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Abstracts of the 17th Transgenic Technology Meeting (TT2022)

Helsinki, Finland, September 17–20, 2022

Paper 1: 3Rs and ethics

Poster

Systematic optimization of CRISPR/CAS9 system using the tyrosinase locus

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Although the CRISPR/Cas9 system is effective for creating genetically engineered mouse models (GEMM), modification efficiency varies for different types of projects (e.g. KO, CKO, CKI, constitutively active KI). Generally, KI projects require a lot of resources to complete given low KI efficiency, especially for large constructs. Thus, performing a systematic evaluation of various factors affecting CRISPR/Cas9 cutting and donor knock-in efficiency is invaluable for the GEMM workflow. To reduce resource utilization, we used an experimental approach that enabled identification of successfully targeted animals based on observable phenotypes (e.g. coat color). Initially, the correlation between phenotype and genotype were determined to demonstrate that phenotype alone is a reliable indicator of genotype. Here, we report results for the following comparisons: guide RNA format (sgRNA vs. crRNA + tracrRNA), RNP storage (fresh vs. pre-complexed and stored at − 80 °C), and guide RNA concentrations (200 vs. 600 ng/ul). In conclusion, this approach enables rapid optimization of the CRISPR/Cas9 system to improve the GEMM workflow.

Paper 2: 3Rs and ethics

Poster

Will my protein work? A compendium of CRISPR-CAS introduced point mutations in commonly used proteins

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A frequent use of CRISPR-Cas technology is to introduce reporter genes or conditional alleles into an animal model. These tend to utilize long DNA repair templates. As chemical synthesis of DNA is error prone, this has resulted in the generation of mice which carry point mutations in sequences commonly introduced. Mutations can also be introduced due to the inexactness of the various repair mechanisms, or in the case of Lox sites, due to their palindromic nature.

In some cases, no founders with the correct sequence are generated, leaving only mutation bearing founders. However, they may still be useful, if it is known whether the protein or sequence retains its function. We have complied a manually curated set of mutations that have been tested for function, including fluorescent markers and Cre recombinase, as well as Lox sites. We are now opening the collection to the public, and invite others to deposit similar information. All target genes or genomic loci are de-identified, so the confidentiality of the research subject is maintained. This compendium should be a useful resource for the transgenic community and should prevent unnecessary repetition of experiments and animal use.

Paper 3: 3Rs and ethics

Poster

Increase the quality of animal research and improve the 3R with CIRS-LAS: research before the study, report during the study and discuss after the study

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In life science everybody is aware of the indispensability of animal transgenic models to validate new developed drugs or analyze disease progression for possible therapies. At the same time the development of transgenic models as well as animal experiments are time-consuming and cost-intensive and represent a great risk for the animals. Unforeseen events and critical incidents (death of animal, technical problems, and unexpected clinical course) may occur and must be handled transparently to prevent the recurrence of potential errors.

In theory we talk about transparency and constructive discussion at all levels in Laboratory Animal Science: from the animal keepers to the veterinarians and the scientists—all aim for successful experiments with highest possible animal welfare. Everybody working with lab animals is aware of the 3Rs. On the way from theory to practice we need tools to implement the 3Rs, transparency and the exchange about ways to improve animal welfare.

CIRS-LAS (Critical Incident Reporting System in Lab Animal Science) provides a practical platform for the reporting of critical incidents, the exchange about possible improvement measures and the discussion about incidents and errors, their causes and consequences for the animal. On CIRS-LAS.org, anyone can enter a case report on a critical event. For this purpose, keywords for a later database search are requested, as well as information on animal species, background of the experiment or general information, a short description of the incident and a classification in a subject area. If improvement or refinement measures have already been taken, these can also be indicated. Registered users can search the database in advance of a planned experiment, preventing the repetition of failed experiments and thus reducing the number of lab animals.

Recognizing critical events and reflecting on them is an important step toward improving conditions and welfare for laboratory animals. And it needs your active support on CIRS-LAS.org!

Paper 4: 3Rs and ethics

Poster

A case study utilising card IASE and IVF for mouse embryo cryopreservation in a high throughput transgenic facility

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The use of inhibin antiserum and eCG (IASe) to "ultra-superovulate" mice, has already been demonstrated by the CARD lab to increase the number of ovulated oocytes per female by roughly threefold compared with the administration of IAS or eCG alone (Takeo & Nakagata 2015). This development was a major step forward for rodent assisted reproductive technologies, enabling transgenic facilities to vastly improve the efficiency of IVFs and thereby reduce the number of animals needed for embryo generation. However, some facilities have been hesitant to adopt the new technology predominantly due to the significantly higher cost of the IASe hormone, commercially available as "HyperOva". After conducting preliminary trials, two years ago our facility made the switch to producing the majority of our embryos for our workflows via hyperovulation and IVF. Here, by looking specifically at our embryo cryopreservation workflow we are able to present a case study for the substantial benefits of using IASe. Across the freezing of over 300 lines we have seen an

important reduction in animal usage; a roughly 40% reduction in number of females needed to archive a line. Despite the high initial cost of the HyperOva, this has in turn, cut the cost of freezing 200 embryos by roughly 65%. We present our data here comparing both animal usage and the cost of embryo generation for cryopreservation via: superovulation and natural mating; superovulation and IVF; and ultra- superovulation and IVF.

Paper 5: 3Rs and ethics

Poster

Maximizing 3Rs impacts: replacement of surgical vasectomy through the use of wild-type sterile hybrids

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For the preparation of embryo transfer recipients, surgically vasectomized mice are commonly used, generated by procedures associated with pain and discomfort. Sterile transgenic strains provide a non-surgical replacement, but their maintenance requires breeding and genotyping procedures.

We have been previously reported the use of naturally sterile STUSB6F1 hybrids for the production of embryo transfer recipients and found the behaviour of these recipients to be indistinguishable from those generated by vasectomized males. The method provides two substantial 3R impacts: Refinement (when compared with surgical vasectomy) and Reduction in breeding procedures (compared with sterile transgenic lines).

Despite initial promise, the 3Rs impact of this innovation was limited by difficulties in breeding the parental STUS/Fore strain which precluded the wider distribution of the sterile hybrid. STUS/Fore mice showed in-breeding depression and refreshment of the colony was no longer possible from the colony source.

The value of a 3Rs initiative is only as good as the uptake in the community. We thus selected a different naturally sterile hybrid, generated from strains which are widely available: the B6SPRTF1 hybrid between Mus spretus and C57BL/6. We first confirmed its sterility and then trialled the recovery of cryopreserved embryos and germplasm within three UK facilities. Distribution of sperm for the generation of these hybrids by IVF was found to be the most robust distribution method, and avoided the need to maintain a live Mus spretus colony.

We then tested the suitability of B6SPRTF1 sterile hybrids for the generation of embryo transfer recipients at these same three UK facilities and found the hybrids to be entirely suitable when compared with surgical vasectomized mice and a sterile transgenic strain. In conclusion, the potential 3Rs impact of this method was confirmed by the ease of distribution and the utility of sterile B6SPRTF1 hybrids at independent production facilities.

Paper 6: 3Rs and ethics

Poster

Recommendations for standardizing the nomenclature of the diploid genotypes in genetically modified transgenic animals

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The genotype of a genetically modified laboratory animal is essential for breeding and experimental planning. Uniform, generally understandable nomenclature rules are a prerequisite for this. They help to avoid errors and the formation of unwanted offspring. They are also necessary for the exchange of transgenic lines between different scientific working groups. Genotyping data can be interpreted and compared more easily. While there are detailed, standardized rules for creating a name for a genetic modification, this has not yet been done for recording the diploid genotype.

We, therefore, propose a routine-oriented, standardized naming of diploid genotypes of genetically modified animals. The nomenclature recommendations are intended to serve as a compact guide for animal care and scientific staff in laboratory animal facilities and as an aid for recording results in the routine genotyping of animals during breeding. This is especially important if several different genotypes are to be expected in the offspring as a result of the mating of different initial lines. Furthermore, the data exchange with external genotyping service providers will be simplified.

Paper 7: 3Rs and ethics

Poster

Automated genotyping improves mouse colony output

Dr. Kelly VanDenBerg[1], Katherine Oravecz-Wilson[2], Lauren Krolikowski[5], Valerie Hill[5], Dr. Pavan Reddy[2,3], **Dr Zachary Freeman[3,4,5,6]**

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Mice have become increasingly popular as genetic tools, facilitated by the production of advanced genetically

engineered mouse models (GEMMs). GEMMs often require in-house breeding and production by research groups, which can be quite complex depending on the design of the GEMM. Identification of methods to increase the efficiency of breeding practices offers opportunities to optimize and reduce the number of animals bred for research while maintaining similar research output. We investigated the use of commercial automated genotyping and centralized breeding management on overall breeding colony productivity in a colony of multiple GEMM lines. This study involved a three-group study design, where the first group continued their standard breeding practices (group A), the second utilized standard breeding practices but outsourced genotyping in place of inhouse genotyping (group B), and a third group outsourced genotyping and had assistance with routine breeding practices from the laboratory animal care team (group C). Compared to standard practice (group A), groups B and C produced more cages and mice over time, which appeared to be driven primarily by an increase in the number of breeding cages in each colony. Higher numbers of breeders correlated with an increased number of litters and generation of new cages. The increases in colony productivity measures were further enhanced in group C compared to group B. The overall cost associated with producing new animals was lowest in group B, followed by groups A and C. Although, by the end of the study, cost to produce new mice was comparable between all three groups. These data suggest that by optimizing breeding practices and management, fewer animals could be utilized to produce the same amount of progeny and reduce overall animal usage and production.

Paper 8: 3Rs and ethics

Poster

Oral swabs as a non-invasive method for automated genotyping in mice

Dr Katharina Gers-Barlag[1], Maria Walter[2], Yassmine El Gourari[1], Dr Miriam Hopfe[1]

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Introduction: Genetically modified mice that are used in research are usually genotyped using an invasive ear or tail biopsy. In line with following the 3R principle (Replacement, Reduction and Refinement) and European regulations there is growing interest in using non-invasive sampling methods for mice genotyping. After having established the genotyping of oral swabs taken from rabbits four years ago in our laboratory for routine analyses, we have now adapted the sampling method of oral swabs taken from mice into our optimized semi-automated genotyping workflow.

Method and Results: DNA samples are collected by gently swabbing the inside of the oral cavity of mice with a slim headed cotton swab. The samples are then left to dry, stored and shipped to the genotyping laboratory. Here, samples are lysed and DNA is extracted for conventional or real-time PCR. We showed that almost 100% of oral swab samples lead to a clear result and that these were comparable with results obtained from ear punches from the same animal. Working in a 96-well format throughout the whole workflow permits the genotyping of a higher number of samples in short time.

Conclusion: We established a method of genotyping mice by using oral swabs and implementing it into our semi-automated workflow. This is important in terms of the 3Rs as it gives us the opportunity to refine the sampling method used for genotyping. In addition to being a non-invasive alternative to ear punches, e.g. when a second biopsy is needed to confirm the genotype, the risk of cross contamination with oral swabs is lower because no sampling device has to be shared and disinfected between different mice. Furthermore, our semi-automated workflow allows for large scale routine genotyping opportunities, e.g. if ear tags or toe tattoos are used for identification.

Paper 9: 3Rs and ethics

Poster

Production of mutant alleles in mouse macrophage and microglial cell lines by CRISPR/CAS9-mediated genome editing

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CRISPR-Cas9 technology represents a significant improvement of traditional genome editing tools, reaching a new level of targeting, efficiency, and easy usability, thereby relieving many steps needed for generating mouse knockout alleles via traditional mouse ES cell technology. But with the restrictions of animal use regulations and housing costs, reduction or replacement of animal research is in great need, and, therefore, designing fast and inexpensive protocols to produce desired genomic modifications directly in the specific cell lines are becoming highly desirable.

In this study we performed genomic modifications of mouse RAW264.7 and IMG cell lines using synthetic sgRNAs and oligo DNA, as well as targeting vectors. We assessed the efficiency of using this method to generate homozygous knockouts and point mutations. We were successful in producing homozygous knockout mutations. However, we couldn't achieve the correct point mutations when we used sgRNAs and oligo DNAs in either cell lines. When targeting vectors carrying selection markers were electroporated into the cells, only heterozygous mutations were obtained. Functional assays confirmed that the knockout cell lines do not express the target RNA, and can be successfully used in downstream functional applications. These cell models are great tools to validate in vivo results, define cell-special function, and carryout in depth mechanistic studies.

Keywords: mutant cell line, CRISPR/Cas9, sgRNA, RAW 264.7, IMG.

Paper 10: 3Rs and ethics

Poster

goGermline™ 2.0: exclusive generation of male gogermline chimeras can double injection efficiency

Dr Frank Koentgen[1], Ms Maya Koentgen[1], Dr Jonathan Gauntlett[1], Ms Jacqui Watts[1], Ms Olivia Delaney[1], Ms Rebekah Hortin[1], Dr Roger Askew[1]

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While goGermline™ (goGermline 1.0) technology already vastly improves the efficiencies of the 3Rs and the timeline for development of gene targeted mouse models, we have further improved these efficiencies with goGermline 2.0 embryos by enabling selective injection of male goGermline embryos so that no female chimeras are produced. For those not familiar with goGermline 1.0, this system enables generation of chimeras in which sperm, and therefore germline transmission (GLT), can only be derived via the injected male embryonic stem (ES) cells. In this situation, the injected ES cells effectively rescue infertility of male goGermline embryos. To achieve this, goGermline 1.0 embryos are engineered to be spermatogenesis deficient. The result is that all fertile male chimeras are germline heterozygotes and exclusively transmit the ES cell genome. This ensures GLT in the first litters of every project and results in overall improvements in the 3Rs, time to GLT, and time to first experiments. However, goGermline 1.0 injection results in production of both male and female chimeras. Since female chimeras cannot transmit the germline of male ES cells, we reasoned that eliminating production of female chimeras was the best opportunity for improving the efficiency of the goGermline system. With this as our goal, we sought to establish a strategy by which only male embryos are harvested and injected. We engineered goGermline 2.0 to distinguish male from female embryos via a fluorescent reporter tag on the X chromosome. Therefore, if only male embryos are injected, only male chimeras will be generated—essentially doubling the current efficiency of chimera production by embryo injection. We will share the detailed strategy, end results and broader implications of this technology on improvements on the 3Rs, injection time, and vivarium space management.

Paper 11: 3Rs and ethics

Poster

Reducing the number of breeding cages and of experimental animals using ivf by accurate prediction of expected germline transmission rate

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The generation of mutant mouse models for biomedical research still plays a major role in understanding processes in the living organism. Mutations are still first integrated in a targeted manner in murine embryonic stem cells (mES-cells). Highly chimeric males, as estimated by coat color mosaicism, are used for further breeding. However, this is often not the case even with visually strong chimerism, but this only becomes certain after several unsuccessful breedings. This "coat color uncertainty" is contrary to the 3R's (Reduction, Replacement, Refinement) that must be considered in experimental science.

A reliable prediction of the success of IVF is possible via knowledge of the chimerism in the sperm by precisely determining the proportion of mES cells. The analysis of STR markers (short tandem repeats) is a universal method for determining sperm-DNA contributors in mixtures, a technique that has been established in forensics for years.

In 2018, we were able to show that with the use of STR's a genomic differentiation of closely related mouse strains and substrains is possible *1. These markers should be able to be used even for closely related to the mES cells recipient animals, such as NTac-based ES cells and C57BL/6NCrl. We adapted this technique for the analysis of chimeric sperm samples.

Based on the results of the sperm analysis, it was possible to identify among candidates the one animal with the highest percentage of transmissible mES genomes and to reliably predict the number of offspring with the desired mutation after successful IVF using the cryopreserved sperm samples.

The achievable reduction of breeding cages and of laboratory animals through this technique is more than 60% and offers a great contribution in terms of the 3Rs.

Paper 12: 3Rs and ethics

Poster

Refined colony management of complex transgenic mouse models using python

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We have designed and created a transgenic mouse platform purpose-built for the discovery of canine therapeutic antibodies. Our Ky9TM transgenic discovery platform has required 35 precise genome engineering steps to insert over two million base pairs of canine immunoglobulin heavy and light chain DNA into a transgenic mouse.

Breeding multi-allele animals to homozygosity at three or more evolving loci can be complex, expensive, time consuming and produce large numbers of mice with undesirable genotypes. Selection of optimal breeding pairs often involves many considerations in addition to calculating mendelian ratios such as preferential allele zygosity and pre-allocation of animals for experimental purposes, which are difficult to consider by hand. While this is manageable for low numbers of alleles, it quickly becomes challenging as the number of alleles increases. For complex transgenic projects with sequential BAC recombination events, generation of the final platform genotype may take many years. It is therefore necessary for

researchers to use functional intermediary genotypes generated as part of the breeding programme for therapeutic discovery projects. Selection of optimal animals for these purposes is often difficult and must consider factors such as age, multi allele zygosity, antigen target gene Knock Outs, naïve usage of canine immunoglobulin genes and whether key mouse loci are inactivated.

Using a sophisticated mouse colony management database and bespoke programming in Python, we have developed methods to rank animals based on the combination and zygosity of caninised alleles. The tool predicts the best future breeding stock, recommends new and replacement mating pairs, and importantly scores the availability of optimal mice based on gene availability for experimental cohorts.

The bespoke programming enhances our application of the 3Rs by reducing our managed excess through an optimised breeding strategy. The tools also help speed up breeding towards a specific goal and to reduce mouse maintenance costs.

Paper 13: 3Rs and ethics

Poster

Oral D-aspartate treatment of sexually immature and mature B6N mice improves the quality and the fertility of spermatozoa

Mrs Manon Peltier[1], Macello Raspa[2], Renata Paoletti[3], Mohamed Majjouti[1], Michele Protti[4], Laura Mercolini[4], Esther Mahabir[1], Ferdinando Scavizzi[2]

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Sperm cryopreservation is the preferred method for archiving mouse lines and is usually performed when males are sexually mature (13 weeks). We previously reported that in vitro D-Aspartate (D-Asp) treatment increases the quality and the fertility of spermatozoa from 9 to 11-week-old C57BL/6 N (B6N) males. Therefore, we investigated the effect of oral D-Asp treatment of B6N males to determine the mechanisms that lead to improved quality and fertility of spermatozoa. A dose of 20 mM sodium D-Aspartate was supplied in drinking water for 2 or 4 weeks to males of different ages so that they were 9 or 16 weeks old when sacrificed. Control mice were not treated. The IVF rate, birth rate, hormone levels (luteinizing hormone (LH), epitestosterone, testosterone), sperm quality, capacitation rate and acrosome reaction were analysed. With spermatozoa from 9-week-old and 16-week-old males, respectively, the IVF rate was significantly higher after 2 and 4 weeks of D-Asp treatment than in controls (68.8% vs. 44.2% and 47.4% vs. 38.7%, respectively) and 54.7% vs. 29.6% and 61.3% vs. 48.2%, respectively. The birth rates were not affected. D-Asp also increased the LH, epitestosterone, and testosterone levels in testes and serum. A general improvement in sperm quality was observed in D-Asp-treated mice with respect to motility, sperm abnormalities, capacitation rate, and acrosome reaction in both age groups. The results indicate that 2 weeks of oral D-Asp treatment is sufficient to improve IVF rates in both sexually immature and mature male mice, possibly due to higher sperm quality. This approach will lead to a reduction in the number of oocyte donors for the production of embryos for many purposes including genetic engineering. It also offers the possibility to use males younger than 13 weeks, thereby saving time and resources.

Paper 14: 3Rs and ethics

Poster

Equine chorionic gonadotropin bioactivity on mouse superovulation outcomes is not impaired after six months freezing

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Equine chorionic gonadotropin (eCG) is a hormone that induces ovarian follicular stimulation in several species. In combination with the human chorionic gonadotropin (hCG) it produces a 6–eightfold increase in the number of mouse oocytes. Commercially available presentations of eCG consists in lyophilized vials containing large doses of the hormone (e.g. 5000 IU) to be resuspended and immediately used. Usually, 5 to 7.5 IU are administered per female for superovulation procedures, therefore, resuspended stocks are maintained at − 20 °C until use. The objective of this work was to evaluate the bioactivity of eCG stocks maintained at − 20 °C up to six months. For this purpose, three different time points were evaluated. At time 0, an eCG vial (Novormon 5000, Zoetis, Argentina) was resuspended and one aliquot injected into females for superovulation, while the other aliquots were stored at − 20 °C for two and six months. At each time point, an additional eCG vial was resuspended and freshly injected as control fresh groups. All the vials had the same lot number and expiration date. A total of 30 C57BL/6 J females (5 animals/group), 3–4 week-old, were subjected to IP administration of 5 IU of fresh or frozen eCG and 48 h later 5 IU of freshly prepared hCG (Chorulon, Boxmeer, the Netherlands). Immediately, females were mated with C57BL/6 J males. Oocytes/zygotes were collected at 0.5 dpc. Number of recovered oocytes/zygotes was 25.8¬ ± 10.2 versus 40.2 ± 10.8 (fresh vs. fresh), 30.2 ± 8.5 versus 26.2 ± 8.7 (2-month frozen vs. fresh) and 41.2 ± 4.0 vs. 27.4 ± 7.6 (6-month frozen vs. fresh). No significant differences were found (P = NS) within the three experimental groups, nor between the experimental and control groups. Our results suggest that eCG stocks can be maintained at − 20 °C and used at least six months after preparation without affecting superovulation outcomes in mice.

Paper 15: 3Rs and ethics

Poster

Inducing pseudopregnancy in female mice without the need for vasectomized males prior to artificial insemination

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Recipient female mice must be induced to a pseudopregnant state for successful maintenance of pregnancy after artificial insemination (AI) or embryo transfer. Traditionally, female mice are paired overnight with vasectomized males and the presence of a copulation plug is assessed the following morning. To increase the efficiency of producing pseudopregnant females for AI, we have standardized a cervical manipulation (CM) technique. The technique replaces the need for vasectomized males and increases the predictability of producing pseudopregnant females. Vaginal cytology was compared for female CD1 and C57Bl/6 mice after mating or CM. By comparing the type of cells obtained from a daily vaginal swab (leukocyte, epithelial, cornified epithelial), a cytology profile was created (N = 20) correlating to the stage of the estrous cycle. While the percentage of cell types found during a particular stage of estrous was highly variable between females, the trends of cell types observed over the estrous cycle were similar. Cytology profiles were created for each strain using pseudopregnant females that had either mated or had received CM. We have determined that the cytology profile of pseudopregnancy induced by CM is indistinguishable from the profile induced by mating. Both profiles mimic the first 10–12 days of a cytologic pregnancy profile. For CD1 mice, efficiency of pseudopregnancy induction using CM was 83% for females in estrus (N = 76) with this technique but only 38% of females in estrus were plugged by vasectomized males. AI recipients receiving CM (N = 76) had a pregnancy rate of 72% and an average litter size of 8.3 pups. Therefore, induction of pseudopregnancy by CM is an efficient and convenient alternative to mating with a vasectomized male when performing assisted reproductive techniques. Use of CM for assisted reproduction techniques provides 3Rs benefits by both reducing the number of animals needed and eliminating the need for surgically altered males.

Paper 16: 3Rs and ethics

Oral: Session 5: Cancer, Europaea Auditorium, September 18, 2022, 15:30–17:00

Inducible dominant negative ERBB2 rat spermatogonial line for generation of transgenic rat model and dissecting ERBB2 tyrosine kinase mediated pathways

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The ERBB2 receptor tyrosine kinase plays an essential role in early organism development and modulation of cell behavior and is estimated to be overexpressed and/or amplified in 15–20% of aggressive breast cancers. Embryonic or perinatal lethality of ErbB2 null mutants in model organisms does not allow the traditional study of the gene function in vivo. We used Sleeping Beauty transposon-based vector to modify donor-derived rat spermatogonial stem cells that express GFP and inducible ERT2CreERT2 recombinase. The construct was designed to conditionally express the truncated kinase-deficient form of the ERBB2 receptor. Clonally derived spermatogonial cell lines were extensively tested in vitro before the production of transgenic animals. By the virtue of stem cell transplantation, we generated chimeric founders of transgenic animal lines. After mating with WT Sprague Dawley females, the transgenic progeny was obtained.

The designed genetic alteration successfully performed inducible competitive inhibition of the normal function of ERBB2. The isogenic nature of induced and uninduced cells allowed the most accurate comparison of cells affected by the interruption of normal function of the ERBB2 receptor in vitro and in vivo.

In addition to investigating ERBB2 biology, our research exemplifies that spermatogonia mediated transgenesis is an absolute champion in all 3Rs. It reduces the number of animals used for the generation of the desired model from a couple of dozens to a couple of animals. Additionally, direct modification of the germ cell line further reduces the number of animals by skipping one generation of breeding required for verification of germline transmission. Furthermore, modified spermatogonia can be thoroughly functionally analyzed and genetically tested for the absence of off-target modifications in vitro prior to transplantation and animal production. Such refinement advances field of mammalian transgenesis by providing unprecedented predictability not achievable by traditional approaches, eliminating mosaicism, while absolutely removing animal strains compatibility issues.

Paper 17: 3Rs and ethics

Poster

Replacing breeding with FlpO mRNA electroporation to induce Flp mediated recombination

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Replacing breeding with FlpO mRNA to induce Flp mediated recombination.

Originally derived from yeast Saccharomyces cerevisiae, the flippase (Flp) recombinase mediates recombination between two identical flippase recognition target (FRT) sites that flank a genomic region of interest. The Flp-FRT site-specific recombination system has become a common tool used for genome engineering. Scientists have used this system for conditional gene manipulation to generate mouse models for their research. Traditionally, breeding is used to induce Flp mediated recombination. This requires the maintenance of a

Flp deleter mouse colony of each commonly used background and two rounds of breeding to generate the desired recombined allele. To bypass this traditional method of breeding, we performed cytoplasmic injection of FlpO mRNA into zygotes. The average Flp recombination efficiency from the five cytoplasmic injection projects was 86.9%. After the electroporation method was established in the lab, we tested FlpO mRNA electroporation into mouse zygotes produced by in vitro fertilization for allele conversion. The use of electroporation reduces damage to the zygotes and is overall less time-consuming. We performed three rounds of electroporation testing, and we observed an average Flp recombination efficiency of 79.4%, which demonstrates that electroporation can also be used to deliver FlpO mRNA into zygotes effectively. This process change not only shortens the project timeline up to 22 weeks and decreases the number of animals required to generate the converted allele mouse model by 69.9%, but it also supports the 3R principles of reduction and replacement by eliminating the traditional method of breeding and maintenance of Flp deleter mouse colonies.

Paper 18: 3Rs and ethics

Poster

A simple and economic protocol for efficient in vitro fertilization using cryopreserved mouse sperm

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The advent of genome editing tools like CRISPR/Cas has substantially increased the number of genetically engineered mouse models in recent years. In support of refinement and reduction, sperm cryopreservation is advantageous compared to embryo freezing for archiving and distribution of such mouse models. The in vitro fertilization using cryopreserved sperm from the most widely used C57BL/6 strain has become highly efficient in recent years due to several improvements of the procedure. However, purchase of the necessary media for routine application of the current protocol poses a constant burden on budgetary constraints. In-house media preparation, instead, is complex and requires quality control of each batch. Here, we describe a cost-effective and easily adaptable approach for in vitro fertilization using cryopreserved C57BL/6 sperm. This is mainly achieved by modification of an affordable commercial fertilization medium and a step-by-step description of all other necessary reagents. Large-scale comparison of fertilization rates from independent lines of genetically engineered C57BL/6 mice upon cryopreservation and in vitro fertilization with our approach demonstrated equal or significantly superior fertilization rates to current protocols. Our novel SEcuRe (Simple Economical set-up for Rederivation) method provides an affordable, easily adaptable and harmonized protocol for highly efficient rederivation using cryopreserved C57BL/6 sperm for a broad application of colony management in the sense of the 3Rs.

Paper 19: Emerging technologies

Flash Poster: Session 3: Poster Flash Presentations, Europaea Auditorium, September 18, 2022, 13:30–13:50

Fusion transcript screening: repurposing the PolyA trap as a fast and robust screen for gene targeted alleles

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We developed a new molecular screen for identifying gene targeted alleles that overcomes limitations of screening by either Southern blot restriction length polymorphism (RFLP) or loss of allele (LA) analysis. The fusion transcript screen (FTS) is based on the unique presence of a fusion transcript designed to be synthesized by correctly targeted cells and provides the specificity of RFLP by Southern blotting and the speed of LAS by qPCR. FTS was developed to overcome a rare and challenging situation in which neither RFLP nor LA could be used for screening of the Sp140 target gene. Sp140 is one of four gene family members for which the degree and length of genomic sequence homology is so great as to preclude development of qPCR primer/probe sets for LA or hybridization probes RFLP. We considered using transcripts unique to the target gene as a surrogate for genomic modification. Next, we identified, enough unique sequence in the 3'UTR to provide target gene specificity located over 30 kb from the targeted modification. We reasoned that driving synthesis of a predictable fusion transcript, from the selectable marker through the 3'UTR, would provide a unique mRNA that could be identified by RT-qPCR. To achieve this, we built a PolyA trap into the targeting vector using the Neomycin resistance (Neo) selectable marker to initiate synthesis of the fusion transcript. The PolyA trap element is designed to be resistant to nonsense mediated decay to ensure stability and availability of the fusion transcript for screening purposes. Using the FTS in a 96 well format we identified several correctly targeted ES cell clones. FTS provides the speed of RT-qPCR and the high confidence level of the RFLP for identification of essentially any type of targeted modification, and is especially suited for rare cases of very high gene family homology.

Paper 20: Emerging technologies

Flash Poster: Session 3: Poster Flash Presentations, Europaea Auditorium, September 18, 2022, 13:30–13:50

Innovative mouse models by replacement or insertion of large genomic fragments (humanization)

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Gene humanization by replacement or site-specific insertion has long been one of the Holy Grail for scientist. Even if few labs have managed to obtain big insertions through microinjection of CRISPR/cas9, ssODNs and BAC in rat fertilized oocytes (Yoshimi et al. 2016), this method has not been successful in many other labs. Here we show that it is now possible to replace or insert at specific location and very efficiently whole genomic fragment (ex: humanization of a gene) using BAC engineering, CRISPR/Cas9 and embryonic stem cells. The BAC can be previously engineered by addition of LoxP (cKO) or a Knock-in (punctual mutation or addition of a reporter). We have managed to humanize complete genes or genomic regions (up to 200 kb) in C57BL/6 N ES cells with high success rates. Recombination frequencies were higher with the use of 5 kb homology arms but ssODNs (with 100 nts homologies) were also efficient. Two successful projects will be presented with the different quality controls that were performed on the selected ES cell clones (PCR, sequencing and copy counting by droplet digital PCR). Germ line transmission was obtained easily for all the projects (when finalized) with the microinjection of approximately 40 blastocysts. This method avoids the use of important quantity of fertilized oocytes (often associated embryo lethality) and thus allows to reduce the number of animals required to obtain a humanized line (in accordance to the 3Rs rule).

Paper 21: Emerging technologies

Poster

A pipeline for improved prime editing in animal and pluripotent stem cell models

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Prime editing (PE) can install precise mutations such as SNV's and small indels with several advantages over current gene editing approaches: (1) does not introduce double-strand DNA breaks (DSBs), (2) does not require DNA repair templates, (3) does not require S-phase of the cell cycle as PE can be used in post-mitotic cells, and (4) does not require silent/wobble mutations to prevent re-editing of the targeted allele. Genome engineering by prime editing minimally consists of an RNA-guided endonuclease such as SpyCas9 nickase tethered to an optimized reverse transcriptase and a specialized guide RNA (pegRNA) containing the target spacer sequence, a primer binding site and the reverse transcription template encoding the desired mutation. Despite several clear advantages of installing genome modifications by prime editing, implementation can be cumbersome as the technique is prone to wide variation in outcomes. For example, PE outcomes may vary considerably in efficiencies depending on installing different types of edits (transitions/transversions/indels), genomic and epigenetic contexts at the target site, and the biomedical animal, organoid, or stem cell model. Introduction of 'passenger' mutations at the target locus is also known to be a frequent problem. Consequently, PE often requires several iterative cycles of optimization before achieving success. To develop a robust pipeline for improved prime editing in animal and pluripotent stem cell (PSC) models, we test a range of technical parameters including delivery formats of prime editing components such as double-stranded DNA plasmids or linear covalently closed DNA vectors that we refer to as DNA ministrings and compare delivery to PE ribonucleo-protein (RNP) formats. For human or mouse PSC models, we optimize PE component delivery by exploring both voltage and pulse duration using a square-wave electroporator to provide transfection conditions that do not require proprietary systems or trade-secret buffers. In human PSCs, we introduce single point mutations at multiple safe harbor sites including AAVS1, ROSA26 and the novel human genomic safe harbor site GSH1 (Aznauryan et al. 2021) to create isogenic normal and mutant human stem cells lines for disease-in-a-dish modeling. We explore the hypothesis that co-selection enrichment and antagonizing DNA mismatch repair (MMR) can improve on-target prime editing outcomes. In summary, we outline strategies for improving on-target DNA-writing by prime editing to endeavor digital genome engineering.

Paper 22: Emerging technologies

Flash Poster: Session 3: Poster Flash Presentations, Europaea Auditorium, September 18, 2022, 13:30–13:50

Establishing a robust and efficient workflow for the generation of complex knock-in alleles, via delivery of AAV donor reagents and CRISPR electroporation

Graham Duddy, Sunita Varsani-Brown, Juliette Horwood, Katherine Courtis, Tina Hodgson, Jessica Olsen, Dr Ben Davies[1], Katharine Mankelow, Ian Rosewell.

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The introduction and mass uptake of CRISPR/Cas9-based genome modification in laboratories worldwide has revolutionised the way in which genetically engineered mouse models are generated. Recent developments have led to a simplification of reagent delivery pipelines, with electroporation of mouse embryos providing an easy alternative to the technically demanding and more time-consuming pronuclear microinjection for the production of simple knock-outs and

small targeted modifications such as SNPs. Interestingly, increased mutagenesis frequencies and improved embryo survival rates have been reported for electroporation, perhaps because the physical damage that occurs when using microinjection techniques is avoided. Electroporation as a gene delivery method also requires considerably less staff training than microinjection.

For more complex targeted integration events, such as reporter gene insertions and conditional alleles, the use of long ssDNA templates has been of particular importance and has been successfully adopted in many labs, including our own. This technique, however, is still reliant on microinjection and the generation and quality control of long ssDNA templates can be challenging. As an alternative we have been exploring the delivery of templates packaged as AAVs, in combination with electroporation of CRISPR reagents, with payloads of between 1.7–4.4 kb for the generation of complex knock-in and conditional alleles. Adopting the CRISPR-READI protocol (CRISPR RNP electroporation and AAV donor infection; Chen et al. 2019), we present our recent production data using this approach at multiple independent gene loci. The data highlight the reliability and simplicity of this method, both in terms of knock-in efficiencies, embryo survival post-delivery and production parameters.

The data suggest that the combination of AAV template delivery with CRISPR/Cas9 electroporation provides a robust alternative to pronuclear microinjection-based methodologies, streamlining the production of complex allele generation and ultimately reducing the animal cost of mouse line development.

Paper 23: Emerging technologies

Oral: Session 9: Neuroscience, Europaea Auditorium, September 19, 2022, 14:00–15:30

Mouse development after embryo culture in HTF medium

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Culture of preimplantation embryos in vitro is an important method for human and mouse reproductive technology. The M16 and, KSOM are routinely used for the culture of mouse embryos. However, in the last several years (related to Covid-19 and others causes) labs have had delays in delivery of media from vendors.

To avoid these problems, we tried to use a medium that was formulated based on the composition of human fallopian tube fluid (HTF) (Quinn et al. 1985) for the culture of mouse embryos. HTF and its modifications were developed for IVF and growth in vitro of human embryos. The mouse embryos serve as an auxiliary tool to test the quality of HTF media for the growth of preimplantation human embryos.

To our knowledge, mouse embryos cultured in HTF were never transferred to fosters to produce litters. To test post-implantation development of C57BL6/NJ mouse embryos after culture we prepared modified HTF (Guan et al. 2014). The embryos were isolated at 0.5 or 1.5 days p.c., and cultured up to blastocyst stage (3.5 days) in HTF, KSOM and M16 media. Embryo growth showed that the percentage of embryos reaching blastocyst stage was similar in all media, however, the embryos in HTF medium reached blastocyst stage 5–6 h later when compared with development in KSOM and M16 medium. The CD-1 fosters after embryo transfer into the uterus demonstrated normal pregnancy and delivery of pups. Newborn mice originating from embryo culture in HTF look normal and have the same body weight on weeks 1, 2, and 3 as those that originated from embryo cultures in M16 or KSOM. Thus, HTF can be used as an alternative medium for mouse embryos culture and for following up with embryo transfer, pregnancy and birth of healthy litters.

Paper 24: Emerging technologies

Flash Poster: Session 3: Poster Flash Presentations, Europaea Auditorium, September 18, 2022, 13:30–13:50

Recovery of live copenhagen rats after 25 years in cryostorage

Miss Kathy Krentz[1], Dr. James Amos-Landgraf[2], Dr. Elizabeth C. Bryda[3], Dr. Melinda R. Dwinell[4]

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Recent genomic accessibility of the rat now permits genetic and molecular approaches to utilize rats for biomedical studies. CRISPR/Cas9 technology has revolutionized rat biology by allowing rapid, reliable, and cost-effective generation of genome-edited rat models. Downstream of initial studies, cryopreservation of rat embryos or sperm is necessary to protect against catastrophic loss or genetic drift as well as decrease animal husbandry costs. The Copenhagen (COP) rat is one of the parental strains in the August by Copenhagen (ACI) rat strain, with ACI being one of the original parental strains in the original NIH heterogenous stock cross. COP is unique in that while ACI is susceptible to various cancers, included estrogen induced mammary cancer, COP are resistant making them a valuable genetic control to resolve complex genetic interactions. Additionally, this strain was included in the Hybrid Rat Diversity Panel because of the genotypic and phenotypic diversity.

University of Wisconsin Animal Models core (UW-AM) in cooperation with Rat Resource and Research Center (RRRC) at the University of Missouri has recovered the COP strain after 25 years of cryopreserved storage. This strain is not available in the US and is currently globally unavailable commercially. Cryopreserved morula were frozen via slow-freezing methods in 1997 at the University of Wisconsin. We were able to use our FreezerworksTM database to easily locate these samples. The RRRC acquired the Wisconsin frozen embryos then

thawed, cultured, transferred and recovered live COP pups in 2022. Simultaneously, the UW-AM thawed, cultured and transferred embryos and recovered live COP pups. Surviving rats were transferred to RRRC for breeding. We believe this is the longest cryopreservation storage recovery of any rat model and this successful recovery supports the value of high-quality.

cryopreservation methods and storage inventory for future needs. We are currently expanding this valuable strain. We plan to have it commercially available through RRRC this fall and are eager to offer it to the research community.

Paper 25: Emerging technologies

Poster

Using a short conditional intron to generate a CDH12 conditional knockout

Ms Margot Linssen[1], PhD Szu-Hsien Sam Wu[2,3], B.Sc. Conny M. Brouwers[1], B.Sc. Jill W. C. Claassens[1], Md, PhD Jaap Mulder[4,5], Prof Bon-Kyoung Koo[2,6], PhD Peter Hohenstein[1]

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In the past, we successfully generated conditional knockout models through the targeting of ES cells. In order to minimize time and animal numbers we attempted direct embryo manipulation for these models using long ssDNA targeting or introduction of loxP sites via electroporation by insertion of one LoxP oligo at a time in two subsequent generations. However, neither gave the required efficiency.

As a new alternative, we are using a Short Conditional artificial intrON (SCON) to generate a conditional mouse model in one step. A SCON cassette, including a splice donor, splice acceptor, branchpoint and 2 × lox2271 sites was designed to be introduced into Exon 2 of the Cdh12 gene. Mouse zygotes were electroporated with RNP complexes along with a 300 bp donor template, including 55 bp homology arms. Within 5 months, with only 1 small electroporation session the first germline transmission animals were available.

So far, animals homozygous for the SCON insertion were found to be viable. Heterozygous animals were bred to a germline Cre-deleter and confirmed successful recombination of the lox2271 sites. Breeding to generate homozygous animals and test mRNA/protein levels before and after Cre-mediated recombination compared to control animals are ongoing.

In conclusion, an artificial intron using the SCON system in comparison to 2 intronic loxP sites promises to be an easy and fast strategy to generate conditional mouse models.

Paper 26: Emerging technologies

Poster

Broadening the scope of long-read sequencing as a tool for animal model validation

Mr Matthew Mackenzie[1], Dr Skevoulla Christou-Smith[1], Mr Jorik Loeffler[1], Mr Krystian Nowicki[1], Mr Edward ONeill[1], Dr Sara Wells[1], Dr Lydia Teboul[1]

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The mouse is the leading mammalian model for understanding human disease and human health, due to the similarities between the species. The CRISPR/Cas9 revolution has enabled more efficient and precise generation of mouse models with reduced cost and timeframes, unlocking the possibilities of producing increasingly complex alleles. We have already implemented long-read sequencing as a tool for the early identification of G0 animals carrying the correct alleles, reducing the instances of breeding false-positive founders (McCabe et al., BioRxiv, 838193). Here, we demonstrate the broadening use of long-read sequencing as a tool for the validation of models. Long-read sequencing has traditionally relied on PCR amplification to provide enrichment of the region of interest. Not all regions are amenable to PCR, particularly those that have been modified with a large/complex construct. In these cases CRISPR/Cas9 can provide amplification-free targeted enrichment of native DNA, allowing the sequencing of the region in a single pass. We have also utilised long-read sequencing in the validation of classical integration transgenic models, identifying and confirming the sites of integration. The random nature of integration associated with transgenesis can result in disruption to coding genes or essential elements, potentially resulting in undesirable phenotypes for downstream experiments. Therefore, thorough characterisation of transgenic animals is required to ensure suitable lines are taken forward. We will present our recent developments of implementing these additional processes within our pipeline.

Paper 27: Emerging technologies

Poster

Development of both mouse and rat preimplantation embryos in a single-medium culture system

Dr Hongsheng Men[1], Dr James Amos-Landgraf[1], Dr Elizabeth Bryda[1], Dr Craig Franklin[1]

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While traditionally, different media have been used for mouse and rat embryo culture, a single medium that could support

both mouse and rat embryo development would simplify operations and improve efficiencies for facilities that perform model creation and/or embryo cryorecovery for both species. In these experiments, we investigated the suitability of a new KSOM for rat embryo culture medium (KSOMr) with additional modifications as a medium for mouse embryo culture. We also investigated the effect of shelf life (up to 6 months) of antibiotic-free KSOM (mKSOM) and antibiotic-free KSOMr (mKOSMr) stored at 4 oC on the development of C57BL/6NJ mouse and Sprague Dawley (SD) rat zygotes respectively. For C57BL/6NJ zygotes cultured in mKSOM, mKSOMr and KSOM, the results showed that there were no significant differences in cleavage rates, blastocyst rates or hatching rates among the three groups. Using mKOSM and mKSOMr media stored for 6 months at 4 °C, the development of mouse C57BL/6NJ zygotes was comparable to the rates seen with control in terms of cleavage, blastocyst formation and hatching. For SD zygotes cultured in mKSOMr using 1- and 6-month-old media, there was no significant difference in cleavage rates, blastocyst rates or hatching rates. C57BL/6NJ blastocysts from mKSOMr (6th month at 4 °C) and KSOM (made monthly) culture were able to develop into live pups after surgical embryo transfers. These pups had no gross abnormalities in morphology and growth. The data demonstrated that mKSOMr is able to support both mouse and rat embryo development in vitro. Additionally, mKSOMr and mKSOM can be stored at 4 °C for at least 6 months without significantly compromising their quality.

Paper 28: Emerging technologies

Poster

Use of CRISPR/Cas9 to Knock-in Next Generation Tags into Zygotes Endogenously

Dr Asif Nakhuda[1], Caroline Wilson[1], Ania Sobczak[1], Bill Mansfield[1], Debbie Drage[1], Katarzyna Wojdyla[2], Hayley Sharpe[2], Teresa Rayon[3], Peter Rugg-Gunn[3]

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CRISPR/Cas9 enables endogenous gene tagging by inducing double-stranded breaks in the genome with presence of a single-stranded DNA (ssDNA, (<200bp)). However, large knock-ins can be challenging, due to lower rates of homology-directed repair (HDR). Targeting embryonic stem (ES) cells can be used to make such models but require extensive cell culture breeding. We created next generation tagged mouse models; Oct4-HaloTag and Ptprc-MiniTurbo using long ssDNA (lssDNA). From F0 pups born, approximately 15% were positive for the whole Knock-in. Positive mice were further bred and tags showed functionality, indicating no errors in protein expression. Our protocol allows for newer experimental tags to be integrated such as Halo- and Miniturbo-tags. Further, we show PCV-Cas9 can be used to enhance HDR efficiency in embryos via electroporation. In conclusion, our protocol allows a new generation of tagged mouse models to be made rapidly.

Paper 29: Emerging technologies

Poster

Versatile applications of adeno-associated virus-based vectors in a transgenic facility

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Adeno-associated viruses (AAVs) are small, non-enveloped, icosahedral viruses with an ssDNA genome of ~ 5 kb, belonging to the genus Dependoparvovirus. AAVs are characterized by their relative safety, long-term expression, and persistence in the nucleus in a form of episome. AAV is a replication-incompetent virus and is dependent on co-infection by another virus, often adenovirus or herpes simplex virus, which supplies AAV with essential replication proteins. In order to decrease risks, recombinant AAVs used in research are produced in Helper-virus free mode where essential replication genes are supplied in trans via a plasmid. When a recombinant AAV (rAAV) particle enters the cell, the viral genome is processed into a double-stranded circular episome. These episomes can form concatemer and remain in the nucleus as a large molecule structure. This event allows for either long-term expression (years) of the AAV genetic information or integration in non-homologous sites with frequency > 0.1%. Due to long-term expression with low integration frequency, rAAV is widely used for gene therapies. In the context of transgenic or disease modeling, AAVs can be used in vivo for pre-clinical studies, model validation, humanization, or generation of CDX models. In addition, specific AAV serotypes can serve as ssDNA HDR donor vectors for zygote transgenesis in a method known as CRISPR-READI. AAV particles are able to penetrate zona pellucida, cellular membrane, and reach the pronucleus without significant negative impact on the viability of the embryo. Subsequent electroporation of Cas9/gRNA RNPs allows for the introduction of DSB and HDR by the ssDNA template. Our data summarize the versatility of rAAV vectors spanning from transgenesis, transient humanization, or transgenic model validation. Furthermore, we introduce a new approach to the production and application of rAAV-based particles, consisting of extracellular vesicles associated with AAVs (EV-AAVs).

Paper 30: Emerging technologies

Poster

CRL4-mediated ubiquitination regulates neural crest cells migration

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Cullin-4 (CUL4a/CUL4b) is a scaffolding protein of CRL4 complex, an E3 ubiquitin ligase involved in ubiquitination and mostly subsequent degradation of target proteins regulating various biological processes, such as genome stability control, cell cycle regulation or development orchestration. CRL4 complex achieves high functional flexibility by interaction of the adaptor protein DDB1 with various substrate receptors (DCAFs) in order to assemble into specific temporal-spatially regulated protein degradation complex. The understanding of how the combinatorial potential of CRL4 complex assembly results in substrate specificity is essential for revealing the molecular function of Cul4 under physiological and pathological conditions.

We focus on the function of CRL4 complex during mammalian craniofacial morphogenesis. Accurate migration of neural crest cells (NCCs) is critical for precise development of forming of cranial bones, cartilages, connective tissues and muscles of the head, as well as nerves. We confirmed the colocalization of Cul4 expression with NCCs using LacZ staining. We crossed the Wnt1Cre2 driver and Ddb1 cKO mouse lineages to assess the role of CRL4 in head mesenchyme originating from the NCCs. We observed that DDB1 loss in neural crest originated cells causes severe malformation of embryonic orofacial structures with particular dysmorphology of facial processes. Mass spectrometry analysis of pulled-down proteins interacting with DDB1 suggests involvement of DNA damage machinery and CRL4-mediated ubiquitination in regulation of NCC migration.

To capture the truly interacting proteins at the physiological condition, in vivo approach to determine protein–protein interactions in live cells is thus the only way. What we would like to accomplish in our work is the preparation of a system based in BioID2, a smaller biotin ligase from Aquifex aeolicus, that would allow to study protein complexes directly on a mouse model generated by a knock-in of this tag. Combination of AAV technology and CRISPR/Cas9 mediated homologous recombination in zygotes, CRISPR-READi, is used to create these strains.

Paper 31: Emerging technologies

Poster

Introducing OZBIG: a robust process for generating 20–240 kb targeted replacement mouse models

Mrs Maarit Patrick[1], Dr Katherine Brechun[2], Dr Harald Kranz[2], Dr Martina Reiss[2], Dr Charles Keller[3], Ms Mattie Clark[3], Dr Jonathan Gauntlett[1], Dr Roger Askew[1]

[1]Ozgene Pty Ltd, Perth, Australia, [2]Gen-H Genetic Engineering Heidelberg GmbH, Heidelberg, Germany, [3]Children's Cancer Therapy Development Institute, Beaverton, USA

Demand for large, genomic humanized mouse models inspired our development of a system, called OzBIG, for robust creation of large, gene-targeted replacement models. This system enables our routine generation of mouse models with very large (20 to 240 kb) genomic replacements. The process for developing OzBIG models is the result of a collaborative effort combining the technological strengths of Ozgene and Gen-H. Large, BAC-based targeting vectors engineered by Gen-H are used to efficiently generate targeted ES cells using conventional gene targeting coupled with a screening platform developed at Ozgene. OzBIG targeted ES cells are then used to develop germline heterozygous chimeras via goGermline^{TM} embryo injection. The targeting method is independent of exogenous nucleases, like CRISPR/Cas9, thereby eliminating the need to analyze for off-target effects. The ability to routinely create large modifications using OzBIG vastly expands the gene set accessible to genomic humanization. Traditional gene targeting using plasmids to deliver the integrative payload has a technical upper size limit of approximately 20 kb, which limits single vector genomic humanization to approximately 10% of all mouse genes. BAC-based OzBIG targeting vectors, while technically more difficult to assemble, can carry up to 240 kb or more of integrative payload. The payload improvement achieved by using our BAC-based targeting vectors increases the gene set amenable to humanization to approximately 90% of all mouse genes. In addition to these large genomic humanizations, we are using OzBIG to build large, non-humanized transgenics, such as complex reporters and synthetic expression systems. As an example, we present development of a conditional Pax7 allele for Cre-inducible expression of the pathognomonic Pax7-Foxo1 chimeric oncogene and the expression marker eYFP. The Pax7-Foxo1 fusion protein drives the childhood muscle cancer alveolar rhabdomyosarcoma and this model will be used to explore the biology of several key aspects of this sarcoma.

Paper 32: Emerging technologies

Poster

Tracking unique adipocyte subpopulations using split-gene reporter systems in transgenic mice

Dr Pawel Pelczar[1], Aline Baur, Sunwoo Chun, Heide Oller, Julia Rositzka, Christian Wolfrum

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Recent studies revealed adipose tissue to be far more diverse in terms of cell types and their respective origins and functions (Sun, et al. 2020). Labelling of distinct cell populations frequently requires the usage of more than one marker that precisely define the given cell type. Building on our split-Cre system (Hermann, et al. 2014), we have developed an intein-mediated split-EGFP system that allows precise in vivo labeling of cell that simultaneously express two complementary EGFP fragments form two different promoters. We have validated the system both in cell culture and transgenic knock-in mice. As a proof of concept we use the system to selectively label a thermogenic subpopulation of beige adipocytes co-expressing the Cyp1e2 and Adiponectin markers.

Paper 33: Emerging technologies

Oral: Session 11: Emerging Technologies, Europaea Auditorium, September 20, 2022, 11:00–12:00

One-step generation of a conditional allele in mice using a short artificial intron

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Despite tremendous advances in genome editing technologies, generation of conditional alleles in mice has remained challenging. Recent studies in cells have successfully made use of artificial introns to engineer conditional alleles. The approach consists of inserting a short artificial intron containing two loxP sites within a coding exon of a gene using CRISPR-Cas9 technology. Under normal conditions, the artificial intron is removed by the spicing machinery, allowing for expression of the gene product. Following Cre-mediated recombination of the two loxP sites, the intron is disabled and splicing can no longer occur. This results in a frame shift, generation of a premature stop codon, and degradation of the mRNA transcript via the nonsense-mediated mRNA decay pathway. Here we describe the application of this technology to engineer conditional alleles in mice using Scyl1 as a model gene. Mice homozygous for the insertion expressed SCYL1 at levels comparable to wild-type mice. Deletion of the cassette via Cre-mediated recombination in vivo inactivated the allele and abrogated SCYL1 protein expression. Our results broaden the applicability of this approach to engineering conditional alleles in mice. The universality of this approach is currently under investigation.

Paper 34: Emerging technologies

Poster

Advanced technologies in correct target insertion screening to increase speed and efficiency for mouse model creation

Mrs Anna Pham[1], Mr Charles Yu[1], Mrs. Lucinda Tam[1], Mr. Roger Caothien[1], Mrs. Merone Roose-Girma[1], Mrs. Tuija Alcantar[1], Mr. Juan Reyes Jr[1], Ms. Natasha Bacarro[1], Dr. Marques Jackson[1], Mr. Diego Diaz[1], Ms. Adriana Castillo[1]

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The Genetically Engineered Mouse (GEM) Lab at Genentech generates many custom mouse models. These models may involve complex and multi-allelic mutations, constitutive or conditional knock-outs, humanized or point mutation knock-ins, and random transgenesis. The CRISPR/Cas9 system enables fast and efficient generation of many knock-out and knock-in mouse models and has become our strategy of choice for model creation while a traditional ES cell strategy remains an important tool for more complex alleles. Regardless of strategy used, technology development and automation play key roles in streamlining our workflows and improving our overall efficiency to deliver models to our scientists faster without losing data integrity. To ensure correct targeting of our custom mouse models at Genentech, the GEM lab uses BioRad's droplet digital PCR and Qiagen's QIAcuity multiplex digital PCR to ascertain copy number to exclude the possibility of random insertion next to the targeted insertion. We present comparative data from both platforms to assess quality and validate these preliminary results with Oxford Nanopore Technology's long-read third generation sequencing platform. These platforms collectively enable the GEM lab to increase screening throughput, reduce timelines, improve ergonomics in the workplace while enhancing the reproducibility of genetic screening results, yielding a fully characterized mouse model up to ten weeks sooner than traditional methods.

Paper 35: Emerging technologies

Poster

Recent progress of CARD reproductive technology

Professor Toru Takeo[1], Dr Satohiro Nakao[1], Mr. Katsuma Yamaga[1], Mr Ryusei Maeda[1], Mr Ryo Kubota[1], Miss Kotono Ito[1], Dr Nobuyuki Mikoda[1], Professor Naomi Nakagata[1]

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Reproductive technology is useful to produce, archive, and transport genetically modified animals. Until now, we have developed the system of mouse repository using reproductive technologies. Recently, genome editing technology and COVID-19 pandemic made a big impact in transgenic and animal core facilities. Genome editing technology promotes to produce genetically engineered rats as new human disease models. Therefore, we developed a technique of rat sperm cryopreservation and in vitro fertilization and launched CARD Rat Bank using the techniques. Under the COVID-19 pandemic, we had to change the way of meeting event, shipment of samples, and priority of technology. CARD Mouse Reproductive Technology Workshops have been held worldwide since 2000. Last year, we developed online system of CARD Mouse Reproductive Technology and had 3 online workshops in Japan, Thailand and Sri Lanka. About the shipment of samples under the COVID-19 pandemic, it was difficult to transport live animals from foreign countries. So, we overcome the problem to receive mouse strains from USA by transporting cold-stored mouse sperm. In addition, we examined the fertility of frozen-thawed sperm which was produced by simple cryopreservation at -80 oC and a new protocol to produce two-cell embryos in vivo using female mice with ultrasuperovulation using inhibin antiserum and gonadotropins. As part of outreach, we made animations about the CARD mouse bank and rat reproduction technology. In this presentation, we will introduce the recent progress of CARD reproductive technology to survive the post COVID-19 era.

Paper 36: Emerging technologies

Poster

Improved methods for large hdr knock-ins using ALT-R™ HDR donor blocks and ALT-R HDR enhancer V2

Mrs Jessica Woodley[1], Bernice Thommandru[1], Ravi Nath[2], Anne Brunet[2], Laura Krueger[3], Ann Morris[3], Sebastien Gingras[4], Mollie Schubert[1], Karthik Murugan[1], Gavin Kurgan[1], Matthew McNeill[1], Ashley Jacobi[1], Garrett Rettig[1]

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CRISPR-based homology-directed repair (HDR) is an invaluable tool to facilitate specific mutations in a genomic region of interest in research studies. While many methods have been reported for improving HDR efficiency, achieving precise changes via HDR remains a challenge particularly for large knock-ins. HDR repair outcomes are most efficient with single-stranded DNA (ssDNA) templates when small insertions, deletions, or SNP changes are desired edits. For these applications, synthetic oligonucleotides (ssODN) have been studied and optimized with modifications for enhanced efficacy in HDR. Larger insertions can be generated via HDR using enzymatically generated ssDNA or double-stranded DNA (dsDNA) donor templates. Here, we present research demonstrating that improved HDR efficiency for large insertions can be obtained when dsDNA donor templates include novel end-modifications. These modifications have been shown to improve the frequency of HDR and reduce homology-independent (blunt) insertion events that can occur at both on- and off-target CRISPR edits relative to unmodified dsDNA. In our experiments, the use of Alt-R modified dsDNA improved the ratio of HDR:Blunt repair events 4.3-fold on average relative to unmodified dsDNA templates for short inserts and reduced blunt insertion of large templates 4.6-fold at a mock off-target site. Experimental results demonstrated further improvement to HDR rates when using Alt-R HDR Enhancer V2, a small molecule that increases the rate of HDR in varied cell types including iPSCs and primary human T-cells. Together the use of Alt-R modified repair templates and the Alt-R HDR Enhancer V2 showed improved HDR rates up to 5- to tenfold across knock-in experiments. Finally, we present investigations into design considerations for large dsDNA HDR templates including homology arm length and the use of Alt-R HDR Blocks in microinjection systems.

Paper 37: Emerging technologies

Oral: Session 11: Emerging Technologies, Europaea Auditorium, September 20, 2022, 11:00–12:00

A novel system for mouse sperm cryopreservation using multilayer insulation and immersion in liquid nitrogen for cooling rate control, using 20 mm microstraw and batch labeling technology to increase storage capacity and labeling efficiency

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Cooling rate and cryoprotectant are two critical factors required for success of animal sperm cryopreservation. Currently, animal sperm cryopreservation is performed in two primary ways. Samples are cryopreserved in a simple container (such as a foam box or liquid nitrogen tank), in which the cooling rate is poorly controlled. Samples are also cryopreserved in a program-controlled freezer, requiring an expensive device. After cooling, the containers need to be packaged inside liquid nitrogen for long term storage. This is dangerous and burdensome, especially when preserving thousands of samples. For mouse, 10 µl sample is not necessary to use 133 mm, 250 µl or 500 µl plastic straws or 2 ml cryotubes and additional medium to prevent floating. The current labeling method need expensive device,need wrap around one by one or hard to recognize handwriting label.

A novel method has been developed (Patent No. US 10,660,327 B2) to overcome the drawbacks of current methods. Sperm is packaged, insulated and sealed in multilayer rigid and/or flexible containers before freezing, and directly immersed into liquid nitrogen to freeze at a controlled ideal cooling rate. The cooling rate is solely controlled by the thermal conductivity of the walls of the containers, the layer(s) of the walls and the quantity of the containers at each layer.

A novel packaging container, MicroStraw, 20 mm in length has been developed to hold 10 µl mouse sperm sample which increase the storage capacity to 7- to 28- fold. For batch labeling, using Microsoft Excel to edit the text and using common office printer to print on a liquid nitrogen resistant label. 22 MicroStraws on a strip can be labelled at same time.

Paper 38: From bench to bedside

Poster

Inactivation of Emilin-1 enhances cardiac fibrosis through TGF-β and IL-1β pathways

Professor Paola Braghetta[1], Dr Nicola Facchinello[1], Dr Giulia Pigato[1], Dr Dario Bizzotto[1], Dr Aurora De Acutis[2], Dr Matilde Cescon[1], Professor Paola Brun[1], Professor Giovanni Vozzi[2]

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Heart failure, that is a major public health issue with a prevalence of over 23 million worldwide, is predominantly linked to cardiac fibrosis, an excessive deposition of extracellular matrix (ECM) proteins in the myocardium. Despite the pivotal role of fibrosis in cardiovascular diseases, the current knowledge of cardiac cellular components and their crosstalk is still modest. As Emilin-1 (E1) is deposited in the heart since early stages of development we investigated the effects of E1 depletion in the adult cardiac tissue. Based on immunofluorescence, biochemical and biomechanical analyses, Emilin-1

knockout mice display increased Collagen type I deposition and decreased Connexin-43 staining, along with increased stiffness in both ventricles, resulting in a more severe phenotype in 9-month-old mice. Aiming at investigating inflammatory contribution we performed cytofluorimetric analyses on whole hearts at 3 and 9 months of age and highlighted a significant age-dependent reduction of macrophages and T cell positive cells in E1 deficient mice. Considering that E1 knockout mice display systemic hypertension linked to enhanced bioavailability of mature TGF-β, reduction in regulatory T-cells suggests interplay between tissue remodeling and profibrotic TGF-β. Furthermore, a decreased expression of IL-4 and increased expression of IL-1β in the heart of E1 knockout mice suggests an inflammatory phenotype linked with tissue remodeling. Moreover, we characterized the fibro/adipogenic progenitor cells (cFAPs) isolated by flow cytometry using specific markers. E1 knockout FAPs cultured in vitro spontaneously differentiate more promptly into fibroblasts as compared to wild type derived ones, as demonstrated by the increase in specific fibrosis marker (Tgf-β, Col1, Col3) by qRT-PCR, suggesting a possible role in the displayed cardiac fibrosis. Finally, we analyzed the effect of a pharmacological inhibition of IL-1β by applying dapansutrile (OLT1177) in cFAPs. Our findings revealed that E1 knockout cFAPs treated with OLT1177 display a decreased expression of the specific fibrosis markers highlighting a strong correlation of inflammatory cells to cardiac fibrosis in the absence of E1. By merging different approaches our results will provide better insight into mechanisms contributing to cardiac fibrosis associated with hypertension and inflammation offering hints for new possible therapies and for the improvement of early diagnosis and prevention of diverse cardiac diseases.

Paper 39: From bench to bedside

Oral: Session 1: Animal Models: From Bench 1756 to Bedside, Europaea Auditorium, September 18, 2022, 1757 09:00 -10:30

Genome editing in an era of precision medicine: generation of personalized human-variant animal models

Dr Daniel Davis[1], Dr Monique Lorson[1], Dr Lane Clarke[1], Ms Rowena Woode[1], Dr Bing Zhang[1], Dr Christian Lorson[1], Dr Elizabeth Bryda[1]

[1]University of Missouri, Columbia, United States

Genome editing in animal models allows scientists to study how genes function by helping them to better understand human diseases caused by specific DNA mutations. Our lab has established an efficient pipeline to create personalized animal models containing human variant alleles to recapitulate specific human diseases. This pipeline includes zygote electroporation of CRISPR/Cas reagents along with single-stranded DNA templates containing the desired human variant allele. Recently, we generated an array of personalized mouse lines modeling various human diseases:

- Spinal Muscular Atrophy with Respiratory Distress Type-1 (SMARD1). These models were generated by introducing specific human variant alleles to mimic diseases linked to 6 different patient-derived single nucleotide polymorphisms (SNPs). These models represent the first SMARD1 mouse strains with an associated respiratory phenotype more closely recapitulating the human SMARD1 disease than past models.
- Cystic Fibrosis (CF). This mouse strain has the complete human CFTR 1213delT exon 8 sequence in place of the corresponding mouse exon. This enables the design of gene therapy strategies that specifically target pertinent human DNA sequences in the context of an animal model. In addition to the mutated human exon, this model includes a mVenus-akaluciferase reporter designed to express only upon correction of the human 1213delT mutation.
- KCNT1-associated epilepsy. This mouse strain was engineered to contain an orthologous missense mutation modeling the human KCNT1 G288S variant. This mutation is linked to both inherited and sporadic cases of a spectrum of epilepsy disorders including autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), malignant migrating focal seizures of infancy (MMFSI) and Leukoencephalopathy.

In conjunction with generating the models listed here, we have created several other personalized mouse and rat models with human variant alleles using this same pipeline. Generation of these types of personalized animal models will further facilitate the power of personalized medicine in the future.

Paper 40: From bench to bedside

Poster

CRISPR-based mouse models made with patient-specific mutations to investigate rare diseases

Dr Almudena Fernández[1,2], Ms. Marta Cantero[1,2], Ms. Ana Guardia[1,2], Dr. Andrea Montero[1,2], Ms. Gema Garrido[1,2], Ms. María Jesús Del Hierro[1], Ms. Julia Fernández[1,2], Dr Lluis Montoliu[1,2]

[1]CNB-CSIC, Madrid, Spain, [2]CIBERER-ISCIII, Madrid, Spain

Albinism is described as a rare disease known for the associated hypopigmentation phenotype. However, the most relevant alterations in albinism are associated with the visual system. Foveal hypoplasia and abnormal chiasmatic connections are the anatomical features characteristic of albinism (Kuht et al. 2022; Bakker et al. 2022). Albinism affects between 1:10,000–20,000 newborns and it is genetically heterogeneous, with up to 22 types and 21 genes described with mutations associated with different types of this genetic condition (Fernandez et al. 2021). In our laboratory we are genetically diagnosing numerous families with members suspected of albinism. And, with some of the mutations we find, because of their intrinsic interest, we are generating the corresponding avatar mouse models using CRISPR-Cas9 genome-editing tools (Seruggia et al. 2020; Fernandez et al. 2020). To date we have generated CRISPR-based mouse

models of several albinism types, including oculocutaneous albinism type 1 (OCA1), OCA2, OCA4, OCA6, OCA7, ocular albinism type 1 (OA1) and FHONDA (foveal hypoplasia, optic nerve decussation defects and anterior segment dysgenesis) type, whose affected genes are Tyr, Oca2, Slc45a2, Slc24a5, Lrmda, Gpr143 and Slc38a8, respectively. At the ISTT—TT2022 meeting we will be updating our progress completing the generation and phenotyping attempts to describe these various new mouse models of albinism.

Paper 41: From bench to bedside

Poster

The international mouse phenotyping consortium (IMPC): mouse high-throughput phenotyping contributes to understanding the role of genes in human disease

Dr Violeta Munoz Fuentes[1], Dr Hamed Haseli Mashhadi[1], Dr Robert Wilson[1], Mr Federico Lopez Gomez[1], Dr Tudor Groza[1], Dr Helen Parkinson[1]

[1]EMBL-EBI, Cambridge, United Kingdom

The International Mouse Phenotyping Consortium (IMPC) is a world-wide initiative which links gene to function using single-gene knockout mice to better understand mammalian gene function and human disease. Currently, more than 8,000 lines have been produced, many for poorly understood genes (the ignorome). A broad phenotyping pipeline, spanning across organismal physiological systems, collects data comprising more than 250 phenotypic parameters of embryos, young adult and aged mice. We will show how the overlap of adult mouse phenotypes with human clinical features has identified over 400 new mouse disease models. We will also show how the combination of viability data generated by the IMPC with human cell viability data generated by other resources has enabled identification of a category of genes that is enriched for disease genes. This showcases the ways in which IMPC data can be used for disease gene discovery and diagnosis. We will also show how the IMPC broad phenotyping pipeline is effectively uncovering pleiotropy, of particular importance when elucidating the genetic causes of syndromic disorders. In our studies, we find wide-ranging sexual dimorphism, hence highlighting the importance of including both males and females in biomedical research. To analyse this complex data set, the IMPC has developed an R package, OpenStats, to address ageing data, and a new methodology, Soft Windowing, which selects control mice proximal in time to mutants and assigns them maximal weight in statistical comparisons. The IMPC is also generating an ever-growing collection of images. Machine learning techniques are allowing us to automatically generate and quality control image annotations, thus aiding phenotyping. With all these efforts, the IMPC is effectively uncovering new mouse models, proposing gene function hypotheses and developing novel methodological approaches that are increasing our understanding of the mammalian genome, forming the basis of new research and identifying new gene-disease associations.

Paper 42: From bench to bedside

Poster

Complex engineering of a transgenic platform for the discovery of species-specific therapeutic antibodies and their use in the treatment of companion animals

Mr Stuart Newman[1], Mr Ross Cook[1], Dr Juexuan Wang[1], Dr Albert Vilella[1], Dr Daniel Bolland[1], Dr Chiara Cossetti[1], Dr Jose Garcia-Bernardo[1], Miss Caroline Sinclair[1], Mrs Madelyn Tooke[1], Mr Jon Burvill[1], Dr Andreas Marquardt[1]

[1]Petmedix Ltd, Cambridge, United Kingdom

Monoclonal antibodies represent a fast-growing class of modern pharmaceuticals in a variety of indications such as cancer, inflammation, and auto immune diseases. Their specificity and affinity to target antigens, combined with natural biophysical properties, qualifies them as efficacious and safe drugs. Over 100 monoclonal antibodies have already been approved for the treatment of human diseases in the US or Europe, companion animals are being underserved. Human monoclonal antibodies are not applicable for the treatment of conditions in non-human species due to a lack of cross-species reactivity and/or the risk of adverse, immunogenic effects Currently there are only 3 USDA/FDA/EMA approved antibodies available for use in companion animals. To address this shortage, PetMedix has designed the proprietary transgenic Ky9TM mouse platform for the discovery of canine therapeutic antibodies. By introducing over 2Mbp of genomic DNA encompassing large regions of the three immunoglobulin loci in dog into the corresponding loci in mouse, we have created a powerful discovery platform that utilises the natural B cell immune response in vivo to facilitate the discovery for highly efficacious canine-specific antibodies. Combined with antigen-specific B cell sorting and a 'sequence first' screening approach using state of the art single cell sequencing technologies, we can select paired immunoglobulin heavy and light chains expressed in the same cell. In silico assessment of individual cell lineages and clusters presents an opportunity to widely sample the repertoire of affinity matured antibodies but also to deprioritise candidates with liabilities concerning their developability. Discovery projects entering preclinical trials clearly demonstrate that the immunisation of our Ky9TM platform with a specific antigen leads to the expression of antibodies with high affinity against a broad range of epitopes, desirable efficacy, optimal biophysical properties, and a safety profile that facilitates fast and successful drug development for the treatment of dogs.

Paper 43: From bench to bedside

Oral: Session 10: Large Animal Transgenesis, Europaea Auditorium, September 20, 2022, 09:00–10:30

Optimised genome engineering of a transgenic mouse platform for the discovery of canine therapeutic antibodies

Mr Ross Cook[1], **Mr Stuart Newman[1]**, Miss Caroline Sinclair[1], Mr Jon Burvill[1], Mrs Madelyn Tooke[1], Miss Sophie Lake[1], Mr Tom Metcalf[1], Dr Andreas Marquardt[1]

[1]Petmedix Ltd, Cambridge, United Kingdom

Antibodies are an excellent class of pharmaceuticals which can address a broad range of indications. Unfortunately companion animals are medically underserved due to the risk of adverse immunogenic effects and lack of cross-reactivity from using huma n therapeutics.

To address the demand for treatments across all major therapeutic areas in dog, we designed the Ky9TM platform, a transgenic mouse line purpose-built for the discovery of canine therapeutic antibodies. We conducted 35 steps of precise genome engineering to introduce more than 2Mbp of canine DNA, encompassing variable genes of the immunoglobulin (Ig) heavy and light chain loci, into the mouse genome. The mice have a fully functioning immune system defined by normal B cell signalling and antibody affinity maturation including V(D)J recombination, junctional diversification of complementary-determining regions, combinatorial pairing of heavy and light chains and hypermutation.

An optimised pipeline for BAC recombination and integration in mouse ESCs and associated lineage tracing across broad clone tree diagrams complemented by a tailored microinjection programme helped facilitate and maintain germ line transmission competency. ESC lineages containing up to 1Mbp of canine DNA for each immunoglobulin heavy, kappa and lambda chain loci were created and, in some cases, required more than 100 passages and 14 steps of genome engineering. The progression of ESC engineering has run simultaneously with large scale ESC microinjection projects which served to verify the maintenance of ESC germ line competency and provided an opportunity to create 'intermediate' caninised platforms for therapeutic discovery. Intermediate canine platforms have been used in multiple successful immunisation campaigns, generating species specific therapeutics which have progressed to pre-clinical trials.

Paper 44: From bench to bedside

Oral: Session 10: Large Animal Transgenesis, Europaea Auditorium, September 20, 2022, 09:00–10:30

Discussing about the variability of the mouse gut microbiota

Loic Lindner[1], Pauline Cayrou[1], Benjamin Eisenmann[1], Dr Marie-Christine Birling[1], Dr Yann Herault[1], **Mr Guillaume Pavlovic[1]**

[1]PHENOMIN-Institut Clinique de la Souris, CELPHEDIA, CNRS, INSERM, Universite de Strasbourg, Illkirch-Graffenstaden, France

Discussing about the variability of the mouse gut microbiota.

In humans, as a potential medication, pathogen, or risk factor, the microbiota is acknowledged as a major health determinant. In the mouse model, the microbiota is also an environmental factor that has an impact on the results of studies. It must therefore be better understood and controlled to ensure reproducibility of experiments between laboratories.

In this presentation, we will review several years of 16S metagenomic data. We will evaluate the stability of microbiota composition in different animal houses over long periods. We will try to dissect different factors and their implication in the variations of the microbiome: sex of the animals, genetic background, cohousing or location in different rooms… We will show how technical factors have a major influence on the 16S results obtained. Finally, we will discuss the potential impact of sampling on the observed variations.

Paper 45: From bench to bedside

Poster

Extensive identification of genes involved in congenital and structural heart disorders and cardiomyopathy

Dr Nadine Spielmann[1], Dr Valérie Gailus-Durner, Prof Martin Hrabe de Angelis

[1]Institute of Experimental Genetics, Helmholtz Centre Munich, Munich Neuherberg, Germany

Clinical presentation of congenital heart disease is heterogeneous, making identification of the disease-causing genes and their genetic pathways and mechanisms of action challenging. By using in-vivo electrocardiography, transthoracic echocardiography, and microcomputed tomography imaging to screen 3,894 single-gene null mouse lines for structural and functional cardiac abnormalities, here we identify 705 lines with cardiac arrhythmia, myocardial hypertrophy, and/or ventricular dilation. Among those 705 genes, 486 have not been previously associated with cardiac dysfunction in humans, and some of them represent variants of unknown significance (VUS). Mice with mutation in Casz1, Dnajc18, Pde4dip, Rnf38, or Tmem161b genes show developmental cardiac structural abnormalities, with their human orthologues being categorized as VUS. Using the UK Biobank data, we validate the importance of DNAJC18 gene for cardiac homeostasis, by showing that its loss of function associates with altered left ventricular systolic function. Our results identify hundreds of previously unappreciated genes with potential function in congenital heart disease and suggest causal function of five VUS in CHD.

Paper 46: From bench to bedside

Oral: Session 1: Animal Models: From Bench to Bedside, Europaea Auditorium, September 18, 2022, 09:00–10:30

Patient-specific mutations reproduce the cardinal characteristics of a rare genetic disorder, Barth syndrome, in a humanised mouse model

Dr Douglas Strathdee[1], Dr David Stevenson[1], Ms Farah Ghaffar[1], Ms Sheila Bryson[1], Dr Cecilia Langhorne[1], Dr Eve Anderson[1]

[1]CRUK Beatson Institute, Glasgow, United Kingdom

Mutation of the gene Tafazzin (Taz) underlies Barth syndrome (BTHS), a serious X-linked inherited genetic disorder. The cardinal characteristics in affected individuals include cardiomyopathy, muscle weakness, neutropenia and growth delay. The enzyme produced by the Taz gene is involved in the remodelling of the mitochondrial lipid, cardiolipin, which is a key component of the mitochondrial membrane. In order to better understand the molecular mechanisms underlying the development and progression of BTHS, we have generated a number of mouse models. Previously we have shown that a knockout (KO) allele of Taz results in similar alterations in cardiolipin levels, to that observed in affected individuals with BTHS.

Although the development of a KO mouse model represents a valuable resource for the understanding of Barth syndrome, there are some differences between the mouse Taz (mTaz) and human Taz (hTaz) genes. For example, there is an additional exon present in hTaz, not present in mTaz. To test if this results in any functional differences, we have generated a mouse line where the entire wild-type mTaz locus has been replaced by the corresponding hTaz genomic locus. In mice carrying the hTaz allele, the human pattern of splicing including the incorporation of the additional exon, is maintained. The clinical signs observed in the mTaz KO strain are completely rescued by replacing the mTaz gene with the wild-type hTaz gene.

As we have a mouse strain expressing hTaz, we can now introduce point mutations identical to those observed in human affected individuals. The patient specific mutations reproduce a number of the characteristics of BTHS and this allows us to study the consequences of the individual mutations. In addition to allowing a better understanding of the disease, this will potentially allow the development and testing of individually tailored therapies.

Paper 47: From bench to bedside

Poster

Generation of mutant hESC by CRISPR/Cas9 electroporation for the study of amyotrophic lateral sclerosis in humanized mouse models

Ms. Sandra Turon López[1], Ms. Joana Garcia Garcia[2], Dr. Rubèn López Vales[2], Dr. Anna Pujol[1]

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It has been described the SOD1 G93A mutations as a cause of ALS (Amyotrophic Lateral Sclerosis). In order to generate isogenic clones only differentiated by this point mutation, hESCs (H9) have been edited using CRISPR/Cas9 technology. The hESCs were electroporated with the Cas9 nuclease, a gRNA and a ssODN carrying the mutation. Previously, electroporation conditions were established using a GFP plasmid. After two experiments, 86 clones were picked up and genotyped by restriction enzyme digestion followed by Sanger sequencing, and 8 positive clones were obtained. These mutant hESC clones may be used to be transplanted into mice to generate humanized mouse models for the study of the neurodegenerative disease.

Paper 48: From bench to bedside

Poster

RNA metabolism changes in LCCS1 disease mouse model

Dr Tomáš Zárybnický[1], Saana Metso[1], Fuping Zhang[2], Satu Kuure[1,2]

[1]Stem Cells and Metabolism Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [2]GM-Unit, Laboratory Animal Center, Helsinki Institute of Life Science, Helsinki, Finland

Tackling rare diseases is recognized not only as a crucial aspect to pave the way for general improvement in access to diagnosis, information, and medical care but also in advancing understanding of similar groups of common diseases. Knowledge of disease pathophysiology is of ultimate importance for understanding of the disease manifestation at the general and molecular and cellular levels. Mutations in the GLE1 RNA transport mediator are responsible for several diseases including amyotrophic lateral sclerosis, lethal congenital contracture syndrome 1 (LCCS1), which is a unique embryonic lethal disease of the Finnish disease heritage presenting hydrops, arthrogryposis, pulmonary hypoplasia, and micrognathia and death of the foetus by the 23rd week of gestation. In my project I focus on LCCS1 and aim at generating novel Gle1 mouse models to provide experimentation-based knowledge of the GLE1 functions in in development and physiology. Previous in vitro cell experiments suggest that GLE1 has versatile functions in the complex nucleocytoplasmic transport machinery, which regulates poly(A) + RNA transport. GLE1 appears also to regulate cytosolic stress granule formation in response to various stimuli. To provide information about in vivo functions of GLE1 and to model LCCS1, we created a novel knockin (KI) mouse model of LCCS1-causing GLE1FinMajor mutation. The KI mouse line is suitable for studying the physiology of RNA transport and LCCS1 disease pathomechanism. KI mice survive until the early-to-mid-adulthood, after which they suddenly die overnight. The characterization of the KI homozygotes at mid-adulthood revealed significant loss of ventral horn motor neurons and hypertrophy of cardiomyocytes in the left ventricle, suggesting neuronal and/or cardiac contribution. To better comprehend the molecular and cellular changes, mouse embryonic fibroblasts were isolated to study the poly(A) + RNA nucleocytoplasmic shuttling, as well as changes in stress granule count upon stress exposure. This will help us not only to comprehend the pathophysiological role of GLE1 in LCCS1 but may also enable identification of cellular cause of the LCCS1 phenotype.

Paper 49: In vivo genome editing

Poster

Improved knockin efficiency in human ips cells and rat embryos by optimized CRISPR/Cas9 molar stoichiometry leads to reduced large deletion

Dr Vanessa Chenouard[1,2], Isabelle Leray[3], Laurent Tesson[1], Dr. Séverine Remy[1], Agnès Fortun[4,5], Dr. Karine Bernardeau[4,6], Dr. Yacine Cherifi[2], Dr. Laurent David[1,3], Dr. Ignacio Anegon[1]

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KI efficiency improvement has been a major objective these past years for the generation of both gene-edited animals and cell lines. KI is dependent on CRISPR/Cas9 double strand break efficacy but also on homology-mediated repair using a DNA donor. Each Cas9 nuclease loads one guide RNA but very diverse concentrations have been described. To optimize ribonucleoprotein complex (RNPc) formation, we have developed an assay using nano differential scanning fluorimetry (nano DSF). We have observed an 8 °C shift between Cas9 alone or with excess of guide RNA, allowing detection of RNPc formation. With optimal conditions (buffer, incubation time…), Cas9/gRNA molar ratio 1/1 leads to undetectable level of apo Cas9. RNPc activated by MgCl2, bound to a DNA target or in presence of a DNA donor also show a different thermostability profile indicating a Cas9 conformational change. We have applied our optimized settings in vivo with a ssODN on a GFP to BFP conversion model on human iPSC and a point mutation on rat electroporated embryos to easily assess edition and KI rates by flow cytometry and genotyping, respectively. To enhance probabilities for DNA donor to be available at the Cas9 cleavage site, RNPc/ssODN molar ratio needs to be in favor of DNA donor. We demonstrated that, with optimized conditions, a molar ratio Cas9/gRNA 1/1 is sufficient and ssODN can be increased to 2 μM. With this setting, relatively low Cas9 concentration (0.4 μM for hiPSC and 0.2 μM for rat embryos achieved high KI rate both on hiPSC and rat embryos (about 50% for both). Moreover, unwanted on-target large deletion events (up to 3.6 Kb) on rat embryos with 0.2 μM of Cas9 were drastically decreased with Cas9/gRNA molar ratio 1/1 (25%) compared to 1/2 (85%). Thus, a rational molar approach allows high KI rate and reduced unwanted events.

Paper 50: In vivo genome editing

Oral: Session 7: Non-Mammalian Models, Europaea Auditorium, September 19, 2022, 09:00–10:30

An optimized workflow for efficient generation of large knock-in mouse models by a combination of IVF and CRISPR-mediated genome editing in C57BL/6 mice

Ms Mitra Cowan[1,2], Ms Jade Desjardins[1,2], Ms Nobuko Honma-Yamanaka[1,2], Ms Marie-Pier Cloutier[1,2], Dr. Yojiro Yamanaka[1,2,3,4]

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Efficient generation of large genome modifications using CRISPR-mediated genome editing in mouse zygotes is still a challenge. Following the report by Gu et al. (2018), our lab modified the published protocol and improved our recombination rates for large knock-ins (KIs) by transitioning to 2-cell nuclear microinjection using Cas9 protein. This method has been very reliable in our lab and has had good success to generate large KIs in a variety of backgrounds, such as hybrid strains (B6C3HF1) and FVB. However, this approach showed some limitations when attempting to target loci in the C57Bl/6 strain. For some constructs, we observed very low efficiency of recombination in the C57Bl/6 strain, but we were able to successfully re-target them in hybrid strains.

Abe et al. (2020) reported a new SPRINT-CRISPR method that delivers genome editing components and donor DNA precisely during the S phase of the zygote to improve large DNA donor KIs. One key element is the generation of synchronized zygotes by In vitro fertilization to permit for precise selection of developmental stages. We implemented this approach and generated all mouse embryos by IVF for 2-cell microinjection. This modification in our workflow improved our recombination efficiency over fivefold. Using this approach, we successfully generated over 10 different models with a variety of donor template sizes of up to 10 kb in multiple genomic locations in both the C57Bl/6 N (CR) and JAX backgrounds. Here, we describe our workflow to generate the IVF embryos, 2-cell microinjections, and donor template designs and preparations for microinjection.

Paper 51: In vivo genome editing

Poster

Comparative study of mouse genome editing tools for small modification insertions at the Tyr locus: from CRISPR/Cas9 to prime editing

Mrs Delphine Cussigh[1], Mr Sébastien Chardenoux[1], Mrs Ilta Lafosse[1], Mr Yohann Sassier[1], Dr Francina Langa Vives[1]

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Since the discovery of programmable nucleases, in particular the CRISPR technology, gene editing field has undergone an incredible breakthrough. It offers the possibility to precisely modify in vivo any potential locus in an unprecedent versatile way thanks to the delivery of different 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 optimising every step of the generation process. For instance, in vivo and ex vivo zygote electroporation techniques have emerged as very effective delivery methods of CRISPR/Cas9 components to mouse zygotes. Prime Editing, on its hand, represents a promising technique to induce small genetic changes without the need of double-stranded DNA breaks and donor DNA. Thus, we decided, within our transgenesis platform, to take advantage of these new technologies to broaden our pipelines of mouse model generation. By using different types of genetic components and delivery methods targeting the tyrosinase gene (Tyr), we compared the efficiency of introduction of small modifications by Non-Homologous-End-Joining (NHEJ), Homology-Directed Repair (HDR) or Prime Editing (PE). We first confirmed, in our hands, the superiority of ex vivo zygote electroporation over classical microinjection techniques due to the improvement in modification efficiency together with reduced effects on embryo viability and development. Then, we successfully managed to induce precise substitutions by PE in mouse zygotes at the Tyr locus and compared its yield to the targeting efficiency obtained via HDR for the introduction of the same mutations. Finally, we are currently trying to optimize the PE tool by using different strategies as for example introducing additional silent mutations into PE template. These implementations allow us to continually set-up optimized workflows that can be proposed to achieve our collaborator's projects.

Paper 52: In vivo genome editing

Poster

A modified DECAI vector allows for the rapid and efficient generation of conditional alleles by zygote electroporation

Dr Stephen Forrow[1], Dr Barbara Tondelli Tondelli[1], Dr Marta Chiodin[1], Dr Maria Isabel Muñoz[1]

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The generation of some classes of mutation via Crispr/Cas9, such as the insertion of long sequences and the creation of conditional mutations, remain technically challenging. For this reason, there is a requirement to devise novel methods for the generation of these mutations. Guzzardo et al. [1] reported the use of a synthetic intron to generate conditional alleles in human cell lines and named this strategy DECAI. The intron comprises a branch point sequence flanked by LoxP sites. Conditional removal of the branch point means that the intron is no longer recognized as such, and also puts one of three stop codons into frame. We generated mouse strains using this DECAI intron, and analysed mRNA expression in tissues from the resulting mice. We show that the DECAI cassette is functional in mice, however, a minor transcript is present which seems to result from transcriptional read through at the intron/exon boundry up to a cryptic splice site in the first LoxP. We speculated that the modification of this DECAI cassette to remove the cryptic splice site should circumvent this problem. Here we report the first results with this modified cassette.

Paper 53: In vivo genome editing

Poster

Electroporation of recombinase mRNA into mouse zygotes as a method to delete Frt/Cre flanked DNA sequences

Dr Stephen Forrow[1], Dr Barbara Tondelli[1], Dr. Marta Chiodin[1], Dr. Maria Isabel Muñoz[1]

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Gene targeting via mouse embryonic stem (mES) cells usually requires that a drug selection cassette is incorporated into the gene targeting vector. Accepted good practice means that this drug selection cassette should be removed from the targeted allele, due to the unpredictable effects of the promoter used in the cassette. Thus, the cassette is commonly flanked by recombinase sites, usually LoxP or Frt, to facilitate its removal via the corresponding recombinase. This is most usually achieved by mating the targeted animal with a recombinase expressing mouse. We have developed an alternative approach in which zygotes can be directly electroporated with mRNA coding for the recombinase to achieve the desired result.

Paper 54: In vivo genome editing

Poster

Genome editing mice for medicine: generating and distributing in vivo mouse models of human disease for developing therapeutic strategies

Mr Alex Fower[1], Dr Gemma F. Codner[1], Dr Alasdair Allen[1], Mr Daniel Archer[1], Dr Rosie K. A. Bunton-Stasyshyn[1], Mr Adam Caulder[1], Dr James Cleak[1], Dr Skevoulla Christou-Smith[1], Gene Delivery Team[1], Mrs Marie Hutchison[1], Mr Jorik Loeffler[1], Connor Macfarlane[1], Mr Matthew Mackenzie[1], Mr Krystian Nowicki[1], Mr Edward O'Neill[1], Mr Peter Price[1], Dr Michelle Stewart[1], Mr Moaz Talat[1], Dr Anna Thamm[1], Dr Susan Varley[1], Dr Sara Wells[1], Dr Lydia Teboul[1]

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The increasing availability of large patient genome sequencing and genome-wide functional genomics datasets is yielding many candidate mutations/genes causative of human disease. Thus, it increases the need for in vivo models for candidate validation and testing potential therapeutic strategies. The Mary Lyon Centre at MRC Harwell has established the Genome Editing Mice for Medicine (GEMM) programme to

facilitate the generation and access to such mouse models. Mutations are nominated by the scientific community and assessed by a cross-disciplinary group of scientific experts. The genome engineering service at the Mary Lyon Centre at MRC Harwell is continuously taking advantage of new genome editing techniques and has already generated and validated 92 mouse models. All models are made publically available for distribution through the European Mouse Mutant Archive (EMMA), one of the world's leading mouse repositories. A minimum of two calls per year over a period of ten years will ensure that models are prioritised in a timely fashion, so the programme answers the current requirements of the community. The GEMM programme can create a wide variety of mutant alleles such as point mutations, conditional alleles, tag KIs, cre drivers and over-expression models in a number of diverse genetic backgrounds. We will present the spectrum of alleles being produced, processes for generation and techniques for validation of these animals.

Paper 55: In vivo genome editing

Poster

High throughput pipeline ROSA26 gene targeting pipeline

Dr Zachary Freeman[1], Galina Gavrilina[1], Wanda Filipiak[1], Corey Ziebell[1], Simon Schweppe[1], Anna LaForest[1], Honglai Zhang[1], Elizabeth Hughes[1], Dr. Thomas Saunders[1]

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ROSA26 is the most highly utilized transgene docking station for inserting genes of interest into the mouse genome. We have developed a methodology to utilize the CRISPR/Cas9 system to introduce genes of interest into the ROSA26 locus. A single guide RNA is used to introduce a double strand chromosome break in intron 1 near the canonical xbal site. A double stranded DNA plasmid donor containing 1 kb 5' and 5 kb 3' arms of homology is then used to introduce the CAG lox puromycin lox gene of interest into the intron cut site. Ribonucleoprotein (RNP) complex is then injected with the targeting plasmid into fertilized zygotes and transplanted into pseudopregnant recipients. We utilized this approach to create 27 different models in C57BL/6:SJL F2 hybrids (F2, n = 21), C57BL/6(B6, n = 3), or FVB/n (n = 3) background strain mice. We injected a median of 315 zygotes per project with an average post injection survival of 86.8%. Overall targeting efficiency averaged 22% (range 5–49%) for the number of transgenic pups compared to total pups born or 4% (range 1–9%) for the number of transgenic pups compared to the total number of zygotes injected. An overall median number of 10 transgenic founders generated per project with medians of 10, 4, and 8 founders per line for F2, B6 and FVB respectively. These data support the continued use of the ROSA26 locus for targeted transgene insertion of the mouse genome in multiple strain backgrounds.

Paper 56: In vivo genome editing

Poster

Do mice with fewer copies of hACE2 exhibit more physiologically relevant disease progression?

Dr Teodora Georgieva[1], Christine Bradshaw[2], Larry Johnson[1], Tama Taylor-Doyle[1], Dr. Thomas Doetschman[1], Dr. Janko Nikolich-Zugich[1,2]

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Genetically Engineered Mouse Model (GEMM) Core is a well-established University of Arizona (UA) Core facility. We provide a wide range of "sequence-to-whiskers" services: transgenic, gene-targeted, and CRISPR-edited mouse models, sperm cryopreservation and storage, IVF, and embryo rederivation. Since 2007, the core has generated hundreds of mouse models for researchers from UA, the rest of the country, and Europe. 90 of these mouse models have been created with the cutting-edge CRISPR/Cas9 gene editing used as a routine method for the generation of simple and conditional knockouts, domain deletions/substitutions, small and fluorescent tag knock-ins, sensor-sensitive tag-knock-ins, and SNPs. With the start of the COVID, 19 pandemic our core reacted fast to complement SARS2 and COVID research of UA scientists with the generation of transgenic mice expressing the hACE2 receptor under the regulation of keratin 18 promoter. We received the transgenic vector by the courtesy of its original creators (P.B. McGray et al., J. Virology 2006) and microinjected it into Black6 NJ embryos to produce 7 founders carrying the transgene. We determined their gene copy numbers and established colonies of one and two-copy transgene models. The expression levels of the hACE2 receptor in the lungs and brains of our models have been characterized in comparison with the 8 transgene copies of the original "COVID" mice distributed by JAX Lab. The transgenic expression of hACE2 in epithelia converts a mild SARS-CoV infection into a rapidly fatal disease in the mice with 8 copies, while our models show an increased survival rate of 32% for two-copy and 100% for one-copy hACE2 transgene with similar viral burden in the lungs. Based on the virus exposure experiments we can conclude that mice with fewer copies of hACE2 exhibit more physiologically relevant disease progression. Currently, our COVID models are available for distribution to interested researchers.

Paper 57: In vivo genome editing

Poster

A doxycycline- and light-inducible Cre recombinase mouse model for optogenetic genome editing

Miss Linda Henneman[1], Miguel Vizoso[1], Colin Pritchard[1], Paul Krimpenfort[1], Jacco van Rheenen[1]

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Several photoactivatable Cre recombinase systems have been developed to control genetic modification in time and space to enable tracing of affected cells individually. However, the combination of inefficient and non-intentional background recombination has prevented thus far the wide application of these systems in biological and biomedical research. Here we used an optimized photoactivatable Cre recombinase system to generate a doxycycline- and light-inducible Cre recombinase (DiLiCre) mouse line, and illustrate its biological applicability for in vivo positional cell-tracing by intravital microscopy after light-induced mutagenesis. As a proof of concept, we show how newly formed mutant HrasV12 expressing cells follow an unnatural movement towards the interfollicular dermis.

Paper 58: In vivo genome editing

Poster

Establishment of a genetic harbor to generate monospecific B-cell receptor expressing mice

Ms Jinke D'Hont[1,2], Dr Stijn Vanhee[1,2], Dr Tino Hochepied[1,2]

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Genetically modified mice expressing predefined monoclonal B-cell receptor (BCR) repertoires are essential tools in immunological research.

Originally, such mice were generated by injecting zygotes with transgenes encoding the BCR that randomly inserted in the genome. Although mice can be generated rapidly in such a way and they have greatly advanced the understanding of aspects of immune regulation, the fact that the BCR is expressed from a nonnative locus has some disadvantages. Firstly, the transgene only carries one isotope, such mice cannot undergo isotype switching, which influences stability and function of the antibody and thus of the immune respons. Secondly, since transgenes frequently integrate in the genome in multiple copies, B-cells of these mice cannot undergo monoallelic somatic hypermutation, a major component of the process of affinity maturation, allowing the immune system to adapt its response to new threats.

A second generation of genetically modified mice expressing predefined monoclonal (BCR) repertoires were generated by knocking in a preassembled heavy or light chain into their respective native loci by homologues recombination in ES-cells. This is much more labor intensive however and two separate mouse strains (one for the Ig heavy chain and one for the Ig light chain) were needed, requiring complex breeding strategies to maintain both chains together upon crossing to other mutant mice.

In the first place, we generated monoclonal BCR expressing mice by using 2 RNPs to remove a part of the endogenous Igh locus and replacing it using a ssDNA repair template containing an endogenous promoter driving the expression of a bicistronic allele encoding both the light and heavy Ig chains of a BCR. This resulted in mice capable of isoclass switching, somatic hypermutation and affinity maturation.

In a second step, using 2 RNPs and a ssDNA repair oligo, we removed the coding regions of the light and heavy Ig chains, leaving only the promoter in position. This creates a genetic harbor to insert other heavy and light chains in a single step using only one RNP and a ssDNA repair template that doesn't need a promoter anymore, turning it in a relative efficient system to generate monoallelic BCR expressing mice relatively easily.

Paper 59: In vivo genome editing

Oral: Session 9: Neuroscience, Europaea Auditorium, September 19, 2022, 14:00–15:30

Creation of the four core genotypes mouse model by inducible CRISPR/dCas9-based systems

Dr. Yang Yu[1], Mr. Zhixing Ma[1], Dr. Yinhuai Chen[1], Dr. Xiaowei Gu[1], Dr. Gowri Nayak-Sarangdhar[1], Mrs. Alexandra E. Iten[1], Mrs. Susan R. Martin[1], Dr. Celvie L. Yuan[1], Mrs. Caitlin E. Schafer[1], Professor Tony DeFalco[1,2], Professor Yueh-Chiang Hu[1,2]

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Sex differences in phenotype have been an important and attractive subject in biomedical research. A sex-biased phenotype can be attributed to the action of sex chromosome complement or gonadal hormones or both. The conventional "four core genotypes" (FCG) mouse model that comprises of XY male, XX female, XY female (lacking the Sry gene on the Y chromosome), and XX male (carrying the Sry transgene on the autosome) has long been the optimal model to tease these

contributing factors apart. However, some limitations are associated with the current FCG mouse model. For instance, Sry deletion that leads to male-to-female sex reversal also excludes its function outside of sex determination, representing an incomplete Y chromosome in XY females. In addition, the position and multiple-copy nature of the Sry transgene in XX males is known to contribute an enhanced and ectopic Sry expression. Therefore, we sought to eliminate these limitations by using the doxycycline (Dox)-inducible and genital ridge-expressing CRISPR interference (Ci) and CRISPR activation (Ca) mice that we created. The CRISPRi and CRISPRa constructs were targeted to a genetic location near the conventional Col1a1 safe harbor locus (CaSH) that we identified to be highly efficient for CRISPR-mediated insertion in mouse zygotes. We also generated the Emx2rtTA3 allele to achieve the genital ridge induction of CRISPRi or CRISPRa upon Dox exposure. Subsequently, we introduced the gRNA transgenes targeting Sry or Sox9 enhancer 13 to generate Sry-Ci and Sox9en-Ca mice, respectively. By feeding pregnant females with Dox diet at time of sex determination in embryos, we knocked down Sry in the genital ridge of Sry-Ci embryos resulting in offspring with complete male-to-female sex reversal (XY female). Similarly, we enhanced Sox9 expression in the genital ridge of Sox9en-Ca embryos to obtain offspring with complete female-to-male sex reversal (XX male). Thus, we have successfully created a novel inducible FCG mouse model with an intact Y chromosome in XY females and no Y-derived genetic material in XX males for studying the sex-biased phenotypes. We can also tune the degree of sex reversal during development to generate adult hermaphrodites. Given the role of Sox9 in Sertoli cell differentiation, we can create larger testes in adult XY males by increasing Sox9 expression in embryos via Sox9en-Ca. Our data also suggest that the Ci and Ca mice are valuable tools for manipulating the expression of critical genes in a time- and space-controlled manner for a wide range of in vivo studies.

Paper 60: In vivo genome editing

Poster

Development of humanized ACE2 mouse and rat models for COVID-19 research

Dr Hongmei Jiang[1], Dr Emma Hyddmark, Sara Gordon, Angela Bartels, Yumei Wu, Dr Joe Warren, Dr Guojun Zhao.

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Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), also called COVID-19 (corona virus disease) has caused more than 6 million deaths globally (https://covid19.who.int/) and now we still in COVID-19 pandemic. SARS-CoV-2 infects host cells by binding its spike protein to the cellular surface protein angiotensin-converting enzyme 2 (ACE2). The predicted 29 amino acid residues of ACE2 that interact with SARS-CoV-2 spike protein receptor binding domain (RBD) vary among species, such as human, mouse and rat. Therefore, wildtype mice and rats show lower SARS-CoV-2 infection rate and mild symptoms compared to human. Small animal models that recapitulate human COVID-19 disease are urgently needed for better understanding the transmission and therapeutic measurement. Currently, scientists use either mouse-adapted SAS-CoV-2 (SAS-CoV-2 MA) models or random transgenic mouse models that artificially express human ACE2 under the control of cytokeratin 18 promoter or a constitutive promoter. SAS-CoV-2 MA may not completely reflect all aspects of the original human-tropic SAS-CoV-2 and the current transgenic human ACE2 mouse models typically have high mortality rate caused by neuroinvasion and encephalitis due to very high human ACE2 expression. To overcome these limitations, we have developed humanized ACE2 mouse and rat models using CRISPR-Cas9. Specifically, we inserted a $\sim$ 3 kb human ACE2 cDNA cassette into the mouse and rat Ace2 gene loci to ensure that human ACE2 expression is under the control of rodent Ace2 promoter and regulatory elements, while simultaneously disabling the rodent Ace2 gene. We confirmed human ACE2 expressed in tissues expressing endogenous Ace2 (such as lung, kidney, and GI tract), while rodent endogenous Ace2 is absent from these tissues in our humanized ACE2 mouse model. Most importantly, these animals displayed obvious symptoms after infection with SARS-CoV-2. In summary, these data suggest that our humanized ACE2 models can recapitulate human COVID-19 disease and will be facilitate the COVID-19 research.

Paper 61: In vivo genome editing

Oral: Session 7: Non-Mammalian Models, Europaea Auditorium, September 19, 2022, 09:00–10:30

Repair of the C57BL/6 inbred mouse genome

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C57Bl/6 inbred mice are most frequently used as mammalian model in genetic and biomedical research. However, resulting from inbreeding their genome harbours numerous homozygous mutant alleles supposed to reduce viability and fecundity as compared to outbred mice. In order to generate an optimized C57Bl/6 inbred mouse line we aim for the repair of point and insertional mutations in 47 genes using CRISPR/Cas9 mediated gene editing in C57Bl/6 zygotes. First we focused on the deletion of endogenous retroviral elements (ERV) that disrupt gene expression by integration of their 5–7 kb genome into intronic regions. Using gRNAs cutting on either side of an element we have currently deleted 29 ERVs as well as the emv2 tumor virus from the C57Bl/6 genome. In addition we aimed to correct point mutations at larger scale without the need for homology-directed repair using A base editors (ABE) combined with the PAM relaxed Cas9 variant SpG. Using ABE-SpG we achieved the correction of point mutations at 17 loci. Multiplexing of gRNAs enabled simultaneous base editing at up to 5 loci in single zygotes. Ultimately all of the repaired alleles will be combined into a new improved C57Bl/6 + line that will be phenotypically characterized in comparison to the parental strain.

Paper 62: In vivo genome editing

Poster

Deletion transcripts are detectable in knockout mouse lines

Ms Lauri Lintott[1], Valerie Laurin, Linda Chan and Lauryl Nutter

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Knockout mouse lines are routinely generated by deleting a critical region such that the modified transcript produced from the altered gene is predicted to be targeted for nonsense mediated decay (NMD). Transcripts targeted for NMD are not expected to be translated into truncated proteins. Truncated proteins may retain some wild-type function or may have new, unexpected functions that could confound phenotypic interpretations. At The Center for Phenogenomics we have generated hundreds of mouse lines that are predicted to give rise to transcripts targeted for NMD. In this study we investigated the transcript profile of 21 recently produced deletion mouse lines and found that in 2/3 of the lines we could detect the deletion transcript by endpoint PCR, indicating that NMD might not be as efficient in knockout lines as predicted. We will present our endpoint PCR results as well as transcript abundance measured by digital droplet PCR to ascertain if deletion transcript levels are comparable to the wild-type transcript levels. Correct interpretation of phenotypes produced in deletion models requires that the molecular consequences of the deletion be clearly defined before the phenotypic consequences can be attributed to a gene knockout.

Paper 63: In vivo genome editing

Poster

Leveraging the Bxb1 integrase for efficient and precise transgenesis of multi-kilobase donors in mouse zygotes

Mr Benjamin Low[1], Dr Vishnu Hosur[1], Dr Simon Lesbirel[1], Dr Michael Wiles[1], Dr Aamir Zuberi[1]

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Humanization of the mouse genome is a critically important tool for increasing our knowledge and understanding of genetics, biology and disease. The classic method for transgenic model generation relies on random insertion of exogenous DNA into the mouse genome, generally at low efficiency and often with detrimental side effects. Multicopy concatemers and transgene rearrangements are common as well as collateral damage to endogenous genes, all of which can distort the phenotype and confound the characterization of the mouse model. To overcome these challenges, we have developed a system to rapidly generate large vector-free, single-copy transgenic alleles up to 43 kb in size, with efficiencies of integration as high as 43%, directly in mouse zygotes. Using CRISPR-Cas9 and homology directed repair, dual heterologous Bxb1 attachment sites are first pre-positioned into the mouse genome (e.g., into the ROSA26 safe harbor locus) on any defined genetic background. Zygotes carrying this "Landing Pad" allele are then targeted for recombinase-mediated cassette exchange (RMCE) by microinjection of the Bxb1 Integrase and the DNA donor construct. This results in a highly efficient, precise, and unidirectional integration of the transgene in a predictable manner without the need for homology arms. Due to the precise nature of the integration, a simple and standardized PCR regime can be used for screening and verification. Complete sequence-verification of the entire transgenic allele, including flanking genomic DNA, can be achieved using nCATS (nanopore based-Cas9 assisted targeted sequencing). Combined, these powerful new technologies allow for the rapid and efficient engineering and verification of large precision transgenes across a range of genetic backgrounds, enabling the creation of improved mouse models of human disease.

Paper 64: In vivo genome editing

Poster

sgRNA testing is required for overall efficiency of genome editing in mouse embryos (or, why efficiency scores aren't enough)

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CRISPR-Cas9 mediated gene editing is widely used in generating genetic engineered mice models. A highly specific and efficient guide RNA is critical for successful genome editing, particularly for gene knock ins. The various current web tools such as CRISPOR are very helpful for choosing guide sequences with high specificity. However, the guide efficiency prediction scores originated from different guide test systems (expression vectors in vitro, in vitro transcribed sgRNAs used in zebrafish embryos) and may not accurately represent guide activity in mouse embryos. In the past several years, we have generated nearly 800 single guide RNAs by in vitro transcription and tested cleavage efficiency in mouse embryos.

While location and specificity are primary criteria, guides with higher activity prediction scores were preferred. In summary, each guide RNA was co-electroporated with control sgRNA (highly efficient guides) and Cas9 protein together into zygotes, then cultured to blastocyst stage in vitro. 10–16 single blastocysts were genotyped by PCR and Sanger sequencing to assess indel creation by the tested guide and 4 to 8 were genotyped for the control sgRNA editing. 364 guide RNAs with control guide indel rate 87.5 to 100% were analyzed based on the actual cleavage efficiency and the predicted cleavage activity by the web tool. Of the 364 sgRNAs, 59 show zero editing activity in embryos, while 27 showed 100% editing efficiency. 170 showed more than 50% editing efficiency and were deemed sufficient for use in editing projects, while 194 showed less than 50% activity. It appears that at least for sgRNAs transcribed in vitro, there is no significant correlation between predicted activity scores and the actual in vivo editing efficiency in mouse embryos. Testing guide activity prior to the targeting experiment is required to save labor and mice.

Paper 65: In vivo genome editing

Poster

Introduction of loxP sites by electroporation in the mouse genome; a simple approach for conditional allele generation in complex targeting loci

Mr Guillaume Bernas[1], Mme Mariette Ouellet[1], Mme Andréa Barrios[1], Mme Hélène Jamann[1,3], Dre Catherine Larochelle[1,3], Dr. Émile Lévy[2,4,5], **Dr Jean-francois Schmouth[1,3]**

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Background: The discovery of the CRISPR-Cas9 system and its applicability in mammalian embryos has revolutionized the way we generate genetically engineered animal models. To date, models harbouring conditional alleles (i.e.: two loxP sites flanking an exon or a critical DNA sequence of interest) remain the most challenging to generate as they require simultaneous cleavage of the genome using two guides in order to properly integrate the repair template. In the current presentation, we describe a modification of the sequential electroporation procedure described by Horii et al. (2017). We demonstrate production of conditional allele mouse models for eight different genes via one of two alternative strategies: either by consecutive sequential electroporation (strategy A) or non-consecutive sequential electroporation (strategy B).

Results: By using strategy A, we demonstrated successful generation of conditional allele models for three different genes (Icam1, Lox, and Sar1b), with targeting efficiencies varying between 5 to 13%. By using strategy B, we generated five conditional allele models (Loxl1, Pard6a, Pard6g, Clcf1, and Mapkapk5), with targeting efficiencies varying between 3 and 25%.

Conclusion: Our modified electroporation-based approach, involving one of the two alternative strategies, allowed the production of conditional allele models for eight different genes via two different possible paths. This reproducible method will serve as another reliable approach in addition to other well-established methodologies in the literature for conditional allele mouse model generation.

Paper 66: In vivo genome editing

Poster

Generation of murine lineage tracing strains using CRISPR

Dr Tripti Sharma[1], Dr Yonglong Wei[2], Ms Yu Zhang[1], Dr Hao Zhu[1,2]

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Single cell sequencing studies have identified many novel cell types within tissues. The relative absence of in-vivo reagents makes it difficult to interrogate these cell types. For example, the cells that contribute most to liver regeneration and the cells that give rise to liver cancer are unclear. In recent years, there has been intense controversy about how much regenerative capacity differs among hepatocyte and cholangiocyte subpopulations and whether or not there is a liver stem/progenitor cell. This is in part due to the lack of diverse lineage tracing strains that label different cellular populations. Here, CRISPR has proven to be an effective tool for precise genome editing for the generation of knockout and point mutation models to the generation of large insertion mouse models involving CreER. At the Children's Research Institute at UTSW Mouse Genome Engineering Core, we generated eleven independent inducible CreER knock-in mouse models that label distinct zonal populations across the portal-central axis in the liver lobule. These novel lineage tracing reagents were used to identify the most important regenerative cell populations during homeostasis and in the context of clinically relevant liver cancer models. Along with answering important questions in liver biology, this effort also served as a paradigm for how investigators studying other tissues can use mouse engineering to capture functional heterogeneity of diverse cell populations.

Paper 67: In vivo genome editing

Poster

CRISPR/Cas9-mediated genome editing in mice: achievable and challenge

Dr Lin Wu[1], Laurie Chen[1], Ying Chen[1], Zhenjuan Wang[1], Sarah Johnson[1]

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Genetically engineered mouse models are essential for elucidating basic biological mechanisms, modeling human disease, testing potential therapies, and facilitating drug developments. Our facility has processed nearly 600 genome editing projects in mice using CRISPR/Cas9 technology. Approximately 77% of the projects were site-specific transgene insertions and ~ 23% were targeted gene disruptions or deletions. We will describe our latest efforts to improve the efficiency of genome editing, share our data on what could be achieved with CRISPR/Cas9 technology on gene KO and KI projects and discuss challenges we have encountered for gene KI.

For gene KO projects, Cas9 protein and synthetic sgRNA are injected to mouse zygotes. To generate gene KI mice, DNA donors are also included in the injection mixture. The DNA donors that we use include oligodeoxynucleotides of 80–200 bases, long ssDNA of 0.3–4.7 kb in length, or plasmid DNA constructs that ranged from 6.7 kb to 21.8 kb.

With these methods, we generated mice with gene disruption or deletions up to 3.2 million bases (Mb), or insertions ranging from a few bp to 12 kb (which is larger than generally reported with CRISPR technology). Based on analysis of the

current data, the efficiency for gene KO through NHEJ averages ~ 60%. The average efficiency of gene KI through HDR or HR varied by method, from ~ 18% using oligo DNA donors, and ~ 34% using long ssDNA donors to ~ 45% using plasmid DNA donors. Our results from more than 200 projects using plasmid DNA donors demonstrates that KI efficiency of ~ 45% is achievable when CRISPR reagents are injected into mouse embryos at 1-cell rather than 2-cell embryos. We have encountered challenges in KI > 3 kb gene sequence with long ssDNA donors and experienced low efficiencies in generating conditional alleles with two oligo DNA donors each carrying a LoxP sequence.

Paper 68: Large animal models

Poster

Establishment of an in vivo model for embryo implantation in ruminants using trophoblast-specific gene manipulation system

Dr Eran Gershon[1], Mrs Maya Ross[1], Mr Alexander Rozov[1], Mrs Ester Karchovski-Shoshan[1], Mrs Alona Klienjan-Elazary[1], Mrs Michal Elbaz[1]

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For a successful pregnancy, the embryo must attach to the uterine wall in a process called embryo implantation. This process is necessary for the blood supply from the mother to the developing embryo. Various embryonic and maternal genes regulate the successful embryo implantation, placenta formation and function. The challenge is to cope with a growing number of endless lists of genes that might be crucial for successful placentation and reproduction. Therefore, there is an increasing need for new models to investigate embryo implantation. In the last few years, viral transgenesis has become a popular approach to deliver genes in different cell types. In order to address the urgent need for molecular in vivo models for embryo implantation in ruminants, we aimed in this study to establish a trophoblast-specific lentiviral gene transfer in sheep. For this purpose, embryos donor ewes were hormonally synchronized. Blastocysts were flushed from the ewe's uteri on day 9. Recovered embryos were incubated in the presence of lentivirus encoding GFP as a marker. We demonstrate that on day 9, GFP can be detected only in the trophoblast cells of the sheep blastocyst and not in the ICM. We further show that at day 23 of pregnancy only the extra-embryonic tissues expressed GFP. Furthermore, Placentomes isolated at day 70 of pregnancy exhibit normal histological appearance. Interestingly, immunostaining using anti-GFP antibody revealed that GFP is localized in all placenta villi cells originated from the trophoblast cells and not in the maternal cells. We demonstrate herein a development of a placenta-specific gene manipulation system in large animals. This model provides a tool to explore in vivo molecular mechanisms that might be significant for embryo implantation in ruminants. The knowledge accumulate using this model might better our understanding of normal pregnancies and pathological conditions in ruminants.

Paper 69: Large animal models

Poster

Comparison of chemically mediated CRISPR/Cas9 gene-editing systems using different transfection mechanisms on the mutation of porcine embryos

Miss Qingyi Lin[1,2], Dr Maki Hirata[1,2], Mr Koki Takebayashi[1,2], Miss Nanaka Torigoe[1,2], Mr Megumi Nagahara[3], Dr Takeshige Otoi[1,2]

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We developed a novel chemically mediated transfection technique that does not require specialized equipment to generate gene-edited blastocysts. Here, we examined the effects of different transfection mechanisms on the transfection efficiency of CRISPR/Cas ribonucleoprotein complexes (RNPs) using transfection reagents that are currently used, including a lipid nanoparticle delivery system (Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent), non-liposomal polymeric system (TransIT-X2® Dynamic Delivery System), and peptide system (ProteoCarry Protein Transfection Reagent). Porcine zona pellucida (ZP)-free zygotes, collected after in vitro fertilization (IVF), were incubated for 5 h with Cas9, guide RNA (gRNA) targeting GGTA1, and one of the above-mentioned reagents. In the first experiment, mutations in blastocysts from zygotes collected at 10 h from the start of IVF at Cas9 protein to gRNA molar ratios of 1:2, 2:2, and 4:2 along with single and double doses of the transfection reagents were examined. In the second experiment, mutations in blastocysts from the ZP-free zygotes collected at 10 and 29 h from the start of IVF were evaluated using optimal molar ratios and transfection reagent doses. For each reagent, a 4:2 molar ratio of Cas9 protein to gRNA, followed by the addition of a double dose of reagent allowed efficient transfection of RNPs into the zygotes. However, the optimal transfection time varied depending on the reagent, and the proportions of blastocysts carrying mutations were < 30%. This newly established chemistry-based technology is still in its infancy and needs further improvement, especially in terms of editing efficiency.

Paper 70: Non-mouse transgenesis

Flash Poster: Session 3: Poster Flash Presentations, Europaea Auditorium, September 18, 2022, 13:30–13:50

Generation and characterization of novel human ACE2 rat models for the study of SARS-CoV-2 and COVID-19

Dr Elizabeth Bryda[1,2], Dr. Daniel Davis[2], Dr. Rachel Olson[3], Dr. Craig Franklin[1], Henda Nabli[1], Mary Shaw[1]

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There is a critical need for animal models that appropriately mimic human COVID-19 disease and can be reliably distributed to the scientific community. Our goal is to provide new rat models for the study of pathogenesis of viruses like SARS-CoV-2 that use human ACE2 (hACE2) for entry into host cells. We performed random transgenesis to integrate copies of hACE2 under control of the ubiquitously expressed CAG promoter into the rat genome in order to create over-expression models in both inbred (F344) and outbred (Sprague Dawley) rats. Transgene copy number was assessed using ddPCR analysis and hACE2 expression has been confirmed at the mRNA and protein levels by RT-PCR and western blot analysis respectively. We are currently creating and characterizing additional models by using CRISPR/Cas9 technology to replace endogenous rat Ace2 with hACE2 under control of its own promoter. To date, we have performed viral challenge studies and demonstrated that wild type rats are not susceptible to high dose SARS-CoV-2 infection by either intranasal or aerosol challenge. In contrast, six-week-old hemizygous F344-Tg-hACE2 rats lost, on average, 84% of their body weight by 6 days post-treatment and displayed overt clinical signs of infection. All rats generated for these studies are available through the Rat Resource and Research Center (www.rrrc.us) to facilitate ready distribution to interested investigators as well as ensure that the models are archived via cryopreservation of germplasm. The availability of these important animal models will advance the understanding of COVID-19 and SARS-CoV-2 pathogenesis as well as accelerate the development of vaccines and antiviral therapies.

Funding

National Institutes of Health P40 OD011062 and P40 OD011062-20 (ECB).

Paper 71: Non-mouse transgenesis

Poster

CRISPR/Cas9 technology opens door to genetic manipulations in the freshwater apple snail, Pomacea Canaliculata

Mr Timothy Corbin[1], Ms Alice Accorsi[1], Ms Brenda Pardo[1,2], Mr Kyle Weaver[1], Mr Brandon Miller[1], Mr Michael Durnin[1], Ms Kym Delventhal[1], Dr Alejandro Sánchez Alvarado[1,3]

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CRISPR/Cas9 has allowed the study of novel genes, genetic pathways, and biological processes in novel research organisms. The freshwater snail Pomacea canaliculata (Gastropoda, Ampullariidae) is an excellent example of an emerging, non-mammalian organism offering unique biological features whose study would bring new and fundamental knowledge in many fields. For example, this snail has camera-type eyes anatomically comparable to vertebrates that can regenerate de novo after complete amputation. To mechanistically understand this process, protocols to genetically manipulate these animals had to be developed. First, we generated protocols to efficiently harvest one-cell fertilized zygotes and culture the dividing embryos ex ovo. The use of conventional microinjection techniques in combination with the WPI MICRO-ePORE^{TM} cell penetrator resulted in 35% survival rate. The microinjection of 10 ng/ul sgRNA/Cas9 RNP into snail zygotes resulted in genetic mutations in the targeted region. Using these tools, we generated F0 mutants for genes that have key roles in embryonic development: Pax6, Dia2 and Pitx. After germline transmission was confirmed by genotyping in F1s, F2s were screened for phenotypes. All the three stable mutant lines generated showed both Mendelian inheritance of the InDels and specific phenotypes. We additionally explored protocols for transgenesis using either Meganuclease-based insertions or CRISPR/Cas9-based knock-ins. While the Meganuclease-based method did not generate any positive samples, the CRISPR/Cas9-based protocol provided us with preliminary evidence of insertion in the targeted region. Further testing and optimization will be needed to increase insertion frequency and confirm complete and in-frame insertions. Altogether, we conclude that CRISPR/Cas9 allowed us to develop stable mutant lines of P. canaliculata for the first time and obtain preliminary positive results about the possibility of generating transgenic organisms. These new tools represent an indispensable resource to study gene functions and unique biological processes in this organism.

Paper 72: Non-mouse transgenesis

Poster

Myoglobin knockdown and knockout discrepancy in zebrafish

Mr Rasmus Hejlesem[1,2], Kasper Kjær-Sørensen[2], Claus Oxvig[2], Angela Fago[1]

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Morpholinos are stable anti-sense oligomers used for gene knockdown in zebrafish by blocking translation or splicing of RNA; however, there is often a discrepancy between phenotypes in morpholino-induced knockdowns and genetic knockouts of a given gene1. Non-specific morpholino knockdown phenotypes are not uncommon and knockout phenotypes may be masked by an early frameshift resulting in a premature termination codon leading to nonsense-mediated decay (NMD) followed by the upregulation of similar genes through transcriptional adaptation (TA)2,3.

We are generating knockout and knockin models of the myoglobin gene (mb) in zebrafish using the CRISPR/Cas9 system. Preliminary results suggest that our early frameshift mb knockout does not show any developmental phenotypes, which contrasts with the enlarged pericardium and ventral body curvature phenotypes reported in morpholino-mediated mb knockdown4. We have generated two additional predicted null variants. One has a large inframe deletion from the middle of ex1 to the 3'end of ex2. The other has a deletion of the in1/ex2 acceptor splice site and is expected to result in skipping of ex2. Both mutants should not result in transcriptional adaptation through NMD, although no-go decay could also lead to

TA. By analysing our three mb mutant models, we aim to establish whether the published knockdown phenotypes are the specific result of mb loss of function or not.

In the KI model, we want to generate a missense mutation of the distal histidine to a glutamine to alter the ligand affinity of myoglobin. In zebrafish the best strategy for donor choice seems to be locus specific. We plan to test the efficiency of several donor templates through a PCR restriction screen currently under development.

When the models have been established, we plan to investigate the possibility of myoglobin acting as a floodgate, protecting a subset of tissues, against the respiratory inhibition caused by hydrogen sulfide.

Paper 73: Non-mouse transgenesis

Poster

Creation and use of genetically modified rabbit models

Zoltán Gál[1], Bálint Bíró[1], Zsófia Nagy[1], Dr. Lilla Bodrogi[1], Tímea Pintér[1], Prof Elen Gócza[1], **Dr. Orsolya Ivett Hoffmann[1]**

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Many aspects of human embryonic development and disease cannot be studied in rodents. This is especially true for early prenatal development, its effects on long-term health, and some economically important diseases such as atherosclerosis or cardiovascular diseases. Transgenic rabbits are excellent animal models for inherited and acquired human diseases, including hypertrophic cardiomyopathy, impaired lipoprotein metabolism, and atherosclerosis. The first transgenic rabbits were models of arrhythmogenesis and, as it turned out, mimic human heart diseases much better than transgenic mice, which clearly shows the importance of creating and using non-rodent animal models. Transgenic rabbits are suitable bioreactors for the production of recombinant protein, either on an experimental or commercial scale. Although genome editing methods have been available for shorter time than other transgenic procedures, they have fundamentally changed the creation of rabbit models. The wide spread and applicability of genome editing methods has accelerated and facilitated the creation of model animals, and the rabbit is no exception. By using the CRISPR/Cas9 system, we can modify the genome in a diverse and yet precise way, which increases the usefulness of rabbit models used for diseases compared to rodent models even more than the physiological similarities.

Paper 74: Non-mouse transgenesis

Poster

Nuclear mitochondrial DNA sequences in the rabbit genome

Bálint Bíró[1], Zoltán Gál[1], Dr. Orsolya Ivett Hoffmann[1]

[1]Hungarian University Of Agricultural And Life, Gödöllő, Hungary

The integration of mitochondrial DNA fragments to the nuclear genome (numtogenesis) is an ongoing process with many interesting genetic and genomic contexts. The exact genomic location of mitochondrial fragments in mammalian genomes (numt) is characterized in more and more however, no such detailed data have been published for rabbits. For researchers working with the rabbit as a model animal the knowledge of its genome is particularly important in term of numts as well. In this study we characterised the mitochondrial insertions and the distinctness of its flanking regions in the rabbit genome.

To identify the numts in the nuclear genome of rabbit, a sequence alignment was performed between the rabbit mitochondrial and nuclear genomes. The alignment significance threshold was calculated as proposed by Tsuji et al. (2012) and thereafter individual characteristics of numts (exact location, GC content, repetitive elements) were analysed. Our analysis placed 153 numts in the rabbit nuclear genome of which nearly a two third were located on unmapped scaffolds. In rabbit, the GC content of numts were significantly lower than the GC content of their genomic flanking regions or the genome itself. Among others, the frequency of three mammalian-wide interspersed repeats (i.e. MIR, MIR3, MIRb) were increased in the proximity of numts. The decreased GC content around numts strengthen the theory which supposes a link between DNA structural instability and numt integration.

Paper 75: Non-mouse transgenesis

Poster

Next generation sequencing of founders is an effective detection tool in the establishment of CRISPR/Cas9 knock-out rat models

Dr James Kehler[1,2], Dr. Ana Vasileva[1,2], Dr. Yu-ting Yang[1,2], Dr. Chia-Lin Wang[1,2], Huaien Wang[1], Nikolay Shargorodsky[1], Anna Chan[1], Allison Maurice[1], Dr. Daniel Davis[3], Dr. Sang Yong Kim[4], Dr. Prem Premsirut[1,2]

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In an effort to optimize the creation of CRISPR/Cas9 knock-out rat models, five loci were targeted in out-bred Wistar and Sprague Dawley rats by three different approaches. In vitro electroporation, In vivo electroporation (GONAD) and Pro-Nuclear Injection (PNI) were used to deliver CRISPR/Cas9 Ribonucleoprotein (RNP) complexes into one-cell embryos and the rates of mutant founder rat creation compared. While all three methods could efficiently delivery RNP's, gene-editing efficiencies were more greatly impacted by the selection of the protospacer adjacent motif (PAM) site and design of the guide RNA (gRNA). Three separate targeting strategies were used to disrupt gene coding sequences in rats using CRISPR/Cas9 mediated to create double-strand break (DSB); Delivery of a single gRNA to rely on mismatch repair to create Insertion/Deletion (INDEL) mutations encoding a

premature termination codon (PTC); Co-delivery of a gRNA with a single-stranded oligodeoxynucleotide (ssODN) template to introduce a defined mutation by Homology Directed Repair; or co-delivery of 2 gRNAs targeting separate exons to effectively delete the intervening sequences, after fusion of separate DSB's. All three strategies successfully generated mosaic founder rats that frequently possessed more than 2 mutant alleles complicating genetic analyses of their PCR products. T7 Endonuclease I assays did not detect 1–2 bp INDELs resulting in PTC's. In addition, minor alleles were frequently obscured in bi-directional Sanger sequencing of PCR reactions. Next Generation Sequencing (NGS) of amplicons spanning predicted PAM sites was the most precise method to determine the full range of mutant alleles present in founder rats. NGS provided a sensitive technique to detect low frequency targeted alleles in founder rats that would normally be missed by traditional screening techniques. Mosaic founders with a desired mutant allele under 5% of total amplicon reads often transmitted this allele to the next generation successfully establishing new knock-out rat models.

Paper 76: Non-mouse transgenesis

Poster

Generation of first genetically engineered marsupials, monodelphis domestica

Dr Hiroshi Kiyonari[1], Ms Mari Kaneko[1], Dr Takaya Abe[1], Ms Aki Shiraishi[1], Ms Riko Yoshimi[1], Mr Ken-ichi Inoue[1], Dr Yasuhide Furuta[1,2]

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

However, lack of technologies to manipulate their genomes have hampered development of in vivo genetic approaches in this group of mammals.

In this study, we have focused on developing methodologies to manipulate the genome of the grey short-tailed opossum (Monodelphis domestica; hereinafter "the opossum") as a potential genetic model system. The opossum is thought to be the ancestor of all marsupials and is the most commonly used experimental marsupial model. The first marsupial to have its whole genome sequenced, the opossum makes a good model animal because its size and breeding characteristics are similar to those of mice and rats.

First, we established reproductive technologies such as ovulation induction, timed copulation, zygote collection and embryo transfer to pseudopregnant females. Further, we demonstrated the generation of gene knock-out opossums at the Tyr locus by microinjection of pronuclear stage zygotes using CRISPR/Cas9 genome editing, and germline transmission of identified mutant alleles to the F1 generation.

This is the first demonstration of the production of genetically engineered animals in the marsupials. This study provides a critical foundation for venues to expand mammalian genetics into the metatherian subclass.

Paper 77: Non-mouse transgenesis

Poster

CRISPR/Cas9 tools in drosophila: pitfalls and success cases

Dr Ana Raquel Tomas[1]

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Since the discovery of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) locus in the 1990s, much has been published on CRISPR/CRISPR-associated protein (Cas)9 system and its applications on genome editing. The ability to precisely edit the genome of a living cell holds enormous potential to accelerate life science research, improve biotechnology, and even treat disease.

Despite the broad list of advantages and applications, the CRISPR/Cas9 system has its limitations. There are several aspects affecting both efficiency and specificity, including Cas9 activity, target site selection and short guide RNA design, delivery methods, off- target effects and the incidence of homology-directed repair.

The Champalimaud Center for the Unknown (CCU) Molecular and Transgenic Tools Platform (MTTP) supports the researchers at Champalimaud Research (CR) on molecular biology techniques, from basic services such as the production of competent bacterial cells, or primer design, to complex clonings of knock-out and knock-in constructs to be delivered into cells/tissues in culture or into one-cell stage embryos of model animals such as zebrafish, fly or mouse, with the purpose of creating transgenic animals. For this purpose we work in close combination with the CCU animal platforms (Fish, Fly and Rodent).

Here we present some CRISPR case studies in Drosophila melanogaster and go through the difficulties and troubleshooting at two levels: the design and at the bench.

Paper 78: Non-mouse transgenesis

Poster

Examination of Venus and mCherry transfected chicken PGCs

Mr Roland Toth[1], Mr Martin Urban[1], Mr Andras Ecker[1], Dr Bence Lazar[1], Dr Stefanie Altgilbers[2], Dr Orsolya Hoffmann[1], Dr Wilfried Kues[2], Dr Elen Gocza[1]

[1]MATE-GBI, Gödöllő, Hungary, [2]Friedrich Loeffler Institut, Neustadt, Germany

The chicken primordial germ cells integrate only into the gonads of recipient embryos. Because of this ability, the PGCs

are the best tool for genome conservation and genome modification in chicken.

We created Venus and mCherry positive PGC lines by Neon electroporation. First, we amplified the plasmid DNA in E. coli competent cells. Then, the isolated DNA vectors were transfected into chicken PGCs by electroporation. We used two PGC lines one male (#1116, ZZ) and one female (#1125, ZW). Two types of plasmid vectors (SB-Venus, mCherry) and SB transposase (pSB100, which contains the hyperactive variant of SB transposase under the CMV promoter) were used. pT2-Venus plasmid encodes the Venus fluorescent reporter gene under CAGGS promoter, flanked by inverted terminal repeats (ITRs) of the SB transposase. We used different molar ratios of transposase and transposon (1:2; 1:6; 1:8). We analyzed the intensity of fluorescency and the number of positive cells in each electroporated cell culture with flow cytometry. The electroporated PGCs were analyzed by a CytoSMART Lux3 FL. The device enables researchers to unravel cellular processes in real-time. Approximately 70–80% of electroporated cells were positive. The fluorescent cells were enriched by BD FACS Canto II (BD Bioscience).

The Venus electroporated cells were collected and injected into 47 recipient embryos of Partridge-color Hungarian breed. 10-Days-old embryonic gonads were dissected. 39 embryos (83%) survived the injection. The isolated gonads of embryos were embedded in gelatin and cryosectioned. The sections were immunostained for DAZL germ cell-specific antibody and with TO-PRO-3 nuclear stain. The integration efficiency was analyzed by confocal microscope. Finally, the cultured PGCs were collected, and we isolated DNA from them. The Venus plasmid integration locus was determined by PCR and DNA sequencing.

Paper 79: Non-mouse transgenesis

Poster

Creation of an RNAi rat model platform and its utility for gene-specific knock-down in vivo

Dr Ana Vasileva[1], Dr. Yu-ting Yang[1,2], Dr. Chia-lin Wang[1,2], Huaien Wang[1], Nikolay Shargorodsky[1], Anna Chan[1], Allison Maurice[1], Dr. James Kehler[1,2], Dr. Thom Saunders[3], Dr Sang Yong Kim[4], Dr. Prem Premsrirut[1,2]

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The vast amounts of genomic data that emerged in the past decade unveiled many potential novel drug targets, which require functional testing to better understand disease processes and select the best points of intervention. Animal models are the gold standard for dissecting disease mechanisms and evaluating experimental therapeutics before human trials. Manipulating and culturing mouse embryonic stem cells enabled mice to become the standard for genetically altered models. The rat still remains the preferred rodent due to their larger size for surgical manipulation, repeat blood sampling, and their cognitive and physiological characteristics that more closely resemble humans than mice.

We have already demonstrated the utility of RNAi mice, and here we draw from our vast experience in mouse model creation. Current CRISPR/Cas9 approaches to manipulating the rat genome result in permanently modified alleles. We created a two-component rat model platform enabling reversible, temporal control of gene expression. The first component was a transgenic rat line containing our RNAi expression cassette and "homing site" for rapid insertion of short hairpin RNAs (shRNAs) targeting any gene of interest into the Col1A1 locus. The second component was an inducible reverse tetracycline transactivator (rtTA) under a constitutive promoter from an alternative safe-harbor locus.

We have used this novel platform to generate a total of 12 gene-specific proof-of-principle models for RNAi-mediated reversible target knock-down. Here we present preliminary data demonstrating the functionality of this system to achieve knock-down of a critical clotting factor and a rat counterpart to the Brd4 RNAi mouse. Induction upon addition of doxycycline resulted in gene knock down, as well as physiological changes corresponding to the role of each gene product. Our data lay the groundwork for future use of RNAi technology for in vivo pharmacology and modeling diseases in the rat.

Paper 80: Resources

Poster

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

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The NCI Mouse Repository, located at the Frederick National Laboratory for Cancer Research (FNLCR), 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.

Paper 81: Resources

Poster

Rat resource and research center

Dr Elizabeth Bryda[1], Hongsheng Men[1], Craig Franklin[1], James Amos-Landgraf[1], Yuksel Agca, Aaron Ericsson[1]

[1]University Of Missouri- Columbia, Columbia, United States

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 540 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, has the ability to make genetically engineered rat models from start to finish using a variety of state-of-the-art technologies including genome editing (i.e. CRISPR/Cas9) as well as traditional methods such as random transgenesis and modified embryonic stem cell microinjection into blastocysts. Our website (www.rrrc.us) allows user-friendly navigation. Current research efforts include generation and characterization of a variety of new rat models, refinement of existing models, characterization of the rat microbiota and its influence on model phenotypes, 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.

Paper 82: Resources

Poster

Presenting the genotyping services at the Mary Lyon Centre at MRC Harwell; a high-throughput mouse genotyping pipeline

Mr Adam Caulder, Ms Rumana Zaman, Dr Debbie Williams, Mrs Sue Morse, Mr Daniel Ford, Ms Laura Richards, Mr Jorik Loeffler, Mr Jan Rainier Sidiangco, Dr Kathryn Birch, Dr Michelle Stewart, Dr Sara Wells, Dr Lydia Teboul

The Genotyping Services at the Mary Lyon Centre at MRC Harwell provides a high throughput Polymerase Chain Reaction (PCR) and quantitative PCR- based genotyping service. We use these PCR based techniques to genotype a wide array of genetically altered mice with the technique employed depending on the type of alteration. The core deals with up to 10,000 samples per month, approximately 75% of which is genotyped via quantitative PCR (qPCR) copy number variation analysis. The high throughput genotyping service has been achieved using a 384 well quantitative PCR machine, which is linked to an automated plate loader and set up by Tecan liquid handling robots. Other PCR techniques employed include allelic discrimination, an end point PCR reaction used to genotype single nucleotide polymorphisms set-up using bench top liquid handling robots. We also utilise short range PCRs, the amplicons of which are visualised using capillary-based electrophoresis. As well as presenting the techniques and establishment of our high throughput platform we will illustrate how the assays we apply are used in zygosity identification for colony management, as well as, mouse line validation and quality assurance.

Paper 83: Resources

Poster

INFRAFRONTIER: resources and services to advance the understanding of human health and disease using mouse models

Reetta Hinttala[1,3], Reetta Vuolteenaho[1,3], Asrar Ali Khan[2,3], Sabine Fessele[2,3], Michael Raess[2,3], Martin Hrabé de Angelis[2,3]

[1]Biocenter Oulu, University of Oulu, Finland, [2]Infrafrontier GmbH, Neuherberg, Germany, [3]INFRAFRONTIER consortium

INFRAFRONTIER is the European Research Infrastructure for the development, phenotyping, archiving and distribution of model mammalian genomes. The INFRAFRONTIER network, currently consisting of 29 academic partners, is engaged in European initiatives to improve its resources and services and closely cooperates with the International Mouse Phenotyping Consortium (IMPC).

The core services of INFRAFRONTIER comprise model generation, specialised phenotyping services, systemic phenotyping of mouse mutants in the participating mouse clinics, as well as archiving and distribution of mouse mutant lines by the European Mouse Mutant Archive (EMMA) that contains more than 7800 mouse strains. INFRAFRONTIER also offers specialized services, such as the generation of germ-free mice (axenic service), and training in state-of-the-art cryopreservation and phenotyping technologies. In addition, INFRAFRONTIER offers trans-national access calls for

projects which are open to the global research community and provides funding based on proposal merit.

Here we want to present our specialised resources for specific research areas like rare diseases, COVID-19 and cancer. INFRAFRONTIER offers tools to discover EMMA strains related to specific disease areas, for e.g., the INFRAFRONTIER Cancer Resource that allows to query more than 50 cancer types. We also provide access to specialised analysis platforms such as our COVID-19 Therapeutics Pipeline. Taken together, the INFRAFRONTIER Research Infrastructure provides access to first-class tools and data for biomedical research and contributes to the understanding of gene function in human health and disease using mouse models.

Paper 84: Resources

Poster

Transgenic and archiving module (TAM): Czech Centre for Phenogenomics

Jana Kopkanova[1], Petr Nickl[1], Irena Jenickova[1], Michaela Krupkova[1], Michaela Vargova[1], Csilla Michalikova[1], Eliska Machalova[1], Sandra Potysova[1], Elena Vikhrova[1], Katarzyna D Solcova[1], Marketa Joskova[1], Anna Tkadlecova[1], Mario Adrian Martinez Monleon[1], Radislav Sedlacek[1]

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Transgenic and archiving module is a key part of Czech Centre for Phenogenomics, responsible for generation of novel genetically modified mice and rats using state-of-the-art technologies. TAM consists of 3 subunits: Targeting, Production, and Genotyping & Breeding subunits, that altogether provide complete service, from the initial gene-targeting design, generation of tools and transgenic rodent models to the genotyping and breeding of desired animal models. The most commonly used genetic background in CCP is C57Bl/6 N, but we are able to generate models on various backgrounds. Vast majority of newly generated mutant rodents are "knock-out" models based on CRISPR/Cas9 targeting tools and zygote electroporation. Also, we have a solid experience with generation of complex "knock-in" transgenic models via pronuclear injection (PNI), CRISPR-EASI or CRISPR-READI. Founder and G1 mice are analyzed to confirm germ line transmission (GLT) and integrity the mutation. Each successfully produced rat/mouse model is cryopreserved (embryo or sperm cryopreservation). Furthermore, we offer mice production with the ES targeting technologies. Routinely we produce models from targeted embryonic stem cells originating from EUCOMM and KOMP repositories. Majority of modifications in these ES cell lines are so called "knockout-first" alleles that represent a LoxP-flanked critical exon with LacZ reporter element.

In cooperation with animal facility of CCP we provide consultation, assistance services, and information on the design and use of genetically modified mice. We also assist in animal rederivation (cleaning of the rodent line), reanimation (creating of the line from frozen embryos or sperms) as well as models import and export using cryopreserved sperm and embryos.

In cooperation with phenotyping module of CCP we provide wide variety of standardized tests and services, including those of IMPReSS (International Mouse Phenotyping Resource of Standardised Screens), mandated by our active partnership in the International Mouse Phenotyping Consortium. Notable is our capacity for conducting comprehensive phenotyping pipelines, providing a wide breadth of clinical information per experimental animal, and thereby minimizing overall animal usage.

TAM provides services to a broad national and international scientific community. As a member of INFRAFRONTIER, we contribute with mouse models generation to the IMPC project that aims to knockout and evaluate all the mammalian genes. We also represent a Czech node of EMMA (European Mouse Mutant Archive), a non-profit repository for the collection, archiving (via cryopreservation) and distribution of relevant mutant mouse strains essential for basic biomedical research.

Paper 85: Resources

Poster

The UAB Center for precision animal modeling (CPAM)

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Background: For many persons with a rare genetic disorder, genome sequencing studies end with identification of "variants of unknown significance" that leave the diagnosis unresolved or with lack of therapeutic options. Disease models, including cells, worms, zebrafish, frogs, mice, or rats that reflect the molecular characteristics of a condition, can aid in understanding a disorder and identification of potential treatments. The University of Alabama at Birmingham has developed a Center for Precision Animal Modeling (CPAM) with expertise in computational and data sciences, human genetics, clinical diagnostics, cellular and molecular biology, animal model generation, and therapeutic modeling and selection.

Methods: We will discuss processes in place to support prioritization, review, analysis, interpretation, generation, and validation of models. Specifics on the nomination process and how individuals can engage with CPAM and submit variants for consideration will also be provided.

Rodent models: CRISPR guides are designed using CRISPOR (crispor.tefor.org) to target the gene of interest. Accompanying donor oligos contain the patient variant and 100 bp symmetrical homology arms (IDT, Inc.). Modified synthetic sgRNAs (Synthego, Inc.) are complexed with S.p.Cas9 Nuclease V3 (IDT, Inc.) before dilution to a final concentration of 50, 100, and 50 ng/ul, of sgRNA, Cas9, and ssDNA repair oligo, respectively. Pronuclear microinjection is

carried out as previously described. Positive samples are confirmed by modified Sanger sequencing.

Results: Multiple patient-specific alleles have been generated in rodents using CRISPR technologies, with models in worm and fish underway. Novel indel alleles will also be characterized if a null model did not previously exist for a given gene.

Conclusion: CPAM is a national resource for efficient and cost-effective analysis of patient-derived gene variants with production and validation of animal models. Development of these models may finally provide answers as to why individuals are sick and help find novel treatments for them.