Flp expressing parents, are not fully protected from developing spontaneous, transgene-induced tumors.

We present mouse data from experiments in which Cre and/or Flp recombinase is delivered by either transgenic crosses or by viral-mediated expression.

Discussion will include the source of Cre- and Flp-independent recombination and risks posed for highly sensitive oncology and immunology models.

P-055: One-step generation of monoallelic TCR knock-in mice targeted to the TCRβ locus results in functional mature T Cells

Ms Juliana Bortolatto[1], PhD student Jana Bilanovic[1], PhD student Susu Duan[2], Pos Doc Fabian Schmidt[1], Research Associate Harald Hartweger[4], Research Associate Valgerdur Bjørnsdottir[5], Associate Professor Amelia Escolano[6], Research Associate Paul G Thomas[2], Associate Professor Gabriel Victora[7], Associate Professor Angelina Bilate[8], Associate Professor Johanne Jacobsen.[5]

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Introduction/Aims

Traditional transgenic mouse models expressing monoclonal T cell receptors (TCRs) have been instrumental in advancing our understanding of T cell biology. However, these models are often hindered by inefficiencies such as random genomic integration, aberrant TCR expression, and the need for extensive founder screening. To address these limitations, we present a novel approach leveraging CRISPR/Cas9 genome editing and adeno-associated virus (AAV) delivery to integrate pre-rearranged TCR sequences into the endogenous TCR locus. This approach ensures physiological TCR expression, rapid generation of monoclonal TCR mice, and consistent functionality in T cell development.

Methods/Results

Our method employs optimized AAV constructs and electroporation techniques for high-efficiency targeting, enabling rapid and scalable generation of founder lines. These mice reliably exhibit normal thymic development, appropriate peripheral T cell homeostasis, and robust differentiation into key helper T cell subsets. The streamlined process minimizes resource requirements while achieving precision and reproducibility, making it suitable for a broad range of immunological studies and preclinical applications.

Conclusion

This approach provides a transformative advance in transgenic technology by overcoming the limitations of traditional TCR transgenics. By ensuring physiological TCR expression

and reducing the complexity of model generation, it establishes a versatile and efficient platform for studying antigen-specific immune responses and evaluating TCR-based therapies. The method represents a significant step forward in creating precise and reliable transgenic mouse models to meet the growing demands of modern immunological research.

P-056: Implementation of Oxford nanopore technology new chemistry to screen large insertions in new and existing mouse models

Ms Mitra Cowan[1,2], Ms. Isabelle Gamache[1,2], Ms. Hedyeh Rahimian[1,2], Dr. Haig Hugo Vrej Djambazian[3,5], Ms. Nobuko Honma-Yamanaka[1,2], Ms. Marie-Pier Cloutier[1,2], Ms. Shu-Huang Chen[3,5], Dr. Yojiro Yamanaka[1,2,4]

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CRISPR-Cas9 technology is now widely used to create genetically modified animal models. Recently, there has been a growing demand for mouse models that incorporate large transgenes (greater than 1 kb) using CRISPR-Cas9 and a donor template. To facilitate the screening, our lab has been working on applying Oxford Nanopore Technology (ONT) targeted sequencing to screen Founder (F0) mice for large transgene knock-ins. Initially, we used a CRISPR/Cas targeted sequencing kit as well as the older R9 chemistry sold by ONT, but this has since been phased out.

In response, ONT has collaborated with New England Biolabs to offer an ''home-made'' version of their previous kit. We have successfully used this protocol with their newer R10 chemistry. With this modified protocol, fortunately, we have enriched to 40X coverage the targeted region and improve the read quality.

In this poster, we will describe our workflow to screen Founder mice, their offspring, existing mouse models and discuss future directions on how this strategy could be multiplexed to decrease costs.

P-057: Rapid screening of founder mice using nanopore long-read technology

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The advent of Crispr-Cas9 genome editing has significantly facilitated the generation of genetically modified animals. However, these approaches often lead to the generation of mosaic F0 animals carrying a mixture of wild-type and modified alleles, which complicates the identification of animals carrying the desired genetic modification. Indeed, traditional screening methods, such as Sanger sequencing, are limited in their ability to detect potential founders of interest, especially in mosaic animals. This is in part due to the limited read length of this approach and the difficulty to interpret overlapping peaks, which make it difficult to efficiently identify mutations present at low frequencies.

To address these challenges, we present an improved screening workflow that uses Nanopore long-read sequencing technology to sequence amplicons followed by alignment of the reads using an openly available automated Nanopore alignment platform (https://www.bioinformatics.babraham.ac.uk/autoalign/). This innovative approach allows for faster and more accurate detection of the desired genetic modification. We will present data demonstrating that this approach is highly efficient for screening conditional alleles, point mutations, large insertions, as well as identifying low frequency mutations in founder mice that would be deemed negative by Sanger sequencing.

This advancement has been crucial in reducing the time and cost associated with screening of newly generated genetically modified animals as it requires no new equipment and the existing staff can perform the analysis. Furthermore, it has helped reduce the number of repeat microinjections, thereby enhancing research productivity and animal welfare.

P-058: Microinjection efficiency versus electroporation efficiency in transgenic animal creation

Ms Eden Dulka[1], M.S. Galina Gavrilina,[1], Dr. Wanda Filipiak[1], Dr. Honglai Zhang[1], Jennifer Leo[1], M.S. Elizabeth Hughes[1], Dr. Thom Saunders[1], Dr. Zach Freeman.[1]

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The creation of transgenic mice is an essential component of biomedical research. Since the advent of CRISPR transformed the field of transgenesis in 2013, an active area of development has focused on methods to streamline the delivery of CRISPR reagents into embryos. Two common methods to deliver CRISPR reagents to fertilized eggs include microinjection and electroporation. Microinjection is an efficient and robust method for delivery of DNA up to a megabase into the pronucleus. Electroporation can also be used to deliver DNA efficiently when molecules are less than 500 bp in size. Despite its size limitation, electroporation is an attractive method to streamline CRISPR workflows because electroporators are cheaper than microinjection rigs and skills required for electroporation can be acquired in weeks rather than the months of practice required for microinjection. To determine if electroporation can replace microinjection, we measured transgenesis efficiencies for identical DNA donors when delivered by electroporation or by microinjection. We directly compared transgene integration success rates in 9 projects: 4 mouse and 5 rat models. We included 6 knockout constructs, 2 short and 1 SNP insertions. An average of 190 eggs per each method for each project were manipulated. Overall, the number

of pups born per 100 eggs manipulated was the same between the two methods. We found no significant difference in the number transgenic animals produced per 100 eggs manipulated (n = 9 studies, $p = 0.6$, student's two-tailed paired t test). These findings demonstrate that both microinjection and electroporation are equally efficient to incorporate genetic changes in mice. Our future analysis of these datasets will examine if differences in species, type of construct, and variability between sessions are present. Thus, the judicious use of electroporation can streamline transgenesis when electroporation can be substituted for microinjection.

P-059: Use of hPREP for gluten degradation in those with celiac disease

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Celiac disease is an autoimmune disorder that occurs when the ingestion of gluten triggers an immune response, damaging the small intestine and leading to malabsorption of nutrients. Gluten epitopes, especially those rich in proline, are recognized by the body triggering an immune reaction. Prolyl endopeptidase (PEP) is an enzyme that can cleave peptide bonds at proline residues, potentially aiding in the breakdown of gluten and reducing immune responses in celiac patients. In this study, we aim to investigate the expression and activity of the hPrep gene, which encodes human prolyl endopeptidase (hPREP). We introduced hPrep cDNA into human mammary epithelial (HME1) cells through lentiviral infection to evaluate its enzymatic activity. After adding Z-Gly-Pro-pNA to the culture media of clonal transfected HME1 cells and incubating for 24 h, we observed a color change in the media to a yellow hue. This chemical reaction indicates the cleavage of the peptide bonds in Z-Gly-Pro-pNA at the proline residues, thereby confirming the activity of the expressed hPREP. No such color change was observed in the control plates. Our next step involves using chitosan wrapping to deliver the hPrep gene into the small intestine of a mouse model for celiac disease (Abadie et al., 2022). We aim to determine whether the hPREP enzyme can be successfully delivered and expressed in this environment. The chitosan coating prevents potential immune responses during gene delivery, enabling effective absorption of the hPREP nanoparticles by the intestines.

P-060: Cryptic transgenic founders discovered with advanced amplicon long read sequencing pipeline

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Detection of correctly targeted animals is critical to determining the efficiency of creating genetically engineered (GE) animal models. Traditional methods for characterizing CRISPR/Cas9 generated founder mice rely on multiple PCR reactions, Sanger sequencing and/or TOPO cloning to confirm the intended modification. Traditional methods can miss correctly targeted founders due to the difficulty of detecting complex transgenes in the context of mosaic founders that carry multiple alleles of the targeted gene. Thus, it is possible for "cryptic" targeting to occur—correct transgene insertion obscured by these and many other factors. We established an advanced long read sequencing (LRS) pipeline that reveals correctly targeted founders that would otherwise be dismissed in traditional genotyping. LRS genotyping delivers long DNA reads in a cost-effective manner allowing for a more complete evaluation of complex transgene integrations present in genetic mosaic founders. We directly compared the number of transgenic founders identified by our traditional genotyping approach and our LRS pipeline on 398 potential founder pups from 9 different CRISPR/Cas9 editing projects. Traditional genotyping identified 44 founders (median 5 founders/project) while our advanced LRS pipeline identified 103 founders (median 9 founders/project). In three cases, our LRS pipeline identified founder animals when no founders were identified by traditional genotyping. Given the substantial number of founders missed by traditional methods, we verified that multiple cryptic founders successfully produced offspring with the correctly targeted transgene. We also used our LRS pipeline to confirm a high degree of correlation for allele frequencies in sperm DNA and somatic tissue DNA collected from correctly edited founders (n = 15). We find that our advanced LRS pipeline is more sensitive than traditional genotyping identifying additional (cryptic) transgenic founders. The widespread application of our advanced LRS pipeline allows for a concomitant reduction in the number of animals required to produce founders.

P-061: DNA preparation methods and its impact on generation of transgenic alleles in mouse embryos

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The preparation and quality of DNA are critical factors that influence the viability and targeting efficiency in mouse embryo genetic modifications. High-quality DNA is essential for successful gene editing techniques towards generating transgenic and knockout mouse models. We have found DNA to be a significant element in embryo targeting noting differences in DNA preparation methods can influence embryo viability, targeting efficiency, and off-target effects. This study explores the relationship between DNA preparation and the success rates of pronuclear injection in mouse embryos. We identified an approach that can increase 2-cell progression by four-fold in sensitive strains such as NOD and another method

to optimize targeting efficiency up to 75% while minimizing off target integrations. Our findings underscore the importance of selecting the best DNA prep method to achieve the desired genetic modification to ensure high efficiency production of modified mouse models, which are indispensable for biomedical research.

P-062: Generic re-targeting of an existing Cre transgene to make a tamoxifen-inducible Cre-ERT2 allele

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Many Cre-expressing transgenic lines exist (> 1000 deposited at JAX) but only a small subset have corresponding alleles for inducible expression (eg. tamoxifen-inducible Cre-ERT2), which are valuable for studies requiring temporal control. Such situations arise when conditional deletion of a developmentally essential gene needs to be delayed due to embryonic lethality; or for lineage-tracing of progenitor cells in adult animals or injury models. We have designed and tested a method for re-targeting a validated Cre-driver strain to append the tamoxifen-responsive ERT2 domain, using a CRISPR-Cas9 gRNA and a repair ssDNA that are agnostic to the locus of insertion and therefore capable of altering any existing Cre line. Compared to de novo targeting, this strategy has the advantages of a high probability of correct expression (by modifying a validated allele); and requiring a smaller cassette (with lower cost and greater targeting efficiency) by re-using the existing Cre coding sequence. Two site-specific knock-ins were identified from 41 Cre/ + live-born candidate founders (4.9%). Conceptually, this strategy could be applied to modify other common transgenes using reusable "generic" targeting constructs to lower costs and speed production.

P-063: Impact of Megamer (ssDNA) versus Ultra HDR (dsDNA) donors on efficiency of targeted transgenesis

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Dr. Sivakumar Jeyarajan, Ms. Jennifer Leo, Dr. Eden Dulka, Ms. Elizabeth Hughes, Ms. Galina Gavrilina, Dr. Wanda Filipiak, Dr. Thomas L. Saunders, Dr. Honglai Zhang, Dr. Zachary T. Freeman.

CRISPR/Cas9 significantly increases targeted transgenesis efficiency via homology directed repair (HDR). Single stranded DNA (ssDNA) donor further improve targeting efficiency over double stranded DNA (dsDNA) donors (PMID: 28,511,701). Data from cultured cells suggests that dsDNA donors (Alt-R™ HDR donor blocks) are as efficient as Megamer™ ssDNA donor fragments in HDR. To determine if this is true for mouse transgenesis we compared ssDNA and dsDNA gene targeting efficiencies in fertilized mouse egg microinjection for two gene targeting projects. sgRNA and Cas9 was combined with either megamer or dsDNA donors to introduce the transgene. The same genotyping protocols were used to identify founders from both donors.

First, we microinjected 450 C57BL/6 J zygotes with a 664 bp donor. Out of 300 eggs injected with the megamer, 68 pups were born, and 10 positive founders were identified. Out of 150 eggs injected with the dsDNA donor, 25 pups were born, and 1 positive founder was identified. Megamer efficiency was 3.33 founders per 100 eggs compared to 0.67 founders per 100 eggs for the dsDNA donor. Second, we microinjected 707 FVB/NCrl zygotes with a 486 bp donor. Out of 415 eggs injected with the megamer, 68 pups were born, and 10 positive founders were identified. Out of 292 eggs injected with the dsDNA donor, 42 pups were born, and 3 positive founders were identified. The megamer efficiency was 2.41 founders per 100 eggs compared to 1.03 founders per 100 eggs for the dsDNA donor. The founders were assessed for both correct targeting and random integration events.

In conclusion: gene targeting with Megamer™ ssDNA donor fragments is 2.4 to 5.0 times more efficient than gene targeting with dsDNA Alt-R™ HDR donor blocks.

P-064: Accelerating transgenic founder characterization and selection by leveraging long-read whole genome sequencing

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Introduction: Random integration transgenesis is a valuable research tool enabling the addition of new features to model organisms. Central challenges to this technique are the inability to control the integration site and limited control over copy number. Transgene function can be affected by the structural composition of the transgene. Furthermore, the integration site can disrupt host genome function by causing structural variants including deletions, duplications, and translocations. Maintenance of lines without a known transgene location is more difficult due to the lack of simple genotyping tests for transgene zygosity.

Aim: Improving characterization of these integration events to overcome some of these limitations through selection of preferred alleles.

Methods: We recently developed new techniques to characterize transgenic integration events to address some of these limitations. Specialized analysis of long-read whole genome sequencing (WGS) data allows us to identify the location, copy number, and internal structure of transgenic alleles more precisely than targeted approaches, like commonly used targeted locus amplification (TLA) without necessarily terminating the animal for sampling.

Results: We sequenced genome DNA from founder sperm using long-read sequencing to characterize transgenic integrations. Using custom analysis to highlight structural variants, we observed founders containing many types of integrations, including large deletions, inversions, duplications, and

complex alleles containing numerous transgene-genome junctions in the same locus. Leveraging the power of WGS, we further identified cases where transgenes integrated within a large genomic locus which itself had been translocated. Targeted sequencing approaches, which were performed in parallel, failed to fully characterize these types of alleles.

Conclusion: These data allow us to select preferred founders for establishing new lines, and to develop specific tests capable of distinguishing the zygosity of different alleles. This method dramatically improves the time and lowers the number of animals needed to develop new lines through the selection of ideal candidate alleles.

P-065: Long read sequencing exposes errors in transgenes during quality control testing

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DNA donors are a critical component of providing a DNA template for introducing targeted or random transgenes. Quality control and assessment of DNA donors is critical to the success of efficient transgenesis. Previous methods of validating transgenes and gene targeting vectors relied on restriction enzyme digests coupled with Sanger sequencing using multiple primers to generate an assessment of the overall sequence of the DNA. Long read sequencing (LRS) has become available for whole plasmid, BAC, and linear DNA fragments for a highly reasonable price. We have used LRS sequencing to characterize 38 plasmid donors and 3 bacterial artificial chromosomes (BACs) for use in either targeted or random transgenesis. For plasmid donors, 33 were cloned and generated in house while 5 were developed by outside labs or companies prior to shipment to the Transgenic Core. DNA samples were purified and submitted to Eurofins for the appropriate LRS sequencing method. Analysis of DNA sequences was performed using Epi2ME from ONT, Eurofins default analytical pipeline or de novo assembly of reads using Flye long read assembly software. We found that for 3 of the plasmids and 1 BAC, LRS DNA sequences did not match the expected transgene designs. For all 3 of these plasmid donors, the plasmids came from outside sources and restriction digests confirmed LRS findings. In the case of one BAC sent from a repository, the sequence and restriction digest pattern were inconsistent with what was published from the source. These data highlight the importance of DNA donor characterization prior to generating transgenic models.

P-066: Compact AAV constructs for virus-mediated gene inactivation with small cas variants

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Gene inactivation is a crucial method for understanding the functions of gene products. Full and conditional knock-out animals serve as important models, however, when studying the nervous system, region-specific studies are often needed in addition to tissue or cell type specificity to gain detailed information on physiological processes, which may require somatic gene transfer. The CRISPR/Cas system is a widely used method for gene inactivation, but the large size of the most commonly used SpCas9 protein does not allow gene transfer to be performed using the simplest rAAV system.

The focus of this study was to develop alternative strategies in order to overcome the size limitations of AAV systems. One approach was based on small functional Cas variants, such as SaCas9, enAsCas12f and AsCas12f-HKRA characterized by high genome editing activity and specificity. Additionally, a compact viral construct was designed which is able to express two distinct gRNAs, the Cas protein and a fluorescent protein, utilizing the full packaging capacity of the virus. The reduced size was achieved by employing micro-promoters specific to both RNA polymerase type II and III. The use of two different gRNAs together enables a higher probability of gene inactivation, or the possibility to inactivate 2 genes in parallel. The constructs were tested on several human and mouse genes ex vivo and in the mouse central nervous system (CNS).

P-067: Precise editing of essential genes efficiently achieved by ribonucleoprotein-mediated prime editing in mouse

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The generation of predefined targeted point mutations is usually achieved in mice by electroporating zygotes with Cas9/gRNA ribonucleoprotein (RNP) complexes and a single-stranded oligodeoxynucleotide (ssODN) repair template. If the DNA double strand break is repaired by the homology directed repair (HDR) pathway, the desired mutations are introduced into the genome. However, HDR pathway is less frequent than the non-homologous end joining (NHEJ) repair pathway which causes random indels. If these indels generate a lethal allele, embryos will not develop properly, hampering the generation of mice with the desired mutations.

This work aimed at testing prime editing to overcome this issue, as this approach does not induce double strand break. It uses a nickase (nCas9) fused with reverse transcriptase (RT) and a modified guide RNA called pegRNA, with a 3' extension carrying the predefined mutations. This extension is reverse transcribed, allowing direct introduction of the edits on DNA.

Tests were conducted for the generation of point mutations by prime editing for two genes for which loss of function is lethal (Dnmt1 and Morc3). Zygotes were electroporated with different RNP concentrations and conditions, using

respectively nCas9-RT or nSpRY-RT (derived from the near-PAMless Cas9 variant) homemade proteins complexed with different synthetic pegRNAs. Efficiencies and mosaicism were evaluated by targeted DNA sequencing of individual blastocysts. The best condition was used to generate an edited Morc3 mouse line.

The optimal protocol produced the expected mutation in 100% (Dnmt1) and 30% (Morc3) of the analyzed embryos. Among these, the proportion of mutated alleles ranged from 40 to 91% (Dnmt1) and from 6 to 31% (Morc3). In vivo, 2 out of 15 pups were born with the predefined Morc3 mutations, which were transmitted to the next generation.

In summary, optimized conditions of prime editing generate predefined mutations in essential genes without producing random lethal indel mutations.

P-068: Bxb1 integrase tools for efficient large transgene integration in mice

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Bxb1 integrase-mediated transgenesis is a highly efficient tool for generating reliable and stable transgenic lines, particularly well-suited for the integration of large transgene inserts. In this study, we utilized the CRISPR/Cas system to insert the Bxb1 attB recognition sequence into various safe harbor loci of mice. With the Bxb1 integrase, we successfully introduced large (10 k and larger) plasmids containing complex transgene constructs into these loci.

To enhance the process, we designed a novel vector backbone that self-excises from the genome via the Dre/Rox recombinase system, offering an efficient alternative to mini-circle vectors for excluding bacterial-derived sequences during integration.

Additionally, we employed a cassette exchange strategy described by [Low, et al.], enabling the replacement of genomic segments flanked by Bxb1 recognition sites, enhancing efficiency and also preventing integration of bacterial backbone sequences.

Current efforts focus on developing tools to modify BAC clones for compatibility with Bxb1 integrase-mediated integration.

These developments provide a platform for efficient and precise genetic engineering.

P-069: Scalable EV-mediated rAAV production for efficient delivery and genome editing

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This study introduces a novel method for the purification of recombinant adeno-associated viruses (rAAVs) utilizing extracellular vesicles (EVs). By leveraging biologically active subpopulations of EVs, specifically exosomes and microsomes, as a matrix for the isolation and purification of rAAV vectors, we developed a new formulation termed EV-AAV. This formulation can be used for both in vivo and in vitro delivery. We demonstrate that the EV-AAV vector efficiently delivers expression constructs and homology templates. Specifically, we assessed the effectiveness of EV-AAV vectors as carriers for homology templates using the CRISPR-READI method. Additionally, we utilized the EV-AAV vector to deliver the PiggyBac transposon system, evaluating its delivery efficiency and potential toxicity. Our results indicate that the delivery efficiency and toxicity profiles of the EV-AAV vector are comparable to those of standard AAV vectors. This work provides a detailed description of the production and characterization processes involved in generating EV-AAV vectors and explores their various downstream applications in transgenesis.

P-070: Using DAJIN2 to analyze founder CRISPR-generated genetically modified mice

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CRISPR-based research methods have transformed the ability to engineer specific genetic variants in the mouse genome. Such methods often generate multiple alleles in tissues of founder (G0) mice, which can confound accurate prediction of individual alleles that might be transmitted to G1 generation offspring. This can pose challenges when trying to decide which animals to select for breeding from many potentially useful G0 mice. Initial strategies to overcome this challenge involved PCR amplification, cloning, and Sanger sequencing to define alleles. Subsequently, computational methods were developed that deconvolve complex sequence chromatograms to predict the composition of alleles (e.g. ICE analysis tool provided by Synthego) which reduced the labor and time required. More recently, second-generation tools, DAJIN2, have been developed that further enhance on-target analysis of multiple alleles in G0 CRISPR'd mice. DAJIN2 is an open source genotyping clustering tool that determines alleles in a gene-edited sample. It processes Oxford Nanopore Technology

(ONT) sequencing data to compare reads to reference sequences. In doing so, it can detect a variety of allelic variants including sequences that match the desired modification and unveil other interesting structural variants. Coupled with barcoding, it can process relatively large numbers of samples, quintessential for rapid G0 animal genotyping and remaining cost effective. We tested the genotyping capabilities of DAJIN2 with 5 different construct designs and found that DAJIN2 detected more alleles and designated allele types more accurately compared to ICE analysis. These findings allow us to make more informative decisions about breeding and colony management. This helps to meet the 3R's by reducing the number of genetically modified mice in the foundational breeding colony. We present results obtained from use of DAJIN2 in our pipeline to streamline development of lines of genetically modified mice.

P-071: Developing chimeric Cas9 proteins for improving CRISPR-mediated homology-dependent recombination in vivo

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Introduction: The CRISPR-Cas9 system has revolutionized the ability of researchers to edit genomes. However, large construct insertion at specific loci remains challenging. Improving large construct targeting efficiency requires the development of new tools. This research project aimed at developing novel smart CRISPR tools for precise in vivo genetic engineering in both zebrafish and mouse embryos.

Aims: (1) To generate novel chimeric Cas9 proteins (2) To validate their mutagenic activity in vivo in zebrafish embryos (3) To confirm their potential to enhance HDR knock-in in vivo in mouse embryos.

Methods: Suitable protein partners that should favour the HDR repair mechanism over the NHEJ in vivo were selected from the literature. The cDNA of these partners were cloned at the C-terminus of the Cas9 endonuclease for in vitro transcription. The Cas9 chimeric mRNAs produced were tested for their mutagenic potential in zebrafish embryos. The most relevant Cas9 chimeric mRNA will be further tested for its ability to integrate large constructs in mouse embryos.

Results: Four protein partners (MRE11, CtIP, RAD51 and RNF138) were selected from the literature to generate five different Cas9-chimeric mRNA. These were first tested for their mutagenic potential in zebrafish embryos. HRM analysis revealed that the five generated Cas9-chimeric constructs retained cleavage activity in zebrafish embryos. Further analyses demonstrated that the chimeric Cas9-MRE11 construct significantly improved small constructs targeting efficiency at the Gabra1 locus in zebrafish. This chimeric Cas9-MRE11 will be used to evaluate its potential to target a mCherry reporter construct at the Actb locus in mouse embryos.

Conclusions: We have generated a total of five Cas9-chimeric constructs; of these, one appears to significantly improve the targeting efficiency of small constructs at the Gabra1 locus in zebrafish. Future work will evaluate the targeting efficiency of this Cas9-MRE11 chimeric construct in mouse embryos, targeting a larger mCherry reporter construct at the Actb locus.

P-072: Oog1-Cre BAC transgenic mice: A Cre tool mice with maternal effect

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Introduction

Maternal effect genes (MEGs) are of maternal origin and influence early embryonic development through proteins expressed in oocytes. Oogenesin 1 (Oog1) is an oocyte-specific MEG expressed from oogenesis through early embryogenesis. One application of MEGs is in the Cre-loxP recombination system for targeted gene manipulation.

Aims

This study aimed to develop and validate a novel Oog1-Cre BAC transgenic mouse strain capable of mediating efficient, ubiquitous, and maternal Cre-loxP recombination to streamline the generation of complete null mice from floxed alleles.

Methods

The mouse BAC clone RP23-256M6 (Chr12: 87,649,458–87,655,620) was modified via RED/ET recombination by inserting an IRES-Cre-polyA sequence into exon 2 and a CAGGS-emGFP-polyA cassette at the 3' downstream. The modified BAC was microinjected into C57BL/6 J embryos, producing two transgenic lines: Tg(Oog1-cre)#14 and Tg(Oog1-cre)#19. Functional validation involved crossing female Tg(Oog1-cre) mice with C57BL/6-Tg(Actb-flEmGFP-FusionRed)/10Sji/Narl males. Cre activity was assessed by fluorescence expression.

Results

The Tg(Oog1-cre)#14 and Tg(Oog1-cre)#19 transgenic lines carrying Cre gene also expressed green fluorescence. After mating the female Tg(Oog1-cre) to male Tg(Actb-flEmGFP-FusionRed), offspring inheriting Tg(Actb-flEmGFP-FusionRed) accompanied with or without Tg(Oog1-cre) all exhibited red fluorescence throughout the body that indicating persistent maternal Cre-mediated excision of floxed alleles.

Conclusions

The newly developed Oog1-Cre BAC transgenic mice exhibit maternal Cre recombinase activity, enabling DNA excision in offspring without inheriting the Cre transgene. This maternal effect eliminates the need for additional breeding to remove Cre, offering a rapid and efficient tool for generating null alleles in genetic studies.

P-073: Use of deactivated Cas9 with active Cas9 to facilitate generation of mouse mutations associated with viability issues.

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Introduction

Genes that are homozygous lethal or associated with viability and welfare issues when mutant pose a challenge for genome engineering. Injecting or electroporating embryos with functional Cas9 enzyme to target these loci can lead to low-to-no pups being born and an absence of the desired homology directed repair (HDR) event. We have investigated whether using deactivated Cas9 (dCas9) in combination with wild type Cas9 may reduce the production of homozygous lethal mutants, whilst allowing the generation of heterozygous mutations.

Aims

To investigate whether different ratios of dCas9:WT Cas9 would improve pup birth rates and intended HDR events and facilitate genome editing in gene associated with lethality or welfare/viability issue.

Method.

Embryos were electroporated with mixes containing either Cas9 or dCas9:WT Cas9 at different ratios, along with guides and ssODNS donor templates. G0 animals were assessed by Sanger sequencing to determine if genome editing events had occurred and whether on-target HDR was evident.

Results

An increase in embryo to pup survival rates was observed with increased dCas9 concentrations, relative to Cas9. Rates of animals with a WT genotype (no mutation detected by Sanger sequencing) significantly differed between groups with the 90:10 dCas9: WT Cas9 having the highest average percentage. Mutation rates of any kind did not significantly differ between groups nor did the rate of on-target HDR.

Conclusions

Genome engineering outcomes can be improved when using an 80:20 dCas9: WT Cas9 ratio to target genes with homozygous mutant lethality. This improvement comes through increasing survival rates of embryos to pups whilst not diminishing Cas9 activity such that only WT alleles are seen. This outcome is likely attributable due to dCas9 recognising and shielding of one target allele whilst Cas9 nuclease generates a double stranded break, which is then modified, in the other allele in cells.

P-074: CRISPR/Cas9-mediated replacement of the mouse Mapt locus with human MAPT in PRX-B6-albino and JM8·N4 mESCs: a general strategy for gene replacement in mice

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Abstract.

Replacement of a mouse gene with its human counterpart produces a powerful in vivo tool to analyze the effect of genetic variants on gene function and organismal biology. However, the upper size limit of gene-replacement via conventional homologous recombination in ES cells is relatively small. The ability to generate targeted double-stranded breaks in the genome via CRISPR offers new opportunities for targeted gene replacement on a larger scale. We combined bacterial artificial chromosome (BAC) derived targeting constructs with CRISPR to replace the mouse Mapt locus with its human counterpart in two different mouse ES cell lines. The BAC targeting construct included two mouse homology arms, two different drug selection cassettes, and a region of human chromosome 17 containing MAPT. The replacement removed 154.7 kb containing the mouse Mapt locus, substituted with 191.6 kb of human chromosome 17 containing MAPT. Despite using the same crRNAs, the efficiency of gene replacement varied in PRX-B6-albino and JM8.N4 ES cells. Subsequent analysis of hMAPT mice confirmed expression of hMAPT mRNA and protein. The same strategy has been used to replace other murine loci with their human orthologs.

P-075: Targeted protein degradation in transgenic mouse models

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Targeted protein degradation (TPD) technology enables protein levels to be rapidly manipulated using drug-like molecules.

When degrader compounds specific to a protein of interest are not available, TPD can be achieved via genetic fusion of degron tags. In recent years, degron tags have become important tools for studying protein function in genetically engineered cells. We and others have recently adapted these systems to enable rapid, tunable and reversible protein degradation in genetically engineered mice. As part of the MRC National Mouse Genetics Network we have developed tools and protocols to support the use of degron tagging in preclinical disease models, including a novel library of degrader compounds intended for use in animal models, and transgenic mouse lines expressing inducible E3 ligases. Degradable biosensor proteins are also providing insights into the relative efficacy of degron tagging systems without complications arising from target-dependent phenotypes.

I will present our ongoing work to evaluate the auxin-inducible degron, dTAG and BromoTag systems in mouse tissues and primary cells. Selecting an optimal strategy for tag fusion remains a significant challenge for some target proteins, and I will highlight mechanisms through which tags can cause unwanted loss or gain of protein stability in the non-induced state. We find that the effects of tag fusion on protein expression is often tissue-specific, and present strategies for allele design that reduce their incidence. Despite these challenges, degron tagging provides exciting opportunities for studying fundamental aspects of protein function, validating clinical drug targets in preclinical models, and understanding cell and tissue-based mechanisms that underpin degrader drug function.

Macdonald, L., et al. (2022). Rapid and specific degradation of endogenous proteins in mouse models using auxin-inducible degrons. eLife 11, e77987.

P-076: Large-scale genome editing using type I-E CRISPR-Cas3 in mice and rat zygotes

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The type I CRISPR system has recently emerged as a promising tool, especially for large-scale genomic modification, but its application to generate model animals by editing zygotes had not been established. In this study, we demonstrate genome editing in zygotes using the type I-E CRISPR-Cas3 system, which efficiently generates deletions of several thousand base pairs at targeted loci in mice with 40%-70% editing efficiency without off-target mutations. To overcome the difficulties associated with detecting the variable deletions, we used a newly long-read sequencing-based multiplex genotyping approach. Demonstrating remarkable versatility, our Cas3-based technique was successfully extended to rats as well as mice, even by zygote electroporation methods. Knockin for SNP exchange and genomic replacement with a donor plasmid were also achieved in mice. This pioneering work with the type I CRISPR zygote editing system offers increased flexibility and broader applications in genetic engineering across different species.

P-078: Establishing guidelines for engineering conditional alleles with short artificial introns

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Engineering alleles with conditional potential remains a significant challenge in functional genomics. To address this, we and others developed a novel approach using a short artificial intron (SAI) to generate conditional alleles. This innovative strategy leverages the Cre-loxP system and consists of integrating a short DNA cassette containing minimal intronic elements, flanked by loxP sites, directly within an exon. In the absence of Cre recombinase, the SAI functions as an intron, enabling proper splicing and normal gene expression. Upon Cre-mediated recombination, the SAI is excised, disrupting the structure of the intron, and preventing recognition by the splicing machinery. The remaining sequences, which contain stop codons in all three frames, persist within the exon, thereby resulting in gene inactivation. While this approach offers a simple and cost-effective alternative to traditional methods for creating conditional alleles its broad adoption requires optimized design parameters to ensure robust functionality across diverse gene targets. To establish these parameters, we engineered a series of SCYL1 alleles in HEK293T cells, strategically varying the placement of the SAI within the gene. We found that intron placement within a gene, but more importantly within an exon is crucial for allele functionality. Moreover, we found that replacing an existing intron with a SAI provides a viable alternative to inserting the SAI within an exon, further expanding the applicability of the approach to genes with small or structurally constrained exons. By refining SAI placement guidelines, this work aims to enhance the versatility and scalability of conditional allele engineering, paving the way for broader application in cultured cells and animal models. This advancement promises to simplify the generation of conditional alleles, facilitating more efficient exploration of gene function and disease mechanisms in biomedical research.

P-079: Application of nanopore sequencing to the validation of genome engineered mouse models

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Oxford Nanopore Technologies (ONT) sequencing is a now well-established sequencing technique applicable to a wide range of genetic research and clinical applications, allowing for the production of long and ultra-long reads. We employ two ONT-based targeted sequencing strategies: (1) PCR-based

amplicon sequencing for regions of interest up to ~ 5 kb and (2) nanopore Cas9-targeted sequencing (nCATS), a PCR-free approach that utilises Cas9 RNPs to enrich libraries for regions of interest, in particular for regions too large to amplify by PCR.

Here we present the application of PCR-based amplicon sequencing and PCR-free nCATS to the quality control and validation of transgenic animals carrying newly generated knock-in or conditional alleles spanning multiple kilobases. We show that mouse models created via combinations of CRISPR/Cas9 editing and homologous recombination on the ES cell level can successfully be validated. We also show applicability to mosaic founders and F1 generations obtained by modification of early mouse embryos with CRISPR reagents and DNA donor. Resulting sequencing depths are sufficient to offset error rates inherent to the technology. ONT sequencing therefore provides an important step in the validation process of transgenic animals, such as in the identification of SNVs as well as potential structural rearrangements that are difficult to identify through conventional PCR methods and Sanger sequencing.

P-080: Investigating the role of C19orf12 in MPAN using CRISPR-engineered disease models

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Mitochondrial membrane protein-associated neurodegeneration (MPAN) is one of the most common subtypes of neurodegeneration with brain iron accumulation (NBIA) caused by mutations in the C19orf12 gene. This rare genetic disorder is characterized by progressive neurodegeneration, abundant alpha-synuclein-positive Lewy bodies, and iron accumulation in the globus pallidus and substantia nigra. However, the pathogenic mechanisms underlying MPAN remain unclear. In this study, we investigated an MPAN disease model based on an autosomal dominant MPAN case in Taiwan with a novel heterozygous frameshift mutation in C19orf12 c.240 G > AA (p.P81Tfs*9) using CRISPR/Cas9 and prime editing (PE) technologies. While recent studies suggest that the role of C19orf12 may be related with lipid metabolism and ferroptosis regulation, our preliminary findings in C19orf12-deficient HEK293T cells revealed no significant differences in sensitivity to ferroptosis inducers. To further investigate the pathogenicity of MPAN, we established isogenic control lines from patient-derived induced pluripotent stem cells (iPSCs) with a CRISPR-based approach. By examining the mitochondrial function, lipid metabolism, and iron metabolism in human iPSC-derived neurons, dopaminergic neurons, astrocyte, and oligodendrocytes, this study aims to uncover the cellular and molecular mechanisms of C19orf12 in MPAN.

P-081: Optimizing CRISPR-Cas9 genome editing experiments considering the contextual DNA sequence to reduce mosaicism

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Preliminary observations served us to confirm that the repairing process after a genome editing event with CRISPR-Cas9 is not entirely random. In other words, replicates tend to display similar profiles. We observed that the cutting efficiency was highly variable, depending on the RNA Guide, with the most common allele usually accounting for 10–20% of all alleles generated and with about 25 additional alleles accumulating already 70% of the repairing profile. We inferred that the most common alleles were deletions/insertions of 1 base pair occurring at + 1/-1 with respect to the DSB (usually a duplication of the nucleotide sitting at -1). The sequence of the region near the DSB seems to be the most important to determine the most likely repairing outcome by NHEJ: insertion or deletion. In particular, the position at -1 is the most crucial. When T or A are in that position, the probability of getting an insertion increases, whereas having a C or a G decreases it. In summary, bioinformatic analyses suggest that the outcome of a CRISPR-Cas9 experiment can be possibly predicted, with a significant probability, simply analyzing the target genomic sequence and placing the RNA guide at a given position. We have explored the optimization of the standard CRISPR-Cas9 genome editing experiments considering the contextual DNA sequence. We have selected 4 RNA guides leaving different bases (A,C,G,T) at position -1, to determine whether we can obtain different levels of mosaicism. This is an in vitro study. To validate these CRISPR strategies we have used mouse embryos, at the blastocyst stage, and genotyped the results by NGS, without the need to generate additional live animals.

P-083: Liveborn rates when culturing to blastocyst stage in mice with CytoSpring HTF + high calcium culturing media

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Introduction

The ability to consistently achieve acceptable liveborn rates following embryo culture to blastocyst stage has been an ongoing problem for many Transgenic facilities. Following Cook Biomedicals discontinuation of Vitro Cleave media, we tested the ability of a potential replacement, CytoSpring Human Tubal Fluid (HTF) + high calcium, to generate liveborn mice after embryo culture to various developmental stages.

Aims

To determine Cytospring HTF + high calcium's culture ability we performed a series of experiments measuring liveborn rates of B6C3F2 and C57BL/6 J embryos following in vitro development to various embryonic stages. Liveborn rates were calculated as pups born/embryos transferred following surgical implantation into pseudopregnant females.

Methods

B6C3F2 embryos were obtained from natural matings and C57BL/6 J embryos were obtained by IVF following super-ovulation of donor females. Starting at 0.5 dpc, embryos were cultured in CytoSpring HTF + high calcium to blastocyst stage, ~ 3.5 days-post-coitus (dpc) for B6C3F2 and ~ 4.5 dpc for C57BL/6 J. Daily throughout culture, a subset of embryos from each strain was implanted into pseudopregnant females to ascertain liveborn rates at progressive stages of development.

Results

Following culture in HTF + high calcium, we achieved a ~ 30% liveborn rate for B6C3F2 embryos throughout all stages of development. Liveborn rates in C57BL/6 J were ~ 25% for 1.5–2.5 dpc embryos and ~ 10% for 3.5–4.5 dpc embryos.

Conclusions

Our findings indicate that embryos cultured in CytoSpring HTF + high calcium are capable of developing into live pups across all tested implantation stages; this challenges the conventional belief that extended culture is universally harmful for embryos and that blastocysts resulting from ~ 3–4 days in culture hold little potential for liveborn. The ability to confidently culture embryos for extended periods grants a broad level of flexibility to Transgenic experiments.

P-084: Derivation, culture and genetic modification of mouse embryonic stem cells (mESC) at PHENOMINICS

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Introduction

Organoid-based experiments are invaluable for studying organ development and disease modeling. These experiments rely on high-quality starting materials, such as mouse embryonic stem cells (mESCs). Our team is specialized in deriving mESC lines from any genetic background, including wild-type, transgenic, and mutant mice. Additionally, we offer custom generation of genetically engineered alleles, including fully humanized alleles, tailored to meet specific research needs.

Aims

We aim to provide researchers with custom mESC lines derived from live or frozen embryos, sperm, or mice, while also creating 'à la carte' genetically engineered mESCs using CRISPR-assisted or traditional approaches.

Methods

Our standardized protocol enables the derivation of mESCs from diverse sources and genetic backgrounds. For cases where no mouse line harboring the desired mutation exists, we modify our proprietary wild-type mESCs to introduce custom alleles. Our team employs advanced genetic engineering tools, including CRISPR and traditional mESC techniques, to generate precise modifications, such as large-scale humanizations.

Quality control measures ensure the integrity of the derived lines, including Southern blotting, long-range PCR, ddPCR for chromosomal anomalies, Giemsa karyotyping, and genotyping.

Results

We have achieved consistent success in all mESC derivation attempts. CRISPR technology has enabled the generation of alleles previously considered challenging or unfeasible, such as complex genetic modifications and large-scale humanizations. Our proprietary mESC lines exhibit a high transmission rate of over 80% per injected clone, supporting efficient in vitro differentiation and downstream applications.

Conclusions

Our expertise in deriving high-quality mESC lines and engineering complex genetic modifications positions us as a valuable resource for researchers in organoid-based experiments and genetic studies. We are committed to providing tailored solutions to advance your research. For inquiries, contact us at mutagenesis@igbmc.fr.

P-085: Rat resource and research center

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The NIH-funded Rat Resource and Research Center (RRRC) serves as a centralized repository for maintaining/distributing rat models and providing rat-related services to the biomedical community. Currently, the RRRC has over 600 rat lines and all are archived by cryopreservation to ensure against future loss. The RRRC distributes live animals, cryopreserved sperm/embryos and rat embryonic stem (ES) cell lines. Quality control measures for all materials include extensive genetic validation and health monitoring. The RRRC has expertise in rat reproductive biology, colony management, health monitoring, genetic assay development/optimization, isolation of germline competent ES cell lines from transgenic rats and can partner as consultants/collaborators. Fee-for-service capabilities include a wide variety of genetic analyses, strain rederivation and cryopreservation, isolation of rat tissues, custom breeding/colony management, microbiota analysis and characterization of genetically engineered rats. The RRRC can make genetically engineered rat models from start to finish using a variety of state-of-the-art technologies including genome editing (e.g., CRISPR/Cas9) as well as traditional methods such as random transgenesis and modified embryonic stem cell microinjection into blastocysts. Our website (www.rrrc.us) allows user-friendly navigation. Current research efforts include generation and characterization of a variety of new rat models, optimizing genetic engineering techniques in rats, and improvements to rat sperm cryopreservation and rat in vitro fertilization. The University of Missouri is home to the NIH-funded MU Mutant Mouse Resource and Research Center (MMRRC), the National Swine

Resource and Research Center (NSRRC), the MU Animal Modeling Core, the MU Metagenomics Center, and the new MU Rat Testing Center for Somatic Cell Genome Editing. Together, these highly collaborative groups provide a variety of animal model-related services across species to facilitate biomedical research.

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P-086: Presenting the mitochondrial genotyping pipeline at the Mary Lyon Centre at MRC Harwell

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With an estimated 1 in 5000 people suffering from a genetic mitochondrial disease, the need for animal models to study these diseases remains high. These diseases can be homoplastic, where all the mitochondrial DNA copies within an individual have the same nucleotide sequence, or heteroplasmic, where an individual has more than one type of mitochondrial DNA. A threshold effect is observed in heteroplasmic mitochondrial mutations. The threshold is the level of mutant mitochondrial DNA copies that causes a disease phenotype. Due to this threshold effect, determining the precise level of heteroplasmy is crucial in studying mouse models of mitochondrial disease. At the Mary Lyon Centre at MRC Harwell, we have compared two different methods to genotype heteroplasmic mutations in mouse models: amplification refractory mutation system quantitative polymerase chain reaction (ARMS-qPCR) and pyrosequencing. From this we concluded that pyrosequencing was the most reliable and reproducible method to genotype precise levels of heteroplasmy in mouse models.

P-087: Progressive impairment of cardiac function in response to a short-term intraperitoneal administration of oil-based vehicle

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The Tamoxifen-Cre/loxP system has been extensively utilized to study gene function, offering researchers the ability to control gene expression with high precision. This system leverages the activity of Cre recombinase, which is activated in response to Tamoxifen (TAM), allowing for spatiotemporal control over gene expression. While the system provides unprecedented control over gene expression, it is essential that experiments using this system are meticulously designed and rigorously controlled to ensure accurate and reliable results. A critical aspect of using the Tamoxifen-Cre/loxP system is the preparation of TAM itself, which must be dissolved in an oil-based solution (commonly sesame or corn oil). However, the potential side effects of these oil-based vehicles have not been thoroughly investigated, raising concerns about their impact on experimental outcomes. Here we show that sesame oil administration induces significant decline in cardiac function over time. Specifically, we show that cardiac function in DBA2J mice remained unaltered at short-term (7 days) post-injection. However, we observed a significant decline in cardiac function in the intermediate (30 days) to long term (> 6 months), accompanied by changes in cardiomyocyte autophagic activity. Additionally, serum triglyceride levels were significantly elevated in the intermediate and long-term post-injection groups, likely contributing to myocardial lipid accumulation, and altered fatty acid metabolism. Mitochondrial dysfunction was also detected in the long-term experimental group, further indicating potential adverse effects of the oil-based vehicle. These findings underscore the importance of conducting properly controlled experiments in preclinical models.

P-088: Rescuing the aceruloplasminemia phenotype in mice via prime editing

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Aceruloplasminemia (ACP) is a rare, adult-onset, autosomal recessive genetic disorder and one of the subtypes of Neurodegeneration with Brain Iron Accumulation (NBIA). ACP is characterized by widespread iron accumulation throughout the body, particularly in the liver and brain. The pathogenesis of ACP is caused by mutations in the CP gene, leading to the absence or dysfunction of ceruloplasmin (CP). CP is a serum ferroxidase that oxidizes toxic ferrous iron (Fe2 +) into non-toxic ferric iron (Fe3 +). Consequently, when CP expression is reduced, ferrous iron accumulates in various organs, leading to iron overload. The current treatment for ACP involves the use of intravenous or oral iron chelators. These treatments can normalize serum ferritin levels, reduce iron overload in the liver, and alleviate symptoms of anemia and diabetes in most patients. However, it remains unclear whether neuropathy can be effectively treated with this approach. Therefore, we aim to address this issue through gene therapy, specifically by directly editing the CP mutation. In this study, we focus on an ACP patient with a mutation at the c.607 + 1 delG site, identified by Dr. Chin-Hsien Lin at National Taiwan University Hospital. We generated a Rosa26-hCP c.607 + 1 delG minigene knock-in allele in an ACP (Cpnull/Cpnull) mouse. Subsequently, we will use lipid nanoparticles (LNPs) or virus-like particles (VLPs) to deliver prime-editing components into the ACP mice to test whether

the human mutation can be corrected and whether ceruloplasmin can be synthesized to reverse the ACP mouse phenotype.

P-089: Human exon replacements in mice to accelerate translation of nucleic acid-directed therapies

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Development of nucleic acid-directed therapeutics would be accelerated by mice with disease-causing mutations in local human sequence context. Such mice could allow testing of the same therapeutic which would be used in patients. To meet this need, we generated CFTR human exon-replacement mice with cystic fibrosis (CF) mutations. Because the human and mouse CFTR genes have 27 exons with matching exon–intron boundaries, we were able to employ an exon-replacement strategy. Individual mouse Cftr exons were replaced by injecting Cas9, gRNAs and dsDNA constructs with the human exon and small segments of flanking introns into zygotes. We tested if wild-type exon-replacements are fully functional by replacing exons 3, 11, 12, 23 or 26 with the corresponding wild-type human exon. All 5 homozygous wild-type human exon-replacement strains are wild type in phenotype with full CFTR channel activity. We generated exon-replacements with CF mutations: E60X in exon 3, F508del in exon 11, G542X and R553X in exon 12, W1282X in exon 23, and Q1411X in exon 26. All 6 mutant exon-replacements have CF phenotypes as homozygotes, with phenotypic severity and channel activity matching expectations from mouse and patient mutations. We restored function of the mutant exon-replacement genes with candidate therapeutic approaches in vitro, demonstrating that the strains are valid models for testing therapeutics. Finally, we corrected the R553X mutation in mice with a base editor delivered to airway basal stem cells by lipid nanoparticles, showing that the exon-replacement strains are powerful models in vivo. We conclude that exon-replacements can accelerate the development of nucleic-acid directed therapeutics for cystic fibrosis. Given our success, we propose that the exon-replacement strategy can be applied to other human genetic diseases where gene structure is conserved.

P-090: 3R method: Electroporation of CRE recombinase to floxed cKO embryos to produce KO mouse models

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Genetically engineered animal models, such as Knock-Out (KO) and Conditional Knock-Out (cKO) models, are crucial in biomedical research for understanding human diseases, studying mechanisms, and developing therapies. KO models allow researchers to study the complete loss of gene function, providing insights into gene roles and disease mechanisms. cKO models enable precise gene disruption in specific tissues or at specific times, offering a more targeted approach to studying gene function and disease progression.

Very often scientists need both models for their studies. Creating the two models individually is possible but involved more project management and genetic characterization resources. Moreover, the KO models generated this way may not always have the exact same mutation as the cKO allele after CRE excision, which can be scientifically less precise.

Another method is to create and genetically characterize the cKO model first, and then modify it to generate the full KO. A traditional way would be breeding the cKO model with a line expressing Cre recombinase in germ cells or early development. This method, technically efficient, requires at least 2 generation to get KO, involve the management of a CRE recombinase model and has a certain animal footprint. The approach described here is using In Vitro Fertilization (IVF) and electroporation (EP) of the Cre recombinase, significantly reducing the number of animals used and the time required.

We performed IVF with cKO males and wild-type females to produce cKO embryos, which are then electroporated with CRE mRNA to induce the KO allele. This approach has demonstrated high efficiency, with up to 100% CRE excision efficiency in both ex-vivo and in-vivo tests. The process requires only one generation and eliminates the need for maintaining CRE colonies, resulting in a 50% reduction in the number of animals used compared to traditional breeding methods

P-091: Lessons learned: errors in mouse genome editing and tools to tackle the challenges of working with different genetic backgrounds

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CRISPR-Cas9 technology has transformed the generation of genetically modified mice, enabling precise genome editing for diverse research applications. However, the success of these modifications depends on the design of guide RNAs (gRNAs), which directs Cas9 to specific genomic sites. A critical but often overlooked factor in gRNA design is the genetic background of the mouse strain, which can significantly influence editing efficiency, specificity, and phenotypic outcomes. Failure to account for these variations can result in inefficient modifications, or unexpected errors in the genetic manipulation process, and not all design tools consider other strains besides C57BL/6 J.

Ensuring precise gRNA design tailored to the genetic background not only improves the efficiency and accuracy of genome editing but also aligns with the principles of the 3Rs (Replacement, Reduction, and Refinement). By minimizing failed experiments, researchers can significantly reduce the number of animals required and enhance animal welfare. Implementing tools that consider genetic variability is a critical step toward more ethical and sustainable research practices.

This poster will explore the importance of using gRNA design tools that consider the genetic background of mice to

enhance precision and reproducibility in CRISPR experiments. We will present a case where a designed target site failed to induce genetic edition due to a SNP variation. Addressing strain-specific differences is essential for advancing the reliability of genetically modified mouse models in biomedical research.

P-092: Disturbed liver zonation is observed in transgenic mouse lines expressing human hepsin

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The liver is the body's most important metabolic organ, performing numerous functions with its unique structure of metabolic zonation. Disorders in liver metabolism may lead to disease such as Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD), which currently has no approved medication. Therefore, there is an urgent need to develop new therapeutic targets. Hepsin, a type II transmembrane serine protease primarily expressed in the liver, has been reported to play a role in liver glucose and lipid metabolism, though much remains unclear. Here, we aim to investigate the physiological functions of hepsin using transgenic mouse models. We previously generated transgenic mouse lines (Tg-hHPN) that specifically express human hepsin in the liver. The expression was under the control of human α1-antitrypsin promoter, ApoE enhancer, and human FIX intron 1 splicing enhancers. The transgenic line was obtained by pronucleus microinjection of a 4.2 kb linear DNA. Two transgenic lines were established: Tg68, expressing wild-type human hepsin, and TgRS, which express active-site mutant form of human hepsin. In the livers of Tg68 mice, immunofluorescence staining of E-cadherin, expressed in the periportal zone, and glutamine synthetase, expressed in the pericentral zone, showed disturbed pattern of liver zonation. Specifically, E-cadherin expanded closer to the pericentral zone compared to non-transgenic littermates. Western blot analysis confirmed an increase in liver E-cadherin protein levels along with a decrease in glutamine synthetase protein levels. Furthermore, elevated levels of HDL, LDL, total cholesterol, and triglyceride in blood were observed, suggesting changes in liver metabolism due to disturbed liver zonation. No difference was observed in TgRS mice. In conclusion, we demonstrated that Tg-hHPN mice aid in investigating the role of hepsin in regulating liver zonation and metabolism.

P-093: Expanding the versatility of the Hipp11 safe harbour locus for transgenic models

Ms Claire Fergus[1], Dr Jill Browning[1], Dr Tania Bezak[1], Dr Ciara Nulty[1], Professor Vincent Kelly.[1]

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Safe harbour loci that enable predictable, stable transgene integration without silencing, tissue specificity bias, or adverse effects on organismal health are invaluable tools for studying gene function, cell-specific expression, and beyond. The Hipp11 intergenic region has previously been identified by our lab and others as an efficient safe harbour locus for CRISPR-mediated transgene integration.

Here, we showcase the versatility of the Hipp11 locus, highlighting its successful application across a range of transgenic models developed over multiple studies in our lab. These include faithful gene expression, complex conditional models, and large-scale integrations of up to 17 kb. We have successfully generated multiple transgenic models in mice using this locus and extended its application to human systems by creating transgenic cell lines at the human orthologous site.

Our optimized CRISPR-Cas9 targeting strategies are tailored to the specific requirements of donor templates (e.g., plasmids, double-stranded linear DNA, and single-stranded oligonucleotides) and the target system (embryos or cells). These strategies employ Cas9 delivered via the pX330 vector or as a ribonucleoprotein complex, with physical delivery achieved through pronuclear microinjection or electroporation.

Here, we present a diverse selection of transgenic models to underscore the adaptability and broad utility of the Hipp11 safe harbour locus for advancing genetic engineering and functional genomics.

P-094: Development of viable rat models using two cell injections to circumvent early lethality

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Advances in genome engineering using endonuclease technology combined with direct embryo engineering have expanded the availability of genetically engineered models for species where the lack of embryonic stem cell technology has previously been a limiting factor. However, an inherent risk of generating mutants using nuclease-mediated embryo targeting is the creation of indel, frameshift, and missense mutations, which can lead to consequential gene knockouts and, in the case of essential genes, lethality.

Knockout of the cardiac-specific troponin Tnni3 gene in mice results in early post-natal lethality due to acute heart failure. Early attempts to develop a disease-causing point mutation using standard nuclease-mediated embryo engineering in rats resulted in a similar phenotype with early lethality due to a large proportion of indels in chimeras. Therefore, alternative strategies were needed to circumvent detrimental effects of inadvertent gene knockouts.

Here, we aimed to overcome these viability challenges for a troponin rat disease model using alternative DNA targeting strategies and direct embryo engineering at the 2-cell stage. Using pinpoint cell penetrator-assisted microinjection, only one cell of the two-cell embryo was targeted for nuclear injection. Successfully injected embryos were then transferred into pseudo-pregnant Sprague Dawley recipients. Careful timing of transfers resulted in live-born pups that survived past the previously described early lethality, were fertile, and could generate germline transmission.

In summary, we describe effective approaches to circumvent issues with inadvertent gene knockouts causing early lethality, thereby expanding the spectrum of models that can be generated with direct embryo engineering in rats.

P-095: Oxford Nanopore adaptive sampling for comprehensive characterization of large, engineered alleles

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Introduction

Confidence in experimental data generated using mutant animals relies on the initial, accurate characterization of these models' induced mutations. Historically, ascertaining the definitive genetic sequence of these mutations within the context of the native locus has been laborious and limited in scope to the immediate vicinity of the modification. These challenges are compounded when mutations involve the introduction of large amounts of exogenous sequence that are intractable to PCR.

Aims.

We sought to streamline and fortify our analysis of mutant alleles using long-read sequencing technologies. Oxford Nanopore adaptive sampling enhances coverage at a specified region based on real-time alignment to a reference sequence; we assessed this technique's ability to analyze the ~ 5.8 kb and ~ 14.7 kb transgenes of two previously generated models.

Methods.

HMW DNA was extracted from heterozygous mutant mouse ear biopsies. After shearing to 15-20 kb, 300 ng of DNA was loaded onto a Nanopore MinION supplied with reference and buffered.bed files. After NanoPlot QC, the resulting fastq files were aligned to the reference using minimap2 to generate aligned BAMs, with and without de novo assembly using Flye/HapDup. Results were visualized using IGV, allowing comparison between each line's engineered and wild-type alleles.

Results

Depending on stringency of alignment criteria, coverage across edited alleles ranged from ~ 10-250x. Alignments allowed comparison of edited sequences consistent with the original design and provided no evidence of unintended CRISPR-associated chromosomal rearrangement.

Conclusions

These experiments evaluated Nanopore adaptive samplings ability to resolve the sequence of two large knock-in alleles. Although the resulting depth of coverage fulfilled this goal to varying degrees, our results definitively determined the edited gene integration patterns and facilitated the development of additional assays enabling full characterization. Adaptive sampling workflow adjustments are ongoing, with optimization of factors (specified.bed files, fastq acceptance metrics, alignment criteria, assembly strategies, etc.) in progress.

P-096: Overview of regeneron genotyping pipeline: building capacity through automation and innovation

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The Genotyping Group determines genotypes of genetically modified rodents produced by Regeneron. To achieve rapid turnaround and high throughput, Real-Time qPCR is employed, which allows for genotype calling by copy number counting. Since 2012 Regeneron has significantly expanded its vivarium capacity, and consequently the demand for genotyping has increased (from 126,144 samples in 2012 to 251,461 samples in 2024). Additionally, genotype complexity has risen, as the number of modified alleles to be genotyped has increased from 2.8 per animal in 2012 to 6.8 in 2024. Therefore, our group has deployed key innovations to build genotyping capacity.

We created workflows to merge our team's workload, use reaction wells more efficiently, and digitally track samples. We automated biopsy sample processing, experimental planning, and execution, as well as data analysis.

New software tools enabled an increase in throughput. These include a database of 2D barcoded qPCR assays, permitting automated retrieval of assay sets, and a custom Excel macro to analyze data and upload results to a database, which stores and disseminates these results. We also implemented a LIMS for assay and sample tracking and built custom software to plan daily genotyping experiments.

Further, we implemented several robotic platforms. An automated biobank freezer enabled storage and retrieval of qPCR assay vials, which are measured by a volume check platform. We added liquid handler capacity: a Hamilton Star for the preparation of allele specific qPCR master mixes (on average 135 per day), and Biomek i7s for combining master mixes and sample DNA into 384 well plates. A hands-free platform, equipped with robotic arms and scheduling software, can distribute 288 plates per day to thermocyclers. Finally, we developed an automated platform to process and consolidate samples for DNA isolation.

These improvements have eliminated bottlenecks, improved risk management, increased productivity, decreased turnaround time, and substantially increased throughput.

P-097: Innovative sequential electroporation strategy for generation of floxed allele in C57BL/6 J mice.

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Introduction: Recent studies demonstrate efficient introduction of loxP sites by sequential electroporation compared to sequential microinjection in hybrid B6D2F1 embryos. Current sequential electroporation protocols lack application in C57BL/6 J embryos which are commonly used mouse strain for genome editing. Advancements in technology enabled genetic manipulations in various mouse strains, including C57BL/6 J embryos utilizing one-directional high-voltage poring pulses (PP) in combination with bi-directional low-voltage transfer pulses (TP). However, there still remains challenges in using sequential electroporation. We hypothesize minimizing exposure to high-voltage shocks by reducing PP followed by low-voltage TP in sequential electroporation can significantly improve survival and development in C57BL/6 J embryos.

Aims: We investigate the capability of efficiently deriving conditional allele mice in C57BL/6 J embryos by introducing PP in sequential electroporation (Aim1) and introducing Rad51 protein, a homologous recombination factor (Aim2).

Methods: Fertilized C57BL/6 J embryos were loaded with RNP complex or fluorescent mRNAs and electroporated at PP: 0–30 V, 3.5 ms length, 50 ms interval, 1, 2, or 4x, 10% decay, + polarity and TP: 5 V, 3.5 ms length, 50 ms interval, 5x, 40% decay, ± polarity. Electroporated embryos were cultured to observe survival and fluorescent expression or were implanted for consequent genotyping for homologous loxp sites in cis orientation.

Results: Our data investigating electroporation efficiency and survival of C57BL/6 J embryos showed potential in successful generation of conditional allele models using optimized PP and Rad51 protein. Cultured blastocysts treated with PP of 30 V showed strong transfection efficiency with overlapping GFP and mCherry fluorescent expression and ~ 67% of embryos retaining GFP expression until blastocysts as well as high survival rate of compared to group from 60 V condition (~ 78 and ~ 7%, respectively).

Conclusions: We have fine-tuned electroporation parameters suitable for successful generation of C57BL/6 J embryos by introducing PP. High PP followed by low TP, in addition to Rad51 protein treatment synergistically propagated efficiency in survival and development.

P-098: Reverse aceruloplasminemia mouse phenotype using LNP-delivered CP cDNA

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Aceruloplasminemia (ACP) is a rare autosomal recessive neurodegenerative disorder caused by CP mutants leading to ceruloplasmin deficiency. This deficiency disrupts systemic and cerebral iron homeostasis, which results in iron accumulation in various organs thus cause subsequent tissue damage as well as neurological dysfunction. As a subtype of neurodegeneration with brain iron accumulation (NBIA), ACP is distinct from other subtypes of NBIA due to systemic iron overload. As for the ACP treatment, it primarily relies on oral iron chelators, such as deferasirox and deferiprone, which are limited by their inability to cross the blood–brain barrier and associated side effects like leukopenia and liver injuries. In addition, enzyme replacement therapy has been proposed as a potential treatment. However, its long-term efficacy is hindered by the development of anti-drug antibodies. In contrast, gene therapy offers a promising alternative by leveraging molecular biology techniques to deliver specific genetic material to patients, which effectively addresses the underlying genetic defects. This study focuses on a CP c.607 + 1 delG mutation identified in ACP patients at the National Taiwan University Hospital, aiming to evaluate the potential of gene therapy as a treatment for ACP. To this end, we established a humanized ACP mouse model and will utilize lipid nanoparticles (LNPs), virus-like particles (VLPs), or herpes simplex viral vectors to deliver CP mRNA in vivo for therapeutic purposes. This approach seeks to restore ceruloplasmin expression and reverse the pathological and phenotypic manifestations of ACP.

P-099: Transgenic tools for Sprague Dawley rats

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The laboratory rat has been an important model organism in biomedical research for a century. However, the adoption of transgenic technologies in rats has lagged behind the mouse due to the more recent development of germline competent rat embryonic stem cells (rESC), a fundamental gene targeting technology. We have derived germline-competent Dark Agouti (DA) rESC; using these cells, we have produced dozens of precisely targeted large modifications in rats using conventional blastocyst microinjection (MI) of DA rESC into albino Sprague Dawley (SD-tyrC) host blastocysts, a process which produces F0 chimeras in which patches of pigmented fur indicate DA rESC contribution. This method is robust, but it limits the use of the SD genetic background, which is preferred in many fields of research due to its many decades of historical data and its high fertility and fecundity.

We set out to take advantage of the SD rat genetic background in our gene targeting/blastocyst MI pipeline by first deriving rESC lines from SD rats. These lines are euploid and are being evaluated for germline transmission and gene targeting efficiency. We have also produced a strain of fully pigmented SDblack rats, to use either as MI hosts or for future rESC derivation. To do this, we used CRISPR technology to produce 2 modifications in SD-tyrC rats. First, we reversed the R299H tyrosinaseC mutation, which blocks melanin synthesis, in SD zygotes, resulting in pigmented SD-tyr + rats that showed the hooded coat color pattern (pigmented fur the head, neck and dorsal midline). Second, we reversed the hooded mutation in SD-tyr + zygotes by deleting an endogenous retrovirus (ERV) in the 1st intron of the kit locus to produce SD-black rats. These can be used as hosts for albino SDtyrC rESC MI and to derive SDblack rESC for use with SDtyrC host blastocysts.

P-100: Powerful new technologies combine to improve efficient generation of precision mouse models

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Introduction

With our ever-expanding knowledge of the genetic causes of disease there is an increased need for advanced, precision animal models to understand disease pathobiology and to serve as platforms for preclinical investigation.

Aims.

To this end, we have established a robust, streamlined platform for the precision engineering of disease causing mutations coupled with humanization of the mouse genome. This platform allows for large-scale modification of the mouse genome in both mouse embryonic stem cells (mESCs) and mouse zygotes and takes advantage of powerful technologies including CRISPR-Cas9 and Bxb1 Recombinase Mediated Cassette Exchange (RMCE).

Methods.

CRISPR-assisted gene targeting is used to pre-position a Bxb1 Landing Pad allele into any locus on a defined mouse strain. Delivery of a donor plasmid and the Bxb1 Integrase mRNA enables RMCE of the cassette at high efficiency, excluding the vector backbone by design. The result is a single-copy precision transgenic allele positioned in a defined genetic locus. Digital PCR enables rapid identification of founder candidates, including low contribution mosaics that might otherwise be missed by standard screening techniques. Copy number verification in the line(s) selected for breeding prevents downstream complications from undesired off-target insertion events. Long-Read Sequencing by ONT ensures that the allele is completely defined, down to the single-base level.

Results

Using this technique, we have created many new mouse lines with genetic modifications ranging from 1.5 kb to 77.3 kb, with an average success rate of 15% for RMCE-positive founders. Our ongoing work with mESCs is showing promising early results for BAC-scale replacements.

Conclusions

Combined, these techniques enable efficient production and verification of the designed allele with confidence in a streamlined, rapid manner.

P-101: Evaluating single and paired CRISPR/Cas9 guide RNA strategies for generation of single nucleotide polymorphisms in rodent models

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Transgenic animal models with human disease associated SNPs are valuable to study the underlying mechanism of disease and for therapeutic evaluation. CRISPR/Cas9 has greatly facilitated generation of SNP transgenic animal models by allowing for easy insertion of the desired alterations. Traditional approaches for generating these models rely on a single guide RNA (gRNA) placed near the desired SNP coupled with short single stranded DNA donors. This approach is highly efficient but requires a PAM adjacent to the desired location and may require silent coding changes that may impact the interpretation of the impact of the human SNP. An alternative approach, termed exon replacement, utilizes gRNAs in the 5' and 3' exon flanking intronic regions coupled with a megamer containing the human SNP inserted into the rodent exon. Advantages of this approach include that sequence changes required to inactivate gRNA are placed in introns with no impact on coding sequence and an increased flexibility in gRNA design. We have evaluated 58 projects that utilized either single gRNA (n = 48) or paired gRNA (n = 10) with exon replacement strategies to successfully create targeted SNPs transgenic mouse and rat models. A median number of 315 zygotes were injected for single gRNA projects and 443 zygotes for exon replacement. Single gRNA projects had a median of 82.5 live pups and 8 transgenic founder animals and exon replacement projects had a median 91.5 live pups and 17 transgenic founder animals. We found that the two approaches were similarly efficient with 2.73 founders per 100 eggs injected with single gRNA and 3.59 founders per 100 eggs injected with exon replacement. Exon replacement has multiple advantages compared with single gRNA approaches while being similarly efficient. These data suggest that a exon replacement should be considered for wider application of introducing SNPs.

P-102: Novel mouse models of baker-gordon syndrome

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Baker-Gordon syndrome is a rare neurodevelopmental disorder characterized by mutations in the synaptotagmin-1 (SYT1)

gene. This gene codes for the protein synaptotagmin-1, which is a neuronal synaptic vesicle transmembrane protein responsible for binding calcium to facilitate exocytosis and neurotransmitter release. Currently, no treatment for Baker-Gordon syndrome exists, and little is known about the factors driving variable symptoms in patients. To better understand how these mutations cause disease, we are developing two distinct mouse models. The first approach uses CRISPR-Cas9 gene editing to incorporate a D365E (corresponding to human D366E) missense mutation into the endogenous mouse Syt1 gene. The nucleotide sequence including and surrounding the mutation in this model is well conserved between mouse and human DNA, which will allow for the evaluation of targeted and translatable gene therapy. The second approach utilizes CRISPR-Cas9 to insert a DNA template including the CAG promoter and Cre-dependent Syt1 D365E mutant cDNA into the mouse Rosa26 safe harbor locus. This model was crossed with a ubiquitously expressing CMV-Cre model to induce mutant Syt1 D365E expression as confirmed with DNA sequencing and RNA sequencing analysis. However, the Cre-dependent nature of this model also allows for spatiotemporal control of Syt1 D365E expression to be manipulated for further evaluation of the mechanism behind pathogenesis. The two models described here can be utilized to further characterize phenotypes, investigate which cell types are driving particular phenotypes, and evaluate potential therapeutics for Baker-Gordon syndrome.

P-103: Evaluating SD-Nanos2em1, an alternative to vasectomized males for inducing pseudopregnancy

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Mating with vasectomized males is one of the most commonly used methods for inducing pseudopregnancy in female rats used as embryo transfer recipients. While vasectomizing rats is not technically challenging, it does require subjecting animals to a surgical procedure. Our lab has generated SD-Nanos2em, a novel rat model that contains a deletion of the Nanos2 protein coding region. This knockout of the Nanos2 gene results in the absence of mature spermatogonia in homozygous mutant males. While these rats are sterile, it is unknown if their genetic mutation also affects their mating behavior. Our hypothesis is that homozygous SD-Nanos2em1 males will exhibit normal mating behavior and can be used to induce pseudopregnancy in female rats. To test this, homozygous mutant males will be mated to hormonally synchronized 8–10-week-old female rats overnight. The next morning, females will be examined for the presence of copulation plugs. Vaginal cytology will be performed over a 10-day period to monitor the estrous cycle and determine if pseudopregnancy has been induced. Additionally, embryo transfers will also be performed to definitively show that this induced pseudopregnancy is sufficient to establish and maintain pregnancy. This would provide validation of the Nanos2 knock out rat as a genetically infertile model that could be used as an alternative to vasectomized males for generating pseudopregnant recipient females for embryo transfer procedures. It would represent a 3Rs refinement by providing an alternative to surgically vasectomized males. (funded by NIH grant P40 OD011062).

P-104: CRISPR-based genome engineering for transgenic model development: overcoming barriers to efficient DNA insertions

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Abstract.

CRISPR-based genome engineering has advanced significantly in the last decade, particularly in the knockout of genes and the insertion of point mutations. However, the efficient knock-in of medium (> 50 bp) and large DNA elements (> 1kp) remains a significant challenge.

In this study, we benchmarked industry-leading CRISPR-based methods to insert small, medium, and large DNA elements into the genome, focusing on their application to the development of transgenic animal models. These methods leverage distinct DNA repair pathways. We present representative examples for each insertion size, identify key design criteria important for knock-in success, and demonstrate editing efficiencies that meet or the exceed current state-of-the-art.

Our results highlight that success is driven by careful design and optimisation, including attention to key metrics and the use of enrichment strategies. Despite these advancements, we emphasise that current CRISPR technologies still require further innovation to consistently achieve high efficiencies for larger DNA insertions across diverse cellular contexts.

P-105: Avatar mouse model of a new genetic variant of autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS)

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Introduction

The first clinical case of a 3-year-old girl with ARSACS was reported in our country in 2023. Exome sequencing analysis revealed two compound heterozygous variants in exon 10 of the SACS gene at chromosome 13. One variant, previously described and classified as "damaging", generates a premature stop codon, 19 codons after a frameshift (p.His1915Argfs*19). The second variant produces a missense mutation (p.Pro2369Leu; UniProt Q9NZJ4). This is a novel variant and has been predicted as pathogenic/damaging by in silico physicochemical scores. Few ARSACS cases have been reported in our region, showing the need to deepen the detection and etiologic characterization of this rare disease.

Objective.

The aim of this work is to use CRISPR/Cas technology to generate a compound heterozygous mouse model for ARSACS carrying the mutations found in the 3-year-old girl ("Avatar mouse").

Construct design and methodology.

The patient's mutations are located in regions where the human and the murine SACS genes are highly similar, making the design of the Avatar mouse reasonably straightforward. Although some additional synonymous mutations had to be included due to the nature of the CRISPR/Cas9 system, the modifications were designed to be as conservative as possible. The methodological plan follows the classic pipeline for producing genome-edited mice. A BTX-ECM-830 electroporator (30 V, 2 pulses of 3 ms, 100 ms interval) has been used to deliver the CRISPR reagents (Cas9/sgRNA RNPs and ssODNs) into C57BL/6 J mouse zygotes.

To obtain the ARSACS Avatar mouse it is necessary to generate two models, each one of them reproducing one of the mutations of the parents. The Avatar mouse will be produced by crossing these lines.

Significance of the project. The molecular, phenotypic, neurochemical and behavioral characterization of the model will allow us to better understand the biology of the disease and, hopefully, test specific treatments in the near future.

P-106: Endogenous protein tagging in mice using a simplified CRISPR/Cas9 knock-in approach

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Title

Abstract

Introduction

The CRISPR/Cas9 system has been used to generate fluorescently labeled fusion proteins via HDR repair in many species. Despite its revolutionary success, there remains a need for increased simplicity and efficiency. Building on previous studies conducted in other models, we are working towards developing a streamlined, high-efficiency, and precise strategy for CRISPR/Cas9-mediated endogenous protein tagging in mice.

Our approach utilizes PCR-amplified donor fragments containing fluorescent reporter sequences flanked by short homology arms (30–40 bp) in combination with a Cas9-RNP complex. We have targeted several loci and achieved promising results. While still optimizing the method, we are excited to share our findings with the ISTT community.

Aims

1. Implement a simplified and efficient CRISPR/Cas9-mediated endogenous protein tagging strategy in mice.
2. Target multiple loci, achieving high efficiency in F0 targeting and germline transmission (GLT).

Methods

1. Test different conditions, such as reagent ratios and delivery methods, to identify the optimal working condition.
2. Design sgRNAs and PCR-amplified donor DNA fragments with fluorescent reporter sequences for different loci and deliver them into mouse embryos.
3. Screen integration events for copy number and proper integration, along with GLT assessment.

Results & Conclusions

We present a cost-effective and accessible approach for endogenously tagging proteins in mice.

While existing gene-editing techniques have shown good efficiencies, they often require considerable time and resources, particularly for producing repair templates with long homology arms. In contrast, our method streamlines the process by enabling the generation of donor templates through a single PCR, making it both affordable and accessible to labs worldwide.

Although further experiments are needed to refine the technique, initial results are highly promising.

P-107: Vitrification of murine zygotes using Spatula MVD in CRISPR/Cas9 technology

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Introduction

Microinjection of CRISPR/Cas9 requires the continuous supply of zygotes, implying animal breeding, superovulation schemes, and embryo collection. Zygote vitrification may allow having ready-to-use embryos and to temporally dissociate the embryo production from microinjection.

Aim.

To compare the outcomes in the production of CRISPR/Cas9 gene edited models in fresh vs. vitrified zygotes.

Experimental design.

C57BL/6 J fresh (F group) or vitrified-warmed (V group) zygotes were microinjected with CRISPR/Cas9 RNP and then transferred to pseudopregnant females at two-cell stage.

Spatula MVD vitrification method was used. Microinjection was carried out in CRISPR projects that were ongoing at the facility and each microinjection session (six per group) was managed as a replicate. Embryo survival, cleavage, pregnancy and birth rates were determined. Efficiency of CRISPR/Cas9 system (mutation rate) was evaluated in terms of generation of indel mutations by heterodimer detection in PAGE and Sanger sequencing.

Results

A total of 1121 C57BL/6 J zygotes were microinjected (F group n = 563, V group n = 558). Embryo survival after microinjection was significantly lower in fresh than in vitrified zygotes (49.2% vs. 62.7%, respectively; $P < 0.05$). Cleavage rate did not show statistical differences ($\sim$ 70%; P = NS). Pregnancy rate (70.0% vs. 58.3%) and birth rate (11.9% vs. 11.2%) were not different between groups (F vs. V group; P = NS). Offspring mutation rate was higher for F than V group, in both heterodimer analysis (73.7% vs. 33.3%, respectively; P = 0.015) and Sanger sequencing (89.5% vs. 41.7%, respectively; P = 0.006).

Conclusions

The use of vitrified-warmed zygotes for CRISPR/Cas9 microinjection showed lower efficiencies in mutation rates comparable to fresh ones. However, the availability of vitrified embryos seems to be a suitable alternative to the production of fresh zygotes when technical staff and time are limited. The cryobanking of zygotes avoids the year-round breeding and maintenance of animals, improves the implementation of CRISPR technology, and complies with the 3R principles.

P-108: Generation of a Cre, Frt and PhiC31 recombinase transgenic reporter rat

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Introduction: Site-specific recombination systems are important tools for spatial and temporal investigation of gene function. However, Cre/loxP is the only recombination system that has been used. The other recombination systems have not been tested in the rat.

Aims: To expand the experimental tools for studying genes and their function in rats, we generated a triple recombinase reporter rat (ALF-mCherry/βgal-EGFP) which could be utilized flexibly to screen and validate vectors or transgenic rats expressing Cre, Flp or PhiC31 recombinases.

Methods: The ALF-mCherry/βgal-EGFP reporter rat, ubiquitously expressed mCherry, which switches to LacZ and EGFP reporter expression after Cre, Flp or PhiC31 recombination, was generated through pronuclear injection of PiggyBac vectors. A novel VASA-Cre transgenic rat line in which Cre is expressed specifically in germ cells (including eggs and sperm) was generated through pronuclear injection for Cre/loxP system.

Results: From a total of 19 live rats, six (31.6%) expressed mCherry when observed under epifluorescence microscopy. When VASA-Cre rats were bred with red fluorescent ALF-mCherry/βgal-EGFP recombinase reporter rats, their progeny expressed the βgal and EGFP transgenes in all tissues. Adenoviruses harboring Flp or PhiC31 recombinases were injected into the muscle of ALF-mCherry/βgal-EGFP rats, resulting in recombination (red to green conversion) at the injection sites. Thus, the ALF-mCherry/βgal-EGFP rat is an effective tool for screening recombinases that will facilitate the development and validation of conditional expression constructs in all rat tissues. The one exception identified is that rat Sertoli cells appear to have endogenous Flp activity, causing the red to green conversion of seminiferous tubules even in the absence of ectopic recombinase.

Conclusion:The ALF-mCherry/βgal-EGFP rats can be used broadly to validate novel Cre, Flp and/or PhiC31 rat lines that that will enable spatial and/or temporal control of targeted genetic modifications to study genes and their functions.

P-109: Abhd18, identified as a genetic modifier of Tafazzin, is a mitochondrial phospholipase and its inhibition restores mitochondrial function in Barth Syndrome

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Barth Syndrome (BTHS) is an inherited rare genetic condition. Some of the symptoms in affected individuals include cardiomyopathy, neutropenia and growth delay. Like most rare diseases a single gene defect, in this case a mutation in Tafazzin on the X-chromosome, has been shown to cause BTHS.

Although a single gene alteration underlies BTHS, the severity of the disorder can vary considerably between patients. One explanation for the variability of symptoms observed in the clinic, is the presence of modifier genes. Modifier genes can increase or decrease the severity of the condition, and in some cases can even prevent onset and progression of the disease. This makes modifier genes potentially attractive therapeutic targets, and their discovery can represent a valuable opportunity for the development of novel therapies.

In order to better understand the the onset and progression of BTHS, we have generated a mouse model. As a result of a Tafazzin mutation, mice develop phenotypes that mirror the clinical signs of BTHS. Furthermore, we could clearly demonstrate the existence of Tafazzin modifier genes in the mouse by showing that the genetic background of the mouse strain alters the impact of the mutation.

One such modifier gene is Abhd18, which was discovered using a genetic screen in cells carrying a Tafazzin mutation. Abhd18 was subsequently shown to function as a phospholipase, upstream of Tafazzin in the synthesis pathway of a unique mitochondrial lipid, cardiolipin. By generating a mutation in the Abhd18 gene, we were able to show that loss of Abhd18 function almost completely rescued the clinical signs of BTHS in the Abhd18KO; TafazzinKO mice. Demonstrating that Abhd18 functions a suppressor of the BTHS

phenotype in our mouse model, has identified a clear opportunity for new a therapeutic path for this rare disease.

P-110: Ehlers-Danlos syndrome biorepository: a key resource for advancing the understanding of genetic connective tissue disorders

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Ehlers-Danlos syndromes (EDS) are a diverse group of 13 hereditary connective tissue disorders first documented by Hippocrates in 400 B.C. Individuals with EDS carry mutations in genes related to collagen or its processing, folding, and stabilization within the extracellular matrix. Although the manifestations of EDS vary significantly among its different types, most patients exhibit some degree of joint hypermobility, hyperextensible ("stretchy") or fragile skin, and fragility of blood vessels and organs. Certain types of EDS are associated with life-threatening complications and/or shortened life expectancy, while all types are characterized by a high prevalence of chronic pain. Currently, there is no definitive treatment for EDS.

To expedite EDS research, MUSC has established a comprehensive mouse biorepository for each of the 13 EDS subtypes utilizing CRISPR-Cas9 technology. These mouse models are provided for free to researchers to accelerate discovery, catalyze new findings, generate additional funding opportunities, and lead to innovative care for patients.

The repository includes all subtypes of EDS incorporating specific known genetic variations. These subtypes and genes include:

Classical EDS (cEDS): COL5A1.
Classical-like EDS Type 1 & 2 (clEDS): AEBP1.
Cardiac-valvular EDS (cvEDS): COL1A2.
Vascular EDS (vEDS): COL3A1.
Hypermobile EDS (hEDS): Gene X.
Arthrochalasia EDS (aEDS): COL1A1, COL1A2.
Dermatosparaxis EDS (dEDS): ADAMTS2.
Kyphoscoliotic EDS (kEDS): FKBP14.
Brittle Cornea Syndrome (BCS): ZNF469.
Spondylodysplastic EDS (spEDS): B3GALT6.
Musculocontractural EDS (mcEDS): CHST14.
Myopathic EDS (mEDS): COL12A1.
Periodontal EDS (pEDS): C1R.

P-111: Efficiency comparison of CRISPR/Cas9-mediated gene Knock-in between different donor DNA condition and ctRNP concentration

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The production of genetically modified mice has entered a mature stage. With the growing demand for CRISPR/Cas9-engineered mice, there is a strong desire to achieve the best results in the shortest possible time. Over the past few years, our core facility has explored various donor DNA design strategies and adjusted the concentration settings for microinjection, aiming to determine conclusive optimal conditions.

Among more than 70 cases we've processed, we found that even when performing gene knokcin at the same genomic locus, the insertion efficiency is not necessarily inversely correlated with the length of the inserted sequence. From the perspective of the GC content ratio in the homologous arms flanking the insert DNA fragment,

in some difficult cases, when the 5' homologous arm contains higher GC%, the knockin efficiency is lower when using the regular Cas9:ctRNA ratio of 1:5 or 1:12. In these cases with higher GC% in 5' homologous arms, we need to adjust the Cas9:ctRNA concentration at the ratio of1:5 to achieves successful knockin.

In summary, we found that the efficiency of gene insertion using the CRISPR/Cas9 system primarily depends on whether the target genomic locus is part of a more open euchromatin structure, which allows effective recognition and cleavage by Cas9/gRNA. Furthermore, even when using the same gRNA at the same genomic locus for gene insertion, the potential secondary or tertiary structures of the donor DNA sequence itself can significantly influence the efficiency of DNA insertion. Finally, the GC% in the 5'-homolgous arm of the donor template can be a factor be be considered when concentration adjustment is required.

P-112: Transgenic models from design to reality: challenges prior to use in R&D

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Transgenic models remain a tool of choice in R&D, not only to better understand the pathophysiology of certain diseases, but also to assess the potential of drug candidates. However, there may be differences between the theoretical design of the model and the effects observed in vivo. These issues, if not identified early in the project, can lead to bias in the evaluation of a therapeutic molecule, extensive breeding of an unusable strain, and loss of time and resources in R&D projects.

The Transgenic In Vivo Models Department at Sanofi is installing a process that integrates early characterisations for all new transgenic strains developed for the different therapeutic areas. This presentation will give an overview of this integrated process, the problems we have identified and the methods we use (molecular biology, flow cytometry or histopathology, etc.) to confirm the suitability of a model for the needs of a project.

P-113: The role of C19orf12 in neurodegeneration with brain iron accumulation

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Neurodegeneration with brain iron accumulation (NBIA) is a rare neurodegenerative disease affecting 1–3 per million people, characterized by iron accumulation in the globus pallidus and substantia nigra. Mitochondrial membrane protein-associated neurodegeneration (MPAN), a subtype of NBIA caused by germline mutations in C19orf12, accounts for 10–30% of NBIA cases. MPAN onset typically ranges from childhood to early adulthood, with symptoms including progressive dystonia-parkinsonism, ataxia, motor axonal neuropathy, optic atrophy, cognitive decline, psychiatric symptoms, and premature death. While initially identified as an autosomal recessive disorder, dominant inheritance has been observed in some cases with C19orf12 C-terminal truncations. However, the function of C19orf12 and its role in MPAN pathogenesis are not fully understood. To investigate MPAN pathogenesis in vivo, we developed autosomal recessive (AR) and dominant (AD) MPAN mouse models and performed comprehensive neurological assessments. Both motor and cognitive deficits were observed in these mice, supporting a causal link between C19orf12 mutations and neurological disease. Furthermore, epitope-tagged C19orf12 variants were used to investigate the protein's molecular properties. Our results suggest that C-terminal truncated C19orf12 exerts a dominant-negative effect by reducing full-length protein levels. Overall, our study demonstrates that C19orf12 mutations lead to neurodegenerative phenotypes and elucidates the mechanism underlying dominant inheritance.

Keywords: C19orf12, MPAN, NBIA, mouse model, dominant-negative.

P-114: Primed for success: unlocking essential gene editing with prime nuclease

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For over a decade, the South Australian Genome Editing (SAGE) Facility has supported researchers by providing custom gene-edited mouse models for biomedical research.

The continuous evolution of CRISPR targeting technologies has driven an increasing demand for more sophisticated disease models, particularly for studying essential genes. While gene editing in non-essential genes is relatively straightforward, modifying essential (e.g. recessive lethal) genes still poses significant challenges due to the risk of bi-allelic mutations disrupting embryonic development.

To address this, we have implemented a novel prime editing nuclease system that incorporates a silent "rescue" allele to preserve cellular function during gene targeting through microinjection of mouse zygotes. This innovative approach not only aims to maintain viability to generate a founder line but also enables precise and reliable gene edits in essential regions.

The ability to perform targeted editing of essential genes represents a major advancement, unlocking opportunities to explore disease mechanisms associated with patient-specific mutations. By generating specific models of essential gene alterations, this technology provides critical insights into the underlying biology of genetic disorders and facilitates the development of targeted therapeutic strategies.

P-115: T cell receptor knockin mice by CRISPR-Cas9 mediated insertion of long single-stranded DNA

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Introduction

Whereas αβ T cell receptor (TCR) transgenic mice, like OT-1 and OT-2 mice, were crucially important for understanding the T cell biology of the αβ T cell lineage, similar mouse models are missing for the γδ TCR. One reason is that antigens for γδ T cells are less well understood. To our knowledge, no orthotropic knockin mice have been generated. This is important for a proper understanding of the thymic development of T cells, however, as TCRα recombination eliminates the TCRδ locus.

Aims.

To overcome the limitations of random integration of TCR genes, mouse models harboring an orthotopic TCRγδ replacement should be generated.

Method.

We targeted the endogenous Tcrδ locus with CRISPR-Cas9 technology. For homologous recombination, two sites were chosen upstream of the first Dδ gene element and downstream of the last Jδ. Recombination was pursued in fertilized oocytes using two gRNAs at the exact recombination breakpoints and an ssDNA DNA template with short homology arms. One TCDδ knock-in mouse and one TCDγδ knock-in mouse were generated. For the latter, the coding region of the full γ-chain was connected to the variable region of the TCRδ chain by a ribosomal skipping sequence.

Results

We obtained TCRδ and TCRγδ knock-in mouse lines with high efficiency. Expression of the TCRδ and TCRγδ chains correctly reflects the normal differentiation pathways in the thymus. The αβ T cell lineage is not affected by the knock-in,

whereas frequencies of γδ T cells in the thymus and peripheral lymphoid organs are enhanced 5–tenfold for the TCRδ mouse and almost 50-fold for the TCRγδ mouse.

Conclusions

Homologous recombination of rearranged γδ TCR genes into the TCRδ gene locus allows the highly efficient analysis of thymic and peripheral differentiation and selection processes. Similar strategies will be evaluated for new αβTCR mouse lines.

P-116: Large gene sequence knock-in to mouse genome via zygote microinjection

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Our core facility has processed more than 820 genome editing projects in mice using CRISPR/Cas9 technology. Approximately 83% of the projects were site-specific transgene insertions (gene knock in, KI) and ~ 17% were targeted gene disruptions or deletions (gene knock out, KO). We will share our experience in generating mouse lines with large gene sequences knock-in to mouse genome at H11, Rosa26, and other loci by zygote microinjection.

We have generated mice with gene disruptions or deletions of up to 3.2 million bases (Mb), or insertions ranging from a few bp to 18.4 kb via mouse zygote microinjection with CRISPR technology. Based on analysis of the current data, the average efficiency for gene KO through NHEJ is ~ 60%. The average efficiency of gene KI through HDR or HR varies by method, from ~ 18% using oligo DNA donors, and ~ 35% using long ssDNA donors to ~ 40% using plasmid DNA donors. Our results from approximately 360 projects using plasmid DNA donors demonstrate that high gene KI efficiency could be achieved with combined efforts in several key steps when CRISPR reagents are injected into mouse embryos at 1-cell stage.

P-117: Engineering and analysis of mutant mice deficient for bitter taste receptors

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Bitter taste receptors (TAS2Rs), a class of G protein-coupled receptors, are expressed in various organs beyond the oral cavity and are known to play critical roles in a variety of biological processes. Emerging evidence suggests that TAS2Rs could serve as highly promising drug targets for the treatment of several diseases, particularly asthma. TAS2R agonists have demonstrated significant therapeutic effects in preclinical models, including robust bronchodilation, suppression of allergen-induced airway inflammation, regulation of airway smooth muscle cell proliferation, and mitigation of airway remodeling. These findings suggest that targeting TAS2Rs might offer a superior therapeutic approach for asthma treatment compared to currently available options.

Despite this potential, an important question remains unanswered: are TAS2Rs themselves directly required for these therapeutic effects, or do TAS2R agonists primarily act through alternative biological pathways? It is possible that these agonists exert their effects via interactions with other cellular receptors or ion channels in the respiratory system, independent of TAS2Rs.

To address this fundamental question, we are systematically deleting all 35 Tas2r genes from the mouse genome, which are distributed across nine genomic regions, including seven located on chromosome 6. To date, 31 of these genes have already been removed, and the final mutant model will feature the complete elimination of all Tas2r genes. This comprehensive strategy overcomes challenges associated with functional redundancy among Tas2r gene clusters, enabling precise functional analyses. The success of these gene deletions has been confirmed at both the DNA and functional levels.

Given the absence of classic orthologs between human TAS2Rs and mouse Tas2rs, these Tas2r-deficient mice provide a foundation for "humanizing" the models with human TAS2R genes. This innovation will drastically facilitate our mechanistic and therapeutic studies of human TAS2Rs in vivo, advancing our understanding of their roles in health and disease.

P-118: The long and short of ROSA26 homology arms: the difference between success and failure of gc rich and repetitive sequence Knockins

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Homology arms (HAs) play a crucial role in the homologous recombination of ectopic gene knock-ins (KIs) in docking harbors such as ROSA26 in mice. We have developed a methodology utilizing the CRISPR/Cas9 system in combination with various donor vectors to introduce genes of interest into the ROSA26 locus. Our core constructs include two CAG-LoxP-STOP-LoxP UMTG ROSA26 targeting vectors: one with an 811 bp right HA (UMTG1) and the other with a 4339 bp right HA (UMTG2), both sharing the same 837 bp left HA.

To compare the effects of different 3' homology arm length on targeting efficiency, UMTG1 and UMTG2 were applied in parallel across three projects involving cDNAs with GC-rich and/or repeat elements. We injected a median of 360 zygotes per targeting vector, achieving an average post-injection survival rate of 90% with UMTG1 and 88% with UMTG2. Birth rates for the UMTG1 vectors were 12%, 20%, and 11%. Birth rates for the UMTG2 vectors were 12%, 15%, and 17%. The same genotyping assays were used to for both targeting vectors. We found that no correctly targeted pups were produced when the UMTG1 vector arms were used. In contrast, with the UMTG2 vector arms, the targeting efficiency of was 5.5%, 8.0%, and 3.2% transgenic pups compared to total number of pups born, or 0.7, 1.0, and 0.5 for the number of transgenic pups per 100 microinjected zygotes.

The use of the long 4.3 kb homology arm for GC-rich and/or repetitive DNA sequences into ROSA26 locus significantly increased knockin efficiency, marking the difference between success and failure.