



# Abstracts of the 18th Transgenic Technology Meeting (TT2023)

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## Oral Presentations

### 0-1 Efficiencies of Different Genetic Modification Techniques in Rat Embryos

**Dr. James McNew<sup>1,2</sup>, Daniel Davis<sup>1,3</sup>, Hongsheng Men<sup>1,4</sup>, Elizabeth Bryda<sup>1,2,3,4</sup>**

<sup>1</sup>Department of Veterinary Pathobiology, University of Missouri, Columbia, United States, <sup>2</sup>Comparative Medicine Program, University of Missouri, Columbia, United States, <sup>3</sup>Animal Modeling Core, University of Missouri, Columbia, United States, <sup>4</sup>Rat Resource and Research Center, University of Missouri, Columbia, United States

Session 1: Precision Animal Models of Human Disease I, November 13, 2023, 09:00–10:55

CRISPR-Cas9 technology has revolutionized our ability to create genetically modified animals. Many animal models require the need to insert (knock-in) preconstructed DNA templates called repair templates. DNA repair templates along with CRISPR-Cas9 reagents can be introduced into embryos by pronuclear injection (PNI), electroporation (EP), or delivery via adeno-associated virus with electroporation (AAV + EP). Currently, no published literature compares the efficiency of these delivery techniques as it relates to DNA insertions via CRISPR mediated genome editing in rats. We used a 400-base pair (bp) repair template consisting of homology arms flanking a floxed short artificial intron designed to target exon 2 of the *Crh* gene. Superovulated Sprague Dawley (SD) female rats mated to SD stud males were used to generate zygotes. Zygotes were randomly assigned into four groups: culture only control, PNI, EP, and AAV + EP. After manipulation, embryos were cultured to the blastocyst stage and submitted for Next Generation Sequencing (NGS) to detect evidence of genome editing. Embryo survival after one day in culture was significantly less following PNI, 58% (101/175), compared to the culture only control, 98% (109/111). Cleavage rates and

development to a 4-cell stage did not differ between embryos that survived 24 h in culture. Knock-in rates for manipulated embryos were 67% (12/18) for PNI, 0% (0/35) for EP, and 63% (22/35) for AAV + EP. We conclude that PNI decreases embryo survivability but not development, and that EP and AAV + EP do not decrease embryo survival or development. Using a 400 bp DNA repair template, we found knock-in rates were similar with PNI and AAV + EP while the template failed to be inserted into the genome with EP only.

### 0-2 Random insertion transgenesis: a fluorescence based selection approach

**Viktor Lang<sup>1</sup>, ShiTing Misaki Hu<sup>1</sup>, Juergen Klawatsch<sup>1</sup>, Ms Tabitha Tombe<sup>2</sup>, TienYin Yau<sup>1</sup>, Dieter Fink<sup>1,3</sup>**

<sup>1</sup>Institute of Laboratory Animal Science, University of Veterinary Medicine Vienna, Vienna, Austria, <sup>2</sup>Vancouver Prostate Centre, Vancouver, Canada, <sup>3</sup>British Columbia Cancer Research Institute, Vancouver, Canada

Session 2: Precision Animal Models of Human Disease II: Tiered Talk, November 13, 2023, 10:55–12:45

Random insertion transgenesis has been proven useful in the past besides its drawbacks regarding position effects, concatenation arrays, and non-controllable genomic rearrangements. Without direct readout of expression patterns, selecting the right founder animals expressing the gene of interest at constant levels, can prove challenging. To render this technique more controllable, we re-designed the lox-stop-lox (LSL) vector that now allows for removal of the fluorescent reporter, utilizing flp recombinase. This way, the mouse lines can be established, the marker removed, and the fluorescent channel is free for other applications. In-vivo imaging systems were used for quantification of fluorescence in founder animals and their offspring. Based on fluorescent levels and educated guess, we selected the respective animals for further breeding. This

allows for segregation of non-linked multiple integration events and to establish the mouse lines. To further demonstrate the power of this technique, we selected low, medium, and high expressing transgenic lines. Within this work, we demonstrate a novel strategy to establish random insertion transgenic mouse lines that simplifies the comparison of transgene expression levels in living mice. Non-bred transgenic F1 animals might be cryopreserved (sperm and ovary) until the selected lines are established. Considering the 3Rs, we reduce the colony size, shorten the timeline necessary for colony establishment, altogether leading to lower animal numbers.

### 0-3 Dual Flex Transgenic Systems: Paired Genetic Switches for Leakproof Conditional Transgenes and Intersection Biology

**Mr Roger Askew<sup>1</sup>, Senior Scientist Jonathan Gauntlett<sup>1</sup>, Resident Doctor Peter Hendrickson<sup>2</sup>, Radiation Oncologist and Clinician Scientist David Kirsh<sup>3</sup>**

<sup>1</sup>Department of Veterinary Pathobiology, University of Missouri, Columbia, United States, <sup>2</sup>Comparative Medicine Program, University of Missouri, Columbia, United States, <sup>3</sup>Animal Modeling Core, University of Missouri, Columbia, United States, <sup>4</sup>Rat Resource and Research Center, University of Missouri, Columbia, United States  
<sup>1</sup>Ozgene, Perth, Australia, <sup>2</sup>Duke University, Durham, United States, <sup>3</sup>Princess Margaret Cancer Center/University Health Network, Toronto, Canada

Session 2: Precision Animal Models of Human Disease II: Tiered Talk, November 13, 2023, 10:55–12:45

We developed a leakproof genetic switch for the purpose of providing tight control of gene expression in conditional On/Off, Off/On, or reversible transgenic alleles.

The Dual Flex system was developed as a solution to recurrent failures using traditional StopFlox (also called Lox-Stop-Lox) elements to regulate the Off/On expression of a potent transgenic oncogene. The problematic model was a conditional oncogenic fusion of CIC-DUX4 using a StopFlox targeted to the Rosa26 or endogenous Cic locus. In three attempts to generate the model, 100% (108/108) of chimeric animals carrying the targeted allele developed tumors in the absence of Cre-recombinase and died before sexual maturity. Using PCR to amplify and sequence across the StopFlox cassette coupled with immunohistochemistry, we confirmed spontaneous (Cre-independent) recombination resulting in CIC-DUX4 mis-expression and metastasis driven mortality. A similar Cre-independent loss of the StopFlox has been observed and reported by others (1).

The Dual Flex system uses two Flex elements, one Cre-based and one Flp-based, each designed to conditionally invert an anti-sense exon into the correct orientation with respect to the promoter and remainder of the CID-DUX4 oncogene. The Dual Flex genetic switch operates similar to multifactor authenticator (or dual key lock), requiring authentic recombination-mediated inversion of both anti-sense exons to activate synthesis of the proper coding transcript and expression of the oncogene. Therefore, this switch should not fail, and leaky

expression of the oncogene by either simple deletion, or by accidental exposure to one of the recombinases, should not occur.

We will present the latest status and characterization the Dual Flex CID-DUX4 model under development. We will also explore possible causes of Cre-independent deletion of the StopFlox.

Reference:

1) Katerina Politi, Ana Kljuic, Matthias Szabolcs, Peter Fisher, Thomas Ludwig and Argiris Efstratiadis ‘Designer’ tumors in mice. *Oncogene* 23, 1558–1565 (2004).

### 0-4 Adaptive sampling long-read sequencing for characterization of CRISPR/Cas9-generated founder transgenic mice

**Dr Zachary Freeman<sup>1</sup>, Weisheng Wu<sup>1</sup>, Laura Burger, Elizabeth Hughes<sup>1</sup>, Thomas Saunders<sup>1</sup>, Shipra Garg<sup>1</sup>, Olivia Koues<sup>1</sup>, Judith Meyers<sup>1</sup>, Suzanne Moenter<sup>1</sup>, Chris Gates<sup>1</sup>**

<sup>1</sup>University of Michigan, Ann Arbor, United States

Session 3: Animal Models in Space Biology I, November 13, 2023, 13:45–15:45

Complete characterization of CRISPR/Cas9-generated animal models presents many challenges that may require advanced sequencing methods. We previously used a long single stranded donor combined with a single gRNA strategy to generate an endogenous iCre knock in the Npvf gene in C57BL/6 J mice. G0 founders were identified by PCR and Sanger sequencing spanning 5' and 3' ends of the insert including the arms of homology. After breeding to wild type, candidate G1 founders were identified using the same PCR strategy, but spanning PCR across the insert were unsuccessful at fully characterizing the entire allele. We used Oxford Nanopore Technology (ONT) long-read sequencing with Adaptive sampling for Chromosome 6 on an individual G1 founder to determine if the entire insert sequence and arms of homology were correct as designed. Reads were aligned to mouse reference genome GRCm38 using the wf-alignment pipeline with the minimap2 v2.24 to align reads. Structural variants (SV) were then called using Sniffles and CuteSV and consensus SVs were called using SURVIVAL v1.0.7. Adaptive sequencing resulted in 58X total coverage with 30X coverage (51.7% allele frequency) of the targeted allele. The insert sequence was identified in the SVs and aligned against the designed megamer sequence. All reads were then segregated into variant supporting and reference supporting reads, which were then assembled into separate consensus sequences using Flye v2.9.1. These two consensus sequences were then aligned individually to the megamer sequence with variant supporting reads matching the megamer sequence in the correctly targeted location. Furthermore, the junctional regions around the arms of homology were correct with no evidence of mutations. These data support the use of ONT adaptive sequencing for the characterization of CRISPR/Cas9-generated animal models.

## 0-5 Long Read Sequencing reveals high frequency of bystander mutations following AAV-driven electroporation of CRISPR RNP complexes in mouse zygotes

**Fabien Delerue<sup>1</sup>, Muhammad W. Luqman<sup>1</sup>, Piroon Jenjaroenpun<sup>2</sup>, Jessica Spathos<sup>1</sup>, Pattaraporn Nimsamer<sup>2</sup>, Thidathip Wongsurawa<sup>2</sup>**

<sup>1</sup>Faculty of Medicine, Health and Human Sciences, Macquarie University, Sydney, Australia, <sup>2</sup>Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand

Session 3: Animal Models in Space Biology I, November 13, 2023, 13:45–15:45

Over the last decade CRISPR gene editing has been successfully used to streamline the generation of animal models for biomedical purposes. However, one limitation of its use is the potential occurrence of on-target mutations that are detrimental or otherwise unintended. These bystander mutations are often undetected using conventional genotyping (i.e., PCR) and routine (i.e., Sanger) sequencing.

Recently, Long Read Sequencing (LRS) has been used in mice to identify the insertion site of randomly integrated transgenes, and to confirm integration following recombinase-mediated cassette exchange (RMCE). However, to the best of our knowledge LRS has not yet been used to perform quality control (QC) following AAV-driven gene editing in zygotes (referred to as “CRISPR-READI”), and the mechanisms by which AAVs release the single-stranded transgenes used as templates for homology directed repair (HDR) remain elusive.

To this end, we performed CRISPR-READI to generate three different types of integrations in two different murine genes (i.e., ACE2 and FOXG1). We generated and analysed these knock-ins (KI) using Oxford Nanopore Technologies (ONT) and identified instances of concatemerisation and partial backbone integration (particularly inverted terminal repeats: ITR sites) in two out of five (40%) lines generated.

Therefore, we recommend using LRS as a stringent QC for KI lines generated using CRISPR-READI (and potentially other methods). Long read sequencing is a powerful, reliable, fast and cost-effective method to assess the outcome of gene editing in animal models. We established a bioinformatics workflow to analyse these outcomes and deliver fully validated mouse lines.

## 0-6 FinnDisMice project: CRISPRing the Finnish Disease Heritage

**Dr Satu Kuure<sup>1</sup>, Dr Tomas Zarybnicky<sup>1</sup>, Dr Sonja Lindfors<sup>1</sup>, Dr Petra Sipilä<sup>2</sup>, Dr Reetta Hinttala<sup>3</sup>**

<sup>1</sup>Univ. Helsinki, Helsinki, Finland, <sup>2</sup>Univ. Turku, Turku, Finland, <sup>3</sup>Univ. Oulu, Oulu, Finland

Session 9: Transgenic Resources, November 15, 2023, 09:00–10:30

Tackling rare diseases is recognized not only as a crucial aspect to pave the way for general improvement to diagnosis but also to facilitate understanding of similar groups of common diseases.

Lack of preclinical models hinders identification of pathophysiological mechanisms of disease and thus development of effective disease modifying strategies. To improve this, we took the advantage of rare diseases enriched in Finland and successfully generated disease models that faithfully genocopy the disease-causing mutation in the mouse genome. Accordingly, nine new mouse models were generated representing rare human fetal/pediatric syndromes, pediatric-onset epilepsies, mitochondrial encephalomyopathy, an adult-onset motoneuron disease, and growth disorder with defective immunity. CRISPR/CAS9 genome editing with single stranded DNA templates was carried out in zygotes or 2-cell stage embryos either by microinjections or electroporation with varying efficiencies that depended on both strategy and genetic locus. The pathophysiological and cellular causes of the models are currently under active investigation. Highlights of our findings so far will be presented mainly focusing on lethal congenital contracture syndrome 1 (LCCS1), which is caused by pathogenic variant in GLE1 RNA transport mediator and in human leads to fetal death before the 32nd gestational week. Human LCCS1 fetuses are akinetic, show hydrops, arthrogryposis, pulmonary hypoplasia, and micrognathia with reported defects in motoneuron development. Phenotype characterization both at morphological and molecular level will be discussed. In generally, the results of our FinnDisMice studies will facilitate understanding of disease-causative pathomechanisms for these rare, incurable diseases. Importantly, the models we have generated are expected to serve as valuable preclinical validation instruments for potential new therapies.

## 0-7 A strategy for the efficient production of CRISPR interference mouse models

**Dr Leesa Sampson<sup>1</sup>, Mrs Jennifer Skelton<sup>1</sup>, Mrs Linda Gower<sup>1</sup>, Ms Katarzyna Jopek<sup>1</sup>, Dr Anna Osipovich<sup>1,2</sup>, Dr Lauren Woodard<sup>1,3,4,5</sup>, Dr Mark Magnuson<sup>1,2,6</sup>**

<sup>1</sup>Center for Stem Cell Biology, Vanderbilt University, Nashville, United States, <sup>2</sup>Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, United States, <sup>3</sup>Division of Nephrology and Hypertension, Department of Medicine, Vanderbilt University Medical Center, Nashville, United States, <sup>4</sup>Department of Biomedical Engineering, Vanderbilt University, Nashville, United States, <sup>5</sup>Department of Veterans Affairs, Tennessee Valley Healthcare System, Nashville, United States, <sup>6</sup>Department of Cell and Developmental Biology, Vanderbilt University, Nashville, United States

Session 9: Transgenic Resources, November 15, 2023, 09:00–10:30

Introduction and Aims: CRISPR interference (CRISPRi) is a promising alternative strategy to more conventional CRISPR knockout and Cre/loxP techniques. CRISPRi mouse models offer specific advantages, such as the ability to repress multiple genes in a single cross, more quantitative control of gene expression levels, and reversibility of gene repression. These advantages are likely to extend the utility of the mouse for studying complex, polygenic diseases like cancer, diabetes, and heart disease. However, there are currently few descriptions of the use of

CRISPRi in mice and methods for the selection and genomic integration of small guide RNAs that reliably repress their targets when in complex with dCas9-KRAB require definition.

**Methods, Results, and Conclusions:** We have developed a rapid and simple strategy for designing, generating, and validating CRISPRi mouse models that utilizes piggyBac transgenesis, commercially sourced reagents, and recently developed constitutive and inducible dCas9-KRAB alleles. Our protocols are easily workable in academic transgenic mouse core facilities capable of pronuclear microinjection. To demonstrate the feasibility of our strategy, we successfully generated seven CRISPRi models targeting five unique genes, resulting in gene repression levels ranging from 50 to 95%. As a proof of concept, we demonstrate the repression of glucocorticoid-induced (Gck) by a dual sgRNA transposon.

### 0-8 Efficient large knockins using rAAV donors in mouse embryos

**Dr Xiaoxia Cui<sup>1</sup>, Dr Monica Sentmanat<sup>1</sup>, Dr Ziteng Wang<sup>1</sup>, Ms Evgenia Kouranova<sup>1</sup>, Mr J. Michael White<sup>1</sup>, Ms Mia Wallace<sup>1</sup>**

<sup>1</sup>Washington University in St Louis, St. Louis, 63108

Session 9: Transgenic Resources, November 15, 2023, 09:00–10:30

Large knockins in mouse embryos are usually a more challenging type of gene editing, compared to KO and introduction of SNPs and small tags, yet necessary to generate models for humanization of a mouse gene, gene overexpression or replacement, reporter lines and conditional knockins (flexing). Using recombinant Adeno-associated virus (rAAV) to deliver donor templates circumvents the need to microinject single cell embryos, when combined with electroporation of Cas9/gRNA ribonucleoprotein complexes. We report here successful creation of tens of mouse models using rAAV donors, overcoming the payload limit of rAAV by using multiple viral donors for sequential insertions in a single round targeting in the embryos, with the largest insertion so far approaching 10 kb. Additionally, we report the development of Nanopore long read sequencing for genotyping, eliminating the need for multiple sets of junction PCRs, to verify the accuracy of insertion and screen against random integration. We will also discuss the overall efficiencies and difficulties we have encountered along the way.

### 0-9 Consideration of surrogate dam gut microbiome to improve animal model rigor and reproducibility

**James Amos-Landgraf<sup>1</sup>, Craig Franklin<sup>1</sup>, Aaron Ericsson<sup>1</sup>**

<sup>1</sup>University of Missouri, Columbia, United States

Session 10: Assisted Reproductive Technologies, November 15, 2023, 11:00–12:00

During transgenic animal production, the scientific community has recognized that the host genetic background can be crucially important to the phenotype of the genetically modified animals. Recent work by our group and others has highlighted that not only the host genome but also the animal's microbiome can significantly contribute to the phenotype as well. The Mutant Mouse Resource and Research Center at the University of Missouri has now examined several well-characterized mouse models of human disease and found quantitative differences in model phenotypes in animals harboring different vendor-derived gut microbiomes (GMs). We have created CD-1 colonies that harbor the GMs of four major commercial vendors' Specific Pathogen Free (SPF) microbiomes that we use as surrogate dams for rederivation or transgenic mouse model production. While all of the resulting animals are SPF, the diversity and richness of the GMs in the animals vary significantly. Using 16 s rRNA sequencing to characterize the GM revealed unique taxa in each of the GMs and significant differences of relative abundances of various shared taxa. Using predictive algorithms and untargeted mass spectroscopy-based metabolomics we identified dramatic differences in the metabolic capacity between the most diverse and complex microbiome and the least. We examined phenotypes in the IL10<sup>-/-</sup>, ApcMin mouse and ApcPirc rat models, as well as established models of behavior, and found significantly different phenotypes as determined by histology, quantitative tumor development, and behavior tests respectively in the various models. It may be beneficial for transgenic cores to inform clients about the source of the recipient dams to inform them about the GM. Additionally, if their animals will be used in crosses with other existing mutants, information about the commercial source of the animals can better inform the choosing of surrogates to more closely match GMs in their colony, leading to reproducible and less variable experiments.

### 0-10 Modeling disease-associated variants in mice using prime editors

**Dr Denise Grant Lanza<sup>1</sup>, Matthew Gonzalez<sup>1</sup>, Lan Liao<sup>2</sup>, Angelina Gaspero<sup>1</sup>, Dr John Seavitt<sup>1</sup>, Center for Precision Medicine Models<sup>3</sup>, Dr Aleksandar Milosavljevic<sup>1</sup>, Dr Lindsay Burrage<sup>1,4</sup>, Dr Jason Heaney<sup>1</sup>**

<sup>1</sup>Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, United States, <sup>2</sup>Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, United States, <sup>3</sup>Baylor College of Medicine, Houston, United States, <sup>4</sup>Texas Children's Hospital, Houston, United States

Session 10: Assisted Reproductive Technologies, November 15, 2023, 11:00–12:00

The Center for Precision Medicine Models leverages Baylor College of Medicine's genome discovery, informatics, and animal modeling programs to develop precision animal models that will end the diagnostic odyssey of patients with undiagnosed, rare, and Mendelian diseases and serve as resources for pre-clinical studies. However, modeling de novo disease-associated variants with traditional knock-in alleles has been

challenging as undesired indel mutations are often generated in trans to a desired variant with biallelic editing causing embryonic or neonatal lethality of founder animals. Moreover, variants alone often cause disease-associated, early-onset phenotypes that lead to founder death prior to weaning. Consequently, time-consuming strategies, such as conditional variant knock-in alleles, are often pursued.

As an alternative, we utilized prime editing in one-cell stage mouse zygotes, employing both PE2 and Pemax editors and PE3 and PE3b approaches. We hypothesized that prime editing would (1) introduce precise genetic alterations in the absence of indel mutations, and (2) produce low-level germline mosaic founders that are viable, fertile, and can be bred to generate heterozygous N1 progeny. To date we have targeted 9 separate loci and successfully generated correctly targeted F0 mice (founders) for 6 knock-in alleles. Founder occurrence varied by locus and often were a small percentage of the total number of F0s genotyped (5–67%). Low-level mosaicism (allele contribution 10–40%) likely contributed to the survival of these founders compared to previous traditional knock-in attempts. Undesired mutations were less common (5–22%) amongst F0 animals and usually not observed in correctly targeted F0s. Of the 6 targeted loci, 4 founders have been bred to generate live-born progeny and 2 have been utilized to generate embryos for an N1 phenotyping screen. Ultimately, the use of prime editors has greatly facilitated model production of embryonic or neonatal lethal variants to produce a stable source of mice for phenotypic evaluation.

## Poster Presentations

### Colony management services (CMS): from administration to validation

**Ms Emma Russell<sup>1</sup>, Mr Clive Da Costa<sup>1</sup>**

<sup>1</sup>The Francis Crick Institute, London, UK

Poster Session 2 (Even Numbers), Imperial West,  
November 14, 2023, 15:30–17:00

The CMS STP offers Cryopreservation, Genotyping, Genetic monitoring, microbiome analysis and Strain sharing/creation services, which require efficient and timely management.

To streamline these processes over the last year we have implemented pipelines and SOPs to ensure all team members carry out the actions via the same method and to provide a constant traceability of strains across the Crick. Using software and tools such as PowerBI and Notion we were able to implement a system that tracks all colony requests through the import and rederivation process. We have also implemented a programme of testing including standard genotyping and genetic monitoring profiles for all strains entering the Crick to ensure the validity of the lines researchers were requesting. Paying particular attention to projects that have improved

genotyping techniques by designing specific genotyping probes replacing the previous generic options. Finally, the introduction of a translations system that part automates our genotyping entry into the mouse record database and provided the CMS team an opportunity to review every single line in the Crick for 'best practice' validation.

The improved traceability of Strains across the Crick has minimised the time taken for the requested lines to be fully rederived into the facility. The more specific genotyping and addition of genetic monitoring checks have identified and rectified issues early on preventing unnecessary breeding and maintenance of lines. The automated translations system has resulted in reduced number of animals kept in the rederivation pipeline unnecessarily and a therefore gained space that is now being used for further project development that was previously on hold.

These advantages have allowed us to maintain the 3Rs principles and we have been visited by multiple institutes across the UK asking us to share this knowledge and help to implement these processes at their facilities.

### Refining euthanasia methods with isoflurane before cervical dislocation for sperm cryopreservation

**Ms Gillian Sleep<sup>1</sup>, Ms Amie Creighton, Ms Valerie Laurin, Ms Maribelle Cruz, Dr Marina Gertsenstein, Dr Lauryl Nutter**

<sup>1</sup>The Centre for Phenogenomics and The Hospital for Sick Children, Toronto, Canada

Poster Session 1 (Odd Numbers), Imperial West, November  
13, 2023, 17:30–19:00

Isoflurane (Iso) is commonly used prior to cervical dislocation (CD) to reduce animal stress. The use of Iso prior to CD is known to reduce the viability of pre-implantation mouse embryos and to negatively impact their development in vitro. However, to our knowledge, systematic investigation of the effects of isoflurane prior to CD on sperm function and embryo development have not been reported. To investigate the effects of Iso prior to CD, we randomly separated CD-1 and C57BL/6 sperm donors into two groups—one euthanized by CD and one for which euthanasia by CD was preceded by isoflurane anesthesia. Pre-freeze and post-thaw motility and swimming patterns were compared, and no statistically significant differences were noted. In vitro fertilization (IVF) was performed for both groups. There was no statistically significant difference in fertilization rate, implantation rate or live birth rate between Iso + CD and CD groups for both CD-1 and C57BL/6 mice. Based on these results, the use of Iso prior to CD does not negatively affect sperm quality or performance and therefore should be used to refine euthanasia by CD prior to sperm collection, in keeping with the 3Rs.

## Comparison of numbers of pups and implantation sites between the left and right uterine horns after embryo transfer of a minimally sufficient amount to induce pregnancy

Kylee Burnside<sup>1</sup>, Corinne Piotter<sup>1</sup>, Judy Hallett<sup>1</sup>, Annemarie Drummond<sup>1</sup>

<sup>1</sup>Transgenic and Genome Editing Facility, Purdue Institute Of Cancer Research, West Lafayette, United States

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

In the field of transgenic technologies, embryo transfers (ETs) involve embryos being surgically transferred into the oviduct or uterus of pseudo-pregnant recipient females to obtain live pups. In an ideal scenario, where there is an excess of embryos to transfer, 18 embryos are typically transferred per recipient, however some difficulties can arise in obtaining embryos. These include, but are not limited to, a low fertilization rate, poor response to superovulation, or low survival post microinjection.

This studies purpose was to compare the survival of embryos in unilateral and bilateral transfers and determine whether the left or right uterine horn is more supportive of embryo development. Preliminary experiments determined that 4, 2-cell embryos appeared to be the minimally sufficient number of embryos to initiate and maintain pregnancy. 2-cell embryos (N = 4) were transferred (unilaterally or bilaterally) into the oviduct of 0.5 dpc (day of plug) pseudo-pregnant 8-16-week-old CD-1 females. For unilateral transfers, all 4 embryos were transferred either into the left or right oviduct, whereas for bilateral transfers they were split evenly between both sides (N = 2 per oviduct). We also examined survivability in relation to which oviduct received embryos first, either right side followed by left (right-left) or left followed by right (left-right). Recipients were euthanized at day 19 post-transfer to determine number and location of pups and implantation sites (total of reabsorption sites and pups).

We determined that the right uterine horn is more supportive of embryo implantation and survivability than that of the left. There was no significance in survivability between the right-left and left-right bilateral ETs. We conclude that when there is a suboptimal number of embryos to transfer, live births can be maximized by favoring embryo transfer into the right uterine horn. Implementing this approach will improve survivability of embryos post-transfer and reduce animal usage in transgenic procedures.

## Addition of LHRH and examination of route of gonadotropin injection in the PMSG/hCG superovulation regime to increase fertilization and ovulation rates in mice and rats

Judy Hallett<sup>1</sup>, Kylee Burnside<sup>1</sup>, Corinne Piotter<sup>1</sup>, Rebecca Houser<sup>1</sup>

<sup>1</sup>Transgenic and Genome Editing Facility, Purdue Institute For Cancer Research, West Lafayette, United States

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

Injection of exogenous gonadotropins to induce superovulation is widely used in several mammalian species. Intraperitoneal (i.p.) injection of Pregnant Mare Serum Gonadotropin (PMSG), to promote follicular growth, is followed by i.p. injection of human Chorionic Gonadotropin (hCG), to induce ovulation. This PMSG/hCG superovulation model, first developed in prepubertal mammals, works well in juvenile mice (3–5 weeks) and rats (4–5 weeks), but less well in older, cycling animals. Luteinizing hormone releasing hormone (LHRH) has been used in cycling mammals to desensitize the pituitary, suppress endogenous gonadotropins and induce cycle synchronicity upon withdrawal; and in anestrous animals to initiate pituitary gonadotropin release and induce ovulation.

This studies objective was to utilize LHRH injection to control the estrous cycle allowing for synchronization of injected gonadotropins with endogenous hormones, thereby increasing follicular development and ovulation. Ovulation and fertilization rates were determined at 0.5dpc and after overnight culture, in mature C57BL/6N (B6) mice and immature Sprague-Dawley (SD) rats, after administration of a standard PMSG/hCG superovulation protocol, with or without prior LHRH injection. We also compared i.p. vs subcutaneous (s.c.) injection of LHRH and PMSG, based on the premise that slower absorption through s.c. administration would closer mimic the sustained release of gonadotropin releasing hormone (GnRH) and follicle stimulating hormone (FSH) in vivo, while faster absorption via i.p. injection would mimic the in vivo LH surge.

We have shown that s.c. PMSG injection increases ovulation rate in prepubertal B6 mice and that LHRH injection improves PMSG/hCG superovulation in adult B6 mice and immature SD rats. These refinements to standard superovulation regimes should reduce the number of mice and rats sacrificed to generate embryos, and eliminate the wastage of surplus or unresponsive mice by allowing them to be reused and superovulated as adults. This should significantly reduce animal usage in transgenic and related fields.

## Precise Plasmid Vector Integration into the Rosa26 Gene of Sheep Fetal Fibroblasts using Integrase Editing.

Dr Iuri Viotti-Perisse<sup>1</sup>, McKailly Adams<sup>2</sup>, Dr Kenneth White<sup>1</sup>, Dr Irina Polejaeva<sup>1</sup>

<sup>1</sup>Utah State University, Logan, USA, <sup>2</sup>Johns Hopkins University, Baltimore, USA

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

'Integrase editing' is a new nomenclature established here to represent a promising and innovative approach in the field of genetic engineering, particularly for large DNA integration. This technique, when combined with CRISPR/Cas9, allows for the precise integration of a large vector into the host genome independent of Non-Homologous End Joining (NHEJ) or Homology-Directed Repair (HDR) pathways. Current integrase editing approaches may be accomplished by using either CRISPR/Cas9, TwinPE, or PASTE editing tools, which will

first introduce the attB sequence in the target locus before a vector integration takes place through an integrase enzyme (i.e., Bxb1). In our study, we developed an approach using CRISPR/Cas9 to insert a 38 bp attB sequence into the Rosa26 locus of sheep fetal fibroblasts, followed by the integration of the px458-SpCas9-GFP plasmid. Initially, we transfected the cells using an electroporation method with Cas9/gRNA Ribonucleoprotein (RNP) and 138 bp of a single-stranded oligodeoxynucleotides (ssODN) to introduce the attB sequence into the Rosa26 locus. Following a 24-h recovery period, we confirmed mutations at the Rosa26 site by using PCR-RFLP and verified the presence of the attB site through PCR amplification with the endogenous gene. Subsequently, the successfully modified cells were cultured in T-25 flasks. Upon reaching confluence, we further transfected the cells with a modified px458 plasmid, containing an attP sequence upstream of the U6 promoter, along with a pCMV-Bxb1 plasmid. After 48 h post-transfection, the cells were analyzed and confirmed by PCR amplification and Sanger sequencing to contain the px458 vector integrated into the Rosa26 locus. Our findings demonstrate the feasibility and efficiency of Bxb1 integrase-mediated gene editing in sheep fetal fibroblasts, providing valuable insights into the targeted integration of large DNA fragments. This approach holds considerable potential for advancing gene editing technologies, offering numerous applications in various scientific fields, including agriculture, medicine, and biotechnology.

### Evidence-based guide to using artificial introns for tissue-specific knockout in mice

**Dr Elena McBeath Fujiwara<sup>1</sup>, Dr Keigi Fujiwara<sup>2</sup>, Dr Marie-Claude Hofmann<sup>1</sup>**

<sup>1</sup>Department of Endocrine Neoplasia and Hormonal Disorders, University of Texas MD Anderson Cancer Center, Houston, United States, <sup>2</sup>National Coalition of Independent Scholars, Brattleboro, United States

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

**Introduction:** Up until recently, methods for generating floxed mice either conventionally or by CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 (CRISPR-associated protein 9) editing have been technically challenging, expensive and error-prone, or time-consuming. To circumvent these issues, several laboratories successfully used a small artificial intron to conditionally knockout (KO) a gene of interest in mice.

**Problem:** However, many other labs are having difficulty getting the technique to work. The key issue appears to be either a failure in achieving correct splicing after the introduction of the artificial intron into the gene or, just as crucial, insufficient functional KO of the gene's protein after Cre-induced removal of the intron's branchpoint.

**Resolution:** Presented here is an evidence-based guide to the selection of a suitable exon and the optimal placement of the recombinase-regulated artificial intron (rAI) within that exon. The primary objective is to avoid disrupting normal gene

splicing after rAI insertion while maximizing mRNA degradation after recombinase treatment. The reasoning behind each step in the guide is also discussed. Adherence to these recommendations should significantly increase the success rate of this easy, novel, and alternative approach for generating conditional KO mice. (Published paper at <https://www.mdpi.com/1422-0067/24/12/10258>).

### Use of “Nanoblades” to target difficult-to-transfect cells

**Ms Katrien Staes<sup>1</sup>, Mr Tino Hocheppied<sup>1</sup>**

<sup>1</sup>TCF VIB-UGent Center for Inflammation Research,

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Delivery of Cas9 and gRNAs for gene editing in eukaryotic cell lines or primary cells can be technically challenging. Several technologies are available, such as transfection, transduction, direct injection or electroporation. Each with its pros and cons. The group of Emiliano Ricci (Mangeot et al., 2019) has developed Nanoblade technology to directly introduce proteins into cell lines or embryos. Nanoblades are engineered murine leukemia virus-like particles which serve as transgene-free protein-delivery particles that allow the transfer of Cas9-sgRNA ribonucleoproteins (RNPs). We have implemented Nanoblade technology in our Transgenic Core Facility to generate knockout cell lines and primary cells. We developed a standardized pipeline to generate knockout cells. Firstly, several gRNA combinations are selected that should generate an out of frame deletion. These gRNA combinations are transfected into LentiX cells to produce the Nanoblades which are then used to deliver their cargo to a cell line of choice. A PCR screening followed by ICE analysis is performed on a pool of cells to check for the presence of cells containing the intended deletions. These pools are single cell sorted over FACS and a 3 primer PCR screening—of which 1 primer is located in the deleted region—is performed to quickly identify which clones contain the intended deletion. We already successfully used Nanoblades on several cell types such as 293 T, THP1, HaCa T, Mefs and human fibroblasts.

Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins. Mangeot et al., Nat Commun 2019 Jan 3;10(1):45. <https://doi.org/10.1038/s41467-018-07845-z>.

### Knock-in genome engineering at novel genomic safe harbor sites in pluripotent stem cell & animal models

**Dr Steven Bischoff<sup>1,2</sup>, Mr Raul Hajiyev<sup>1</sup>, Leonard Vaccaro<sup>1</sup>**

<sup>1</sup>NovoHelix, Miami, United States, <sup>2</sup>Foundry for Genome Engineering & Reproductive Medicine, Miami, United States

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Knock-in genome engineering refers to the process of precise and targeted insertion of DNA fragments or payloads and is a powerful reverse genetics tool for manipulating the genomes of human pluripotent stem cells and biomedical animal models. A handful of genomic safe harbor (GSH) sites are widely used for the integration of large DNA fragments, often several to tens of kilobases in size, that may contain regulatory elements such as enhancers, promoters or synthetic transgene circuits, or may contain genetic modifications that recreate a disease phenotype. Overall, the choice of GSH site for generating cellular and animal models will depend on the experimental criteria, but to qualify as an authentic safe harbor, the integration site should yield minimal disruption to the neighboring and endogenous gene expression patterns; and importantly, any disruption should not lead to genotoxic stress, abnormal cellular functions, developmental abnormalities, and disease. The most common GSH include: ROSA26, AAVS1, COL1A1, H11, and CCR5. Recently, Aznauryan et al., 2022 discovered two novel regions of gene insertion, eponymously named Rogi1 and Rogi2, that are GSH on human chromosomes 1 and 3, respectively. Using bioinformatics, we discover and map new Rogi1 orthologous GSH sites in both mice and swine. Additionally, we develop genome engineering reagents such as CRISPR-Cas9 high-performing sgRNAs and PASTE<sub>v3</sub> editing with integrases Bxb1 and Bacillus cereus (BceINTa) suitable for the insertion of the large DNA payloads at Rogi1. Using pluripotent stem cells from mice including embryonic stem (ES) and iPS cells, and porcine expanded potential stem cells (EPSCs) we demonstrate high-level and ubiquitous expression at Rogi1 safe harbor using a CAG promoter driven StayGold. We have microinjected the StayGold genetic reporter into both mouse and pig zygotes to monitor transgene expression in whole tissues and validate Rogi1 as a GSH in both small and large biomedical animal models.

### EV-AAV Vector: A Novel Application of the Compound Vector for Efficient and Non-Invasive Delivery In Vitro and In Vivo

**Mr Petr Nickl<sup>1</sup>, Mrs Maria Barbiera<sup>2</sup>, Mr Jacopo Zini<sup>2</sup>, Mrs Tereza Nickl<sup>1</sup>, Mrs Irena Jenickova<sup>1</sup>, Mrs Jana Kopkanova<sup>1</sup>, Mrs Aki Ushiki<sup>3</sup>, Mrs Marjo Yliperttula<sup>2</sup>, Mr Nadav Ahituv<sup>3,4</sup>, Mr Radislav Sedlacek<sup>1</sup>**

<sup>1</sup>Institute of Molecular Genetics, Prague, Czechia, <sup>2</sup>University of Helsinki, Division of Pharmaceutical Biosciences, Helsinki, Finland, <sup>3</sup>Department of Bioengineering and Therapeutic Sciences, University of California San Francisco, San Francisco, USA, <sup>4</sup>Institute for Human Genetics, University of California San Francisco, San Francisco, USA

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In this study, we introduce a novel application of the EV-AAV vector, a compound vector that combines the strengths of extracellular vesicles (EVs) and adeno-associated virus (AAV).

Our research focuses on an innovative application of this compound vector as a non-invasive and efficient delivery system into zygotes and cells. We successfully evaluated the efficiency of EV-AAV vectors as carriers for homology templates using the CRISPR-READI method and demonstrated that the EV-AAV compound vector has the potential to deliver an AAV genome exceeding the five kilobases. Additionally, we conducted further investigations into the potential of the EV-AAV vector in delivering the PiggyBac transposon system, enabling us to explore delivery efficiency and potential toxicity. Our results show that the EV-AAV vector outperforms the standard AAV vector, exhibiting superior delivery efficiency and reduced toxicity profiles. In conclusion, this study highlights the remarkable potential of EV-AAVs as an innovative delivery platform for diverse genetic materials. These vectors represent a convergence of unique advantages, encompassing simple production, characterization, and adaptable cargo delivery.

### Where is my Transgene? Long-range Sequencing to Determine the Integration Site of a Transgene in the Mouse Genome

**Ms Elizabeth Hughes<sup>1</sup>, Ms Galina Gavrilina<sup>1</sup>, Dr Wanda Filipiak<sup>1</sup>, Dr Thomas Saunders<sup>1</sup>, Dr Zachary Freeman<sup>1</sup>**

<sup>1</sup>Univ. Of Michigan Medical School, Ann Arbor, United States

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A triple-transgenic mouse line (3DR) carrying drug resistance for neomycin, puromycin and hygromycin was generated by co-injection of 3 transgenes into pronuclei of fertilized FVB-background mouse eggs. Incorporation of the transgenes into the genome was confirmed by PCR amplification of the individual transgenes. G0 founders were subsequently bred to wild type FVB with the 3 drug resistance transgenes co inherited on G1 offspring, confirming close linkage. This transgenic mouse line was subsequently used in preparation of mouse embryonic fibroblasts for use as feeder layers for mouse embryonic stem cells in gene-targeting experiments. We sought to identify the genomic location of transgene integration using long read sequencing. Genomic DNA was extracted from 3DR mouse embryonic fibroblasts and submitted to the University of Michigan Advanced Genomics Core for Oxford Nanopore Technology (ONT) Sequencing. Initial whole genome sequencing ONT achieved 3X coverage and after alignment identified potential integration sites in Chromosome 4 and X. ONT Adaptive sampling real time sequencing which allows for real time enrichment based on alignment to a reference sequence enhancing overall target read depth. Chromosome 4 and X were used for references and sampling confirmed all three transgenes were co integrated into Chromosome 4 at position chr4:125440180. The transgenes were each integrated in a single copy in the following order: hygromycin, puromycin, and neomycin. These methods represent a promising way to identify transgene insertion sites.

## Optimization of Prime Editing approach for reversion of mouse albino phenotype

**Mrs Delphine Cussigh<sup>1</sup>, Mr Sébastien Chardenoux<sup>1</sup>, Mr Yann Sassier<sup>1</sup>, Mr Gwendal Kerzerho<sup>1</sup>, Dr Francina Langa Vives<sup>1</sup>**

<sup>1</sup>Institut Pasteur, Paris, France

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The discovery of programmable nucleases, in particular the CRISPR system, has revolutionized the field of genome modification. Versatile and efficient, this tool offers the possibility to precisely modify *in vivo* any potential locus thanks to the delivery of various exogenous genetic components. Since its implementation in gene-edited mouse model generation, there has been a constant effort to build an even easier, faster, and highly efficient system by developing new innovative CRISPR-based techniques. For instance, Prime Editing (PE) has recently emerged as a promising technique to induce small genetic changes without need of double-stranded DNA breaks nor donor DNA. *Ex vivo* zygote electroporation, for its part, represents a very effective delivery method of CRISPR/Cas9 components into mouse zygotes. Thus, we decided to take advantage of these new technologies to broaden our pipelines for mouse model generation within our transgenesis platform. First, we successfully managed to induce precise substitutions into the tyrosinase gene via mouse zygote microinjection of PE system under the form of RNA molecules. This technique corrected the mutation at the origin of mouse albino phenotype with good efficiencies and reduced off-target effects compared to strategies based on Homology-Directed Repair (HDR) pathway often accompanied by undesired mutations triggered by Non-Homologous-End-Joining (NHEJ) mechanism. Encouraged by these results, we then generated Tyr-edited mouse embryos by zygote electroporation delivery of PE system under the form of ribonucleoprotein complexes reaching up to 40% efficiency. Finally, we are currently refining the PE system by optimizing each component. These improvements take advantage of strategies like, for example, introduction of additional silent mutations into PE templates or use of *in vitro* optimized Prime Editors and PE template RNA molecules.

## An Efficient Approach for Generating Genetically Modified Mice Expressing Predefined Monoclonal B-Cell Receptor Repertoires

**Ms Jinke D'Hont<sup>1,2</sup>, Dr Stijn Vanhee<sup>2,3</sup>, Dr Tino Hochepped<sup>1,2</sup>**

<sup>1</sup>Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium, Ghent, Belgium, <sup>2</sup>VIB Center for Inflammation Research, Ghent, Belgium, Ghent, Belgium, <sup>3</sup>Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium, Ghent, Belgium

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Genetically modified mice expressing predefined monoclonal B-cell receptor (BCR) repertoires play a pivotal role as essential tools in immunological research. Traditionally, such mice were generated through transgene insertion into zygotes, resulting in expression from nonnative loci and limitations in isotype switching and affinity maturation.

In this study, we present an innovative and efficient methodology for generating monoclonal BCR-expressing mice. Leveraging the power of Ribonucleoproteins (RNPs) and single-stranded DNA (ssDNA) repair templates, we successfully eliminated a part of the endogenous *Igh* locus and replaced it with a bicistronic allele encoding both the light and heavy Ig chains of the BCR, under control of an Ig-V promoter. This novel approach enabled mice capable of isoclass switching, somatic hypermutation, and affinity maturation.

Furthermore, through the utilization of RNPs and ssDNA repair oligos, we removed the coding regions of the light and heavy Ig chains, leaving the Ig-V promoter in place, creating a genetic harbor for the insertion of other heavy and light chains in a single step. By implementing this streamlined technique, we significantly reduced labor and complexity, ultimately leading to the generation of monoallelic BCR-expressing mice with precision and ease.

The implications of this methodology empower researchers to tailor BCR repertoires with specific characteristics, shedding light on intricate aspects of immune regulation and immune response adaptation to new threats.

## Enhancing the Efficiency of Fragment Knock-in Efficiency in CRISPR/Cas9-mediated genome edited mouse model by using of HDR enhancer

**Dr Ching-yen Tsai<sup>1</sup>, Mei-Ling Chang, Lu-A Lu, Hao-Chun Chen, You-Xuan Zhu, Chun-Ru Lin, Chun-Feng Wu**

<sup>1</sup>Transgenic Core Facility, Inst. of Molecular Biology, Academia Sinica, Taipei, Taiwan

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The application of CRISPR/Cas9-mediated genome in mic model production was first demonstrated in 2013. Among the modifications of mouse genome, the needs of direct knockout mediated by bases insertion and deletion (Indel) are much higher than precise knock-in (KI) mutation. For the past decade, researchers have demonstrated several approaches to perform CRISPR/Cas9-mediated homology-directed repair (HDR), including PITCH (Precise-Integration into Target Chromosome), Easi-CRISPR, Homology-mediated End Joining (HMEJ) and 2C-HR CRISPR etc. However, sometimes in some difficult cases, it is still hard to get a precise KI mutant no matter which approach was used.

In this study, we first demonstrated a commercialized *in vitro* HDR enhancer was able to use *in vivo*, i.e. in mouse embryos. We tested the embryo toxicity of the HDR enhancer and used it at a non-toxic concentration. We tested the HDR

enhancer in 54 individual cases, the results showed the HRD enhancer indeed enhances the KI efficiency significantly ( $24.0 \pm 2.7\%$  vs.  $16.42 \pm 2.8$ ). Among the 54 cases, three of them were extreme difficult to obtain precise KI founders. By using the HDR enhancer, the KI efficiencies were dramatically increased from 0 to 61.1%.

In summary, we first demonstrated the commercial in-vitro HRD enhancer was applicable in-vivo. The precise KI efficiency is significantly increased, furthermore, in those loci that are difficult to be edited, the HDR enhancer shows a positive effect on the HDR events. This enhancement of the HDR enhancer can increase the KI efficiency therefore the less animal amount was used, which comply with the 3R's.

### Applicability of Oxford Nanopore Cas9-capture in the validation of transgenic mouse models

**Mr Jorik Loeffler<sup>1</sup>, Mr Krystian Nowicki, Dr Skevoulla Christou, Mr Matthew Mackenzie, Dr Alasdair Allen, Dr Rosie Bunton-Stasyshyn, Dr Gemma Codner, Dr Lydia Teboul**

<sup>1</sup>Medical Research Council, Harwell OX11 0RD, United Kingdom

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Oxford Nanopore Technologies (ONT) sequencing is a now well-established technology applicable to a wide range of genetic research and clinical applications. Even though a major advantage of ONT sequencing is that it allows for the processing of long and ultra-long reads, amplicons multiple kilobases in size to be sequenced can be difficult to attain via conventional PCR methods. Recently we have adopted ONT Cas9 capture as an alternative, PCR-free approach that utilises the DNA-cleaving activity of Cas9 nuclease to capture and subsequently sequence regions of interest too large to enrich via conventional PCR.

Here we present the applicability of ONT Cas9 capture in 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 by Cas9 capture. We also show applicability to mosaic founders and F1 generations obtained by pronuclear microinjection of one-cell embryos with CRISPR reagents and DNA donor. Resulting sequencing depths are sufficient to offset error rates inherent to the technology. Cas9 capture therefore provides a crucial 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 PCR and Sanger sequencing.

Due to its complex and elaborate laboratory procedure, successful implementation as well as data acquisition can be challenging to achieve and require careful and detailed attention to a wide range of experimental parameters. We therefore also highlight several key technical choices, such as DNA extraction and preparation approaches, guide cleavage

conditions and final library assembly considerations that can improve input sample quality and ultimately provide robust data output of ONT Cas9 capture.

### Use of ddPCR to Determine Copy Number in Founder mice made with AAV6

**Mr Asif Nakhuda<sup>1</sup>, Miss Caro Wilson, Mr Bill Mansfield, Dr Louise Webb, Dr Michelle Linterman, Dr Peter Rugg-Gunn**

<sup>1</sup>Babraham Institute

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Introduction: Knock-in mouse models serve as tools to study in vivo biology. Generating knock-in mouse models is inefficient but the use of AAV6 to deliver template DNA has improved gene editing outcomes significantly. However, since of use AAV6 as template DNA is relatively new, mice generated in this way haven't undergone full QC. Due to the high permeability of AAV6 to enter the nucleus, the increased frequency of random integrations has not been determined. Further, we assessed the effect of volume on AAV6 infected media on copy number integrations in embryos.

Methods: We generated multiple Dre recombinase mouse lines using AAV6 by infecting mouse zygotes at the 1-cell stage ( $5.33\text{--}8.64 \times 10^9$ ) and electroporating them with CRISPR/Cas9. We used digital droplet PCR (ddPCR) to accurately measure the number of integrations in founder mice.

Results: We identified positive mice with Dre integrations in the correct loci using traditional long range PCR in two lines. In these same mice we determined the number of copies of Dre with median copy number of  $\sim 1$  per mouse. However, a few founders had more than 3 copies. We also compared different volumes of AAV6 infection media on embryos and found lower volumes increased copy number per founder mouse.

Conclusions: Use of AAV6 at sensible concentrations does not cause excessive random integrations. In scenarios where low titre AAV is produced, reduction of total infection media may enhance AAV exposure. Overall, It is important to track copy number when choosing F0 mice to breed further.

### Generation of Novel Mouse Model for GGGGCC Repeat-Expanded Humanized C9orf72 Allele and Analysis of the Repeat Instability

**Ms Nada Kojak<sup>1</sup>, Dr Daisuke Kajimura<sup>1</sup>, Junko Kuno<sup>1</sup>, Kristina Solarino<sup>1</sup>, Ambereen Khan<sup>1</sup>, David Wenger<sup>1</sup>, Roberto Donnianni<sup>1</sup>, Michael Glasser<sup>1</sup>, Yajun Tang<sup>1</sup>, Terrence Turner<sup>1</sup>, Roxanne Ally<sup>1</sup>, Jose Rojas<sup>1</sup>, Charleen Hunt<sup>1</sup>, Jean Siao<sup>1</sup>, Eric Chiao<sup>1</sup>, Brian Zambrowicz<sup>1</sup>**

<sup>1</sup>Regeneron, Tarrytown, US

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Microsatellite expansion diseases are a group of genetic disorders that are characterized by an expansion of short nucleotide homopolymer. GGGGCC repeat expansion is the most common cause of familial Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD), but the mechanisms of how this microsatellite expands or contracts, i.e. repeat instability, are largely unknown. Recapitulating microsatellite expansion diseases in experimental animals is challenging due to the technical difficulty in manipulating repeat-containing sequences *in vitro*. In this study, we successfully generated targeted humanized C9orf72 mouse with  $96 \times$  GGGGCC repeats to study repeat instability and explore mechanisms involved in expansion. Tissue analysis in mouse showed somatic repeat instability varied depending on age and tissue. When Msh2, a critical gene in Mismatch repair (MMR) pathway, was deleted from the C9orf72 humanized mouse and blocked MMR pathway, this manipulation stabilized somatic repeat expansions, indicating MMR pathway is the major driver for the GGGGCC somatic repeat expansion. In our effort to generate larger C9orf72 repeat alleles, we found a DSB adjacent to the repeats, introduced by CRISPR-Cas9, induced large-scale repeat expansion, enabling us to generate up to  $550 \times$  GGGGCC alleles. Importantly, this CRISPR-Cas9-induced repeat expansion occurred independently from MMR pathway. Our findings suggested that C9orf72 GGGGCC expands through multiple pathways. Also, CRISPR-Cas9 induced repeat expansion may be applicable to model other microsatellite expansion diseases.

### Using Cas9 electroporation and AAV templates to generate knock-in and conditional alleles in mice

**Ms Marina Gertsenstein<sup>1</sup>, Ms Lauri G. Lintott<sup>1,2</sup>, Ms Valerie Laurin<sup>1,2</sup>, Ms Linda Chan<sup>1,2</sup>, Ms Qing Fan-Lan<sup>1,2</sup>, Mrs Sandra Tondat<sup>1</sup>, Mr Amit Patel<sup>1</sup>, Ms Maribelle Cruz<sup>1</sup>, Dr Lauryl M.J. Nutter<sup>1,2</sup>**

<sup>1</sup>The Centre for Phenogenomics (TCP), Toronto, Canada, <sup>2</sup>The Hospital for Sick Children, Toronto, Canada

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**Introduction:** Genome editing with Cas9 significantly simplified generation of genetically modified mice. However, targeted insertion of large fragments and one-step generation of loxP-flanked conditional alleles remain challenging. Recently, co-incubation of recombinant adeno-associated virus (rAAV) with rodent embryos has been used to deliver repair templates for Cas9-mediated genome editing. Coincident or subsequent delivery of Cas9 ribonucleoprotein (RNP) by electroporation was reported to efficiently generate knock-in alleles in treated embryos.

**Aim:** Our aim was to test and adopt these protocols to establish a workflow to efficiently generate knock-in and conditional alleles.

**Methods:** We tested several variables to establish our workflow, including different rAAV-embryo co-incubation times, different Cas9 RNP concentrations for electroporation,

and electroporation of zygote and two-cell stage mouse embryos. Pups born from these experiments were screened with short- and long-range PCR, Sanger sequencing, and template copy number assessment by quantitative real-time or digital PCR to identify founders. Germline transmission test breeding was done by crossing founders with wild-type mice of the same strain background.

**Results:** All combinations of rAAV co-incubation times to deliver repair templates with electroporation to deliver Cas9 RNP successfully generated founders with either knock-in or loxP-flanked conditional alleles. Template-specific PCR and template copy number assessment demonstrated that multicopy rAAV integration can occur both on- and off-target. Evaluation of screening results allowed us to develop a founder screening pipeline that eliminates the most mice at early steps, minimizing the number of animals that need to be screened at each stage. Using founders that passed our founder quality control for germline transmission test breeding enabled us to establish mouse lines with the desired allele in each case tested.

**Conclusions:** We conclude that template delivery by rAAV co-incubation and Cas9 RNP electroporation is a robust and easily adopted protocol to generate knock-in and conditional alleles.

### Optimization of superovulation and zygote electroporation to edit immunodeficient NSG mouse genome

**Mr Kuei-Liang Chen, Mr Shih-Fan Wu, Ms Te-Hsien Liu, Mr Guan-Rong Lai, Mr Wei-Nien Chen, Ms Hao-Yun Kao, Mr Cheng-Ju Wang, Mr Shao-Fu Lo, PhD I-Shing Yu, PhD You-Tzung Chen, PhD Shu-Wen Lin**

<sup>1</sup>National Taiwan University, College of Medicine, Department of Clinical Laboratory Science and medical Biotechnology, Taipei, Taiwan

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**Background:** Immunodeficient NSG mice are commonly used in xenograft transplantation and humanization. Genetic modification of the NSG mice can create better xenograft models to study human diseases by improving the success rates of human cell transplantation.

**Aims:** This study aims to develop a protocol for efficient genome modification in NSG mice.

**Methods:** We tested various amounts of a superovulation reagent called CARD HyperOva® in female mice under 8 weeks (wks) of age. The gene-modified NSG mice were generated by the combination of *in vitro* fertilization (IVF) and the CRISPR/Cas9 technology. We used IVF to gain sufficient numbers of NSG mouse embryos for genetic manipulations. Several electroporation conditions were tested with mixtures of Cas9 protein and sgRNA into the embryos.

**Results:** We found that when a full dose Hyper-OVA was used, even old females (> 24 wks) could give an average 22 oocytes per mouse ( $n = 26$ ), medium age females (8 ~ 24 wks) gave 51 oocytes ( $n = 152$ ) and young females (< 8 wks) gave more than 3 times as many oocytes (75 oocytes/mouse,

n = 18) as that of the old females. Given Hyper-OVA plus PMSG at a 1:1 ratio, the number of oocytes recovered were 35 ~ 38 per mouse at either young or medium age. In terms of electroporation, we optimized several electroporation conditions for introducing sgRNA into fertilized eggs and found that the condition of 2 pulses of 1 ms at 27 V with a 1000 ms interval enabled CRISPR/Cas9-based genome editing with high efficiency (37.5 ~ 50%, n = 16) and high survival rate (46 ~ 50%, n = 16). The birth rate of electroporated NSG embryos (n = 1693) was 24.5%, which was comparable to that of un-electroporated ones (n = 1650, 23.6%).

Conclusion: We established a novel procedure to efficiently modify the NSG mice by optimizing the superovulation protocol, IVF, and electroporation conditions to introduce the Cas9/sgRNA into zygotes.

### Is the custom creation of GMO mouse models using the CRISPR-Cas9 system possible today?

**Mr Thomas Bagarre<sup>1</sup>, Mme Naïs Philippe<sup>1</sup>, Mme Corine Arnoux<sup>1</sup>, Mme Mireille Richelme<sup>1</sup>, Mme Cécile Garcia<sup>1</sup>, Mme Diane Lalaina<sup>1</sup>, Mr Fabien Angelis<sup>2</sup>, Mr Aziz Adda-Benatia<sup>2</sup>, Mr Yannick Berkane<sup>2</sup>, Mr Robin-Nicolas Lacombe<sup>2</sup>, Mr Elyes Tavernier<sup>2</sup>, Mr Frédéric Fiore<sup>3</sup>**

<sup>1</sup>Microinjection and animal husbandry service, Marseille, FRANCE, <sup>2</sup>Molecular and cellular biology service, Marseille, FRANCE, <sup>3</sup>Department leader of Genetic Engineering and Mouse Transgenesis (GEMTis), Marseille, FRANCE

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Since 2012, a new technological tool has appeared in the transgenesis market: the CRISPR-Cas9 system. In the context of providing the creation of genetically modified mice, it is now classically used on the C57BL/6 genetic background. Despite everything, we occasionally encounter drops in performance when obtaining certain models. This is observed more frequently when it comes to a less commonly exploited genetic background.

Depending on the type of genetic modification to be made (e.g. Knock-Out, Knock-In of a point mutation, conditional Knock-Out, large Knock-In) and the targeted locus, different strategic choices are proposed, which directly influence the rate of success and the effectiveness of the means implemented. In this poster, we will present a quantitative comparative study of our work carried out in a service provision context. We will evaluate the impacts of the choice of technique (microinjection or electroporation) and the type of embryos (from natural mating or cryopreserved) used on different genetic backgrounds (BALBc/J, NOD, C57BL/6N and C57BL/6J). We will list in our materials and methods the equipment, the reagents, the concentrations of the crRNA complex as well as the animals that we use, which are all modifiable variables that have a major impact. This assessment now allows us to optimize our strategies by adapting our techniques and protocols according to the requested model.

### Can we use historical data to predict optimal targeting strategies for efficient knock-in generation of Mouse models

**Ms Aline Baur<sup>1</sup>, Mr Sunwoo Chun<sup>1</sup>, Ms Désirée Gronostay<sup>1</sup>, Ms Annette Nebenius<sup>1</sup>, Dr Anna Rüegg<sup>1</sup>, Dr Pawel Pelczar<sup>1</sup>**

<sup>1</sup>University of Basel, Basel, Switzerland

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Over the last few years, a multitude of CRISPR-based methods have been developed and used to generate knock-in mouse models. Is it possible to use historical data to predict the most effective targeting strategy for creating knock-ins at specific genomic loci? We conducted a comprehensive analysis of our transgenic core facility's data collected over the past three years. In this, we were focusing on the correlation between our success rate and the targeting techniques used, the conformation and DNA methylation status of the targeted locus as well as the assumed conformation of the inserted DNA fragment.

### Evolving the analysis pipeline for CRISPR/Cas9 mediated, embryonic stem cell derived, mouse models

**Dr Alasdair Allan<sup>1</sup>, Dr James Cleak<sup>1</sup>, Ms Ellen Hyde<sup>1</sup>, Mr Akash Mukhopadhyay<sup>1</sup>, Mr Connor Macfarlane<sup>1</sup>, Mr Krystian Nowicki<sup>1</sup>, Ms Hannah Dobbs<sup>1</sup>, Mr Christy Greenwood<sup>1</sup>, Mrs Sue Varley<sup>1</sup>, Mr Jorik Loeffler<sup>1</sup>, Mr Matthew Mackenzie<sup>1</sup>, Dr Rosie K. A. Bunton-Stasyshyn<sup>1</sup>, Dr Gemma F. Codner<sup>1</sup>, Dr Lydia Teboul<sup>1</sup>**

<sup>1</sup>MRC Harwell, Harwell, UK

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The advance of transgenic techniques is revolutionising the modelling of human genetic diseases with ever greater complexity. The Mary Lyon Centre at MRC Harwell is at the forefront of generating mouse models with which to study human genetic diseases for both the UK and global research community. As part of our production pipeline, we have a laboratory dedicated to the production of materials for complex alleles via the embryonic stem (ES) cell to mouse conversion route. In order to increase efficiencies, we have been implementing traditional gene targeting vector electroporations with the aid of CRISPR/Cas9 technology to cut the genome at the desired integration site. This approach has been shown to enhance the incorporation of large and/or complex genetic modifications into ES cells in comparison to traditional electroporation techniques.

The increasing complexity of our models in turn requires an increasing complexity towards determining the accuracy of modified alleles. Here we will present our current quality control pipeline for the analysis of CRISPR-mediated ES cell lines using a range of traditional and contemporary methods. This pipeline is designed to quickly eliminate clones that are

not of biological interest while also confirming, in as robust a manner as is practicable, the genetic sequence of both the targeted locus and the surrounding sequence both in ES cells and the subsequent mouse line.

## Re-engineering the mouse ROSA26 targeting vector for improved efficiency

**Dr Honglai Zhang<sup>1</sup>, Elizabeth Hughes, Galina Gavrilina, Wanda Filipiak, Thomas Saunders, Zachary Freeman**

<sup>1</sup>Transgenic Animal Model Core, Univ. Of Michigan Medical School, Ann Arbor, United States

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

The ROSA26 (R26) locus is a widely used safe harbor locus for transgene integration in the mouse genome. We previously reported creating more than 35 R26 models using the pR26 CAG AsiSI/MluI targeting vector (Chu et al. 2016, PMID: 26,772,810) with a median of 3 transgenic founders per 100 eggs injected. We received data from one investigator demonstrating evidence of leaky expression of a cDNA cloned behind the floxed stop (LSL)cassette in the vector. mRNA expression of the cDNA was present at levels similar to the endogenous gene which interfered with the investigator's research. We responded by designing new R26 targeting vectors to overcome this limitation. We report the development of a new ROSA26 vector with 1 kb arms of homology and an enhanced stop cassette that prevents unwanted expression of cDNAs. Furthermore, our new R26 vector is compatible with the sgRNA targeting the canonical ROSA26 XbaI site (Chu et al.). We present data from two projects in which knockin mice were generated with both the pR26 CAG AsiSI/MluI targeting vector and our new UMTG R26 targeting vector. We compare integration rate, efficiency, and specificity of our new vector in generating ROSA26 knockin mouse models. In one project, when a cDNA behind the LSL cassette included a suspected embryonic lethal human SNP, we identified no founders with the pR26 CAG AsiSI/MluI vector, while we successfully generated a founder with our new UMTG R26 vector. A series of UMTG ROSA26 targeting vectors: CAG-LSL-MCS-bGH pA, CAG-MCS-bGH pA and non-CAG version were cloned for mouse and rat targeting. Our results suggest that these new ROSA26 targeting vectors are a powerful new tool for transgenic animal model creation.

## A novel electroporation-based CRISPR strategy for generation of conditional knockout alleles

**Mr Juan Reyes<sup>1</sup>, Natasha Bacarro<sup>1</sup>, Tuija Alcantar<sup>1</sup>, Deborah Siler<sup>1</sup>, Maria Martinez<sup>1</sup>, Carol Cain-Hom<sup>1</sup>, Emily Hunley<sup>1</sup>, Hetal Patel<sup>1</sup>, Sanjeet Auja<sup>1</sup>, Areum Yoon<sup>1</sup>, Taylor Hunsucker<sup>1</sup>, Lisa Lima<sup>1</sup>, Soren Warming<sup>1</sup>**

<sup>1</sup>Genentech, South San Francisco, USA

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

Conditional knockout (CKO) mouse models provide valuable insight into the etiologies of human disease and are therefore critical for the development of novel therapies. As such, improving methods to efficiently and rapidly generate CKO mice is invaluable. Here, we compared a novel approach to previously reported strategies that utilized CRISPR technology in combination with electroporation to successfully produce floxed mice. Horii et al. (2017) reported using sequential electroporation, which involves knocking in one loxP in zygotes and retargeting the same embryos at the 2-cell stage to knock in the second loxP. Alternatively, Sentmanat et al. (2022) utilized simultaneous electroporation of zygotes to knock in both loxPs in one step. Our modified simultaneous electroporation approach in zygotes combines 5' and 3' loxP oligos, Cas protein/sgRNA #1 in a complex (RNP) and Cas9 mRNA plus sgRNA#2. Taking advantage of delayed mRNA translation, we separate the two DNA cutting events, thereby reducing dropout rates and increasing our rate of successful floxing.

## Evaluating Cas9 ribonucleoprotein (RNP) stability for mouse genome editing

**Ms Lauri Lintott<sup>1</sup>, Qing Fan-Lan<sup>1</sup>, Valerie Laurin<sup>1</sup>, Linda Chan<sup>1</sup>, Nimisha Assudani<sup>1</sup>, Maribelle Cruz<sup>1</sup>, Sandra Tondat<sup>1</sup>, Marina Gertsenstein<sup>1</sup>, Lauryl Nutter<sup>1</sup>**

<sup>1</sup>The Centre For Phenogenomics, Toronto, Canada

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

Introduction: Mouse genome edits are commonly introduced by electroporation (EP) of zygotes with RNA-guided endonucleases such as Cas9 or Cas12a. At The Centre for Phenogenomics, we typically use a ribonucleoprotein (RNP) complex, which we make shortly before EP; however, this workflow can be operationally limiting since the RNP is prepared outside the barrier by different staff than those treating the embryos.

Methods: RNP complexes were prepared up to seven days before EP and stored at 4°C. Same-day RNP complexes were made up to 1 h before EP. On the day of EP, zygotes were randomly split into two groups and each group was electroporated with same-day or earlier-day complexes. Pups were assayed by end-point PCR to identify the desired genome edit.

Results: In pilot experiments using RNP complexes prepared up to 7 days before EP, we found high genome editing rates in GFP reporter mice, as assayed by the absence of GFP signal in blastocysts and day 10.5 embryos. We then compared founder rates for targeted deletions using same-day and earlier-day RNP complexes. Both conditions produced founders in all experiments except for one that yielded no founders. While the average number of deletion founders was somewhat lower when RNP was prepared before the day of EP, we obtained sufficient founders to establish the new mouse line.

Conclusion: *S. pyogenes* Cas9 RNP complexes can be prepared up to seven days before EP, allowing more flexibility for scheduling mouse genome editing experiments.

### Production of genetically modified mice through microinjection of eggs from vitro fertilization

**Ms Hslao-hui Chang<sup>1</sup>, Mr Weilun Huang<sup>1</sup>, Mr Howard Hao Wang<sup>1</sup>, Mr Hsien-pin Chiu<sup>1</sup>, Ms Ying-ying Wu<sup>1</sup>, Ms Yu-shan Yeh<sup>1</sup>, Dr Monica Meng-chun Shih<sup>1</sup>, Dr Hsiang-suan Sung<sup>1</sup>, Ms Pei-Yu Liao<sup>1</sup>**

<sup>1</sup>National Applied Research Laboratories, National Laboratory Animal Center, Taipei City, Taiwan

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

Microinjection technology of embryos at pronuclear stage combined with various gene constructs is a well-established methodology for generating genetically modified animals. The conventional approach involves sourcing pronuclear stage embryos through the natural mating of superovulated female mice with their male counterparts. Subsequent microinjection introduces the genetically modified material, followed by the transfer of viable embryos to the oviduct, facilitating their successful development into viable mice. The conclusive phase entails genetic analysis to discern and validate the founder(s) harboring the intended modifications.

In recent years, advances in genetic research and the creation of immune-humanized animal models have necessitated the production of genetically modified mice within precise inbred strains. However, procuring embryos from these strains via natural breeding mandates dedicated housing for individual stud mice. Furthermore, suboptimal mating efficacy among male mice often yields a limited embryo count for subsequent microinjection procedures. In contrast, *in vitro* fertilization (IVF) techniques, conventionally employed to harvest embryos at the 2-cell stage for cryopreservation purposes, present an avenue to obtain ample fertilized eggs without reliance on live studs. This study exploits the potential of IVF-derived pronuclear stage eggs for microinjection procedures, effectively engendering genetically modified mice across strains such as C57BL/6JNarl, NOD.CB17-Prkdcscid/JNarl and other strains. An assortment of constructs, encompassing plasmids, BAC DNA, *lsDNA*, *ssODN*, Cas9 RNA/RNP, and mRNA, were also assessed. These constructs, formulated for diverse objectives and constituents, consistently yielded stable generation of genetically modified mice utilizing IVF-derived embryos. This investigation showcases the successful utilization of IVF-derived pronuclear stage eggs for microinjection procedures, facilitating the consistent production of genetically modified mice across various strains. The comprehensive assessment of diverse constructs underscores the versatility and reliability of this approach in generating stable genetic modifications.

### Generation and comprehensive analysis of CRISPR-mediated NHEJ profiles in F0 founders

**Jade Zhang<sup>1</sup>, Ms charleen hunt<sup>1</sup>, Michael Kelley<sup>1</sup>, Michael Ceriello<sup>1</sup>, Suzanne Hartford<sup>1</sup>, Heather Brown<sup>1</sup>, Jarrell Wiley<sup>1</sup>, Clarissa Herman<sup>1</sup>, Brittany Lee<sup>1</sup>, Jessica Kuhnert<sup>1</sup>, Thomas Kehrer<sup>1</sup>, Marine Prissette<sup>1</sup>, Virginia Hughes<sup>1</sup>, Jennifer Schmahl<sup>1</sup>, Nicolas Gale<sup>1</sup>, William Poueymirou<sup>1</sup>, Eric Chiao<sup>1</sup>, Brian Zambrowicz<sup>1</sup>, Guochun Gong<sup>1</sup>**

<sup>1</sup>Regeneron, Tarrytown, United States

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

The delivery of CRISPR/Cas9 (CC9) nucleases to one-cell stage mouse embryos is a powerful approach for rapidly generating gene knockouts (KOs) bypassing the need to generate modified embryonic stem cells. One long-standing concern regarding CRISPR KOs is the generation of mosaic alleles in founder mice. Here, we describe our systematic approach to optimizing CRISPR non-homologous end joining (NHEJ) outcomes associated with high-throughput embryo electroporation. We sought to gain a better understanding of typical CRISPR-mediated NHEJ events by employing next generation sequencing technologies to thoroughly characterize KO allele sequences in eight adult F0 founder tissues with comparison to cognate P7 tail snips. We further characterized embryonic and adult F0 founder phenotypes relative to TaqMan and NGS genotypes to highlight the utility of F0 KO animals for rapid, go/no-go, decisions.

### Idiopathic accumulation of subcutaneous fats in CYP17A1 knockout rats

**Mr Beomjin Jeon<sup>1</sup>, Mr Donghyeok Kwon<sup>1</sup>, Ms Heekyoung Kim<sup>1</sup>, Mr Goo Jang<sup>1</sup>**

<sup>1</sup>Laboratory of Theriogenology, College of Veterinary Medicine, Seoul National University, Seoul, South Korea

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

Most obesity models, including transgenic animals (*ob/ob* mouse, *DIO* rats, etc.), are being studied in relation to visceral fat, and the association of subcutaneous fat with metabolic syndrome has yet to be thoroughly investigated due to a lack of a suitable subcutaneous research model. The *CYP17A1* gene, which encodes an enzyme with 17,20-lyase and 17 $\alpha$ -hydroxylase activity, is crucial for the body's ability to synthesize steroid hormones like androgen and glucocorticoids. Recent research revealed a connection between the *CYP17A1* gene and atherosclerosis, prostate cancer, congenital adrenal hyperplasia (CAH), and polycystic ovarian syndrome (PCOS). Here, we generated *CYP17A1* knockout rat using CRISPR/Cas9 via ribonucleoprotein (RNP) electroporation. Pre-incubated gRNA and Cas9 RNP was electroporated into 1-cell stage embryo, and 2-cell stage embryos (number: 20 ~ 25) were transferred

to oviducts of recipients. Total 281 embryos were transferred to 19 recipients, 48 pups were born, and 41 pups had CYP17A1 indel mutation (85.42%). In order to identify depot-specific fat accumulation, we sampled CYP17A1 knockout rats, which demonstrated a sex-reversed and obese phenotype. Inguinal fat (subcutaneous fat) was found to accumulate excessively in CYP17A1 KO rats compared to wild-type rats when body weight or visceral adipose tissue mass were normalized. A number of tests, including the insulin tolerance test, the oral glucose tolerance test, the blood biochemistry test, and the blood pressure analysis, were used to examine the metabolic symptoms of CYP17A1 KO rats. These tests revealed that CYP17A1 KO rats are normal in insulin and glucose tolerance, and also have no hypertension, hyperglycemia, hyperlipidemia, or hypercholesterolemia. In conclusion, it demonstrated that the CYP17A1 knockout rat model will aid in understanding the relationship between steroid hormones, subcutaneous fat accumulation, and metabolic syndrome.

### Humanizing the NSG mice for adopting human blood cells and producing specific inhibitory antibodies against blood clotting factors

Mr Li-Fu Chen, MD Sheng-Chieh Chou, Ms Yi-Hsuan Wu, Mr Shih-Fan Wu, Ms Te-Hsien Liu, Mr Kuei-Liang Chen, Mr Chien-Hong Liu, PhD I-Shing Yu, PhD You-Tzung Chen, PhD Shu-Wha Lin

<sup>1</sup>National Taiwan University, Taipei, Taiwan

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

Background: Humanized mouse models by transplanting human peripheral mononuclear cells (PBMCs) into NSG mice are valuable tools for they can provide an opportunity to study the mechanism(s) contributing to the eradication of the inhibitory antibodies in autoimmune or alloimmune diseases. However, these PBMC-engrafted mice typically die within 1–2 months due to graft-versus-host disease (GvHD).

Aims: This research aims to modify the hemophilia A (HA)-NSG mice to minimize GvHD and to effectively harbor anti-factor VIII (FVIII) antibody-producing immune cells for long term.

Methods: The NSG-HA mice we have generated by the CRISPR technology (Yen et al 2016, *Thrombosis J* 2016) were further modified to knockout mouse MHC class I (H2-K1, H2-D1) and class II (H2-IA) genes. Two guide RNAs (sgRNAs) are designed at the 5' and 3' end of each target gene respectively. Inhibitor-positive HA patients' PBMCs (3 × 10<sup>6</sup>/mouse) were transplanted into the spleen of mice. Engraftment efficiency was examined by flow cytometry. Inhibitory antibodies were analyzed by neutralization assays.

Results: The novel NSG-KDIA(null)-HA mice were generated by three rounds of CRISPR/Cas9 technology. Human CD45<sup>+</sup> cells (white blood cells) can be detected in the periphery of mice 2 weeks after engraftment into the NSG-KDIA(null)-HA mice and persist for > 20 weeks. The mean hCD45<sup>+</sup> cells reached 17.41% at week 12. The spleen and bone marrow of the highest recipient mice were 87.8% and

32.2%, with 3.31% and 3.68% of B cells. Mean FVIII-inhibitor titers are 1.93 BU (range 0.22–9.68 BU, week 6).

Conclusion: We have established a novel NSG-HA mouse model that can harbor HA patients' PBMCs for long term without showing GvHD phenotype and can produce a significant amount of functional human antibodies.

### Establishing a pipeline for validating VelociGene's humanized animal models

Katie Huling<sup>1</sup>, Jay Zhong<sup>1</sup>, Susannah Brydges<sup>1</sup>, Sean Trzaska<sup>1</sup>, Marina Maiuri<sup>1</sup>, Michelle McAlister<sup>1</sup>, Ming Huang<sup>1</sup>, Michael Toner<sup>1</sup>, Kevin Bugge<sup>1</sup>, Nicole Oristian<sup>1</sup>, Greg Polites<sup>1</sup>, Jan Roos<sup>1</sup>, Jun Zhong<sup>1</sup>, Marine Prissette<sup>1</sup>, Haibo Qiu<sup>1</sup>, Jean Siao<sup>1</sup>, Ning Li<sup>1</sup>, Eric Chiao<sup>1</sup>, Albert Torri<sup>1</sup>, Brian Zambrowicz<sup>1</sup>

<sup>1</sup>Regeneron Pharmaceuticals, Tarrytown, United States

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

The VelociGene department of Regeneron Pharmaceuticals creates animal models for scientific study. Humanizations (humIns) involve the complete or partial replacement of a mouse or rat gene with human homologous sequence. These animal models are used for various study needs, such as therapeutics development, antibody testing, and modeling human disease. Proper expression from the humIn allele is therefore vital. We present here our pipeline for validation of humanized mRNA and protein expression during early cohort breeding. By evaluating F1 animals heterozygous for a humIn allele, we can confirm that engineered mRNA and protein are made in the tissue locations and amounts necessary or halt further study for redesign if the allele does not perform satisfactorily. This early validation saves time and animal cage space. Here we present mRNA and protein results from a series of humIn alleles.

### A novel 61 base-pair intronic deletion of Snrpb is crucial for Snrpb regulation and normal development in mouse

Dr Marie-claude Beauchamp<sup>1</sup>, Dr Sabrina Shameen Alam<sup>1,2</sup>, Dr Loydie Jerome-Majewska<sup>1,2,3,4</sup>

<sup>1</sup>Research Institute-McGill University Health Center, Montreal, Canada, <sup>2</sup>Department of Human Genetics, McGill University, Montreal, Canada, <sup>3</sup>Department of Anatomy and Cell Biology, McGill University, Montreal, Canada, <sup>4</sup>Department of Pediatrics, McGill University, Montreal, Canada

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00.

Mutations in a common core spliceosomal factor called SNRPB causes cerebrocostomandibular syndrome (CCMS). Most CCMS patients have point mutations that increase levels of transcripts containing a pre-termination codon (PTC)

containing alternative exon 2 (AE2). Herein, we generated a mouse line with a 61-base pair intronic deletion upstream of AE2 ( $\Delta 61$ ) and show that 10% of heterozygous and homozygous embryos ( $\text{Snrpb } \Delta 61/+$ ;  $\text{Snrpb } \Delta 61/\Delta 61$ ) had abnormalities similar to those found in CCMS patients.  $\Delta 61$  mutants had microcephaly, defects in the bones of the craniofacial region and ribs and 18/47 die from 4 weeks of age onwards. These embryos also had a significant increase in expression of the AE2 and a reduction in  $\text{Snrpb}$  levels. In parallel, we generated a conditional mutant mouse carrying loxp sequences flanking exons 2–3 of  $\text{Snrpb}$ . We used mesoderm-specific  $\text{Mesp1-Cre}$  to delete  $\text{Snrpb}$ , and showed that a fraction (5/40) of heterozygous  $\text{Snrpb loxp/+}$ ;  $\text{Mesp1-Cre } \pm$  embryos are abnormal at E9.5. They have a narrow frontonasal prominence, a smaller 2nd pharyngeal arch, an enlarged heart, and begin to die at E12.5 where 50% are found alive. In these mutants,  $\text{Snrpb}$  expression was not changed whereas expression of the AE2 was half of controls, suggesting a potential compensatory increase of the  $\text{Snrpb}$  wild-type allele. To test if the  $\Delta 61$  mutation fails to complement  $\text{Mesp1-Cre}$  mediated deletion of  $\text{Snrpb}$ , we generated  $\text{Snrpb loxp}/\Delta 61$ ;  $\text{Mesp1-Cre } \pm$  mutant embryos. At E9.5, all of the recovered double heterozygous embryos showed an unlooped heart, misshapen somites and failed to turn. These embryos had a significant reduction in  $\text{Snrpb}$  levels without a change in AE2 expression. Our findings suggest that the 61-bp intronic region regulates AE2 inclusion and plays an important role in  $\text{Snrpb}$  regulation. Thus, these sequences should be investigated in CCMS patients that do not carry mutations in  $\text{SNRPB}$  coding exons.

### A reverse genetic approach in geckos with the CRISPR/Cas9 system by oocyte microinjection

**Dr Takaya Abe<sup>1</sup>, Ms Mari Kaneko<sup>1</sup>, Dr Hiroshi Kiyonari<sup>1</sup>**

<sup>1</sup>RIKEN BDR, Kobe, Japan

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

Reptiles are important model organisms in developmental and evolutionary biology but are used less widely than other amniotes such as mice and chickens. One of the main reasons for this is that has proven difficult to conduct CRISPR/Cas9-mediated genome editing in many reptile species despite the widespread use of this technology in other taxa. Certain features of reptile reproductive systems make it difficult to access one-cell or early-stage zygotes, which impedes gene editing techniques. Recently, Rasys and colleagues reported a genome editing method using unfertilized oocyte microinjection that allowed them to produce genome-edited *Anolis* lizards. This method opened a new avenue to reverse genetics studies in reptiles. Here, we report the development of a related method for genome editing in the Madagascar ground gecko (*Paroedura picta*), in which we have established genome and transcriptome resources, and describe the generation of Tyr and Fgf10 gene-knockout geckos in the F0 generation.

### A new experimental animal model in mammalian genetics, “The grey short-tailed opossum”

**Ms Riko Yoshimi<sup>1</sup>, Dr Hiroshi Kiyonari<sup>1</sup>, Dr Takaya Abe<sup>1</sup>, Ms Mari Kaneko<sup>1</sup>**

<sup>1</sup>Laboratory for Animal Resources and Genetic Engineering, Riken Center For Biosystems Dynamics Research, Kobe, Japan

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00.

Marsupials represent one of three extant mammalian subclasses with several very unique characteristics not shared by other mammals. Most notably, much of the development of immaturely born neonates takes place in the external environment.

The grey short-tailed opossum (*Monodelphis domestica*; hereinafter “the opossum”) is thought to be the ancestor of all marsupials. The opossums are most used as experimental marsupial model because opossums are similar in body size and breeding characteristics to popular rodent models such as mice and rats. Moreover, in 2007, the opossum was the first marsupial species to have its whole genome sequenced. Like other marsupials, the opossums give birth after a short gestation period of 14 days to immature pups that are the equivalent of 13- to 15-day embryos in mice and rats or 40-day embryos in humans. Interestingly, although the opossum is a marsupial, females don’t have a pouch. After birth, the baby clings to the mother’s nipples until weaning. As a result, it has been reported that the maturity of each organ varies greatly before and after birth, with the forelimbs, jaws, tongue, and sense of smell being highly developed at birth, and the digestive, reproductive, and lymphatic systems, ears, eyes, and hindlimbs being developed after birth.

Despite these unique characteristics, the lack of technology to manipulate their genomes has hindered the development of in vivo genetic approaches in this group of mammals. Recently, we have successfully generated tyrosinase gene knockout opossums by genome editing using CRISPR/Cas9. This is the first demonstration of the production of genetically engineered animals in marsupials. This study will be able to provide a critical foundation for the venue to expand mammalian genetics to the metatherian subclass.

### Implementing in vitro fertilization for rat model cryo-resuscitation from frozen-thawed sperm

**Dr Hongsheng Men<sup>1,2</sup>, Mr Payton Oswalt<sup>1,2</sup>, Dr Elizabeth Bryda<sup>1,2</sup>**

<sup>1</sup>Rat Resource and Research Center, University of Missouri, Columbia., United States, <sup>2</sup>Department of Veterinary Pathobiology, University of Missouri, Columbia., United States

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

Intracytoplasmic sperm injection (ICSI) is currently the most commonly used method to resuscitate rat models from frozen

sperm due mainly to the lack of efficient and repeatable sperm cryopreservation and in vitro fertilization (IVF) protocols. The ICSI procedure requires expensive equipment and extensive technical training. Therefore, optimization and implementation of IVF as a cost-effective alternative for cryo-resuscitation will provide the biomedical community with more accessibility to cryopreserved rat models. Since significant improvements in both sperm freezing and IVF have been accomplished in recent years, we investigated the feasibility of IVF for rat model cryo-resuscitation at the Rat Resource and Research Center which serves as a repository for a large number of rat strains/stocks generated and used by the research community. Two transgenic and two knock-in lines were used in this pilot study. Cauda epididymal sperm were frozen in an egg yolk-lactose-Equex STM based freezing medium. The frozen thawed sperm were then used to fertilize oocytes from superovulated wild type immature females in vitro. The in vitro development potential of embryos resulting from IVF was used to assess the efficiencies of the IVF procedure. Preliminary results showed that the fertilization rates ranged from 44.4% to 91.3% and blastocyst rates ranged from 0% to 71.4% with significant variation among different mutant lines and even different IVF attempts within the same mutant line. However, within three replicates, we achieved blastocyst rates ranging from 45.8% to 71.4% in all four lines. Cryo-resuscitation only requires a few animals to establish breeding pairs, therefore, the preliminary results support implementation of the use of IVF for cryo-resuscitation with ICSI as a back-up procedure for lines with repeated IVF failures. Future work will include additional mutant lines and embryo transfers to assess their in vivo developmental potential (funded by NIH grant P40 OD011062-22S1).

## Management of mouse sperm freezing and quality control

**Mrs Nicole Kuepper<sup>1</sup>, Mrs Julia Mock<sup>1</sup>, Mrs Sonja Schavier<sup>1</sup>, Mrs Ursula Sorg<sup>1</sup>, Mr Klaus Pfeffer<sup>1</sup>**

<sup>1</sup>Heinrich Heine University Duesseldorf, Institute of Medical Microbiology, Duesseldorf, Germany

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

Generally, in research an increasing set of gene-modified mouse lines and intercrosses thereof is maintained in the animal facility. Due to changing research some mouse lines are not used in experimental settings. These lines have been bred in a “maintenance mode” to secure the genetic modification, especially if the gene modification will not be considered by repositories. However, even with a minimal number of breeders, offspring is constantly arising, ultimately limiting animal capacity for “active lines”, increasing costs and animal numbers. The management of gene-modified mouse lines, considering three “Rs” in project design and animal maintenance therefore requires management strategies to reduce the number of breeding lines, typing work and expenses.

To achieve these aims, systematic sperm freezing was introduced, based on the protocol of Takeo & Nakagata for

mouse lines on a C57BL/6N background. Moreover, different media and conditions were evaluated. Report and data forms were developed for documentation and quality control of cryopreservation and in vitro fertilization (IVF). Currently, more than 25 lines have been cryopreserved and successfully rederived by IVF of mouse oocytes (C57BL/6N). To test sperm viability and gene modifications, PCR conditions for different alleles were optimized based on the method of Scavizzi et al., allowing typing of fertilized embryos already at the blastocyst stage. This reduces the numbers of foster mice and born offspring required 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 available, enabling simplified transfer of mouse lines, and providing a significant reduction of animals required in research.

## Facilitate Cryopreservation and Re-derivation of Genetically Modified Mice via Automated Sperm Cryopreservation

**Mrs Ralica Zamfirova<sup>1</sup>**

<sup>1</sup>Regeneron Pharmaceuticals, Tarrytown, United States

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

The emergence of efficient genome editing technologies significantly increased the need for fast and reliable cryopreservation methods to cryopreserve extensive number of genetically modified mouse lines which are no longer needed for breeding. The various cryopreservation tools available have significantly reduced the cost and ethical concerns associated with animal studies. However, despite the progress made in this field in the past two decades, sperm cryopreservation remains a slow manual process that requires tedious and time-consuming sample aliquoting into cryo vials or straws, which can also lead to human errors and inconsistent volume and quality of the cryopreserved samples.

Here we present the development and implementation of a novel automated sperm cryopreservation procedure which utilized a STARlet Liquid Handling System and a LabElite Automated Screw Decapper. The automated sperm cryopreservation had adapted slow rate freezing with skim milk/raffinose cryoprotectant (1) to freezing in 2D barcode 200 ul Matrix tubes. We conducted a comprehensive comparison of the automated sperm cryopreservation techniques to the original manual method, which outlined significant increase of the speed of the cryopreservation process while maintaining sperm viability and post-thaw motility parameters. The procedure can be used to freeze sperm from up to 40 males in less than 90 min. It minimizes the chance of human error and enhances the reliability of the aliquoting and freezing steps. Post implementation data from IVF procedures utilizing

cryopreserved sperm showed that the automation can improve the fertilization rates leading to more successful outcomes in subsequent sperm cryopreservation recovery procedures. Furthermore, the automation approach allows to cryopreserve large amount of genetically modified mouse lines in a short period of time. Using it, we were able to reduce significant burden on our vivarium by cryopreserving 640 males in ten weeks in a response to the Covid19 pandemic in 2020.

### Using extended shelf-life HTF to replace Cook's discontinued fertilization medium in an affordable state-of-the-art mouse IVF protocol

**Dr Magdalena Wigger<sup>1,2</sup>, Marco Schneider<sup>1,2</sup>, Anni Feldmann<sup>1,2</sup>, Sonja Assenmacher<sup>1,2</sup>, Prof Dr Branko Zevnik<sup>1,2</sup>, Dr Simon Tröder<sup>1,2</sup>**

<sup>1</sup>Cluster of Excellence Cellular Stress Responses in Aging-associated Diseases (CECAD), University of Cologne, Cologne, Germany, <sup>2</sup>in vivo Research Facility, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

Sperm cryopreservation is becoming increasingly popular for the archiving of genetically engineered mouse lines, offering cost, time, and animal welfare advantages. For the rederivation of these lines by IVF, we previously developed SEcuRe, an affordable state-of-the-art IVF protocol. Based on the principles of the leading CARD method, our universal SEcuRE protocol allows efficient fertilization with sperm from all major cryopreservation protocols as well as freshly harvested sperm. A key component was Cook's® "Research Vitro Fert" (RVF) fertilization medium. However, its unexpected discontinuation disrupted the applicability of our approach and other IVF protocols dependent on this fertilization medium, necessitating a suitable alternative.

Here we present our updated SEcuRe 2.0 protocol using HTF instead of RVF as a basal fertilization medium. Comparison of RVF and HTF during IVFs with cryopreserved C57BL/6 sperm from a variety of genetically engineered mice revealed equal fertilization rates, validating our approach. In addition, we demonstrate that HTF has a substantially extended shelf-life by utilizing commercial HTF that was six months past its expiration date. Expired HTF did not affect fertilization or subsequent embryonic development, underscoring the economic value of our modified approach. In summary, we demonstrate that extended shelf-life HTF can be used in place of the now-discontinued RVF medium to ensure the applicability of SEcuRe and other IVF protocols employing Cook's® RVF. With a media composition identical to the successful CARD protocol, our enhanced SEcuRe 2.0 offers IVF laboratories an easily adaptable and 3R-compliant method to efficiently archive and distribute genetically engineered mouse models with minimal costs.

### Evaluating CRISPR/Cas9 guide RNA activity in rodent blastocysts

**Ms Hailey Maresca-Fichter<sup>1</sup>, Elizabeth Hughes<sup>2</sup>, Honglai Zhang<sup>2</sup>, Wanda Filipiak<sup>2</sup>, Galina Gavrilina<sup>2</sup>, Dr Thomas Saunders<sup>2</sup>, Dr Zachary Freeman<sup>2</sup>**

<sup>1</sup>College of Veterinary Medicine, Michigan State University, East Lansing, United States, <sup>2</sup>Transgenic Animal Model Core, Biomedical Research Core Facilities, University of Michigan, Ann Arbor, United States

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Guide RNA (gRNAs) are a critical component to determining CRISPR/Cas9 activity when generating transgenic animal models. A bioinformatic approach is commonly used to identify gRNA with current pipelines relying heavily on in vitro results in immortalized cell lines coupled with to protospacer adjacent motif (PAM) sequence identification to predict on target activity. It remains unclear how the in vitro and in vivo results correlate with the expected success of gRNA identified by these methods. We have evaluated more than 950 unique gRNAs over the past 7 years utilizing an ex vivo pipeline to test gRNA activity prior to generating transgenic rodents. Briefly, gRNAs are microinjected into fertilized zygotes, allowed to develop to blastocysts, and then characterized at an individually blastocyst level for on target cutting activity. gRNAs are determined to be active if evidence of cutting is present in more than 33% of tested blastocysts. We aimed to understand from this data how current gRNA selection tools predicted on target cutting in zygotes. We hypothesize that the relationship between guide scoring and blastocyst cutting activity are not strongly correlated. We found that only 61.3% of gRNAs with predicted activity were active in our assay while 38.7% of guides were inactive. Furthermore, the percent active guides remained constant over time and was similar across different variants of Cas9 enzyme. We next evaluated Moreno-Mateos (MM) and cutting frequency determination (CFD) scores in active vs inactive gRNA, as metrics for gRNA cutting activity and off-target events, respectively. We found no difference in MM and CFD scores for active vs inactive gRNAs. These data suggest that current gRNA selection tools may need improvement for application to the generation of transgenic animal models.

### Evidence of Gram-negative bacteria in the reproductive tract of embryo donor female mice after copula

**Ms Maria Noel Meikle<sup>1</sup>, Ms Geraldine Schlapp<sup>1</sup>, Dr Jorge Pórfido<sup>1</sup>, Dr Tamara Fernández-Calero<sup>2</sup>, Dr Natalia Rego<sup>2</sup>, Dr Hugo Naya<sup>2</sup>, Dr Martina Crispo<sup>1</sup>**

<sup>1</sup>Laboratory Animal Biotechnology Unit, Institut Pasteur de Montevideo, Montevideo, Uruguay, <sup>2</sup>Bioinformatics Unit, Institut Pasteur de Montevideo, Montevideo, Uruguay

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

Previous findings of our laboratory have shown bacterial contamination in the culture media drops of *in vivo* produced murine embryos from different mouse strains. Although all reagents and materials used were tested, no contamination could be detected. The aim of this work was to determine if the source of this bacterial contamination could come from the female's reproductive tract after male copula. Twelve C57BL/6 J females (4-week-old) were superovulated with 5 IU of eCG injection followed by 5 IU of hCG injection 48 h later. Immediately, six females were mated with C57BL/6 J adult males (1 × 1; mating group), while the others were not mated (control group). Next morning all females were euthanized by cervical dislocation, and their oviducts were individually collected in 200 µL of M2 medium. Thirty minutes later, 50 µL of M2 medium were placed in M16 drops under paraffin oil (two replicates/female) and maintained in 5% CO<sub>2</sub>, 37 °C for 96 h. M16 drops were observed under a Zeiss Stemi 508 stereomicroscope (50x), using rear-lit. After 24 h, motile bacteria were detected in the mating group samples but not in the control ones. Samples from the contaminated drops were Gram stained and a preliminary study of the 16S rRNA amplicon sequencing was performed using MinION (Oxford Nanopore). For this approach, bacteria were enriched in Luria–Bertani broth and Monarch® Genomic DNA Purification Kit was used for DNA extraction. Gram-negative bacteria were found in the sample smears. Taxonomical classification of the sequences using SINA (SILVA Incremental Aligner) with the SILVA ribosomal RNA database revealed bacteria from the Enterobacteriaceae family, particularly *Escherichia-Shigella*. Preliminary results of our group show Gram-negative bacteria in the reproductive tract of females after copula, which probably comes from their vagina (Parr, 1985). More studies are being performed to determine the effect of these bacteria on embryo development.

### Optimizing the isolation of murine bone marrow cells: A direct comparison of three protocols

**Mrs Sonja Schavier<sup>1</sup>, Mrs Nicole Küpper<sup>1</sup>, Mrs Julia Mock<sup>1</sup>, Prof. Dr Klaus Pfeffer<sup>1</sup>, Dr Ursula Sorg<sup>1</sup>, Prof. Dr Stefanie Scheu<sup>1</sup>**

<sup>1</sup>Institute of Medical Microbiology and Hospital Hygiene, Heinrich Heine University Duesseldorf, Duesseldorf, Germany

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

In primary cell culture, bone marrow (BM) cells are often used to differentiate various hematopoietic cell types by cultivation with the appropriate growth factors. Optimizing the protocol for BM cell isolation increases cell viability and yield, saves time and reduces the number of mice required.

Here, we evaluate three methods for the isolation of murine BM cells.

1) Flushing: Both ends of prepped femurs and tibiae are carefully severed with scissors and the BM is flushed into a 5 ml petri dish with a 23 G needle.

2) Crushing: Prepped, meticulously cleaned and dried femurs and tibiae are placed in a mortar. After cracking the bones into 2–3 pieces with a pistol, buffer of choice is added and bones are gently crushed. The liquid is then collected. These steps are repeated until the bones appear pale.

3) Centrifuging: The distal ends of prepped femurs and the proximal ends of prepped tibiae are severed with scissors. Both are placed with the open end downwards into a 0.5 ml tube which has been pierced at the bottom with a 18 G needle. This tube is inserted into a 1.5 ml tube and both are centrifuged, expelling the BM.

Time wise, centrifuging is the best option, as the bones do not need to be cleaned extensively. They can be processed in parallel and tubes can be prepared beforehand. Also, there is far less detritus, higher yield and better viability compared to the crushing protocol. While flushing also yields adequate numbers of viable cells, it is more time intensive than centrifuging.

In conclusion, in our hands centrifugation of femurs and tibiae is the ideal protocol to quickly isolate the maximum number of murine BM cells without compromising the viability. In addition, the protocol can easily be adapted for other bones.

### Comparison of medium formulations in derivation of mixed background mouse ES cell lines

**Ms Junko Kuno<sup>1</sup>, John Nuara<sup>1</sup>, Craig Grant<sup>1</sup>, Jan Roos<sup>1</sup>, Ralica Zamfirova<sup>1</sup>, Mayumi Isaka<sup>1</sup>, Michael Brown<sup>1</sup>, Joseph Hickey<sup>1</sup>, Marine Prissette<sup>1</sup>, Guochun Gong<sup>1</sup>, Bill Poueymirou<sup>1</sup>, Brian Zambrowicz<sup>1</sup>, Eric Chiao<sup>1</sup>**

<sup>1</sup>Regeneron Pharmaceuticals, Inc., Tarrytown, United States

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

Although simple genetic modifications can be achieved by introducing the CRISPR/Cas9 system directly in mouse embryos, large modifications still require targeting via homologous recombination in mouse embryonic stem (ES) cells. To support and accelerate the generation of mouse models with multiple genetic modifications, we routinely derive new mouse ES cell lines that are homozygous for multiple large, engineered alleles. Historically, we have used 2i medium to derive mixed background ES cell lines that consist of various percentage contributions of the C57BL/6, 129S6, and Balb/c strains. Following microinjection of the modified ES cells into 8-cell embryos, 100% ES cell-derived mice can be produced. From 2008 to 2016, we have used those lines derived in 2i medium in more than 500 microinjections, transferring approximately 24,000 injected embryos into pseudo-pregnant surrogates, resulting in an overall average of 6% of the

transferred embryos giving rise to 100% ES cell-derived live-born F0 animals.

Recently, we performed parallel ES cell derivations from C57BL/6, 129S6, and Balb/c mixed background mice, comparing the standard 2i medium with 2 newer published medium formulations: N2B27-LCM medium and N2B27-LCC medium. Here we report the details and conclusions of these experiments. In 5 out of 6 independent mouse derivations, N2B27-LCCDM was the most optimal medium which yielded a 15% to 45% range of 100% ES cell-derived mice following microinjection into 8-cell embryos. From these observations, we have standardized the use of N2B27-LCCDM in our derivation pipeline for C57BL/6, 129S6, and Balb/c mixed background mice.

### Speeding up the pipeline to phenotyping cohort generation

**Mr Anthony Gagliardi<sup>1</sup>, Shirley Chen<sup>1</sup>, Pei Yi Huang<sup>1</sup>, Eric Chiao<sup>1</sup>, Brian Zambrowicz<sup>1</sup>**

<sup>1</sup>Regeneron Pharmaceuticals, Inc., Tarrytown, United States

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

Mouse embryonic stem cell gene targeting efforts usually consist of two independent electroporations. One to introduce the targeting vector and another to remove the drug-selection cassette found within the vector. In between these two events, considerable time is required to characterize and identify correctly targeted clones, also testing their ability to contribute to chimera production & germline transmission to choose the clone which will be used for cassette removal. This process can take months to produce the cassette-deleted targeted clones desired to move forward with cohort production for phenotyping.

Here we describe a shortened timeline to produce these targeted cassette-deleted clones by removing the need to identify cassette-containing clones before proceeding to cassette removal step. After the initial electroporation and drug-selection, the resulting colonies are used directly for cassette removal. The standard process requires the picking of colonies after each electroporation while this new workflow requires colony picking only once. These colonies are then characterized using LOA Taqman screening technology to identify clones having been both targeted and having the drug-selection cassette removed. Our results indicate the cassette removal step is very efficient and the success of this new workflow is dependent on the initial targeting event taking place.

This much time-constrained timeline allows for the identification of clones desired for cohort production in weeks, compared to the months previously required. Less effort and time are required in colony picking, clone expansion and Taqman screening. Further, by eliminating the need to test clones for germline transmission, less animals are required during this process. The time savings realized throughout allows for earlier than expected phenotyping.

### Comparison of genotyping approaches for conditional knock-Out and Cre Mouse breeding lines

**Ryan Gotcher<sup>1</sup>, Cecile Pfaff<sup>3</sup>, Hong Lei<sup>1</sup>, Qiang Zhou<sup>2</sup>, Merle Lindstrom<sup>2</sup>, Daniel Breustedt<sup>3</sup>, Fanny Decarpentrie<sup>3</sup>, Dr Kevin Forbes<sup>1</sup>**

<sup>1</sup>Novartis Institutes For Biomedical Research, Cambridge, USA, <sup>2</sup>Novartis Institutes For Biomedical Research, La Jolla, USA, <sup>3</sup>Novartis Institutes For Biomedical Research, Basel, CH

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

Conditional knock-out (cKO) mouse models aid in the detailed analysis of gene functions in a tissue- or temporally specific fashion. Most cKO mouse models follow the Cre/loxP system to control these genetic changes involving a floxed (Two-flanking loxP sites) mouse allele model cross-bred to a Cre mouse model that expresses the enzyme to control the tissue- or temporally-specific ablation of the floxed allele. While cross-breeding these mouse lines, it is key to identify wild-type, heterozygous or homozygous genotypes for the cKO allele and for the presence or absence of the Cre transgene. Traditional end point PCR, gel and sequencing analysis methods used for this genotyping, can be laborious and time consuming. Here we compare our efforts to use Capillary Electrophoresis (CE), multi-plexed (fluor) quantitative PCR and outsourcing for genotyping cKO and Cre mouse lines. When compared to traditional methods, these alternatives allow for quicker turnaround times on making genotype calls ultimately leading to a reduced mouse cage footprint.

### The NCI Mouse repository: cancer models and miRNA-ES cell resource

**Mrs Roackie Awasthi<sup>1</sup>, Ms Debra Fitzgerald<sup>1</sup>, Dr Stephen Jones<sup>1</sup>**

<sup>1</sup>Leidos Biomedical Research, Inc., Frederick, United States

Poster Session 1 (Odd Numbers), Imperial West, November 13, 2023, 17:30–19:00

The NCI Mouse Repository, located at the Frederick National Laboratory for Cancer Research (FNLRC), Frederick, Maryland, is an NCI-funded resource of approximately 160 genetically-engineered mouse cancer models and associated strains, including mice bearing conditional and point-mutant alleles in cancer-related genes. In addition, the Repository houses a unique collection of over 1500 different mouse ES cell clones bearing conditionally-activated miRNA transgenes to facilitate in vivo exploration of miRNA functions. The NCI Mouse Repository's mouse strains and mESCs are available to all members of the scientific community (academic, non-profit,

and commercial). The mouse models and ES cell clones are cryo-archived and distributed as frozen germplasm or cells.

Requests may be placed through the NCI Mouse Repository website (<https://ncifrederick.cancer.gov/Lasp/MouseRepository/Default.aspx>). In addition to the request form, this website includes detailed descriptions for each strain accepted into the Repository and the associated publications provided by the donating scientist. The miR-harboring ESCs, originally generated at the Cold Spring Harbor Laboratories for the NCI, are described in full detail and include validation data for each miRNA ES cell clone. These resources are available for nominal cost to NCI, NIH, and other US government-funded investigators, as well as to Investigators at non-profit organizations.

## Rat resource and research center

### Dr Elizabeth Bryda<sup>2</sup>

<sup>1</sup>University Of Missouri, Columbia, USA, <sup>2</sup>Rat Resource and Research Center, Columbia, USA

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

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 575 rat lines; 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, microbiota analysis and characterization of genetically engineered rats. The RRRC, in conjunction with the MU Animal Modeling Core, makes 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](http://www.rrrc.us)) allows user-friendly navigation. Current research efforts include generation and characterization of a variety of new rat models and improvements to rat in vitro fertilization. The University of Missouri is home to the NIH-funded MU Mutant Mouse Resource and Research Center (MMRRC) and the National Swine Resource and Research Center (NSRRC) as well as the MU Animal Modeling Core and MU Metagenomics Center. Together, these highly collaborative groups provide a variety of animal model-related services across species to facilitate biomedical research. Funding: NIH 5P40 OD01106.

## The NCI Mouse repository: cancer models and miRNA-ES cell resource

### Mrs Parirokh Awasthi<sup>1</sup>, Ms Debra Fitzgerald<sup>1</sup>, Dr Stephen Jones<sup>1</sup>

<sup>1</sup>Leidos Biomedical Research Inc., Leesburg, United States

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

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## Generation of a mouse line with a cAMP FRET sensor

### Dr Jorge Pórfido<sup>1</sup>, MSc Maria Noel Meikle<sup>1</sup>, MSc Geraldine Schlapp<sup>1</sup>, Dr Vanesa Piattoni<sup>2</sup>, Dr Matías Machado<sup>3</sup>, Dr Cecilia Abreu<sup>2</sup>, Dr Mariela Bollati<sup>2</sup>, Dr Marcelo Comini<sup>4</sup>, Dr Sergio Pantano<sup>3</sup>, Dr Martina Crispo<sup>1</sup>

<sup>1</sup>Laboratory Animal Biotechnology Unit, Institut Pasteur De Montevideo, Montevideo, Uruguay, <sup>2</sup>Cell Biology Unit, Institut Pasteur de Montevideo, Montevideo, Uruguay, <sup>3</sup>Biomolecular Simulations Laboratory, Institut Pasteur de Montevideo, Montevideo, Uruguay, <sup>4</sup>Redox Biology of Tripanosomatids Laboratory, Institut Pasteur de Montevideo, Montevideo, Uruguay

Poster Session 2 (Even Numbers), Imperial West, November 14, 2023, 15:30–17:00

CUTie (cAMP Universal Tag for imaging experiments) is a cAMP FRET-based sensor designed by computational techniques in order to be targeted to different macromolecular

complexes, with equal cAMP sensitivities in different environments. This characteristic allows direct comparison of cAMP signals at multiple subcellular sites.

These sensors have been already tested in cells with good results, allowing accurate detection of compartmentalized cAMP near certain subcellular complexes [1].

Our aim is to develop a genetically modified mouse model harboring one variant of CUTie with ubiquitous expression in (nearly) every cell of the animal. If successful, this mouse model will serve as a source of reporter cells and/or organoids for multiple studies involving cAMP signaling.

In the first trial, the construction was targeted to Rosa26 locus employing the corresponding sgRNA, Cas9-mSA and a biotinylated-PCR as a template, injected in two-cell embryos. Four potential founder animals were obtained out of 30 born pups. However, three of them were random insertions and the one that was inserted on-site had a rearrangement and had to be discarded. As an alternative strategy, we are currently injecting in zygotes a plasmid with Cas9/sgRNA cutting sites flanking the template sequence and homology regions, with the aim to guide the modification to H11 safe harbor locus. One hundred and forty-seven 2-cell embryos were transferred to seven B6D2 F1 pseudo pregnant females, but no pups were born.

Improving the design in order to succeed in the generation of this model is mandatory as a starting point to produce future mouse lines harboring FRET-based sensors.

1. Surdo NC, Berrera M, Koschinski A, Brescia M, MacHado MR, Carr C, et al. FRET biosensor uncovers cAMP nano-domains at b-adrenergic targets that dictate precise tuning of cardiac contractility. *Nat Commun.* 2017;8. <http://doi.org/10.1038/ncomms15031>.

## The laboratory animal biotechnology unit—more than 15 years' experience in south America

Ms Geraldine Schlapp<sup>1</sup>, María Noel Meikle<sup>1</sup>, Ana Paula Arévalo<sup>1</sup>, Gabriel Fernández-Graña<sup>1</sup>, Jorge Pórfido<sup>1</sup>, Andrés Pereyra<sup>1</sup>, Sergio Ancheta<sup>1</sup>, Ismael González<sup>1</sup>, Andrea Massa<sup>1</sup>, Pedro Casaravilla<sup>1</sup>, Martina Crispo<sup>1</sup>

<sup>1</sup>Institut Pasteur De Montevideo, Montevideo, Uruguay

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The LABU was created in 2006 as a regional platform for the generation of genetically modified (GM) mouse models at Institut Pasteur de Montevideo. Since then, the whole unit has been in continuous evolution, incorporating new equipment, staff, services and mouse lines. Currently, 120 scientists employ animals at some point in their research, and LABU is producing 15 times more mice than in early stages, maintaining the same SPF microbiological status. At present, more than 50 GM lines are bred at the SPF area to cover a variety of research projects in the fields of cancer, metabolic disorders, and neurodegenerative and infectious diseases. Since 2014 we have introduced the CRISPR technology that has replaced the use of ES cell microinjection, increasing the efficiency and reducing the time needed to produce GM-edited mice. Additionally, ART's improvements, for instance, the use of IVF instead of conventional superovulation and natural breeding, and ultra-superovulation schemes have boosted embryo and sperm cryopreservation and rederivation techniques. Special emphasis to develop a preclinical research area that includes an *in vivo* imaging lab, surgical procedure's room, 2 behavioral testing rooms, 2 BSL3 cabinets, blood and biochemical profile determination and polyclonal antibody production has been carried out. Last but not least, our unit has organized 11 national and 8 international courses including use and welfare of lab animals, genome editing, cryopreservation and alternative methods. We have fruitfully combined services and research that are reflected in more than 50 international articles. The continuous effort and dedication of our staff ensures the excellence in research for our country and region, being the center of reference for many scientists working in these fields.

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