Transgenic Res (2014) 23:827–909
DOI 10.1007/s11248-014-9820-1

ABSTRACTS

Program and Abstracts of the 12th Transgenic Technology Meeting (TT2014)

The Assembly Rooms, Edinburgh, Scotland, United Kingdom, 6–8 October 2014

6-8 October 2014 Edinburgh, Scotland,UK

ISTT www.transtechsociety.org
International Society for Transgenic Technologies

The TT2014 Meeting is hosted by: The Roslin Institute, The University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, Scotland, United Kingdom.

ROSLIN

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TT2014 Scientific Program (July, 2014)

Sunday, October 5, 2014

10:00–13:00	ISTT Council Meeting
16:00–18:00	Registration at the Conference Venue The Assembly Rooms 54 George Street Edinburgh EH2 2LR
18:00–22:00	Pre-Meeting Social Event—Scottish Dinner and Ceilidh Lessons at Ghillie Dhu Ghillie Dhu, 2 Rutland Place Edinburgh EH1 2AD

Monday, October 6, 2014

07:30– Registration at the Conference Venue

Opening

09:00–09:15 Welcome Address by the ISTT President and Organisers

Session 1: Opening Keynote Lecture
Chair: David Hume

09:15–10:00 **Rudolf Jaenisch** (Whitehead Institute for Biomedical Research, Cambridge, USA)
iPS technology, gene editing and disease research

10:00–10:30 Coffee Break, Posters and Exhibitors

Session 2: Embryonic Stem Cells
Chair: Peter Hohenstein

10:30–11:00 **Jos Jonkers** (The Netherlands Cancer Institute, Amsterdam, The Netherlands)
Rapid in vivo *validation of cancer genes and drug targets using ESC-GEMMs of human cancer*

11:00–11:30 **William Skarnes** (Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK)
The International Knockout Mouse Consortium (IKMC) resource: Finishing the genome-wide collection of mouse knockouts

11:30–12:00 **Austin Smith** (Wellcome Trust-Medical Research Council Stem Cell Institute, Cambridge, UK)
Embryonic stem cells: capture of the ground state

Scottish Science Showcase Presentation

12:00–12:15 **Ian Chambers** (MRC Centre for Regenerative Medicine, Edinburgh, UK)
Transcription factor function in pluripotent cell states

Session 3: Poster Teasers 1

12:15–12:45 Poster teasers 1–6 (to be selected from abstracts)

12:45–14:45 Lunch, Posters and Exhibitors

13.00–13.40 **Sponsored Lunchtime Symposia 1**

13:45–14:45 **Poster viewing session 1**

Session 4: Round Table Discussion

14:45–16:15 *What does the Future of Our GA/transgenic facilities look like?*
Chair: James Bussell (Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK)
Inken Beck (Institute of Molecular Genetics of the ASCR, Prague, Czech Republic)
Transgenic and Archiving Module of the Czech Centre for Phenogenomics—a young and one of the largest transgenic core facility in central Europe
Brendan Doe (Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK)
Mouse production of GA animals in a large-scale production centre: What does the future hold?
Lynn Doglio (Feinberg School of Medicine, Northwestern University, Chicago, USA)
Transgenic Core Facilities: Past, Present and Where Do We Go From Here?
Xin Rairdan (*Genentech Inc., South San Francisco, California, USA*)
The Future of the Large Scale Animal Facility with Complex Mouse and Rat Models

16:15–16:45 Coffee Break, Posters and Exhibitors

Session 5: Large-Scale Phenotyping
Chair: Ian Jackson

16:45–17:15 **Jaqueline White** (Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK)
High-throughput mutant mouse PHENOTYPING is a powerful tool to generate novel hypotheses

17:15–17:45 **Stephen Murray** (The Jackson Laboratory, Maine, USA)
Large-scale resource development at the Jackson Laboratory: KOMP2 and beyond

Session 6: ISTT Prize

17:45–17:55 Introduction to the *10th ISTT Prize for Outstanding Contributions to Transgenic Technologies*
Lluís Montoliu (National Center of Biotechnology, CSIC, Madrid, Spain; ISTT President)
Award Ceremony, with **Alexandre Fraichard**, CEO genOway

17:55–19:00 **Janet Rossant** (The Hospital for Sick Children, University of Toronto, Canada)
Manipulating the mouse embryo: from cells to genes to stem cells and back again

19:00–20:30 Reception & Lord Provost Welcome at the Assembly Rooms

Tuesday, October 7, 2014

Session 7: Animal Biotechnology
Chair: Helen Sang

08:45–09:15 **James Murray** (University of California, Davis, California, USA)
Transgenic Animals for Agriculture—Where in the world are we now?

09:15–09:45 **Angelika Schnieke** (WZW Center of Life Science, Freising-Weihenstephan, Germany)
Modelling human cancers in pig

Scottish Science Showcase Presentation

09:45–10:00 **Michael McGrew** (The Roslin Institute, Edinburgh, UK)
Transgenic chickens: New birds in the coop

Scottish Science Showcase Presentation

10:00–10:15 **Adrian Sherman** (The Roslin Institute, Edinburgh, UK)
Generating transgenic chickens

10:15–10:45 Coffee Break, Posters and Exhibitors

Session 8: Rodent Models
Chair: Douglas Strathdee

10:45–11:15 **David Adams** (Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK)
Cancer Gene Discovery in the Mouse

11:15–11:45 **Kai Schönig** (Central Institute of Mental Health, Heidelberg University, Mannheim, Germany)
Transgenic rats: catching up with the mouse

Scottish Science Showcase Presentation

11:45–12:00 **Daniel J Murphy** (The Roslin Institute & MRC IGMM, Edinburgh, UK)
Temporally tractable systems for interrogating tumour progression in vivo

12:00–12:15 **Short Talk** (To be selected from abstracts)

Session 9: Poster Teasers 2

12:15–12:45 Poster teasers 7–12 (to be selected from abstracts)

12:45–14:30 Lunch, Posters and Exhibitors

13.00–13.40 **Sponsored Lunchtime Symposia 2**

13:45–14:45 **Poster viewing session 2**

Session 10: New Technologies
Chair: Bruce Whitelaw

14:45–15:15 **Ignacio Anegon** (*Center for Research in Transplantation and Immunology, Nantes, France*)
Editing the rat genome using gene-specific nucleases

15:15–15:45 **Pawel Pelczar** (Institute of Laboratory Animal Science, Zürich, Switzerland)
Tracking Elusive Cells Using High-Resolution Lineage Tracing

15:45–16:15 **Francis Stewart** (Biotechnology Center of the TU Dresden, Germany
Genome engineering combining CRISPR with the best of the rest

16:15–16:45 Coffee Break, Posters and Exhibitors

16:45–18:45 **ISTT General Assembly**

19:30–23.55 Conference Banquet at Dynamic Earth
Dynamic Earth, Holyrood Rd, Edinburgh EH8 8AS

Wednesday, October 8, 2014

Session 11: Zebrafish models and transgenesis
Chair: Robert Kelsh

08:45–09:15 **Stephen Ekker** (Mayo Clinic, Rochester, Minnesota, USA)
The Zebrafish Molecular Genetics Programme Training Toolbox

09:15–09:45 **Koichi Kawakami** (National Institute of Genetics, Shizuoka, Japan)
The Tol2 transposon technology in vertebrates

09:45–10:15 **Keith Joung** (Massachusetts General Hospital, Charlestown, USA)
Targeted Genome and Epigenome Editing Using CRISPR-Cas and TALE Technologies

Scottish Science Showcase Presentation

10:15–10:30 **Elizabeth Patton** (MRC Human Genetics Unit & MRC IGMM, University of Edinburgh, UK)
A novel WT1 response to notochord injury and vertebra development in zebrafish

10:30–11:00 Coffee Break, Posters and Exhibitors

Session 12: Animal Ethics and Welfare
Chair: Aimee Stablewski

11:00–11:30 **Sara Wells** (MRC Harwell, Oxfordshire, UK)
Breeding the unknown- the benefits and issues of breeding new genetic combination

11:30–12:00 **Jan-Bas Prins** (Leiden University Medical Centre, The Netherlands)
Surplus animals in transgenic breeding: Bred but not used!

Scottish Science Showcase Presentation

12:00–12:15 **Peter Hohenstein** (The Roslin Institute & MRC IGMM, Edinburgh, UK)
Transgenic tools to reduce animal numbers

12:15–12:30 **Short Talk** (To be selected from abstracts)

Session 13: Poster Teasers 3

12:30–13:00 Poster teasers 13–18 (to be selected from abstracts)

13:00–15:00 Lunch, Posters and Exhibitors

13.15–13.55 **Sponsored Lunchtime Symposia 3**

14:00–15:00 **Poster viewing session 3**

Session 14: Imaging
Chair: Jan Parker-Thornburg

15:00–15:30 **Anna-Katerina Hadjatonakis** (Sloan-Kettering Institute, New York, USA)
Seeing is believing: quantitative imaging of cellular dynamics in early mouse embryos

15:30–16:00 **Vasilis Ntziachristos** (Technische Universität München, Munich, Germany)
The amazing opportunities from integrating optoacoustic and transgenic technology

16:00–16:30 Coffee Break, Posters and Exhibitors

Session 15: ISTT Young Investigator Award
Chairs: Lluís Montoliu and Douglas Strathdee

16:30–16:40 Presentation of the 2nd ISTT Young Investigator Award
Changhong Pang (Director of Molecular Biology, inGenious Targeting Laboratory)

16:40–17:00 Short Lecture by the recipient of the 3rd ISTT Young Investigator Award
Feng Zhang (Harvard & MIT, Cambridge, MA, USA)
Development and Application of CRISPR-Cas9 for Genome Editing

Session 16: Closing Keynote Lecture
Chairs: Lluís Montoliu and Douglas Strathdee

17:00–17:45 **Alexandra Joyner** (Sloan-Kettering Institute, New York, USA)
Interdependence of cellular networks ensures scaling of cell types during cerebellum development

17:45–18:00 **Concluding Remarks**
Presentation of Diplomas for the ISTT Registration Awards
The ISTT Best Poster Awards, sponsored by Charles River Laboratories International, Inc. with **David Grass, Ph.D.** (Senior Director of Gen Test Embryology services, Charles River)
Presentation of the TT2016 meeting
Radislav Sedlacek (Institute of Molecular Genetics, Prague, Czech Republic)

18:00 **End of TT2014 meeting**

TT2014 Hands-on Workshop 'An Introduction to Zebrafish Transgenesis'

The Queen's Medical Research Institute, The University of Edinburgh 8–10 October 2014

TT2014 Workshop Committee

Carl Tucker (Biomedical Research Resources, University of Edinburgh, UK) **Chair**
Matthew Sharp (Biomedical Research Resources, University of Edinburgh, UK) **Co-Chair**
Elizabeth Patton (MRC Human Genetics Unit & MRC IGMM, University of Edinburgh, UK)
Karthikeyani Paranthaman (MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, UK)
Peter Hohenstein (The Roslin Institute & MRC IGMM, Edinburgh, UK)
Douglas Strathdee (CRUK Beatson Institute, Glasgow, UK)

Wednesday, October 8, 2014

Session 1

18:15–18:30 **Elizabeth Patton** (MRC Human Genetics Unit & MRC IGMM, University of Edinburgh, UK)
Welcome & overview of workshop

18:30–19:00 **Carl Tucker** (Biomedical Research Resources, University of Edinburgh, UK)
Introduction to zebrafish, its basic husbandry and requirements

18:45–19:00 Set up for practical workshop

Thursday, October 9, 2014

Session 2

09:00–09:30 **Tim Czopka** (Centre for Neuroregeneration, University of Edinburgh, UK)
Introduction to microinjection: practices and principles

Practical Workshop Day 1:

09:30–12:30 *Microinjection of morpholinos*

12:30–13:30 Lunch

Session 3

13:30–14:30 **Koichi Kawakami** (National Institute of Genetics, Shizuoka, Japan)
Tol2 transgenic technologies & zebrafish

14:30–15:30 **Stephen Ekker** (Mayo Clinic, Rochester, Minnesota, USA)
Cre recombinase-revertible gene-breaking transposon mutagenesis

15:30–16:00 Coffee Break

16:00–17:00 **Keith Joung** (Massachusetts General Hospital, Charlestown, USA)
Targeted genome editing using TALENs and CRISPR-Cas RNA-guided nucleases

17:00–18:00 **Henry Roehl** (Department of Biomedical Science, University of Sheffield, UK)
Development of multilox technology in zebrafish

18:00–18:30 **Robert Kelsh** (Centre for Regenerative Medicine and Department of Biology and Biochemistry, The University of Bath)
Neural crest development in 'glorious Technicolor'!

18:30–19:00 **Martin Denvir** (British Heart Foundation Centre for Cardiovascular Science, The University of Edinburgh UK)
Cardiac regeneration with modalities for measuring cardiac function

19.15–22.00 Workshop Dinner

Friday, October 10, 2014

Practical Workshop Day 2 (Part 1):

09:00–12:00 *Microinjection of eGFP constructs*

12:00–13:00 Lunch

Session 4

13:00–14:00 **David Lyons** (Centre for Neuroregeneration, University of Edinburgh, UK)
ENU mutagenesis and forward genetic screening in zebrafish

14:00–14:30 **Dirk Seiger** (Centre for Neuroregeneration, University of Edinburgh, UK)
Understanding cellular signalling mechanisms through imaging of transgenic zebrafish

14:30–15:00 **Yi Feng** (MRC Centre for Inflammation Research, University of Edinburgh, UK)
Understanding inflammatory function through imaging of transgenic zebrafish lines

15:00–15:30 Coffee Break

Practical Workshop Day 2 (Part 2):

15:30–16:30	*Examination of morpholino and eGFP genetically modified zebrafish*
16:30–17:30	**Karthikeyani Paranthaman** (MRC Institute of Genetics and Molecular Medicine, University of Edinburgh) *Cryopreservation of zebrafish stock and IVF (to incl. demonstration)*
17:30–18:00	Workshop discussion & concluding remarks
18:00	**End of TT2014 Hands-on Workshop 'An Introduction to Zebrafish Transgenesis'**

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TT2014 Invited Speakers Abstracts

1. iPS cell technology, gene editing and disease research

Rudolf Jaenisch

Whitehead Institute for Biomedical Research and Department of Biology, Cambridge, MA, USA

The recent demonstration of in vitro reprogramming using transduction of 4 transcription factors by Yamanaka and colleagues represents a major advance in the field. However, major questions regarding the mechanism of in vitro reprogramming need to be understood and will be one focus of the talk. A major impediment in realizing the potential of ES and iPS cells to study human diseases is the inefficiency of gene targeting. Methods based on Zn finger- or TALEN-mediated genome editing have allowed to overcome the inefficiency of homologous recombination in human pluripotent cells. Using this genome-editing approaches, we have established efficient protocols to target expressed and silent genes in human ES and iPS cells. The most recent advance comes from the use of the CRISPR/Cas9 system to engineer ES cells and mice. This technology allows the simultaneous editing of multiple genes and will facilitate establishing relevant models to study human disease. We have used this technology to generate isogenic pairs of cells that differ exclusively at a disease-causing mutation. The talk will describe the use or isogenic pairs of mutant and control iPS cells to establish in vitro systems for the study of diseases such as Parkinson's and Rett syndrome.

2. Rapid in vivo validation of cancer genes and drug targets using ESC-GEMMs of human cancer

Jos Jonkers

The Netherlands Cancer Institute, Amsterdam, The Netherlands

Genetically engineered mouse models (GEMMs) have proven invaluable tools in cancer research. However, generation and validation of new GEMMs carrying multiple mutant alleles is both laborious and time-consuming. We have developed a novel approach for the generation of compound mutant GEMMs, which is much faster than conventional methods. Three elements are central for this system: (1) the efficient derivation of authentic embryonic stem cells (ESCs) from established GEMMs; (2) the routine introduction of transgenes of choice in these GEMM-ESCs by Flp recombinase-mediated integration; (3) the direct use of the chimeric mice in tumour cohorts.

We have used this approach to generate GEMM-ESC lines from our well-established GEMMs of BRCA1-deficient hereditary breast cancer and E-cadherin mutated lobular breast cancer. We have used these GEMM-ESC lines to study the function of several cancer genes (Myc, Myb, Met, Pik3ca, Akt1) in these two subtypes of breast cancer. The results from these studies demonstrate the utility of the GEMM-ESC strategy for in vivo analysis of the role of (candidate) driver mutations in tumour development, progression and therapy response.

3. The International Knockout Mouse Consortium (IKMC) resource: finishing the genome

Bill Skarnes

Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK

The International Knockout Mouse Consortium provides a public resource of thousands of lacZ-tagged knockouts in C57BL6 ES cells. This effort requires the design and construction of vectors and the production of targeted ES cells on an unprecedented scale, beyond the scope of conventional methodologies. Several high-throughput pipelines have been established for the generation of reporter-tagged conditional alleles[1], gene deletions[2] and trapped alleles[3]. To date, knockout alleles are available for more than 16,000 protein-coding genes. For this program, highly germline-competent ES cells from the C57BL/6N substrain were established[3–6] and validated by the generation of more than 3,600 lines of mutant mice. In my talk, I will describe the resources available from the IKMC and explain how researchers can search for and access material generated by the IKMC through a common web portal (www.mousephenotype.org)[7,8]. Prospects for finishing the entire collection of knockout alleles in mouse protein-coding genes will be discussed.

1. Skarnes, W. C. et al. A conditional knockout resource for genome-wide analysis of mouse gene function. Nature 474, 337–342 (2011).
2. Valenzuela, D.M. et al. High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. Nature Biotechnol. 21, 652–659 (2003).
3. Hansen, G. M. et al. Large-scale gene trapping in C57BL/6N mouse embryonic stem cells. Genome Res 18, 1670–1679 (2008).
4. Pettitt, S. J. et al. Agouti C57BL/6N embryonic stem cells for mouse genetic resources. Nat Methods 6, 493–495 (2009).
5. Poueymirou, W. T. et al. F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses. Nat Biotechnol 25, 91–99 (2007).
6. Gertsenstein, M. et al. Efficient generation of germline transmitting chimeras from C57BL/6N ES cells by aggregation with outbred host embryos. PLoS One 5, e11260 (2010).
7. Ringwald, M. et al. The IKMC web portal: a central point of entry to data and resources from the International Knockout Mouse Consortium. Nucleic Acids Res 39, D849–855 (2011).

8. Koscielny G. et al. The International Mouse Phenotyping Consortium Web Portal, a unified point of access for knockout mice and related phenotyping data. Nucleic Acids Res. 42(Database issue):D802–9 (2014).

4. Embryonic stem cells: capture of the ground state

Austin Smith

Cambridge Stem Cell Research, Cambridge, UK

Pluripotency, the capacity to generate all cell types of the body, lies at the foundation of development in mammals. In 1981, scientists discovered that pluripotency could be maintained in the laboratory in cells called embryonic stem cells. Study of these unique cells over the past 30 years has uncovered the molecular machinery that governs pluripotency. These studies have also highlighted significant differences between ES cells from rodents and from humans. So what makes an authentic embryonic stem cell?

5. Transcription factor control of transitions in pluripotent cells

Ian Chambers[1,2]

[1]MRC Centre for Regenerative Medicine, Edinburgh, UK; [2]University of Edinburgh, Edinburgh, UK

Embryonic stem (ES) cells are defined by two key characteristics: the ability to differentiate into cells of all three germ layers (pluripotency) and the ability to undergo apparently symmetrical self-renewing cell division, essentially indefinitely. Pluripotent cell identity is governed by the action of a gene regulatory network centred on Oct4, Sox2 and Nanog.

I will discuss findings that shed light on the operation of the central components of this network, including the contribution made to function by target genes and protein interacting partners of the central components. Interestingly, while Oct4 and Sox2 are expressed relatively homogeneously in ES cell culture, Nanog is heterogeneously expressed. We and others have shown that Nanog is under autorepressive control, and this autorepression contributes to Nanog heterogeneity. In addition, we have found that ES cells expressing an average of 50 % of the wild-type Oct4 levels maintain pluripotency robustly. Significantly, these cells do not require BMP, serum or pharmacological modulators of signalling pathways to propagate the pluripotent state. As these cells express the same level of Oct4 as single cells in a wild-type ES cell population, these findings suggest that robust pluripotency originates from a subset of cells present within wild-type cultures and that the full range of parameters present in wild-type cultures enables effective differentiation.

6. TT2014 round table discussion: what does the future of our GA/transgenic facilities look like?

James Bussell

Wellcome Trust Sanger Institute, Cambridgeshire, UK

Over the past four decades, animal facilities for genetic research have continuously adapted to the requirements of the science they support. Often at the cutting edge, these adaptations are driven by requirements of healthier animals, 3R's, statistically relevant data and influences of the scientific direction and tools. Facilities have progressed from open barriers holding locally bred inbred strains to complex infrastructures and programs controlling everything from the animal's environment to their genetic integrity. Underpinning these requirements are advances in the techniques to manipulate the animals genomes. In 1970s chemical mutagenesis and insertions of DNA fragments were the available tools for mutating genomes, predominantly those of mice. The advent of ES cell technology in the 1980s and 1990s brought a shift in direction with targeted gene disruption and replacement an achievable goal. Large-scale KO programs ensued and now see the mouse as the tool of choice to understand our genome. Such programs are ambitious and require the mutation of some 20,000 protein-coding genes and as such either the scale of the research must increase or new experimental methods allowing easier targeting of the genome must be employed. These factors, however, place new burdens on the facilities to ensure good management and the success of the programs of research. The advent of CRISPR/Cas technologies once again makes us question our approaches and is set to bring a new set of challenges for us and access to a wider range of species. The immediate and mid-term impacts of these strategies require us to think not only about the attractive utility of such techniques but also the broader implications of uncontrolled its use. This round table discussion looks to explore these topics from the stance point of four facilities with differing drivers and requirements.

7. Transgenic and archiving module of the czech centre for phenogenomics: a young and one of the largest transgenic core facility in central Europe

Inken M. Beck, Bjoern Schuster, Anna Lastuvkova, Jana Kopkanova, Irena Barnetova, Trevor A. Epp, Radislav Sedlacek

Czech Centre for Phenogenomics, BIOCEV, Institute of Molecular Genetics of the ASCR, Prague, Czech Republic

The Transgenic Unit at the Institute of Molecular Genetics in Prague was established in 2008/2009, and during the last years grew up into Transgenic and Archiving Module of the Czech Centre for Phenogenomics (CCP, http://www.phenogenomics.cz/). Starting with mouse rederivation and pronuclear injection, our core facility offers now a comprehensive range of services. Current services include customized targeted mutagenesis and transgenesis, blastocyst injection, pronuclear microinjection, sperm and embryo cryopreservation, in vitro fertilization, and breeding on demand. Over 140 service projects were completed in 2013. Full operation of standardized phenotyping services is expected in middle of 2015 upon first occupancy of the newly constructed CCP building. The new €24 million CCP building will have a floor space of 7,200 m^2 with a maximum capacity of 13,000 cages for mice and 4,000 cages for rats and will have over 70 employees.

Employing new technologies based on customized programmable nucleases (mainly TALENs and CRISPR/Cas-based approaches), we see turnaround times with increase over

75 % from conventional methods. Not surprisingly, the use of these technologies is an area of rapid growth within our facility—in 2013, we have successfully generated nearly 30 new mouse strains using nucleases.

Our core facility within The Czech Centre for Phenogenomics (CCP) is uniquely combining genetic engineering capabilities, advanced phenotyping and imaging modalities, specific pathogen-free (SPF) animal housing and husbandry, as well as cryopreservation and archiving, all in one central location—located within the new BIOCEV complex (Biotechnology and Biomedicine Center of the Academy of Sciences and Charles University in Vestec). This concentration of specialized infrastructure and expertise will provide a unique and valuable resource for the medical research community.

8. Mouse production of GA animals in a large-scale production centre: what does the future hold?

Brendan Doe

Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK

As Mouse Production Manager I am responsible along with many other interacting people for the mouse production at the Wellcome Trust Sanger Institute which is one of the largest non-commercial mouse production centres in the world. Annually we produce 350–400 unique mouse strains for large-scale international projects such as the IKMC (International Knockout Mouse Consortium) and the IMPC (International Mouse Phenotyping Consortium) as well as our own faculty members. In addition, we carry out sperm cryopreservations at a rate of 450 lines per year with associated IVF quality control for these lines. Further to this, our lines are deposited at large-scale repositories such as EMMA/Infrafrontiers and MMRC for use by the wider scientific community.

With respect to mouse production on this scale, we are always looking to make processes more efficient to save both resources and animals. I will discuss how some of the new technologies CRISPR/Cas, Cell permeable treatments and other new ideas might impact on the way in which we think about mouse production in the future? and how these might give us savings in efficiencies and animals and the impact these new technologies and the changing funding landscape may have on large-scale funded projects?

9. Transgenic core facilities: past, present and where do we go from here?

Lynn Doglio

Northwestern University, Chicago, Illinois, USA

The Northwestern University Transgenic Facility was established in 1989 as part of the Markey Program in Developmental Biology to provide a resource for generating transgenic mice by pronuclear microinjection. At the time, most academic core facilities originated in individual PI's labs and expanded into independent cores as the need to consolidate resources into a centralized infrastructure became apparent.

Today, with increasing pressure for research cores in general to adopt a business model and tighter federal regulations, the expanded Transgenic and Targeted Mutagenesis Laboratory (TTML) provides a broad range of services to investigators, including generation of transgenic mice, gene targeting of mouse embryonic stem cells, ES cell microinjection, cryopreservation and long-term storage of mouse sperm and embryos, recovery of mouse lines from cryopreserved germplasm, IVF line rescue, and rederivation of mouse lines.

The role of the facility began to shift with the inception of international consortiums to create and distribute targeted ES cell clones and improved cryopreservation methodology that made transport, and thus accessibility, of mouse lines global. Newer technologies, such as ZFN, TALEN, RMCE and CRISPR/Cas9, which allow direct mutagenesis in the embryo have begun shifting the emphasis of the core back towards microinjection, bringing us, in a sense, full circle.

The relative simplicity of these techniques, particularly CRISPR/Cas9, will likely result in an upsurge of new investigators to the transgenic field, investigators who have been reluctant to use transgenic mice in their studies due to the perceived costs and complexities traditionally associated with the generation these animal models. For investigators already in the field, there are thousand of easily accessible mice lines into which, conceptually, a 2nd, 3rd or multiple mutations can be introduced with relative ease. A technique with the potential to have the greatest impact on transgenic core facilities is the generation of transgenic mice by testicular transgenesis.

10. The future of the large-scale animal facility with complex mouse and rat models

Xin Rairdan, Rhonda Wiler

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The Genentech Transgenic Technology Department is one of the largest state-of-the-art animal facilities in the world with a capacity of 54,000 mouse cages. This operation currently has over 20,000 mouse cages occupied containing over 850 active compound genetic mouse colonies, produces 30,000 pups analyses over 40,000 genotypes per month, generates 60–80 new mouse and rat models by microinjection annually, cryopreserves, rederives and resuscitates over 300 colonies per year.

Since its discovery in 2009, Nuclease Technology for gene editing is transforming the landscape for creating complex genetically modified mouse models and has made targeted genetically modified rat models obtainable. This is reflected in our facility where the number of projects using ZFN or CRISPR/Cas9 technology is quickly outpacing the use of targeted ES cells, especially for making compound mutant mouse models with two or more modified alleles. In our hands, using CRISPR/Cas9 protocol has resulted in an 85 % reduction in the number of animals used and has resulted in an eightfold reduction in timeline for generating single mutation, 2.5-fold reduction for double mutation and a threefold reduction for triple mutations. Additionally, this technology enables the creation of targeted genetic modification models in other species. The Genentech Transgenic Technology Department is now generating knockout rat models by using the CRISPR/Cas9 system. We also believe this new technology will expand

animal models used in future research from mice to rats, rabbits and variety of other species.

Nuclease Technology is advancing quickly, and it is poised to replace ES cells and can be used to create targeted genetic modification in any species. Resources required to create genetic modifications in other species requires careful consideration due to the different requirements such as embryo generation and manipulation, creation of recipient females, as well as husbandry needs. In addition, the Microinjection and IVF/cryo lab personnel need to optimize processes for each species. Another consideration is F0 founder screening is heavily reliant upon sequencing, and achieving sequencing results and analysing the data before weaning is critical. More genetic analysis assays will need to be designed for cases where more than one founder with different mutations are generated and bred. The reduction in timeline, reduction in animal use and the ability to genetically modify other species makes Nuclease Technology very appealing and we are very enthusiastic about its future potential.

11. High-throughput mutant mouse phenotyping is a powerful tool to generate novel hypotheses

Jacqueline White, Carl Shannon, Chris Lelliott, Antonella Galli, Anneliese Speak, Joanna Bottomley, Edward Ryder, Brendan Doe, Ramiro Ramirez-Solis, David Adams

Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire, UK

As part of the International Mouse Phenotyping Consortium (IMPC), the Sanger Mouse Genetics Project has generated over 1,100 knockout mouse lines. Viability and fertility of each line is assessed during colony expansion; 24 % of lines have been found to be embryonic lethal at postnatal day 14, with a further 12 % classed as sub-viable, and 5.2 % of lines homozygous for the targeted allele present with fertility issues. Following expansion, dedicated groups of mice are phenotyped through an extensive and standardised battery of tests assessing development, physiology and metabolism, and yielding data on >300 parameters for every line. To date, over 800 of our knockout lines have completed this phenotypic characterisation. For homozygous lethal lines, heterozygous animals are phenotyped. While lines homozygous for the targeted alleles present with a higher hit rate (64 % of lines assessed in the homozygous state presented with $\geq$1 hit and averaged 3.9 hits per line), lines assessed in the heterozygous state were still remarkably informative (42 % of lines assessed in the heterozygous state were classed as phenodeviant with an average of 1 hit per line) and represent a rich source of information, all of which is openly available on the IMPC website (http://www.mousephenotype.org/).

A significant proportion of genes have been selected based on the absence of available mutant mouse characterisation. Examples will be presented for a number of lines in which phenotyping results have seeded hypothesis generation, such as mice with metabolic abnormalities, skeletal and behavioural changes, haematological alterations and developmental defects. Our data shows that high-throughput phenotyping is a powerful tool to generate novel hypotheses and that the majority of knockouts have robust phenotypes, few of which could be predicted a priori.

12. Large-scale resource development at the Jackson Laboratory: KOMP2 and beyond

Stephen Murray, Matthew McKay, Brianna Caddle, James Denegre, Emily Gordon, Adrienne Mehalow, Melissa Berry, Leslie Goodwin, Susan Kales, Rachel Urban, Rick Bedigian, Elissa Chesler, Juliet Ndukum, Robert Taft, Robert Braun, Karen Svenson, Leah Rae Donahue

The Jackson Laboratory, Bar Harbor, ME, USA

The overarching goal of the Knockout Mouse Phenotyping Program (KOMP2) and its partners in the IMPC International Mouse Phenotyping Consortium (IMPC) is to generate an encyclopedia of gene function through genome-wide generation and phenotyping of knockout mice. KOMP^2 offers an unprecedented opportunity to apply a parallel, standardized set of broad phenotyping modalities to a genome-wide set of knockout strains, eliminating the variability and ascertainment bias intrinsic to analysis in individual labs. Currently halfway through our ultimate goal of 833 strains, JAX has built and implemented a high-throughput pipeline of 25 tests comprising several hundred phenotypic parameters to characterize adult mice. Because up to 30 % of all strains are predicted to be embryonic lethal, we have built an additional embryo phenotyping pipeline, which includes the use of high-resolution 3D imaging for high-throughput generation of rich datasets. Thus far, JAX has observed $\sim$30 % combined partial and complete prewean lethality, including both novel genes and novel findings for previously published gene knockouts. Additionally, we are developing an atlas of gene expression through comprehensive analysis of in situ LacZ reporter activity, revealing numerous novel gene expression patterns in both embryonic and adult tissues. All of the data are made publically available as it is generated at www.mousephenotype.org. Progress towards improving production efficiency and speed, including a large-scale pilot to incorporate the use of the CRISPR/Cas9 system, will also be presented. With the scale and throughput of this platform in place, further bolstered by the potential of genome-editing technology, we will look towards its current and future application for modelling the current flood of human disease genetic data.

13. Manipulating the mouse embryo: from cells to genes to stem cells and back again

Janet Rossant

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I began my research career fascinated with the mouse blastocyst as a model for understanding how the earliest lineage decisions are made in embryonic development. I remain fascinated by this problem and have been able to see our understanding progress from lineage description to defining lineage commitment to understanding the signalling pathways and transcriptional networks that specify early cell fate. But in order to make these advances, new technologies have had to be developed. I have been lucky to be involved in a number of these. First, chimeras as means to follow cell fate and cell

commitment: we helped develop in situ marker systems to follow cell fate. Second, gene knockout and gene trap technology to identify and mutate developmental genes. Third, tetraploid complementation to assess stem cell potential. Fourth, lentivirus infection systems to knockdown gene expression in the blastocyst. The interpretation of the results of these and other technologies depend on a full understanding of the development of the early mammalian embryo. Too often we hear that the blastocyst injection assay is the gold standard for assessing stem cell potential. I will argue that this is not the case and discuss why comparative analysis of different mammalian embryos and stem cells is going to redefine what we mean by pluripotency.

14. Transgenic animals for agriculture: where in the world are we now?

James Murray

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As soon as the first publications came out reporting the effects of human growth hormone in transgenic mice speculation began about how genetic engineering could be applied to improve livestock for use in agriculture. However, 30 plus years later, this promise has yet to be realized as there are no GE animals approved for food consumption anywhere in the world. The reasons for this will be discussed and included a number of possible explanations. Initial work mostly concentrated on the production of animals (sheep and swine) with deregulated growth hormone (GH) genes. While a variety of GH transgenic lines of livestock were successfully produced, most had compromised health and were not suitable for further development. However, there were a number of lines of transgenic farm animals and fish produced during the 1980s and 1990s that did not have health issues and were potentially useful genotypes for use in production agriculture. To date none of these lines have yet been approved for human consumption and thus have not made it into production. Many of these lines have been placed in frozen storage or have been lost and would need to be redeveloped. The reasons for this are varied and include simply retirement of the developer, insufficient funds, pressure from anti-biotechnology activists, lack of a regulatory process, public opposition or lack of a perceived need by industry. The combination of these factors has led to a virtual cessation of research and development of transgenic animals for use in agriculture in Europe and Australia, early leaders in developing this technology, and the emergence of programs in places like Brazil, China and Argentina. Over the last 15 years, the development of gene targeting allowed by cloning and now the emergence of targeting nucleases such as TALENs or CRISPR has set the stage for a new round of development of animals for use in agriculture. The growing demand on animal agriculture to increase production to keep pace with the increase in the world's population, while coping with less land and water inputs, suggests there is a need for GE technologies and the resulting animals in agriculture. However, it is still not clear when GE animals will be adopted to any significant degree for use in agriculture.

15. Modelling human cancers in pigs

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Most animal models of solid cancers are in rodents, particularly genetically engineered mice. However, mice differ significantly from humans in size, lifespan, physiology, anatomy and diet, limiting their usefulness for some studies. Pigs are increasingly recognised as a valuable adjunct to pre-clinical research. Our aim is to provide a series of genetically defined pigs that model serious and common human cancers (1). These will allow new diagnostic and therapeutic strategies to be investigated at human scale, and longitudinal studies under conditions that mimic the human patient. We are thus engaged in a program of gene targeting to replicate in pigs a series of genetic lesions known to underlie human cancers. Somatic mutations of the major tumour suppressor gene TP53 are present in most human cancers; therefore, we have created gene-targeted knockout pigs and pigs carrying a latent TP53R167H mutant allele orthologous to human mutant TP53R175H that can be activated by Cre recombination (2). In order to prove Cre-activity in a defined organ, we have placed a Cre-inducible dual fluorescent reporter by gene targeting at the porcine ROSA26 locus to achieve ubiquitous expression (3).

To model colorectal cancer, we have generated gene-targeted cloned pigs carrying two different nonsense mutations in APC (APC1061 and APC1311). Histological and molecular analysis showed that the porcine model recapitulates all major features of early-stage human familial adenomatous polyposis (FAP) (4). We will report on the progress in the derivation of porcine models for human cancer, the disease phenotype and preliminary results regarding biomarkers for early stages of colorectal tumourigenesis.

16. Transgenic chickens: new birds in the coop

Mike McGrew

The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh, Edinburgh, UK

Studies using the chick embryo have made significant contributions to our understanding of vertebrate development. This is due to the wide range of experimental manipulations that can be carried out *in ovo* at early developmental stages. What have been lacking in avian model systems are efficient tools for generating transgenic models. For the chicken, the primordial germ cells, the early precursors of the germ cell lineage can be propagated for extensive periods in vitro and retain germline competency. Cultured chicken germ cells hold the promise to form a stem cell-based system for the investigation of germ cell development, advanced transgenesis and for use in the

conservation of poultry genetic resources. The propagation of chicken primordial germ cells uses a complex medium similar to the initial culture conditions used to propagate mouse embryonic stem cells. To develop standardised culture conditions, we have developed a feeder-free and serum-free medium for the long-term propagation of germline-competent chicken primordial germ cells. These cells will be useful for the conservation of avian genetic resources and transgenesis.

We have previously demonstrated that chicken primordial germ cells can be efficiently modified in vitro using DNA transposons. Recently, it has been shown that targeted chicken knockouts can be produced using standard homologous recombination in primordial germ cells. We present our recent efforts to use site-specific nucleases to increase targeting efficiencies in primordial germ cells and generate knockout birds.

17. Generating transgenic chickens

Adrian Sherman, Helen Sang

The Roslin Institute Edinburgh, UK

The Transgenic Chicken Facility, now part of the National Avian Research Facility at the Roslin Institute, has produced a number of transgenic chicken lines containing transgenes under the control of ubiquitous and tissue-specific promoters. Examples of the work of the facility include the demonstration of oviduct-specific expression and incorporation of pharmaceutically relevant proteins in egg white and the investigation of methods to potentially increase resistance to commercially important diseases such as avian influenza. We have also established transgenic lines that ubiquitously express fluorescent proteins localised to the cytoplasm or cell membranes to provide a source of marked cells for a variety of developmental biology applications where the chick is used as a model organism.

The efficient generation of transgenic chickens has been a challenge owing to the difficulty in visualising the pro-nuclei in the yolky cytoplasm and the inaccessibility of the newly fertilised ovum. However, the development at Roslin of an efficient method for the genetic modification of chickens using lentiviral vectors to transfer genes of interest to the early embryo has enabled the production of germline transgenic birds at high frequency with reliable transgene expression.

The method involves the microinjection of lentiviral particles, containing the transgene of interest, into the subgerminal cavity below the developing embryo in newly laid eggs followed by their incubation to hatch in a host-shell culture system. The aim is to genetically modify the embryonic primordial germ cells and so the gametes in the mature bird. Founder birds are mosaic for the transgene and transgenic lines are established by breeding to produce hemizygous transgenic offspring.

The successful culture of manipulated eggs to hatch using surrogate 'host' shells requires that the embryo experiences conditions as close to natural incubation as is possible to achieve. In addition to the vital incubation parameters of temperature, humidity and turning, the size and quality of the host shell are important. Successful development in this ex vivo environment requires careful management.

An overview of the technique showing the viral injection, embryological manipulations and incubation in the host-shell system will be presented.

18. Cancer gene discovery in the mouse

David Adams

Wellcome Trust Sanger Institute, Hinxton, UK

Cancers are characterised by numerous somatic mutations. However, distinguishing mutations that promote tumourigenesis from inert lesions can be a challenging task, particularly those mutations that occur at lower frequencies. The growing availability of cancer genome datasets from thousands of tumours provides an unprecedented opportunity to uncover these low-frequency putative cancer drivers. Thus, by interrogating the genomes of 7,651 human cancers, we identified inactivating mutations in the homeodomain transcription factor gene *CUX1* (cut-like homeobox 1) in $\sim$1–5 % of various tumours. Furthermore, analysis of 2,519 cases of myeloid malignancies revealed disruptive *CUX1* mutations that were associated with poor clinical outcome. To validate *CUX1* as a *bona fide* tumour suppressor, we used mouse transposon-mediated insertional mutagenesis and *Drosophila* cancer models. Mechanistically, we found that *CUX1* deficiency activates phosphoinositide 3-kinase (PI3K) signalling through direct transcriptional downregulation of the PI3K inhibitor *PIK3IP1* (phosphoinositide-3-kinase interacting protein 1), leading to increased tumour growth while exposing susceptibility to PI3K–Akt inhibitor therapy. Thus, our complementary approaches identify *CUX1* as a pan-driver of tumourigenesis and uncover a potential strategy for treating *CUX1* mutant tumours. This study also highlighted a number of additional putative tumour suppressors that may be clinically relevant across a broad range of cancer types and warrant further investigation.

19. Transgenic rats: catching up with the mouse

Kai Schönig[1], Jost Seibler[2], Sonja Ortmann[2], Simone Janzen[2], Jon Chesnut[3], Lena Wendler[1], Ariana Frömmig[1], Brigitte Pesold[1], Dusan Bartsch[1]

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The rat is the lab animal of choice in many areas of biomedical research. However, the development of rationally designed, sophisticated rat models has been hampered for a long time by the lack of advanced genetic tools for the targeted modification the rat genome. Only recently, the development of individually tailored sequence-specific nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided engineered nucleases (RGENs) has changed the game, allowing target-oriented reverse genetic genome modifications and the accurate insertion of transgenic sequences in the genome of this species.

We have established ZFN-, TALEN- and RGEN-based genomic tools for efficiently targeted modification of the rat

genome: ZFNs were applied for the reproducible insertion of transgenic expression vectors in the rat Rosa26 (rRosa26) locus, leading to strong and ubiquitous expression, similar to the Rosa26 expression pattern in mice. TALENs and the RGENs have been applied for the targeted modification of genomic loci by non-homologous end joining (NHEJ) and homologous recombination with the help of oligonucleotides. The established technology can be used either for the straight knockout of genes of interests, insertion of SNPs or for the overexpression of transgenes, including endogenous or synthetic microRNAs or long non-coding RNAs. In combination with brain-specific Cre lines generated in our laboratory (e.g. Drd1a-Cre, Dat-Cre, Cam-CreERT2 or TPH2-Cre), this technology allows the generation of neuronal subtype-specific rat models for studying neuronal gene functions. Furthermore, we demonstrate that Cre mRNA injections in fertilized oocytes and a newly generated Cre deleter line lead to germline deletion of a LoxP-flanked STOP sequence in rRosa26-targeted rats and in the CAG-LoxP.EGFP Cre reporter line.

Taken together, nuclease-based targeting approaches strongly simplifies and speeds up the generation of transgenic rat models for studying gene functions or human diseases, which are often superior to mouse models.

20. Temporally tractable GEMMs to reveal mechanisms of tumour progression and maintenance

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Genetically engineered mouse models of Cancer uniquely fill a vital gap between tissue culture-based experimental models and actual clinical disease. The advantages afforded by GEMMs over culture-based approaches allow for the faithful recapitulation of the entire natural history of a nascent tumour, embedded for the entirety of that history within its natural physiological environment, replete with all of the appropriate homotypic and heterotypic intracellular interactions, the presence of physiological substrata and physical barriers, exposure to appropriate physiological cues and stresses including dynamic changes to circulating growth factors, hormones, vital gasses and nutrients, as well as the continuous removal of waste and debris. The continuous refinement of GEMMs in recent years using conditional alleles of mutations present in human cancers has facilitated the development of models that accurately and faithfully recapitulate human disease at the levels of histological appearance and complexity, gene expression signatures and indeed response to both generic and targeted therapeutics (for a recent review, see Singh, Murriel and Johnson, Cancer Research 2012, doi:10.1158/0008-5472.CAN-11-2786). By harnessing temporal control over allele activation, it is possible to now trace the entire course of tumour evolution in situ from inception through to metastasis and migration. Temporal control thus uniquely enables researchers to interrogate tumours in their dynamic state at early stages that are typically inaccessible to clinical scientists, shedding much needed light on the mechanisms of early disease development, identifying new candidate biomarkers for early detection and new points for therapeutic intervention. Our recent advances in understanding progression of lung cancer from benign to invasive disease will be discussed.

21. Editing the rat genome using gene-specific nucleases

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The rat is a widely used experimental model but the lack of KO and KI mutants has been an obstacle to fully develop the potential of rat models. Rat ES cells have been described but their use is still difficult. Since 2009, we and others have used microinjection into rat zygotes of ZFNs to readily generate several KO and a few KI rats (1). We generated rats KO for heavy and light chain antibody genes using ZFNs (2). These rats were then crossed with transgenic rats in which human immunoglobulin genes were introduced by BAC and YAC microinjection. Human monoclonal antibodies were generated from these immunoglobulin humanized rats that showed comparable diversity and high affinity to the ones from wild-type rats (3).

We have TALENs also used to generate KO rats (4) and recently to generate KI rats on three different loci (5).

Results on the generation of KI rats using CRISPRs/Cas9 will also be presented.

The availability of gene-specific nucleases provide an extremely useful tool to generate KO and KI rats for the analysis of gene function, generate disease models and biotechnological applications.

1. A. Geurts et al. Science 2009
2. S. Ménoret et al. Eur. J. Immunol. 2010
3. M. J. Osborn et al. J. Immunol. 2013
4. L. Tesson et al. Nat Biotechnol; 2011
5. Rémy et al. Genome Res. 2014.

22. Tracking elusive cells using high-resolution lineage tracing

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Conditional mutagenesis and expression approaches that use tissue-specific Cre-driver genes are often limited by the lack of truly cell type-specific promoters. In order to increase recombination specificity, we developed two dual-promoter-driven

conditional mutagenesis systems optimized for greater accuracy and high efficiency of recombination. Co-Driver consists of a recombinase cascade of Dre and Dre-respondent Cre, which processes LoxP-flanked alleles only when both recombinases are expressed in a predetermined temporal sequence. This unique property makes Co-Driver ideal for sequential lineage tracing studies aimed at unravelling the relationships between cellular precursors and mature cell types. Co-InCre relies on highly active transsplicing inteins and promoters with simultaneous transcriptional activity to reconstitute native Cre recombinase from two inactive precursor fragments. Both Co-Driver and Co-InCre significantly extend the utility of existing Cre-responsive alleles.

23. Genome engineering combining CRISPR with the best of the rest

A. Francis Stewart

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Sequence-specific cleavage of the genome with designer nucleases is fundamentally changing genome engineering. The advent of Cas9/CRISPR has further simplified the technology and many genomes are now accessible for precise mutagenesis. Straightforward mutagenesis by site-directed damage is now achievable in any genome where microinjection can be used to access the zygotic genome. Similarly site-directed point mutations can be inserted at workable frequencies by co-injection of an oligonucleotide for incorporation by homologous recombination at the cleavage site. Cas9 also raises the possibility that the advanced engineering strategies hitherto limited to the mouse genome can be widely applied. Furthermore, Cas9 may facilitate formerly difficult exercises such as large insertions/replacements.

We are working to combine Cas9 with two advanced genome engineering applications—conditional mutagenesis and humanizations. Conditional mutagenesis employing site-specific recombinases is a remarkably powerful aspect of mouse genetics, and the methodology to establish conditional alleles has been applied at high-throughput utilizing recombineering pipelines (1). For Cas9-assisted targeting, we have recently developed a one-step recombineering method to make short-arm conditional targeting constructs. In addition to the same knockout first allele design that is employed by the International Knockout Mouse Consortium (1), we are also implementing conditional mutagenesis based on the auxin degron. This degron utilizes a protein tag that conveys degradation upon binding of a small auxin ligand. Auxin degron alleles are established by short-arm knockin targeting assisted by Cas9 cleavage near either the initiating methionine or stop codons of the target gene.

Concomitant with its remarkable properties, Cas9 has been reported to also provoke unwanted cleavage at off-target sites. Concerns about off-target mutagenesis require one of two types of control. Either two independent founding mutations are examined or the mutation is rescued by a transgene. We recommend rescue using BAC transgenes preferably integrated by transposition, which ensures full-length, single-copy insertions at genomic sites that are easy to identify by splinkerette sequencing (2). Rescue by BAC transposons also offers a flexible platform for the evaluation of target gene mutations that is less work than making each mutation by Cas9 *de novo*.

1. Skarnes et al. (2011) A conditional knockout resource for genome-wide analysis of mouse gene function. Nature 474, 337–42.
2. Rostovskaya et al. (2013) Transposon-mediated BAC transgenesis via pronuclear injection of mouse zygotes. Genesis 51, 135–141.

24. The zebrafish molecular genetics programme training toolbox

Stephen Ekker

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The firehose of data that is modern human genetics biology generates a flood of data and at the level of the individual patient. How to interpret that information for optimal prevention and treatment is a major next hurdle in this era of personalized or precision medicine. With the advent of powerful new tools, the zebrafish has the potential for diverse genetic and behavioural studies to assign biological significance to critical sequence variations. From morpholinos to gene-breaking transposons to custom nucleases, the zebrafish field is now established for core testing of nuclearly encoded genes. We have generated a publicly available collection of molecularly characterized and revertible mutant zebrafish gene-break transposon lines at the Mayo Clinic and in collaboration with labs around the world as a part of the International Protein Trap Consortium. We currently have over 700 lines, with 1,000+ more in process (up-to-date info at zfishbook.org). New, tissue-specific Cre lines have been recently reporting, opening the door to conditional somatic gene testing in the zebrafish. Custom restriction endonucleases offer a complementary approach, targeted genome modification. We use the highly active GoldyTALEN system to conduct genome-editing applications in zebrafish, including the introduction of small changes suitable for modelling human disease using the zebrafish. We generated a web-based TALEN design software (talendesign.org) as well as a follow-up Golden Gate TALEN assembly toolbox. We are deploying TALEN-based targeting to address patient-based sequence variation questions at the Mayo Clinic, using the zebrafish as the pioneering in vivo model system.

25. The *Tol2* transposable element and its applications to genetic studies in vertebrates

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Tol2 is a transposable element identified from the genome of the Japanese medaka fish. We identified an autonomous member of the *To2* transposon that encodes a gene for a

functional transposase, and also characterized the minimal *cis*-sequence that is essential for transposition. These enabled us to apply the *Tol2* transposition system to genetic studies in vertebrates, including mice, chicken, frog and zebrafish.
Zebrafish has been a powerful model vertebrate amenable to genetic studies. We developed a transgenesis method using the *Tol2* transposition system in zebrafish, which is highly efficient. Furthermore, we developed BAC transgensis, gene trap and enhancer trap methods and the Gal4–UAS method using *Tol2*. By employing these methods, we have performed large-scale genetic screens and generated more than 1,000 transgenic lines that expressed Gal4FF, an engineered Gal4 transcription activator, in specific cells, tissues and organs. By crossing these fish with UAS reporter and effector fish, proliferation, differentiation and migration of the Gal4FF-expressing cells can be visualized and manipulated.

We aimed to apply these methods to the study of functional neural circuits that control larval and adult fish behaviours. First, we constructed UAS effector fish carrying improved versions of GCaMPs, a DNA-encoded calcium indicator to visualize the neuronal activity during behaviours. We expressed GCaMP in various brain regions via the Gal4–UAS system, and successfully imaged the brain activity during fish's behaviours in real time. Second, we constructed UAS effector fish carrying the tetanus or botulinum neurotoxin gene downstream of UAS to inhibit the activity of Gal4-expressing neurons. By using this system, we inhibited the activity of neurons that are essential for fear conditioning in zebrafish and proposed that it may be a functional homolog of the mammalian amygdala.

Thus, the transgenic fish generated via the *Tol2*-transposon-mediated genetic methods are useful for the study of developmental biology, organogenesis and neuroscience.

26. Targeted genome and epigenome editing using CRISPR–Cas and TALE technologies

Keith Joung

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Targeted genome and epigenome-editing technologies have recently emerged as important tools for modifying a wide variety of cell types and organisms. In this talk, I will summarize our recent efforts on the clustered regularly interspaced short palindromic repeat (CRISPR) RNA-guided nucleases, an important platform for introducing targeted genome sequence alterations. I will describe our next-generation CRISPR-based platforms that exhibit reduced off-target mutation frequencies relative to the original first-generation platform. I will also present our work on new technologies for modifying specific epigenomic marks on histones and DNA and show that these targeted modifiers can be used to induce alterations in endogenous human gene expression. Taken together, these methodologies provide important tools for modifying whole organisms and for understanding human biology.

27. A Novel WT1 response to notochord injury and vertebra development in zebrafish

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Wilms' Tumour (WT) 1 is a master transcription factor of mesenchyme-derived tissues, with critical roles in development, regeneration, homeostasis and cancer. Here, we discover a novel subpopulation of wt1-expressing cells following injury of the zebrafish notochord. The notochord is the axial skeleton of embryonic chordates that later becomes ossified to form the vertebrae and contributes to the intervertebral discs. Using a fine needle to induce precise damage, we discover local upregulation of wt1 expression follow notochord injury in living zebrafish. Time lapse imaging reveals loss of sagff214a:gfp-expressing vacuolated notochord cells at the injury site. Instead, we find wt1b:gfp cells are col2a1a:dsRED-expressing notochord sheath cells that migrate into the damaged area to replace absent vacuolated cells, thereby sealing the injury site. Cartilage forms at the injury site, followed by recruitment of entpd5:gfp expressing osteoblasts. Bone mineralization continues to be associated with wt1b:gfp cells during vertebra formation and is associated with the ecoptic vertebra formation. We are currently perfoming lineage tracing analysis to determine the cellular origin of the ectopic vertebra. In summary, transgenic technologies in zebrafish have enabled the visualization of a novel process in living vertebrates that may have important implications for vertebrae injury and repair.

28. Breeding the unknown: the benefits and problems of creating new GA lines?

Sara Wells

MRC Harwell, OXON, UK

The last few years have seen many technical changes, advances in scientific knowledge and a more intense scrutiny of animal research than ever before. Mouse models for the study of human disease have become ever more sophisticated, as researchers embrace technologies such as conditional transgenesis, RGEN and whole-genome sequencing.

More than ever, there is a desire to investigate the consequences of combinations of genetic alterations within a single animal. Indeed, mouse lines carrying altered alleles of several different genes have already been heralded as more appropriate models for the study of specific, complex diseases such as Alzheimer's and are replacing strains with monogenic alterations. Furthermore, the importance of the genetic integrity of mice used in research should be a priority for all researchers. The need to work on coisogenic or congenic backgrounds of mice, avoiding mixed and undefined genomes,

has recently been a driving theme for many. The mouse community in general is recognising the importance of controlling and standardising mouse strains as a key factor in increasing reproducibility.

Crossing strains carrying different genetic alterations or indeed simply backcrossing an established transgene to a different genetic background may not, however, be as straightforward as mating two strains together. The crossing of two different mouse lines does not only produce different combinations of transgenic alleles, but potentially an entirely novel and different mixture of all the genes in the respective background strains. These types of crosses may produce unexpected and undesirable adverse effects. These may range from complete non-viability or infertility to subtle phenotypic traits, possibly unrelated to the system being investigates. Identifying early indicators of when a combination of genes may be detrimental is pivot for reducing the numbers of animals bred, refining animal care and experimentation and enhancing the scientific outcome.

29. 'Bred but not used', a subject of debate and policy making in the Netherlands

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October 2013 in international expert workshop was held in the Netherlands to discuss the number of animals produced in GA breeding programmes that are subsequently not used in animal experiments. These include animals that are not suitable because of their genetic makeup, age and/or sex to name the most prominent reasons. In the Netherlands, the number of animals 'bred but not used' have increased steadily over the past 5 years. In 2012, their numbers reached to 524,735 very close to the number of animals used in procedures which amounted to 579,338 that year. The figures have raised concern with the public at large and the Dutch authorities. Therefore, the authorities invited twenty-two international experts on 3Rs, animal welfare, ethics, colony management, molecular biology and laboratory animal science to discuss trends and possible actions. During the 2 days of intense discussions, the following themes were addressed: morality and ethics; management and technology; education, training and communication; and alternatives. The deliverables of the workshop included recommendations to the authorities. These were presented in a report to the authorities. The chairs of the workshop presented these to the public at large during a public meeting organised by the ministry. Since then the debate has shifted to parliament where the minister presented her action plan 'Animal testing and alternatives' which included the full workshop report including recommendations.

30. Transgenic tools to reduce animal numbers

Peter Hohenstein

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After declining for many years, the last few years have seen a steady increase in the numbers of animals used in research. For ethical, financial and practical reasons, this is an undesirable development. Detailed analysis of the numbers shows this increase can for a big part be attributed to the breeding of genetically modified mice. Mendelian inheritance of alleles when combining multiple alleles through breeding will generate a surplus of animals that is 'bred but not used' and that increases with the complexity of the crosses. It is important to understand the reasons for this increase and try to develop solutions that will not affect scientific research. Recent technological developments in the transgenesis field, such as gene-editing technology (CRISPRs/TALENs/ZFNs), iPS cells and improved culture methods to make and grow embryonic stem cells, are rapidly extending the possibilities of many model organisms for genetic analyses. Care should be taken that this breeding surplus problem is not extended to these species as well. At the same time, transgenic (and other) tools could be used to minimize the surplus breeding. I will discuss ongoing projects to develop these sorts of transgenic tools in mice and what additional ways could be available to improve the efficiency with which animals are being used.

31. Imaging cellular dynamics and fate in the early mouse embryo

Kat Hadjantonakis, Manuel Viotti, Sonja Nowotschin

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Gastrulation is a paradigm for the coupling of cell fate specification and tissue morphogenesis. In the mouse embryo, gastrulation transforms a cup-\ structure comprising two tissue layers (epiblast and visceral endoderm) into one comprising three tissue layers—the definitive germ layers—ectoderm, mesoderm and gut endoderm. By exploiting transgenic reporter lines with high-resolution imaging approaches, we are investigating the cellular dynamics that drive the emergence of the gut endoderm, the precursor tissue of the respiratory and digestive tracts and associated organs. Our transgenic imaging studies have revealed that morphogenesis of gut endoderm in the mouse embryo involves a dynamic widespread intercalation between two-cell populations, definitive endoderm and visceral endoderm. This morphogenetic event results in the formation of an epithelium on the surface of the embryo, comprising cells of two distinct origins. By combining imaging with the analysis of mutants in which gut endoderm morphogenesis is perturbed, we are developing a mechanistic understanding of the dynamic cell behaviours regulating this process. Recent progress will be presented.

32. The amazing opportunities from integrating optoacoustic and transgenic technology

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Optical imaging is unequivocally the most versatile and widely used visualization modality in the life sciences. Yet, it is

significantly limited by photon scattering, which complicates imaging beyond a few hundred microns.

For the past few years, however, there has been an emergence of powerful new optical imaging methods that can offer high-resolution imaging beyond the penetration limits of microscopic methods. These methods can prove essential in cancer research. Of particular importance is the development of multi-spectral optoacoustic tomography (MSOT) that brings unprecedented optical imaging performance in visualizing anatomical, physiological and molecular imaging biomarkers. Some of the attractive features of the method are the ability to offer 10–100 microns resolution through several millimetres to centimetres of tissue and real-time imaging.

In parallel, we have now achieved the clinical translation of targeted fluorescent probes, which opens new ways in the interventional detection of cancer in surgical and endoscopic optical molecular imaging.

This talk describes current progress with methods and applications for in vivo optical and optoacoustic imaging in cancer and outlines how new optoacoustic and fluorescence imaging concepts are necessary for accurate and quantitative molecular investigations in tissues.

33. Development and application of CRISPR–Cas9 for genome editing

Feng Zhang

The Broad Institute, Cambridge, MA, USA

The Cas9 endonuclease from the microbial adaptive immune system CRISPR can be easily programmed to bind or cleave specific DNA sequence using a short RNA guide. Cas9 is enabling the generation of more realistic disease models and is broadening the number of genetically tractable organisms that can be used to study a variety of biological processes. The Cas9 nuclease can also be modified to modulate transcription, alter epigenetic states and track the dynamics of chromatin in living cells. In this presentation, we will look at the latest developments and applications of the Cas9 nuclease for understanding the function of the mammalian genome. We will also look at the ongoing challenges as well as future prospects of the technology.

34. Interdependence of cellular networks ensures scaling of cell types during cerebellum development

Alexandra Joyner, Ryan Willet, Alexander Wojcinski

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During development of the brain, the different neurons and glia that make up each neural circuit must be produced in the correct proportions (scaled) in order for each circuit to function properly. In addition, the neurons must migrate to their correct positions and choose their appropriate synaptic partners. The cerebellum is an ideal brain structure for determining how scaling is achieved during development, as it has a complex foliated structure, yet a simple layered cytoarchitecture with only three major neuron types and several interneuron subtypes and glia. The cerebellum is not only critical for skilled motor performance, but also contributes to cognitive and social functions through connections with the neocortex (forebrain). Accordingly, the cerebellum is associated with many debilitating developmental diseases including autism. We have used the engrailed homeobox genes (*En1/2*) and the sonic hedgehog (SHH) pathway that signals through the GLI transcription factors as entry points for studying cerebellum development. I will described a series of experiments combining several conditional knockout approaches, mosaic mutant analysis with spatial and temporal control of recombination (MASTR) and genetic inducible fate mapping (GIFM) that have revealed insights into how the different neurons/glia are scaled during cerebellum development, and the cellular basis of regeneration of one-cell type in response to genetically encoded injury.

TT2014 Submitted Abstracts (Posters)

35. An archive of embryonic stem cells with complex genotypes for fast generation of new mouse models of cancer

Jessica Del Bravo, Rahmen Bin Ali, Colin Pritchard, Tanya M Braumuller, Martine H van Miltenburg, Linda Henneman, Ewa M Michalak, Paul Krimpenfort, Anton Berns, Jos Jonkers, Ivo J Huijbers

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The Netherlands Cancer Institute (NKI) has created a unique archive of embryonic stem cells (ESCs) derived from validated genetically engineered mouse models (GEMMs) of cancer (Huijbers et al., EMBO Molecular Medicine 2014). These GEMM-ESCs, with often multiple modified alleles, form the basis for further genetic engineering either by Flp recombinase-mediated integration, gene targeting or CRISPR/Cas9 to allow for the evaluation of altered target gene expression in a spontaneous tumour model. The culture and manipulation of GEMM-ESC clones is performed entirely under feeder- and serum-free conditions using the defined N2B27 medium with LIF and the two inhibitors (2i), CHIR99021 and PD0325901, as originally described by the group of Austin Smith, Cambridge, UK. Once the desired genetic alteration is introduced in a GEMM-ESC clone, chimeric mice can be produced using the classic ESC injection protocols that are routinely applied in transgenic facilities. Interestingly, the chimeric mice can be directly used to monitor tumour development as they contain the same genetic alterations as the original GEMM including the altered target gene expression. This GEMM-ESC approach allows for rapid and scalable target gene validation in vivo. The GEMM-ESC archive is housed at the Mouse Clinic for Cancer and Aging (MCCA) in Amsterdam, the Netherlands, and the archive is hosted at www.infrafrontier.eu and contains several mouse models of cancer. New GEMM-ESC clones are routinely derived and validated.

36. Developmental studies for therapeutic approaches using endothelial cells derived from mouse embryonic stem cells

Anita Golec[1], Sven Becker[1], Rory Morty[1,2], Werner Seeger[1,2], Robert Voswinckel[1,2]

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Underlying principles: Endothelial cell structure and functional integrity are essential in the maintenance of the vessel wall and its circulatory function. Endothelial cell injury, activation or dysfunction is a feature of many pathologic states, just to mention inflammation or effects on the vascular tone. Regenerative medicine, including cell replacement therapy, is a promising alternative for disease treatment. The application of in vitro-generated (and if required) genetically amended cells could help to recover the function of damaged parts of the specific organ. One of the approaches is to establish or restore normal function of affected endothelial cells by therapies based on embryonic stem cells (ES). However, generation of these cells is ethically debatable. Development of induced pluripotent stem cells (iPS)—from somatic cells—emerged as a solution. This enables the generation of patient- and disease-specific iPS cells, which may produce therapeutic cell populations without immune rejection and moral dispute.
Methods: To investigate the sites of integration of ES cell-derived endothelial (progenitor) cells, we generated new cell lines from murine ES cells using lentiviral (LV) transduction. In order to achieve our aim, we decided to test different promoter-resistance-gene combinations. Therefore, a set of vectors containing murine promoters: VE-Cadherin or VEGFR2 in connection with GFP and antibiotic resistance genes: hygromycin B, neomycin and puromycin had been produced and validated.
Results: We were able to generate and screen a cell line within a few weeks. The newly established cell lines are suitable for monitoring of endothelial differentiation and selection by means of antibiotic resistance.
Conclusion: Previous experiments demonstrated that for an effective antibiotic selection of desired cell type; further investigations are required. The production of murine iPS cell lines, containing abovementioned transgenes, is a subject of ongoing work. Carefully chosen murine endothelial cell subsets will be used for in vivo studies in tumour angiogenesis model. It is essential in our search for the therapeutic agents to use animal models.

37. Functional hepatocytes differentiation from human embryonic stem cells based on gene-targeting technology

Ming Yin, Huan Yang, Dexue Li, Yan Shi

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Human hepatocytes are invaluable tools for the evaluation of drug absorption, distribution, metabolism and excretion/toxicity (ADME/Tox) ability in the liver, which has great potential for application in drug development. However, one major challenge for the pharmaceutical application of these cells is how to regulate and maintain their metabolic functions, which could be impaired dramatically when culture in vitro. Currently reliable tools to identify signalling pathways regulating the metabolic functions of human hepatocytes in vitro are still lacking. The other obstacle is the shortage of donor human hepatocytes. Surrogates such as other species hepatocytes or cell lines differentiated from human hepatocellular carcinoma are not very representative system for recapitulating hepatocyte function and response to xenobiotics. Embryonic stem cells (ESCs) essentially proliferate infinitely and maintain the ability to differentiate into a variety of tissue cells, including hepatocytes. Thus, ES cells can serve as an inexhaustible cell source for hepatocyte.

CYP3A4 is the major cytochrome P450 present in adult human liver and is involved in the metabolism of over 50 % of therapeutic compounds currently in use. In this study, we report that transcription activation-like effector nuclease (TALEN)-based gene targeting and directed hepatic differentiation of human embryonic stem cells (hES cells) could be combined to identify candidates promoting expression of CYP3A4. The CYP3A4 gene was efficiently targeted using TALEN, resulting in expression of green fluorescent protein (GFP) under the control of the endogenous CYP3A4 promoter. The CYP3A4: EGFP knockin hES cell line was further differentiated into human hepatocytes in vitro, GFP as a reporter of CYP3A4 enabled the identification of epidermal growth factor (EGF) as a signalling protein that efficiently promotes and maintains CYP3A4 expression in human hepatocytes in vitro. The addition of EGF at the differentiated hepatic progenitor stage increased the percentage of $CYP3A4^{+}$ hepatocytes up to 40.2 %. The differentiated hepatocytes were transplanted into URG(Tet-uPA/$Rag2^{-/-}$/$Il2rg^{-/-}$) mice, which has controllable Alb-uPA damage liver; after 2 weeks, human serum ALBUMIN was detected in URG mice, which shows the derived hepatocytes' function of liver reconstitution and humanization.

In conclusion, our study developed an intuitive, reliable tool to identify matured and metabolic functional human hepatocytes system in vitro, and use this system further to identify EGF is a novel regulator that is critical for CYP3A4 gene expression in human hepatocytes. And the combination of TALEN-based gene targeting and directed hepatic differentiation of hES cells would potentially provide useful tools for generating human hepatocytes with stable metabolic functions in vitro.

38. An inducible shRNA approach to mTORC2 biology in ES cells

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TOR is a highly conserved kinase exerting a wide number of functions in all the organisms ranging from yeast to mammals. In mammalian cells, mTOR can be found as a part of two complexes: mTORC1 and mTORC2. While the functions of the first complex are currently well understood, mTORC2 pathway represents an important and yet poorly characterized signalling way in both normal and tumour cells. The complex has been linked to several cellular and tissue specific processes, such as cytoskeleton reorganization, migration and metastasis.

However, the early embryonic mortality of mTORC2 null mice and the lack of pathway-specific inhibitors complicate greatly the work necessary to dissect the complex's activity in-vivo. Here we propose an inducible shRNA-based approach aimed to unravel the functions of mTORC2 in both normal and tumour environment. We used the modified Col1a1 locus as an entering site for a Doxycycline-inducible shRNA, targeting the rapamicyn insensitive companion of mTOR (Rictor). Rictor knock-down results in the specific inhibition of the formation of mTOR complex 2. The Tet-ON/OFF system allows us to modulate the expression of the transgene in a controllable and reversible way, resulting in a better control of the trans-regulation of the complex. A Rictor inducible knock-down mouse model will help us to overcome the hurdles connected to the study of the pathway in-vivo and will ultimately contribute to shed a light on the activities of mTORC2 in established murine tumour models. Using both general and tissue specific expression of the transgene, the model will also allow us to characterize the physiologic functions of the complex in the adult organism and it may lead to a better understanding of the role of mTORC2 in embryogenesis and tissue differentiation.

39. EZ mouse model method for generating entirely ES cell-derived mice using C57BL/6 and GEMMs-ES cells by tetraploid complementation

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The C57BL/6 (B6) mouse strain is the most widely used strain for transgenesis and mutagenesis. However, gene targeting in B6 embryonic stem (ES) cells is not very efficient and therefore is currently done using 129SvE and F1 hybrid ES cells, which then require extensive backcrosses to B6 mice to establish a pure genetic background. Typically, ten backcrosses are needed, a process that can take up to 2 years and is therefore costly and inefficient. To make the generation of mouse models more rapid and obtain high throughput, we have developed 'EZ mouse model method' in which gene-targeted C57BL/6 ES cells are microinjected into tetraploid blastocysts, and reexpanded blastocyst embryos were transferred into pseudopregnant female for generating heterozygotes that are entired ES cell derived from microinjected ES cells. This method allows to generate entirely ES cell-derived mice (ES mice) directly from C57BL/6 inbred, F1 hybrid and genetically engineered mouse models rederived ES cell lines (GEMMs-ESC). This EZ mouse model method have a number of advantages over those already available. First, they can eliminate the chimeric mice production, and does not require the germline transmission test and genotyping, which means first, maintaining a large breeding colony can reduce both costs and number of animals needed each experiment. Second, this method can produce a high percentage of ES mice that are entirely injected ES cells. Third, this method can be used to rapidly generate mice that are homozygous for the gene of interest. Forth, this method can be used for rapid generation of complex mutlialleic ES mice without future intercrossing for in vivo validation of cancer gene. These advantages indicate that these EZ mouse model method facilitate the rapid generation of mouse models in a cost-effective and efficient manner.

40. Comparison of aggregation and microinjection using outbred ICR (CD-1) morulae for generation of germline chimeras from C57BL/6N ES cells

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Generation of chimeras from genetically modified embryonic stem cells (ESC) from publicly available libraries such as the International Knockout Mouse Consortium (IKMC) that is one of the services offered at the TCP Transgenic Core. Together with the Canadian Mouse Mutant Repository (CMMR) and the Canadian contribution to the IKMC ESC library, we are integral partners in the Genome Canada-funded NorCOMM2 and the NIH-funded KOMP2-DTCC projects. These projects are part of the International Mouse Phenotyping Consortium (IMPC), a collaboration of 13 international production and phenotyping centres, that has generated >2,000 knockout mouselines and aims to produce and phenotype 5,000 mouselines by the end of 2016. While Cas9 RNA-guided nuclease (Cas9-RGN) has offered another method of mouse genome-editing, standardized alleles enable comparison of phentoypes and reproducibility which will address the recent criticism of mouse models' studies. The IKMC resource provides a library of single-gene knockouts for >15,000 genes, each with a lacZ reporter and many with elements to convert to conditional alleles. In order to take advantage of this resource and to fully realize its utility, it is necessary to have efficient methods for generation of germline chimeras.

We have been successfully using outbred morula aggregation for years and more recently reported combining this expertise with culture of C57BL/6N ESC in medium containing Knockout Serum Replacement™ (KOSR) and 2i inhibitors: MEK (PD0325901) and GSK3 (CHIR99021). >80 % of the clones produced chimeras with >50 % ESC-derived coat colour contribution. However, germline transmission (GLT) rates vary between parental ESC lines: from ~30 % for JM8A1.N3 and JM8A3.N1 to ~60 % for VGB6 and ~75 % for C2. We compared microinjection with aggregation using outbred morulae as host embryos for >600 ESC clones. No significant difference in GLT rates was observed between methods for JM8.F6, JM8A1.N3 or JM8A3.N1 clones. Aggregation was more efficient than microinjection for VGB6 (67 vs. 57 %) and JM8.N4 (62 vs. 20 %) clones. The methods were not extensively compared for C2 ESC as the vast majority of chimeras were produced by aggregation with a 77 % GLT rate. Overall, 48 % of aggregated and 37 % of injected clones resulted in GLT. Taken together, these data indicate that the use of KOSR + 2i culture medium prior to aggregation or microinjection of ICR(CD-1) morulae results in efficient GLT from most parental ESC lines. For centres using microinjection, outbred ICR (CD-1) 8-cell stage morulae can provide an economical alternative to C57BL/6-Tyrc or BALB/c blastocysts as host embryos for C57BL/6 ESC.

41. Production of chimeric common marmosets

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Introduction: The common marmoset (*Callithrix jacchus*) is the only non-human primate that can be used to produce genetically modified animals with germline transmission of transgenes. Although several species of non-human primate stem cell, including marmoset embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have been established, their pluripotency has not been confirmed. Indeed, chimeric non-human primates could not be produced using ESCs (Tachibana M et al. 2012) and target gene knockout non-human primates have not been generated by pluripotent cell blastocyst injection. In this study, marmoset naïve-like pluripotent cells, named induced pluripotent ESCs (iPESCs), were established by introducing six pluripotency-related transcription factors into marmoset ESCs, and chimeric marmoset production experiments were performed.

Materials and methods: Marmoset iPESCs were established by transduction of six doxycycline (Dox)-inducible transcription factors—Oct3/4, Klf4, Sox2, Lyn28, Nanog and c-Myc—together with Kusabira-Orange (KO) (as a fluorescent protein marker) into marmoset ESCs. These iPESCs were cultured with marmoset iPESC medium (knockout DMEM, 20 % KSR, 10 ng/ml LIF, 1 ng/ml Dox) on an extracellular matrix (GELTLEX). For production of chimeric marmoset embryos, iPESCs expressing KO were injected into the perivitelline space in pre-compacted marmoset host embryos using a piezo drive. The chimeric embryos were cultured for 2 days and transferred non-surgically to surrogate mothers. Pregnancy was confirmed in the recipients by plasma progesterone measurements and ultrasonography of the uterus.

Results and conclusion: The established iPESCs had several naïve cell characteristics, such as LIF responsiveness, single-cell tolerance to trypsin–EDTA, X chromosome activation and feeder-free survival. Five chimeric embryos were injected with iPESCs and transferred to five surrogate mothers at 2 days after injection. While three surrogate mothers became pregnant, two of them aborted during the early and middle stages of pregnancy; the remaining recipient delivered a neonate. To validate the chimera production ability of the iPESCs, we performed genomic PCR and RT-PCR using placental tissue and neonate blood and hair samples to detect KO. The KO gene was detected in the placenta but not in the neonate tissues, suggesting that iPESCs can contribute to the placenta but not the embryo. This result was different to findings for mouse ESCs, which do not contribute to the placenta. To produce chimeric marmosets, unlike in rodents, pluripotent cells, LIF responsiveness, single-cell tolerance and X chromosome activation were not sufficient. Further molecular mechanisms must be elucidated.

42. Effect of FSH storage and treatment dose for marmoset oocyte collection

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The common marmoset (*Callithrix jacchus*) is the only non-human primate that can produce a transgenic model with transgene germline transmission. In transgenic marmoset production, adult female marmoset ovaries were stimulated with follicle-stimulating hormone (FSH) to collect oocytes. In this study, we examined the most suitable FSH treatment for marmoset oocyte collection. To determine the appropriate FSH storage temperature, storage temperature of a 50 IU FSH solution was compared at 4 °C, −20 °C, and fresh non-storage sample as control. The FSH solutions were stored at each temperature for 3 days to 1 week, and then, the FSH concentration was measured in each solution with an AIA-900 enzyme immune assay system. To validate frozen-thawed FSH activity, FSH was administered to marmosets at 25 or 50 IU/head for 9 days to stimulate follicular maturation. The day after the last FSH treatment, human chorionic gonadotropin (hCG) (75 IU/head) was injected intramuscularly and follicular aspiration was performed at 20 h post-hCG injection. The germinal vesicle stage pre-matured oocytes were cultured for 24 h in POM medium for in vitro maturation (IVM). The metaphase II stage oocytes were incubated for 16 h in TYH medium for in vitro fertilization (IVF). The embryos were cultured in ISM1 medium until the 8-cell stage and were then cultured in Blastassist medium supplemented with 10 % foetal bovine serum and 2 mM L-glutamine until the blastocyst stage. As a result, FSH activity values after thawed samples were 8.8 ± 1 m IU/ml at 4 °C and 126.5 ± 14.2 m IU/ml at −20 °C. The fresh control was 112.5 ± 13.9 m IU/ml. These results show that −20 °C was suitable for storing the FSH solution. The average numbers of collected oocytes were 19 ± 13.9 in the 50 IU FSH/head group and 22 ± 12.7 in the 25 IU FSH/head group. Ovulation was observed frequently in the 50 IU FSH/head group when follicular aspiration was performed. The oocyte maturation rates were 65.9 % (25 IU/head) and 64.3 % (50 IU/head). The developmental rates to the 2-cell stage were 90.3 % at 25 IU/head and 74.5 % at 50 IU/head ($P < 0.05$, χ^2 test). The developmental rates to the blastocyst stage were 13.1 % (25 IU/head) and 9.8 % (50 IU/head). These results indicate that the most effective FSH dose to stimulate marmoset follicles was 25 IU/head. This FSH administration protocol will contribute to appropriate animal experiments, reduce the number of animals used and refine the method for '3R' compliance.

43. Comparative analysis of germline transmission from morulae cultured to blastocyst stage in KSOM or M15 and injected with EUCOMM/KOMP clones

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M15 and KSOM media were used to culture C57BL/6J-Tyrc-Brd Albino moruae from natural mating to the blastocyst stage for injection of ES clones from the EUCOMM/KOMP resource. Here, we looked at how different culture conditions for morulae influenced the outcome of key parameters such as pregnancy, birth rate, chimera formation, numbers of heterozygotes in litters and germline transmission rates. These results were also compared to the parameters obtained from those embryos that were already at the blastocyst stage of development without further culturing. Here, we report our results that show in vitro cultured moruale that develop to blastocyst may be used successfully for the germline transmission from EUCOMM/KOMP clones and that media and culture conditions when coupled with ES cell number injected may influence outcomes and therefore choices for culturing conditions in future experiments.

44. A new method to pick targeted ES cell clones more efficiently

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One way to generate a KO mouse line is the usage of genetically modified ES cells. In brief: the ES cells are electroporated with a DNA construct and grown under selective conditions to enrich the clones with the desired genetic modification. The surviving clones are then separated manually to genotype them. The individual clones have to be scraped from the plate (picked) in PBS and the cells afterwards separated in trypsin–EDTA solution, both for 15–30 min minimum.

We have developed a new, fast and easy method to isolate separate ES cell clones cultivated on feeders. The ES cell colonies are trypsinized on the 10-cm dish for 1 min only to detach the clones from the plate thereby producing a mixture of single colonies. The trypsin is inactivated with medium, and the intact colonies are then collected in culture medium instead of PBS in a very gentle, efficient and fast way. Since a suspension of single colonies is prepared, it is easy to extract and transfer them to a 96-well plate with a mouth pipette. At the end, every well can easily be checked for the presence of a single clone under the microscope. To generate single-cell suspensions, the isolated clones are trypsinized for 7 min and diluted 1:3 on the next day.

With practice, the whole procedure takes half the time of the classical way of picking clones and the incubation time under inappropriate conditions is reduced to a minimum.

To address the problem of mixed clones after picking, we will present data where we co-cultivated lacZ-positive and lacZ-negative ES cells in the picking plate, isolated them with the method described and check for mixed clones after picking with our new method.

45. IKMC EC cell resources: conversion into mice for the IMPC phenotyping pipelines

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ESC (embryonic stem cells) to mouse conversion has become a major component of the IMPC (International Mouse Phenotyping Consortium) project that brings together centres across the world in an effort to generate and phenotype mouse null mutants for every gene in the genome. The generation of these mouse mutants relies on the use of targeted ES cells from various production centres also linked to this significant international effort.

At Harwell, we have developed a pipeline system to allow for the prompt and efficient assessment of the ESC material received and the timely use for microinjection and generation of mutant mice that feed the phenotyping pipeline.

46. Derivation of haploid ES cells from genetically modified mice of interest in cancer research

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Mouse embryonic stem (ES) cells represent an extremely versatile biological system: they are not only the vehicle for introducing targeted mutations in the mouse germline, but in addition, they constitute an essential tool for studying the molecular basis of pluripotency, self-renewal and cell differentiation. Moreover, genome-wide genetic screenings in ES cells allow exploring the genetic basis of human disease and the functional annotation of the mammalian genome. However, the diploid karyotype of ES cells represents a limitation for genetic screenings, especially for the identification of recessive mutations. Several groups have shown that it is possible to derive haploid ES cells from mouse parthenogenetic and androgenetic embryos, expanding the possibilities of genetic screenings in mammals. We aim to apply the potential of genome-wide screenings in mouse haploid ES cells to cancer research and in particular to preclinical drug development.

In the CNIO, we have generated a large collection of mouse models of cancer as well as genetically modified mouse lines carrying targeted alleles of interest for cancer research, from

which we aim to establish the first collection of haploid ES cells and mutant ES cell libraries for genetic screenings in the context of cancer. With these lines, we aim to combine the power of targeted conditional mutations in cancer-related genes with the potential of genetic screenings using the haploid genome. The properties of the ES cells will allow us also setting up genetic screenings for ES cell functional assays of differentiation, pluripotency, in vivo growth/differentiation, etc., which will expand the repertoire of applications of the haploid ES cell lines derived from this project. We will present our results on the derivation of the first haploid ES cell lines derived from gene-targeted mouse lines of relevance in cancer research.

47. The derivation of mouse embryonic stem (ES) cells in a transgenic facility: technology improvement produces science benefits

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The use of small-molecule inhibitors to maintain ES cells in the 'ground state' has in recent years made ES cell derivation fast and reliable. At the London Research Institute (LRI), ES cell derivation has developed from an occasional technique to a key provision of the Transgenic Facility yielding a service that has seen significant development in the numbers of projects requested and the uses to which the finished ES cells are applied.

To ensure the ES cells produced are productive, some form of quality control would be useful though in practice, options are limited by their usefulness and cost. Chromosome counting is one relatively easy and affordable means to 'weed out' cell lines that have low counts and may not prove productive. Performing chromosome counting revealed that the use of a MEK inhibitor (PD0325901) alone provided the highest and the more reliable chromosome counts under the conditions employed.

The initial use for the ES cells, besides of course for stock and targeting, were produced with a focus on the generation of chimeras for in vitro and in vivo assays for the study of blood vessel formation. In this way, the role of specific genes in this process could be evaluated. Further projects have sought to generate chimera or embryo cohorts for a variety of experimental purposes. Results will be shown that summarise the scientific benefit that have flowed from the development of ES cell derivation technology.

48. Ensuring the quality of ES cells injected at MRC Harwell: development and implementation of a novel ddPCR-based karyotyping screen to complement traditional gene targeting validation

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MRC Harwell, Harwell, UK

The Mary Lyon Centre (MLC) at MRC Harwell provides the global research community with a wide variety of tools and services for mouse functional genomics. As part of the International Mouse Phenotyping Consortium, MRC Harwell will convert 500 embryonic stem (ES) cells bearing targeted mutations into transgenic mice between 2012 and 2016. To ensure that researchers receive high-quality mouse models, a stringent quality control pipeline has been developed to confirm both the integrity of the gene-targeting event and the karyotypic quality of the ES cells prior to injection.

Firstly, DNA from each ES cell clone is extracted at a point of growth equivalent to microinjection and undergoes a karyotype screen using ddPCR (digital droplet PCR). We will present how this ddPCR screen was developed in-house and validated against traditional karyotype G-banding data. The screen uses TaqMan probes situated on chromosomes 1, 8, 11 and Y. Each assay is multiplexed with a control (assay against Dot1 l) to determine the copy number of each these chromosomes, enabling rapid identification of aneuploid samples that are unsuitable for injection.

Euploid clones are further expanded in order to provide DNA for long-range PCR of the target region, the product of which is sequenced, to confirm the identity of the targeted allele in the construct. Gene-specific restriction digests are performed and a non-radioactive Southern blot analysis employing universal internal probes carried out to verify that the construct has integrated correctly into the genome. These three methods of analysis ensure that only good-quality clones are passed on to the microinjection laboratory, thus optimising animal usage. Here, we present data derived from our quality control laboratory.

49. Characterization of high-quality mESC lines derived in 2i by a simplified method

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Mouse embryonic stem cells (mESC) have become an essential object of study to understand the molecular mechanisms involved in early development and to investigate new cell-based therapies. They are also a powerful tool to generate genetically modified mouse models. Traditionally, the establishment of mESC lines in culture has been an inefficient procedure. However, in the last years, the use of defined culture media and inhibitors of the main differentiation pathways (2i conditions) have largely increased the efficacy of mESC derivation. Here, we compare the efficiency of mESC derivation from different genetic backgrounds and using different culture media. To derive mESC in 2i conditions, we follow a simplified version of Nagy and Nichols' protocol (Springer, 2011), which omits the immunosurgery step but still provides a high derivation efficiency. As many uncertainties exist about the convenience of using 2i-derived mESCs routinely for chimaera generation in a transgenic mouse facility, we have characterized in detail several cell lines derived and grown in 2i conditions. We found that all of the mESC lines tested were euploid and germline competent. These cells, even at high passages, contributed to high-pigmentation-percentage chimaeras when injected into early-stage embryos. Moreover, the vast majority of chimaeras produced by multiple cell injection into 8-cell morulae were 100 % ES-derived, judged by coat colour, and all

tested chimaeras showed 100 % germline transmission. We have used these cells to generate mouse models by random transgene integration and by both homologous recombination-based and CRISPR-induced gene-targeting strategies with the same good results. The simplicity and efficiency of this derivation protocol, the feeder-free culture conditions and the high-quality of the mESC lines obtained make this powerful tool accessible and suitable to any transgenic mouse laboratory.

50. 100 % Coat-coloured chimaera generation by single es cell injection

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Murine embryonic stem cells (mESC) are defined as 'pluripotent' i.e. they can generate all cell types of the adult mouse as well as the germ cells. The gold standard assessment of pluripotency is the generation of chimaeras by reintroducing mESCs into pre-implantation embryos. Traditionally, multiple cells have been injected into blastocysts or, more recently, into morulae. The extent of chimaerism is routinely scored as the percentage of coat pigmentation, and this can vary dramatically between individual mice and experiments, reflecting the heterogeneity of mESC cultures. Here, we investigate the pluripotency of mESC derived and maintained in different conditions, using a very stringent test where single cells are injected into early embryonic stages. We have found that a single cell can give rise to 100 % coat-coloured chimaeras when injected into 2-cell embryos. In these animals, the contribution of the injected ES cell to all the main organs is comparable to that found in 100 % coat-coloured chimaeras generated by multiple cell injection. Remarkably, only serum-free culture conditions (KOSR + LIF and 2i + LIF), but not serum-containing medium, support this ability of the cells when injected individually. This work reveals that serum-free conditions enhance the clonogenicity of mESCs and describes a rigorous technical procedure to assess this ability of the cells.

51. Novel tools to discern the pluripotency gene network establishment

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Not only does the reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) by exogenous transcription factors remain highly elusive, but there is also characteristic discrepancy among the discoveries of different labs.

In order to address this, first we developed a system for a robust and reproducible systematic mechanistic analysis of reprogramming. Briefly, we generated a single targeting vector carrying both the reverse tetracycline-controlled transactivator (rtTA) and the doxycycline (Dox)-inducible reprogramming factors (2A-peptide-linked c-Myc-Klf4-Oct4-Sox2 = MKOS) for a novel genomic locus that assured optimal transgene expression. One round of targeting with this vector makes any ES cell (ESC) line a source of Dox-inducible reprogrammable cells/mouse.

Intriguingly, there is a variety of reprogramming cassettes used by different labs to perform mechanistic analysis; thus, we compared the existing cassettes, and to our surprise, they generated different number of both fully reprogrammed and partially reprogrammed colonies. Keeping that in mind, we used our novel reprogramming system to generate Nanog-GFP ESC lines with the reprogramming factors in a different order (TNG MKOS/OKMS ESCs). TNG OKMS reprogramming gave rise to $\sim$10 times more colonies upon the addition of Dox, but with poor activation of the Nanog-GFP reporter, compared to the TNG MKOS one. CD44 and ICAM1 expression, which we recently utilized to dissect the reprogramming process, along with E-cadherin staining, enabled us to identify a clear roadblock in OKMS reprogramming. Finally, RNA sequencing of these reprogramming intermediate subpopulations provided us with many candidate genes which potentially affect the pluripotency acquisition.

The tools described here enable the precise analysis of the reprogramming intermediates and will make a large contribution to reveal the molecular mechanisms for the establishment of the pluripotency gene network and to shed light to the existing conflict.

52. Characterization the developmental potential of cultured chicken primordial germ cells

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The avian embryos are widely used for studying early embryogenesis. The chicken embryos are also important model system for studying primordial germ cell (PGC) development. Researchers used the avian model to reveal the cellular origins and migratory pathways of PGCs. The most recent work focuses on the molecular mechanisms regulating early germ cell development. Avian PGCs have significant potential as a cell-based system for the study and preservation of avian germ cells. Long-term maintenance of avian PGCs in vitro has tremendous potential because it can be used to deepen our understanding of the biology of PGCs and also a promising option for gene banks and genome preservation.

Avian embryos have also proven to be useful organisms for transgenic research. PGCs can be cloned and maintain the germline competency, so they are a good source for modifying the chicken genome and producing chimeras. This provides enormous benefits in advancing animal biotechnology and aids

in the development of unique technologies for bioreactor production and experimental model development.

In our laboratory, we adapted the method of feeder-free in vitro cultivation of PGCs. We isolated the PG cells from the blood of HH16 chicken embryos and investigated the expression profile of stem cell and PGC-specific genes. cPouV, cNanog, cSox2, cGATA6, cGATA4, cCDX2, cKlf4, cDAZL, cBrachyury, cDDX4, cStra8 expression were checked in chicken embryos and cultivated PGCs. We also analysed the expression profile of stem cell-specific miR-302 cluster. miRNAs regulate the lineage-specific differentiation of cells and maintain the pluripotency in stem cells. The post transcriptional regulation through miRNAs are also important role for the control of differentiation in the chicken PGCs. Using SSEA-1 and EMA1 immunostaining, we annotated that our cultivated cells are PGC cells.

In order to check the viability and functionality of the cultured PG cells in vivo, cultivated GFP expressing PGCs were injected back to the HH16 embryos and followed their way to the developing gonads.

53. Rapid generation and screening of high-percentage mouse chimeras utilizing CRISPR modified ESCs

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Our current project combines new advances in CRISPR technology, ES cell chimera technology and targeted phenotyping to generate mouse mutants within 2 months, for a fraction of the current cost of mouse knockouts. This technology enables large numbers of disease candidate genes to be inexpensively and quickly assessed by individual labs for their relevance to human disease. The screen is not intended to define the definitive phenotype associated with candidate genes rather the screen is designed to quickly determine whether the candidate gene has disease-related function that can be further pursued in follow-up experiments with traditional knockout approaches. RFP + host embryos were used to determine the chimeric contribution of modified ESC to early developmental disorders. We determined that using RFP + host embryos facilitated rapid detection of RFP + host-derived tissue still present in high percentage of chimeric pre- and postnatal animals.

54. Identification of rabbit microrna expression profile in rabbit pluripotent stem cells

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In the context of biomedical research, precise genetic engineering in rabbits (*Oryctolagus cuniculus*) would be precious model to generate genetically defined rabbit models of human diseases. Rabbit embryonic stem cells (ESCs) would be invaluable tool for both creating second-generation transgenic models of human diseases and testing stem cell therapies for human applications.

We identified new mirna sequences in several rabbit samples sequenced by solid^{TM} System and validated them using real-time quantitative PCR and Custom TaqMan® Array Plates. Then, we aimed to compare the mirna expression patterns of rabbit samples with the read numbers of solid^{TM} sequencing. Our results underlined that solid^{TM} sequencing is really good at identifying new mirna sequences, but it could be established that the read numbers of solid^{TM} sequencing cannot be directly compared to the relative expression levels of real-time PCR analysis.

Using our new rabbit specific mirna sequences, we analysed the expression profile of pluripotency-associated mirnas in rabbit embryos and embryonic stem-like (ES-like) cells. The rabbit specific ocu-miR-302, ocu-miR-290 clusters and three homologs of human C19MC cluster (ocu-miR-512, ocu-miR-520e and ocu-miR-498) were identified in rabbit preimplantation embryos and pluripotent stem cells. The ocu-miR-302 cluster was highly similar to its human homolog, while ocu-miR-290 revealed a low level of evolutionary conservation with its mouse homologous cluster. The expression of ocu-miR-302 cluster members began at 3.5 dpc early blastocyst stage and they stayed highly expressed in rabbit pluripotent stem cells. In contrast, high expression level of ocu-miR-290 cluster members was detected during preimplantation embryonic development, but low level of expression was found in rabbit ES-like cells.

55. Czech centre for phenogenomics: new research infrastructure for transgenic mouse model production and phenotyping

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The Czech Centre for Phenogenomics (CCP), a newly set up infrastructure, is combining genetic engineering capabilities, advanced phenotyping and imaging modalities, specific pathogen-free (SPF) animal housing and husbandry, as well as cryopreservation and archiving, all in one central location—located within the new BIOCEV complex (Biotechnology and Biomedicine Center of the Academy of Sciences and Charles University in Vestec) and the hosting institution, the Institute of Molecular Genetics (IMG) in Prague.

The Czech Centre for Phenogenomics (CCP), through its memberships in INFRAFRONTIER and the International Mouse Phenotyping Consortium (IMPC), is a partner in a collective global network that aims to comprehensively and systematically analyse the effect of loss-of-function gene mutations in mice. The goal is to produce a comprehensive 'encyclopedia' of gene function that will help identify causative factors of human diseases as well as novel targets for therapeutic intervention.

Current services in model production include custom-targeted mutagenesis and transgenesis, blastocyst injection, pronuclear microinjection, sperm and embryo cryopreservation, in vitro fertilization and breeding on demand. Over 80

transgenic generation projects were completed in 2013, from both local and international customers, and this number should further increase over next few years as we build towards full functionality. Full operation of standardized phenotyping services is expected at the end of 2015, corresponding to the full functionality of the newly constructed CCP building. The new €24 million building will have a floor space of 7,200 m^2 with a maximum capacity of 12,000 cages for mice (30,000 when combined with existing capacity) and 4,000 cages for rats, and will have over 70 employees.

56. LincRNA *Pint* knockout mice display early ageing-associated phenotypes

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Large intergenic non-coding RNAs (lincRNAs) encodes for transcripts that resemble protein-coding mRNAs in structure but do not code for proteins. To elucidate the biological functions of this new class of molecules, we employed VelociGene® technology to create knockout mouse lines with a LacZ reporter for 20 selected lincRNAs. The targeted lincRNA genes showed unique and diverse lacZ expression profile in both embryos and adults, and of the 20 lincRNA genes targeted, the most remarkable expression pattern we observed was for *linc-Pint*, which exhibited an increase in the extent and intensity as the mice aged. This striking age-associated pattern prompted us to conduct a longitudinal analysis for growth and any overt signs of health abnormality. The *linc-Pint*$^{-/-}$ mice appeared healthy and normal at birth but as the mice aged, they exhibited progressive hair loss and sign of muscle weakening, severe lordokyphosis, reduced body fat, bone mineral density, and a significant decline in body weight and survival in comparison with wild-type littermates. Interestingly, *linc-Pint* heterozygous mice exhibited an intermediate or a delayed onset of phenotype. This spectrum of age-associated pathologies in the *linc-Pint* knockout mice, along with the unusual increase in gene expression with age, implies that mice may require a critical dose of *linc-Pint* for the maintenance of health and tissue function in vivo and points to potential role of LincRNA in physiological ageing process with potential implications in human diseases.

Our aim in initiating this work was not only to shed light on the functions of the 20 particular lincRNAs whose genes we chose to mutate, but also to obtain a better understanding of the general properties of lincRNAs as a class. This study reveals that lincRNAs, like proteins, serve diverse roles in development, physiology, and homeostasis. This collection could serve as a seed for a larger-scale effort to mutate many more members of the lincRNA gene family.

57. Integrating large-scale phenotyping data into the mouse genome informatics database

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For the past 25 years, the Mouse Genome Informatics database (MGI, www.informatics.jax.org) has been collecting and integrating data on mouse mutations and phenotypes. This has resulted in the creation of a dataset that contains over 52,000 mouse genotypes (alleles plus strain background) annotated to over 270,000 Mammalian Phenotype (MP) terms. Most of these data comes from published reports and direct data submissions. Frequently, these phenotype descriptions are highly detailed in a single morphological or physiological system or a few systems, but lack a breadth of coverage across all systems. Newly released phenotype data from the large-scale phenotyping projects undertaken by the International Mouse Phenotyping Consortium (IMPC) will be integrated to add increased scope to MGI's phenotype data. Of the 436 genes currently listed as completed for phenotype on the IMPC site (July 2014), over 250 already have mutant alleles with phenotype data in MGI and 34 are annotated as a model of at least one human disease. Approximately half of the genes with phenotype data have associations with no more than three of the MP morphological or physiological system header terms. An example of the benefit of the addition of breadth is seen for Mybbp1a where the sole published allele has only embryonic lethality-related phenotypes in homozygous mutant mice. The IMPC data adds information about homeostasis and hematopoietic phenotypes in heterozygous mutant mice. Conversely the power of the depth of annotation can be seen for Myo7a, where the IMPC data reports only abnormal brainstem auditory evoked potentials. The phenotype data in MGI includes extensive details on the defects in ear morphology and physiology that underlie to the hearing defects. Overall, integrating the broad phenotype data coming from the IMPC with the phenotype data already in MGI will allow users to easily access the full range of known phenotypes for mutations in a gene. The ability to compare the effects of different mutations in the same gene or the same mutation in different strain backgrounds will facilitate a more comprehensive understanding of the function and impact of the gene in the context of the whole genome. Supported by NIH Grant HG000330.

58. The INFRAFRONTIER research infrastructure and EMMA: the european mouse mutant archive

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INFRAFRONTIER aims to build a world-class research infrastructure that provides the biomedical research community with the tools needed to unravel the role of gene function in human disease with the following objectives: (1) shaping the European Research Area in the field of mouse functional genomics and thereby make an important contribution to the study of human disease; (2) setting standards for systemic phenotyping of mouse models and for archiving and distribution of mouse mutants in Europe; (3) offering highest quality services and cutting-edge technologies provided by the leading labs in Europe; and (4) disseminating knowledge by state-of-the-art training courses. The INFRAFRONTIER Research Infrastructure has currently 23 scientific partners from 15 European countries and Canada. The INFRAFRONTIER network is coordinated by Professor Martin Hrabé de Angelis who also heads the HMGU/IEG in Munich. The European Mouse Mutant Archive (EMMA) is included within INFRAFRONTIER, as a non-profit repository for the collection, archiving (via cryopreservation) and distribution of relevant mutant strains essential for basic biomedical research. The EMMA network is currently formed by a partnership of 16 nodes in 13 different countries. EMMA's primary objective is to establish and manage a unified repository for maintaining biomedically relevant mouse mutants and making them available to the scientific community. Therefore, EMMA archives mutant strains and distributes them to requesting researchers. At present, EMMA holds more than 3,000 mouse strains, corresponding to transgenic mice, different type of mutants, gene traps, knockins, knockouts and also including some targeted alleles from Deltagen, Lexicon and EUCOMM projects. EMMA's technology development programme is focusing on improving sperm cryopreservation methods by implementing CARD-University of Kumamoto protocols, setting ICSI protocols, and has explored different techniques to allow the shipment of refrigerated, unfrozen mouse embryos and frozen sperm in dry ice, without liquid nitrogen. EMMA also hosts cryopreservation courses, to promote the use and dissemination of frozen embryos and spermatozoa. All EMMA procedures and all required information to deposit or request mouse lines from EMMA are easily available through the INFRAFRONTIER web site at: www.infrafrontier.eu. EMMA is supported by the partner institutions, national research programmes and by EC FP7 Capacities Specific Programme. EMMA has already secured funding for the next 4 years (2013–2016) through the INFRAFRONTIER-I3 EC grant, where the production of new transgenic lines, associated phenotyping activities and cryopreservation/archiving tasks are jointly addressed by a larger group of partners. EMMA is a founding member of the Federation of International Mouse Resources (FIMRe).

59. Efficient shipment of refrigerated mouse embryos across Europe

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The distribution of mouse mutant lines between distant research institutions is normally achieved through the shipment of frozen mouse embryos, cryopreserved at archives, and using liquid nitrogen dry shippers. This routine and robust procedure is, however, expensive and requires special containers for shipment and the use of knowledgeable courier services that are used to handle this precious and fragile material. More importantly, the recipient institution must have the required skills to reproducibly thaw the frozen mouse embryos they receive. Therefore, the shipment of unfrozen mouse embryos

that are ready to be transferred to suitable recipient pseudo-pregnant females upon arrival would be a preferable method. At EMMA, within the INFRAFRONTIER-I3 EU Project, we decided to explore the use of the CARD cold transportation kit (Kyudo Co., Ltd./Cosmo Bio Co., Ltd.) to ship refrigerated mouse embryos across Europe, as a cheaper and simpler shipment procedure. We set out to distribute 2-cell IVF-derived wild-type mouse embryos of two different genetic backgrounds, C57BL/6J and albino outbred Crl:CD-1(ICR), recently thawed from the Spanish EMMA node at CNB-CSIC in Madrid to seven other EMMA nodes, located in Orleans (France), Harwell (United Kingdom), Strasbourg (France), Monterotondo (Italy), Munich (Germany), Vienna (Austria) and Oulu (Finland), under refrigerated (+4 to +8 °C) conditions. The linear distances from Madrid to all these seven EMMA nodes ranged from 945 km (Orleans) to 3,308 km (Oulu). Seven CARD cold transportation kits that had been used for teaching purposes at the previous CARD-CNB cryopreservation course, held in Madrid, in October 2013, were used according to the recommended protocol (CARD Manual, March 2013; CARD web site: http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/lowtemptransp.html). We used standard courier services (Federal Express) under refrigerated conditions (labelled as +4 °C) and the boxes were delivered between 24 and 48 h after departing from Madrid, therefore, within the 72 h confidence limit where the mouse embryos can be maintained at refrigerated temperatures without loosing viability. Refrigerated mouse embryos were transferred upon arrival to the oviduct of suitable pseudo-pregnant females and, in all seven EMMA nodes, pups from both genetic backgrounds were successfully obtained, confirming the adequacy of this protocol for the robust shipment of unfrozen refrigerated mouse embryos. In contrast, most of the refrigerated embryos that were received by the EMMA nodes but cultured to the blastocyst stage before transfer failed to produce pups thus indicating that the immediate embryo transfer is the recommended procedure to follow upon receiving refrigerated mouse embryos.

60. Robust nuclease activities in Salmon trout ooplasm as a main transgenesis hurdle

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In this study, we aimed to examine whether simple incubation of sperm, oocyte or embryo could be a plausible approach for making transgenic Salmon trout. We used pDB2 plasmid containing enhanced green fluorescent protein (EGFP) under human cytomegalo-virus promoter. Lipofectamine2000 was used as the cationic lipid reagent for the transfection assay. First, we evaluated whether the fish oocyte does have any type of nuclease activity. We incubated 3 μg DNA from Escherichia coli as a prokaryote, pig and sheep as eukaryotes, pDB2 and pTn5 as plasmids, and two single strand oligonucleotides (each 64 nucleotide) with 100 μl of oocyte cytoplasm at 25 °C for 30 min. DNA from any sources was fragmented into 50–1,000 bp segments after 30 min incubation. However, during 24-h incubation, all of the DNAs from various origins were completely removed. Then, 3 μg of pDB2 was incubated with oocyte cytoplasm aspirated from one, three or five oocytes for 1, 10 and 30 min at 25 °C. Following one minute incubation of the ooplasm-DNA mixture, the entire foreign DNA was removed when ooplasm from 5 oocytes were incubated. However, after 30-min incubation of ooplasm from only one oocyte, the entire exogenous DNA was degraded. Then, we evaluated whether transfection of sperm cells with/without cationic lipids can be helpful to protect foreign DNAs from the oocyte nuclease activities. Three micrograms of pDB2 plasmid was incubated with 10, 100, 1,000 and 1,000,000 sperm cells from either freeze-thawed killed or fixed groups, and then treated with or without 100 μl of oocyte cytoplasm. The fish oocyte nucleases not only completely degraded the exogenous DNA, but also partly degraded the sperm genome in both frozen-thawed and fixed sperm cells. These results clearly showed the presence of an extraordinarily robust nuclease activity of egg cytoplasm so that could degrade any types of foreign DNAs including both naked plasmid DNAs and intensively compact sperm genome. Finally, we incubated fish sperm cells, oocytes and fertilized eggs with liposomes comprising Lipofectamine2000 and pDB2 plasmid to see the transgenesis efficiency. Stringent PCR results on DNAs extracted from the treatment groups versus untreated negative control group showed that none of the four-month-old fishes produced from the sperm-, oocyte- and embryo-treated groups ($n = 150$, 114, and 30, respectively) carried the EGFP transgene. In conclusion, the strong nuclease activity of Salmon trout oocplasm could be an important stumbling block for fish transgenesis.

61. Bypassing chicken egg albumen nucleases by simultaneous injection of lipoplexes and EDTA could lead to efficient transgenesis

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This study was aimed to examine (1) possible interaction between exogenous DNA and chicken egg white and (2) chicken embryo ability in up-taking *in ovo* injected exogenous DNA. First, we evaluated whether the exogenous DNA can durably last in egg albumen to be taken up by chicken embryo. We incubated genomic DNA from Escherichia coli, wild boar and sheep, pDB2 and pTn5 as naked DNA, and two single strand DNA amplicons (each 64 nucleotide) with 100 μl of egg albumen at 37.5 °C for 30 min and 24 h. Double-strand DNAs from all sources fragmented after 30 min incubation and completely removed after 24 h incubation, except for the pTn5

plasmid. The 50-bp resistant fragments from pTn5 plasmid were cloned into T-vector and sequenced successively. In addition, single strand DNAs was completely resistant to the egg albumen nucleases during even 5-day incubation. Then, we assayed different inactivation approaches for the chicken albumen nucleases. One-hundred microlitre of egg albumen was mixed with 5 μl EDTA 0.5 M, treated with 20 μl of ProteinaseK (20 mg/ml) for 1 h, or heated at 65 °C for 5 min and 38 °C for 24 h. Afterwards, the mixtures were incubated with 3 μg of pDB2 plasmid at 37.5 °C for 1 h. Egg albumen nuclease activity was not affected by Proteinase K, while both EDTA pre-treatment and the heating approaches could strongly inhibit the nuclease activities. Furthermore, the egg albumen was incubated with Lipofectamine2000 (1, 2, 3 μl):pDB2 (1 μl) complex with or without EDTA for 24 h. Even though the lipoplexes were resistant to the egg albumen nucleases, supplementation of EDTA could robustly enhance the protection assay. Finally, the embryo-mediated transgenesis was evaluated by Day0 *in ovo* injection of 3 μg pDB2 plasmid, containing enhanced green fluorescent protein (EGFP) under human-CMV promoter, along with 3 μl Lipofectamine2000, 3 μl EDTA 0.5 M, and both Lipofectamine2000 and EDTA in the vicinity of the embryo. Distilled water injection and non-injection eggs were considered as control groups. None of embryos/chicks from the lipoplex ($n = 22$) or EDTA ($n = 25$) carried the EGFP plasmid. However, rigorous PCR analysis from the lipoplex-EDTA group showed that 6 out of 11 embryos at Day4 of incubation and 3 out of 10 chicks were positive for EGFP transgene. Mosaic expression of EGFP transgene was evident in two chicks by Fluorescent imaging. In conclusion, concomitant *in ovo* injection of lipoplexes and EDTA could be very efficient approach for bypassing chicken egg albumen nucleases and making transgenic chicks.

62. A novel vector configuration dramatically improves recovery of gene-targeted clones

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Recent advances with targeted nucleases have greatly simplified genome modification in a wide variety of model organisms, but there are still situations where gene targeting by classical homologous recombination is preferred, often where there is concern about off-target mutations. If targeting efficiency is poor, cell handling and screening is labour-intensive. Constraints associated with precise manipulations like conditional alleles or humanizations can make this difficult to avoid, thus any strategy that improves recovery of targeted clones is of value. Here, we describe a novel targeting vector based on the pJAZZ linear plasmid system that dramatically improves targeted clone recovery due to an order of magnitude improvement in negative selection power. This greatly reduces the number of clones that have to be handled and facilitates recovery of targeted clones with constructs that normally display very low targeting efficiencies.

63. An efficient high-throughput system for screening adeno-associated virus administered via an intravenous temporal vein injection in mice

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MAPS (Mouse Animal Production Service) was commissioned by the CanEuCre (Canada European Cre) Initiative to create a bimonthly production of postnatal day (P) zero pups to test adeno-associated viruses (AAV). By scaling up the method of Foust et al. 2009, we were able to establish a reproducible, reliable and extremely successful pipeline that can be used throughout transgenic core facilities for multiple purposes.

To produce timed births, 8 females were crowded for 10 days to induce the Lee-Boot effect; then, they were set up with studs and plug checked for 5 days. To increase the likelihood of pregnancy, all females were left with their male counterpart for the 5 days. This setup created a convenient Monday to Friday injection schedule. On the 5th day post setup, all the females were rehoused with a nanny (a non-pregnant female companion). We began checking the females for litters twice a day at 18 days post-setup. Frozen virus was diluted in room-temperature PBS to a titre of 10^{13} genome copies/mL, and 50 mL were injected intravenously into the temporal vein of P0 B6129F1-*Gt(ROSA)26Sor*^{tm1Sor} pups. Before the start of injections, the litter was moved to a clean cage, and the cage was placed on a heating pad. During injections, the dam and nanny were removed from the room. Pups were gently restrained by one person, while a second person injected into the temporal vein. Pressure was then applied at the injection site with a cotton swab until the bleeding stopped. Post injection, when dams, nannies and pups were reunited in their original cage, the dams were scruffed and made to urinate on the pups to facilitate acceptance. Litters were health checked for a minimum of 3 days post injection. Mice were allowed to mature to set time points, and then brains, eyes, hearts and spinal cords were collected and stained for promoter expression analysis.

By this method, we were able to achieve a 72.1 % pregnancy rate and a 98.3 % pup survival rate for 3 days post injection. Different injection ages were tested, and P0 injections were found to optimize expression in the brain, while P4 injections optimized expression in the retina. Different harvesting times were also tested, and increased age showed increased expression. P56 was chosen as a suitable optimization of expression and experimental duration. In just over a year, the MAPS group tested 42 unique AAV, using this mouse production system.

64. Lentiviral and transposon methods for fluorescent transgenic chicken production

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The chicken embryo is an established developmental biology model. Integrated single-copy transgenes in the chicken genome are stably and uniformly expressed, in contrast to fluorescent protein reporter transgenes delivered transiently by electroporation. Embryos carrying a single-copy GFP transgene, from the Roslin Greens ubiquitous GFP transgenic chicken line, have been used for developmental biology applications such as cell lineage tracing (McGrew et al. 2008).

Notch signalling regulates neurogenesis in the vertebrate neural tube. We are developing a fluorescent reporter transgenic chicken line in which Notch signalling can be tracked in embryonic tissues throughout development. A Venus fluorescent reporter driven by the Hes5-1 promoter was developed in 2011 by Vilas-Boas and Storey (Vilas-Boas et al. 2011) as a dynamic reporter of Notch signalling in the developing chicken neural tube. We cloned this reporter construct into a lentiviral vector. After lentiviral packaging, the pseudovirus containing the transgene was injected into chicken embryos from newly laid eggs which were then incubated to hatch. One surviving chimeric transgenic male was identified and bred to produce transgenic offspring. Two G1 transgenic males obtained are being bred to produce G2 transgenic embryos for analysis. We describe progress in validation of these transgenic embryos as Notch signalling reporters.

Injection of transgenic primordial germ cells (PGCs) is underway to generate a transgenic chicken line that ubiquitously expresses the red fluorescent protein tdTomato. A transposon vector was used to introduce transgenes into PGCs. Transfected cells were cultured until only cells with stable transgene integration expressed tdTomato. Red fluorescent PGCs were selected by FACS, allowed to proliferate in vitro and injected into the aorta of chicken embryos at 2-day incubation. These embryos were incubated to hatch. Fluorescent cells were seen in embryonic and post-hatch gonads, indicating that the injected PGCs had colonised the gonads. Other transgene constructs in transposons are being tested in PGCs, with the aim of generating 'Brainbow' transgenic chickens that can express different fluorescent proteins in different cells.

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Vilas-Boas, F., Fior, R., Swedlow, J., Storey, K. and Henrique, D. (2011) 'A novel reporter of notch signalling indicates regulated and random notch activation during vertebrate neurogenesis', *BMC Biology* 9(1): 58.

65. Reprogramming meiotic recombination in the mouse

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Meiotic recombination between paternal and maternal chromosomes allows the transmission of new combinations of alleles and ensures the accurate segregation of chromosomes. Instead of occurring at random across the genome, distinct hotspots of recombination are found which correlate with a particular pattern of histone modification (H3K4me3).

PRDM9, a zinc finger DNA-binding protein, has been implicated in defining the hotspot position as the distinct DNA motif bound by the zinc finger domain is present at the centre of the majority of hotspots. Furthermore, PRDM9 contains a methyl transferase domain and has been shown to generate the H3K4me3 mark found at preferred sites of meiotic recombination.

The zinc finger domain of PRDM9 shows a remarkable sequence diversity with large numbers of variants being present within populations and substrains. Each variant correlates with an alternative meiotic hotspots motif, providing further evidence that PRDM9 is central to defining the pattern of meiotic recombination. Interestingly, the gene has also been implicated in hybrid sterility with certain combinations of PRDM9 zinc finger domains being incompatible with progression through meiosis. Consequently, PRDM9 has been implicated in the molecular mechanism underlying speciation by reproductive isolation.

To explore PRDM9 biology and its role in defining the recombination landscape in more detail, we have used gene targeting to reprogram PRDM9 to bind at different sites within the genome. We have replaced the mouse zinc finger domain of *Prdm9* with that of human and show that the pattern of meiosis moves entirely to the human hotspot motif, thus confirming that the DNA-binding characteristics of PRDM9 control the pattern of meiotic recombination.

Using a PhiC31 integrase cassette exchange methodology, we are now embarking on further manipulations of the zinc finger domain to redirect recombination hotspots. Alternative zinc finger domains addressing different motifs have been incorporated, and we have replaced the zinc finger domain with a TALE domain to facilitate the reprogramming of PRDM9. As well as providing insights into PRDM9 biology, directing the meiotic machinery against specific sequences may facilitate a new type of genome engineering and allow the inheritance of specific combinations of alleles and traits to be controlled.

66. Integrases for genome engineering

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The irreversible nature of the recombination mediated by the serine integrase family makes these recombinase attractive candidates for genome engineering. Targeted integration of DNA constructs into the genome can be achieved at high efficiency, and serine integrases lend themselves well to recombination-mediated cassette exchange approaches.

The integrase from Streptomyces phage PhiC31 has been widely used over the last decade, but few other members of this family have been adapted for use in mammalian systems. Despite the widespread use of PhiC31, evidence exists to suggest that PhiC31 integrase may not be the most appropriate enzyme for genome manipulation as the enzyme has a tendency to destroy its substrate recombination sites through incomplete reactions. Furthermore, there is some evidence to suggest that high levels of expression may result in toxicity, possibly due to pseudosite recombination within the mammalian genome.

The aim of this study was thus to establish whether there are any alternatives to PhiC31 integrase. Towards this goal, we have explored the utility and compared the efficiency of 15 members of the serine integrase family for genome engineering within mammalian cells.

PhiC31, Bxb1 and W-beta integrases revealed the highest activity in ES cells and were able to mediate accurate attP x attB-mediated deletion events and RMCE events at high efficiency. R4, SPBc, TG1, TP901-1 and BT1 revealed more moderate activity in mammalian cells but were still able to mediate attP x attB deletion events. MR11, A118, Phi370, PhiC1, RV, BL3 and K38 were inactive in our hands.

ES cells manipulated using PhiC31, Bxb1 and W-beta were used to generate proof-of-concept animal models, and germline transmission was obtained with all three integrases. These three enzymes are thus suitable for the genome engineering of mouse strains and their expression within ES cells is entirely compatible with germline transmission.

67. *R26Fucci2aR:* A global Cre-inducible cell cycle reporter mouse allowing live imaging of cell cycle progression during mouse embryonic development

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Fucci (Fluorescent Ubiquitination-based Cell Cycle Indicator) is a system for the visualisation of cell cycle progression by the use of two fluorescently tagged probes, whose abundance is regulated in relation to the cell cycle. Cells are labelled red during G1, yellow at the G1/S transition and green during late S/G2 and M phases. The system allows live imaging of cell cycle progression. However, existing Fucci mouse models are limited by requiring the crossing of multiple lines, by transgene inactivation and variegated expression and by lack of inducible potential. To overcome these limitations, we have designed the Fucci2a bicistronic construct by fusing the two Fucci probes into a single construct separated by the *Thosea asigna* virus self-cleaving peptide sequence (T2A). This allows for equal expression of both probes from a single construct. We characterised the Fucci2a construct in a stable 3T3 cell line demonstrating that the localisation and cell cycle-specific expression of the probes is not altered by the addition of the T2A sequences. Subsequently, we generated the *R26Fucci2aR* transgenic mouse line by targeted recombination to the *Rosa26* locus, a proven site of constitutive, ubiquitous gene expression. In *R26Fucci2aR* animals, Cre recombinase-inducible expression is driven by the CAG promoter, a strong synthetic promoter incorporating the cytomegalovirus early enhancer element; the promoter, first exon and first intron of the chick beta actin gene; and the beta-globin splice acceptor sequence. Our Fucci2a construct and *R26Fucci2aR* mouse line therefore overcome the limitations of previous Fucci systems and are powerful tools for the investigation of cell cycle dynamics in vitro and in vivo. We demonstrate the utility of Fucci2a using tissue-specific and ubiquitous Cre-expressing mouse lines and live confocal imaging of organotypic cultures.

68. Concurrent production of multiple targeted transgenic mouse lines with C57BL/6N genetic background by improved PITT

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Pronuclear injection (PI) of foreign DNA is the simplest and most widely used method to generate transgenic (Tg) mice. However, the method is always associated with random integration of multiple-copy transgenes that often causes unreliable transgene expression. Recently, we and another group overcame this pitfall by developing the '*PI*-based *T*argeted *T*ransgenesis technology', called PITT, which allows targeted integration of single-copy transgene into the predetermined genomic locus such as *Rosa26* using Cre-*LoxP* or PhiC31-*attP/B*-mediated genetic integration system. The resulting targeted Tg mice generated with PITT exhibited reliable transgene expression. Unfortunately, the mice made by the PITT method were in mixed genetic background, which often causes problems in interpretations on the results of gene function studies. Here, we constructed a transgene carrying mutant *LoxP*s, FRTs and *attP* sites as a new combination of landing-pads with the aim of widening the choice of transgene integration system, thereby being expected to be compatible with a variety of transgenes. The resulting construct was targeted

into the *Rosa26* locus in C57BL/6N ES cells. We then validated these targeted insertion systems by introducing a donor vector together with recombinase/integrase expression plasmid into the gene-engineered ES cells and confirmed that Cre-*LoxP* and PhiC31 systems worked as expected. Even though the FLP-FRT system also worked, its efficiency was relatively poor compared to the other two systems. The results also indicated that the combinational use of Cre-*LoxP* and PhiC31 systems can improve targeted integration efficiency. The C57BL/6N-based seed mice generated through chimeric mouse production via engineered ES cells were then validated for PITT by injection of donor vector into zygotes together with mRNA for iCre recombinase and/or PhiC31o integrase. Combinational in vivo use of Cre-*LoxP* and PhiC31 systems also resulted in improved targeted integration efficiency of transgene similar to the in vitro experiment. The integration rate was 7.1–61.5 % of F0 embryos/foetuses and 1.7–19.5 % of injected zygotes when hemizygous zygotes were subjected to the improved PITT using both systems. We also performed simultaneous injection of multiple transgenes containing donor vectors using the improved PITT, and a single injection of transgenes into 89–181 zygotes resulted in production of as many as three distinctly targeted Tg mice. The strategy and the resources developed in this study would be useful for high-throughput, cost-saving and animal-friendly generation of Tg mice with C57BL/6 N genetic background in which reliable transgene expression is guaranteed.

69. Targeting of embryonic stem cells through the use of new tools and generation of C57BL6 diploid ES cells

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Embryonic stem (ES) cell technology remains an important approach to making mouse models with complex targeted loci. The targeting of ES cells has been a time-consuming bottleneck, and at Genentech, we have focused on streamlining the process to improve efficiency. Here, we will present details of efficient, viral delivery of recombinases as well as use of recombination-mediated cassette exchange (RMCE) in ES cells. In addition, we will describe how we derive diploid mouse ES cell lines for retargeting purposes.

Generation of ES cells with complex targeted loci typically involves the utilization of FRT or LoxP recombination sites. ES cells are usually selected via drug resistance cassettes that subsequently need to be removed using mouse crosses. We developed an efficient delivery method for Cre and FLP recombinases that uses a modified adenovirus, termed Ad(RGD). This virus contains an arginine-glycine-aspartic acid (RGD) motif genetically engineered into the HI loop of an adenovirus fibre knob domain. This Ad(RGD) motif allows for efficient binding to integrins that are ubiquitously expressed on the cell surface of ES cells. In tests of 24 different alleles, we obtained 62.5–91.7 % recombination efficiency for Cre recombination and 41.7–70.8 % efficiency for FLP recombination. Furthermore, we were able to show that the ES cells remained multi-potent after microinjection produced normal birth rates and germline transmission. This new method is vastly more efficient than plasmid-introduced recombination by electroporation and bypasses the need for time-consuming mouse crosses to remove unwanted drug cassettes in the targeted loci.

The *Rosa26* locus is a preferred site for introduction of gene expression cassettes. Here, we describe the generation of *Rosa26* acceptor ES cell clone with a puro cassette flanked by FRT recombination sites and one 5′ LoxP site. Expression cassettes with a stop codon and second LoxP site can be efficiently transferred into these ES cells through recombination mediated cassette exchange (RMCE). This method bypasses the challenging cloning into the *Rosa26* locus and streamlines generation of *Rosa26*—based mouse models.

Diploid mouse ES cell lines derived from *mutant* mouse strains can produce cell lines with unlimited self-renewal and with the potential or to either retarget or differentiate into specialized cells for further study. We will present the details of our methods for diploid ES cell derivation and how we utilize these *mutant* ES cell lines for further cell biological studies in forward and reverse genetics.

70. In vivo promoter analyse platform in mouse embryos

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Promoters contain specific DNA sequences and elements that is recognized by transcriptional factors and RNA polymerase. Polymerases can transcript mRNA according DNA sequences. There are many DNA elements on the promoter, as we know the interactions between transcriptional factors and DNA sequences achieve the differential gene expressions at various stages and tissues. Although we can use a lot of methods to verify gene expressions in vitro, but it is very difficult to trace promoter activity in live animals.

We demonstrated a platform by combining the ROSA26R mouse strain and the transgenic techniques to analyse the effects of DNA elements in 14.5 days mouse embryos. Briefly speaking, we used the homozygote ROSA26R male mice to mate with C57BL/6 female mice, and flushed 0.5-day mouse embryos for microinjection. We used a transient Cre/ROSA26R transgenic mice system to examine the regulation of CYP11A1 promoter in the brain. We found that the 5′-flanking region between 3.5 and 3.2 kb contains a crucial sequence for the activation of CYP11A1 promoter in the olfactory epithelium, diencephalon and the anterior part of midbrain in the developing brain.

71. A second-generation inducible and non-leaky system for gene expression in transgenic mice

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The Tetracycline (Tet)-controlled inducible system is the most widely used reversible system for transgene expression in mice

with over 500 lines created to date. Although this system has been optimized over the years, it still has limitations such as residual transgene expression when turned off, referred to as leakiness. Here, we present a series of new Tet-OFF transgenic mice based on the second-generation tetracycline-responsive transactivator system. The tTA-Advanced ($tTA2^S$) is expressed under control of the neuron-specific Thy1.2 promoter (Thy-OFF), to regulate expression in the mouse brain. In addition, we generated a LacZ reporter line, utilizing the P_{tight} Tet-responsive promoter (P_{tight}-lacZ), to test our system. Two Thy-OFF transgenic lines displaying two distinct patterns of expression were selected. Oral doxycycline treatment of Thy-OFF/P_{tight}-lacZ mice demonstrated tight transgene regulation with no leak expression. These new Thy-OFF mice are valuable for studies in a broad range of neurodegenerative diseases such as Alzheimer's disease and related forms of dementia, where spatial and temporal control of transgene expression is critical to understanding mechanisms underlying the disease. Furthermore, P_{tight}-lacZ reporter mice are widely applicable.

72. Sperm-mediated anti-freeze protein gene transfer in tilapia

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Gene targeting is a DNA-editing techniques which is based on the principle of homologous recombination in order that the foreign gene in the genome can be stably expressed and inherited to the next generation. The zinc finger nuclease (ZFN)-mediated gene targeting develops rapidly in recent years, and its high efficiency of gene targeting has been confirmed in many species. The site-specific targeting technology in which specific zinc finger proteins could identify and cut genomic DNA in the specific sites in genome,which induces the homologous recombination between the exogenous DNA and genome DNA.

Our previous study has established a technique of 'multi-locus gene targeting' which identifying the internal transcribed spacer (ITS) of ribosome DNA (rDNA) genes in human genome proved to be with efficient site-specific integration and stable expression of foreign genes. In present study, ZFN expression vector and the multi-locus gene-targeting vector were combined to implement gene targeting of tilapia in vivo.

First of all,the sperm-mediated exogenous gene was inserted into the genome and 114 tilapias in three batches were obtained. Genomic DNA was extracted and PCR was used to detect the integration of exogenous gene (Anti-freeze Protein Gene). Nine fishes were found carrying the GFP and AFP of exogenous gene and were labelled as the experimental group, while the tilapia without, the control group. The effect of anti-freeze was detected by observation of incubation overnight at 15 °C temperature. No significant difference was found between the experimental group and the control group. Nine experimental fishes were dissected and the fluorescence protein expression on visceral was observed. Strong fluorescence could be found in the liver of three fishes. At the same time, the targeting vector and expression vectors were co-transfected into the Epinephelus coioides cell lines. Fluorescence expression in these cells could be observed, and the integration of exogenous gene (AFP and GFP) were also found by PCR analysis.

Our results suggested that transgenic tilapia by sperm-mediated gene transfer was successfully obtained.

Keywords: Tilapia; Transgenic technology; Zinc finger nuclease; Anti-freeze protein; Gene targeting

The present work was supported by NSFC (No. 8127 2552 and No. 81101537), National Science and Technology Major Project (No. 2014ZX08010-012B) and Guangdong provincial high-tech development special projects (No. 2012A030400066).

73. En masse lentiviral gene delivery to mouse single-cell embryos via laser perforation of the zona pellucida

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The zona pellucida (zona) is a multi-functional porous matrix of glycoproteins that envelopes oocytes and preimplantation-stage embryos to limit environmental interactions and polyspremy. While protecting the embryo, it creates a barrier that complicates gene delivery to embryos. Herein, we utilize the Xyclone laser, typically used to perforate zona for in vitro fertilization, to allow access of our lentiviral vectors to the embryos in culture. A single laser carved hole in the zona is sufficient for gaining access to embryos and lentiviral gene delivery without microinjection or micromanipulation. It takes a matter of minutes to carve a batch of 10–20 embryos while they are resting freely on the bottom of the culture dish. Again, no special techniques or equipment are required for immobilizing or handling the embryos in culture. The lentiviral vectors are then pipetted directly into the culture media containing the perforated embryos allowing for coordinated infection and transduction of the whole batch of embryos. The embryos are cultured with the virus until they develop into blastocysts during which time we can assess viability and even gene expression in the case of fluorescent indicator-tagged constructs. We then utilize non-surgical embryo transfer (NSET) to introduce the treated blastocysts into the uterus of pseudo-pregnant dams and generate transgenic animals. We demonstrate that lentiviral transduced cultured mouse embryos generated by this method illicit no damage and can develop to term for creation of transgenic animals. We typically use 1ul of concentrated lentiviral vector with a titre of around $1 \times 10e9$ transducing units per ml and easily achieve nearly 100 % transduction of 10–20 embryos as visualized by GFP expression after 2–3 days in culture. In all cases so far, we have utilized the EF1 alpha promoter for driving expression of our transgenes partly because of its known ability to drive expression in the early embryo and thus a useful tool for investigating early embryogenesis. We are also investigating the use of this method to deliver lentiviral CRISPR for generating knockout animals.

74. Using layered double hidroxides in ovine sperm-mediated gene transfer

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Layered double hidroxides (LDH) are chemical materials that can be synthesized from many different substances, have double lipid layers in their strucutres and it is highly important to determine their sizes, working pH values and synthesis methods in order to use them as transfection agents. When DNA molecules are being kept in between double lipid layers, it is aimed that it releases the DNA molecule in order to achieve a successful transfection in cells. LDH's are used in some cell transfection researches but they have not been used as a new vehicle in sperm-mediated gene transfer (SMGT) yet.

In our study, we used oocytes obtained from ovaries of slaughtered sheeps and used two different kins of LDH's Mg-Al-Cl_LDH and Ca-Al-Cl-LDH with the concentration of 2.5 ng/μL. pDNA (plasmid DNA) for the transfection of ram spermatozoa LDH's were mixed with DNA at 37 °C for 4 h, after that LDH-DNA complexes were incubated with spermatozoa for 1 h at room temperature. ICSI (intracytoplasmic sperm injection) was used to fertilize the MI ovine oocytes. We used EGFP (enhanced green fluorescent) gene to determine transgenesis under attempted with transfection. The controls of transgenic embryos are achieved according to the green luminescence status under fluorescent attached invert microscobe. All the embryonic development procedures were made as in Birler et al. 2010, except we used only ionomycine activation and put oocytes in fertilization SOF media after ICSI.

Our results showed that in both Mg-Al-Cl_LDH and Ca-Al-Cl-LDH groups we obtained had transgenic embryos 13.04 % and 21.87 %, respectively, but not statistically important ($p > 0.05$). The cleavage ratios were 74.19 % and 71.11 % also the degeneration rates were 25.80 and 26.66, respectively, but not statistically important ($p > 0.05$). The embryonic development ratios of the LDH groups were not lower than our control group.

In conclusion, we determined that both Mg-Al-Cl_LDH and Ca-Al-Cl-LDH can be used for SMGT in ovine transgenesis. They did not affect embryonic development negatively, and their transgenesis efficiency can be elevated with more researches in this field.

75. A vaginal pessary-based system for pseudopregnancy induction in laboratory mice

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Successful pseudopregnancy induction is key to implementing streamlined, effective protocols for the preparation of recipients for embryo transfer in transgenic/line rederivation-based embryo transfer programmes. This neuroendocrine induction hinges around a combination of vagina/cervical mechanical stimulation by coitus and reproductive tract mucosal biochemical stimulation by seminal plasma. While good success rates are obtained by mating recipients with a vasectomised male, this approach has its own disadvantages, ranging from the ethical (increased animal usage, surgery) to the pragmatic (rest time/ardency-related variable success rates). The study therefore aimed to develop an alternative, male-free, vaginal pessary-based method for pseudopregnancy induction which artificially mimics natural mating.

Four-mm-diameter pedunculated nylon porous vaginal pessaries were created using selective laser sintering technology. Virgin 4- to 6-week-old CD-1, Whitten effect-synchronised females were randomly allocated to mating with a vasectomised male ($n = 25$) or to the use of pessary-based system with/without loading with a seminal plasma mimic ($n = 5$ and $n = 26$, respectively). Laparotomic unilateral oviductal embryo transfers were performed the following morning using 15 zygotes (obtained by mating superovulated donors to syngeneic stud males) per female. Recipients were individually housed on day 16 to litter down. Pregnancy rates, litter sizes, birth weights and pup fertility in adulthood were recorded. Data were analysed by C^2 analysis.

No pregnancies were achieved with unadulterated pessaries, while there was no significant difference in pregnancy rates (56 vs. 48 %; vasectomised male-mated and loaded pessary groups, respectively). Litter sizes and birth weights were comparable across these groups, and all pups were proven fertile when mated to syngeneic animals at 5 weeks.

This method offers a viable and ethically sound alternative to current vasectomised or sterile male mating-based approaches for pseudopregnancy induction. While the total replacement of males is of doubtless ethical value in reducing animal usage, it also has financial benefits (obviating the need to house additional animals) and pragmatic advantages (independence from male rest periods, no need to vasectomise/breed sterile males, more predictable numbers of pseudopregnant recipients available). This novel method therefore has the potential to improve workflow and reduce cost/dedicated staffing time, thus making embryo transfer more accessible to service users.

This method is protected by patent GB1314452.2.

76. RepLiCre: *Rep*orter-*li*nked-*Cre* transgenic mice for efficient use of conditional knockout mouse resources

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Many organizations worldwide have joined hands to establish large-scale programs such as IKMC (International Knockout

Mouse Consortium) to create knockout mouse for every protein coding gene-specifically Cre-LoxP-based conditional knockout (CKO) models. The efficient use of CKO models, however, depends heavily on the community-generated tissue-specific Cre models. While many Cre lines are available, the majority lack fundamental and rigorous quality control assessment, miss clear, comprehensive and reliable annotation of expression, and are deficient in robust functional characterization. Further, few are distributed from bona fide public repositories. Thus, this situation coupled with the necessity to breed them to another *transgenic* (reporter) mouse is becoming a bottleneck in efficient use of *knockout* models. Added complication is the extensive time required to breed multiple mutants (at least three alleles; CKO, tissue-specific Cre driver and a reporter) further impedes the speed of multi-allelic mutant mouse research.

A novel method called **p**ronuclear **i**njection-based **t**argeted **t**ransgenesis (PITT) developed by us addresses both of these problems. (1) PITT directs the transgene to a pre-determined locus to generate highly reliable transgenic lines. Using this approach, we have shown that a single transgenic line is sufficient, and thereby obviates the need to screen multiple lines. (2) The technology we propose here, termed **PITT-*Plus***, addresses the second issue of multi-allelic mutant mouse research through the ability to create two linked transgenes in one mouse that significantly decreases the time and resources needed for mouse breeding.

The PITT-*Plus* approach works as follows: First, a universal prototype PITT seed mouse line is created in which a Cre-inducible reporter (e.g. Lox-Stop-Lox EGFP) is targeted to *ROSA 26* locus along with a tandemly linked *attP* site. In a second step, the *attP* site landing pad is used to insert a tissue-specific Cre cassette through the PhiC31-based PITT approach. The resulting mouse, called **RepLiCre** mouse, will have a ***rep***orter cassette genetically ***li***nked to a tissue-specific ***Cre*** cassette: the two transgenes in the RepLiCre model always reliably express and always co-segregate during breeding with a CKO mouse. Furthermore, the prototype seed mouse can be used for creating large array of RepLiCre lines for various tissue-specific Cre mouse models. PITT-Plus approach is expected to lead into generation of several add-on tools to enhance and extend the use of vast array of existing mutant mouse models as well as the CKO models generated through IKMC resources.

77. Transgene inheritance in transposase-mediated transgenesis

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Transgene inheritance is one of the most important issues of transgenesis. Founders often show mosaicism, since transgene integration often occurs after the first cell divisions. Due to this phenomenon, transgenes are not always passed on to all offsprings.

Transposase-mediated transgenesis is highly efficient way to generate genetically modified animals. Another advantage of this method is that transgene integrates as a singly copy to a limited number of genomic loci. Sleeping beauty (SB) 100 × mRNA and the circular CAG-Venus SB construct was microinjected into the pronuclei of fertilized FVB/Ant mouse eggs. Quantitative PCR was used to genotype founders and their offspring. Detailed analysis showed that transposase-mediated integration occurred in various copy numbers at late stages of embryogenesis and the founders were mosaics for the transgene. Tissue distributions of integrated transgenes are variable; inheritance of the transgene is higher than expected. Copy numbers of transgenes are reduced from generation to generation because single transgene units are integrated in several independent loci.

Even though the CAG promoter is considered strong and ubiquitous, in our hands, CAG-promoter-driven transgene expression is highly dependent on integration site and especially the neuronal expression of the marker gene is very limited.

78. Proposal of a new method of gene transfer for large transgenes: inter-pronuclear cytoplasmic microinjection

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Aim: To raise survival rates of zygotes in gene transfer of large constructs, I have proposed inter-pronuclear cytoplasmic microinjection (IPCM), in which a solution containing a high concentration of large transgene is microinjected into a narrow space between two pronuclei, instead of the pronuclear microinjection delivering large transgene, which frequently causes such a fatal event that a micropipette pulls out pronuclear components. IPCM was evaluated in this study.

Materials and Methods: (1) Two solutions of approximately 300 kb BAC DNA with tdTomato as a reporter gene were made at concentrations of 7.5 and 2.0 ng/μL. (2) A total of 615 zygotes obtained from 36 BDF1 female mice were divided into two groups: one received the 7.5 ng/μl solution (high-dose group) by the vibratory microinjection and the other was given the 2.0 ng/μL solution (low-dose group) mainly by ordinary microinjection. (3) All microinjections were conducted only at a compensation pressure of 40–60 hPa. (4) All the zygotes were cultured for 6 days after microinjection. Embryonic development was assessed periodically.

Results: (1) On each experimental day, we managed to finish all microinjections with only one micropipette (58 eggs in average) by breaking the tip of a micropipette when it was clogged. Ten of 12 micropipettes used in both groups were broken: 9 intentionally and 1 accidentally. (2) The survival rates of zygotes after microinjection in high- and low-dose

groups were 77.7 % (310/399) and 79.6 % (172/216), respectively (NS, χ^2 test), and the rates of embryos developing to the blastocyst stage were 72.9 % (291/399) and 79.6 % (172/216), respectively ($P = 0.0632$). The rate of blastocysts expressing tdTomato in high-dose group was significantly higher than that in low-dose group: 35.8 % (143/399) versus 6.0 % (13/216) ($P < 0.0001$). Furthermore, when the low-dose group was divided into 2 subgroups according to injection volume, the subgroup receiving larger volumes expressed tdTomato more frequently ($9/64 = 14.1$ % vs. $4/152 = 2.6$ %) ($P = 0.0023$) although it showed significantly worse survival and blastocyst rates.

Conclusions (1) Together with the two groups, IPCM reached a survival rate of 78.4 % and a blastocyst-developing rate of 75.3 % by precluding the pulling-out event. These results also indicated that a high concentration of 7.5 ng/μl BAC DNA did not cause any deleterious effects when it was injected into the cytoplasm. (2) A considerably high concentration of BAC DNA solution is needed to raise the probability of expressing the transgene in embryo in IPCM.

79. Transformation of chicken spermatogonia cells in vivo using retroviral vectors

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Using cells of the gonads is considered as one of the promising methods for producing transgenic birds. As part of optimization and improvement of individual stages of this technology, we have investigated the introduction of recombinant DNA into the spermatogonia stem cells of the chicken testes. As a vector system to introduce the transgenes, it was applied a retroviral vector derived from Moloney murine leukaemia virus that encoded the reporter gene EGFP.

To determine the age of roosters in which the maximum number of spermatogonia are presented in their testes, we carried out histological studies of the testes in males aged from 1 week to 12 months. It was found that the optimal age of roosters for introduction of retroviral vectors into their testis is between 1.5 and 3 months, when the number of spermatogonia from the total number of spermatogenic epithelium reached 66.4 %.

Introduction of the retroviral vectors into the chicken testis was performed at the age of 2.5 months. The transformation efficiency of target cells was evaluated after 1 month, using immunohistochemical analysis of histological sections of testis. The percentage of transfected cells in the seminiferous tubules ranged from 1.8 to 32.2 %. The overall efficiency of transformation of the seminiferous tubules (ratio of the number of transformed seminiferous tubules to the total number in all studied sections, expressed as a percentage) was 13.3 ± 2.3 %. The transformation efficiency of spermatogenic layer (ratio of the number of transformed cells to the total number of cells in all slices studied, expressed as a percentage) was 1.3 ± 0.3 %.

The study of the transgene inheritance was performed in six roosters. At least embryos were obtained and analysed from the each rooster. These experiments showed that the amount of the resulting transgenic embryos ranged from 1 to 7 % (on average 4.2 %). The results demonstrate that retroviral vectors can be successfully used for gene transfer into the testicular cells in vivo.

80. The study of conditions for effective gene transfer into avian embryonic cells using lentiviral vectors

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The factors that affected the transgenesis efficiency of the avian embryonic cells in vivo have been studied. The modified lentiviral vectors of second generation were used for gene transfer. The lentiviral vectors contained EGFP marker gene under the control of the constitutive transcriptional regulatory elements (RSV (Rouse sarcoma virus) promoter or hybrid CAG enhancer promoter) enhancer of early genes of human cytomegalovirus and promoter of β-actin gene of chicken)). For an introduction to the embryos, the vector preparations were concentrated to give titres of 2×10^9 colony-forming units per millilitre (CFU/ml). The concentrated viral solutions were injected into the developing embryos after 50 h of incubation. After 6 days of incubation, the eggs were opened and the degree of development of the embryos and the efficiency of gene transfer into the cells were examined. Evaluating the effectiveness of gene transfer was performed by analysing of EGFP expression in the embryonic cells using FACS analyser. According to the results, virus preparations with titres of 5×10^8 CFU/ml or less did not show any significant cytotoxicity. Efficiency of gene transfer for viral preparations with this titre was about 30 % and varied slightly with an increase of virus titre. The next step of our research was to determine the developmental stage of the chicken embryos which is most sensitive to infection by lentiviruses. The gene transfer efficiency was evaluated by detection of copy number of viral vectors using real-time PCR and by analysing EGFP expression using FACS analysis. It was shown that the efficiency of gene transfer evaluated by both methods was in agreement when the infection of embryos was carried out after 50 h of incubation. On the other hand, more than half of the embryos infected at the early stages of development contained one or more copies of the viral vector per diploid cellular genome whereas only 30–35 % of the cells expressed the marker gene (according to FACS analysis). Since the infection of embryos at various developmental stages was carried out with the same virus preparations (5×10^8 CFU/ml), it could be expected that the embryo cells at the earlier stages were infected with a lot of virus particles. These results suggest that the chicken embryos can be infected with lentiviral preparations even at the very early stages of development, and the efficiency of gene transfer using these vectors can be predicted.

81. Chimera production using laser-assisted injection of ES cells into morula-stage embryos

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In our facility, superovulated C57BL/6OlaHsd females are used as embryo donors. In our hands, the yield of morula-stage embryos at E2.5 is in average nearly double the yield of blastocyst-stage embryos at E3.5. To test whether the number of donor females could be reduced, we set up the Zyrcos laser system for morula injections. ES cells obtained from KOMP and EUMMCR as well as PRX-B6 N ES cells targeted in our facility were injected by this method. Injected embryos were transferred into oviducts of foster females on the day of injection in most cases. In average, 23 % of transferred morulae developed to live pups, whereas the proportion was 41 % from blastocyst transfers. The lower yield was, however, balanced by a higher number of chimeras obtained from morula injections. We are currently analysing how laser-treated morulae survive cryopreservation.

The laser-assisted morula injections are technically less demanding and thus faster to perform than blastocyst injections. Importantly, the number of embryo donors needed for the project to be completed is about half of that used in blastocyst injections; therefore, we currently prefer the morula injection method for the production of chimeric mice.

82. Generation of HHO-1/HA20/GGTA1-ko pigs by using sleeping beauty transposon and zinc finger nucleases

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Genetically modified pigs lacking the α1,3-galactosyltransferase gene (GGTA1-KO) and/or expressing human complement regulatory genes prevent the hyperacute rejection associated with pig-to-non-human primate xenotransplantation. On this basis, additional xenoprotective strategies are required to overcome the acute vascular rejection (AVR). This includes integration of human anti-apoptotic and anti-inflammatory genes, such as heme oxygenase-1 (hHO-1) or A20 (hA20), into the porcine genome. We recently established two such pig lines. Our hHO-1 transgenic pigs demonstrated protection from xenograft rejection in ex vivo kidney perfusion with human blood. The hA20 pig line revealed in vitro protection from TNFα-induced apoptosis in porcine aortic endothelial cells (PAECs) but expressed hA20 only at low levels and restricted to heart, skeletal muscle and PAECs. In the present study, we generated pigs co-expressing hHO-1 and hA20 on a GGTA1-KO background.

Foetal fibroblasts from our initial hHO-1 pig line were electroporated to integrate a zinc finger nuclease-based biallelic GGTA1-KO. In a next step, hHO-1/GGTA1-KO foetal fibrolasts were co-transfected with a new hA20-expressing vector based on the Sleeping Beauty transposon system and the SB transposase 100X plasmid (kindly provided by Dr. Zoltán Ivics). Antibiotically selected fibroblasts (5 μg/ml puromycin for 10 days) served as donor cells for somatic cell nuclear transfer. 25 days after embryo transfer, one recipient was sacrificed and six foetuses could be obtained. One of them was used for recloning. Four out of six recipients (66.7 %) who received an average of 97 embryos became pregnant. One sow lost pregnancy. The other recipients delivered a total of five live born and six stillborn piglets. Two piglets survived and developed normally. Fibroblast cultures of the six hHO-1/GGTA1-KO/hA20 foetuses and ear fibroblast cultures of the two recloned pigs were analysed for hA20 and hHO-1 expression by real-time PCR. HHO-1 expression was detected in foetuses and live pigs. The hA20 mRNA expression levels in the six foetuses were increased from 16- to 33-fold compared to the previously generated hA20 transgenic pig line and remained high in the recloned pigs. Flow cytometry confirmed the absence of Gal epitopes in all tested individuals. Forthcoming ex vivo kidney perfusions with human blood will unveil xenoprotective properties of the two pigs. One pig served as donor for recloning and embryo transfer into ten recipients to establish the hHO-1/GGTA1-KO/hA20 pig line as basis for further genetic modifications to finally generate the ultimate multi-transgenic pig for clinical xenotransplantation.

This project was funded by DFG TR CRC 127.

83. Mouse embryos from in vitro fertilization for microinjection

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Pronuclear microinjection is by far the most preferred method for genetically modified rodent production due to time and cost-effectiveness. With the recent technique breakthrough, pronuclear microinjection can now facilitate not only the production of gene overexpression animals, but also knockdown and knockout rodents with striking efficiency. However, to perform efficient and cost-effective transgenic services rely on not only steady demand, and more importantly, the number of animal to provide enough material is essential. Particularly, the number of both donor female and fertile male need to reach critical mass for stable harvest of fertilized eggs and other subsequent procedures. Thus, most transgenic mouse production cores often provide services based on only one (or few) common strain(s) to maintain optimal operation efficiency. Nevertheless, there is an increasing trend from researchers requesting genetic manipulation on unique genetic background or on specific genetic modified strain. It becomes a challenge for a core facility to set up experiment merely for defined project. Consequently, cage space expansion, inefficient animal use and unstable outcome seem unavoidable. In vitro fertilization (IVF) is one of the common assisted reproductive technologies widely used in mice embryos cryopreservation, colony expansion, strain recovery, rescue and rederivation.

In this report, we tried to verify the feasibility of using embryos from IVF in microinjection services for animal of less common backgrounds. We had tested plasmid DNA pronuclei microinjection and CRISPR/Cas9 RNA gene-

editing experiments using embryos from IVF. Thus far, no obvious difference was observed on rate of microinjection survival, live pups from transferred embryo, and genetic manipulation confirmed mutation mice of the live pups. Our results show IVF can easily provide embryos in large number of most mice strains for subsequent injection thus surpass constrain of fertile male number. Our data shows the quality of embryos from IVF are equivalent to embryos obtained by other common means for many manipulations. The advantages from using embryos from IVF provide alternative for core operations, furthermore, allow new opportunities for diverse experimentations.

84. Efficient, cost-effective and fast generation of transgenic mice by lentivirus-mediated modification of embryos

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The generation of transgenic animals using lentiviral vectors has been proven to be an efficient and cost-effective method allowing the generation of founders in only 3 months. This approach is also advantageous because it is technically easier to perform and more efficient than the ES cells and the DNA pronucleus injections techniques.

Here, we define an improved protocol to this approach that even more increase the efficiency of the generation of transgenic mice. Using highly concentrated (>1E9 TU/ml) and very pure lentiviral suspensions, we have been able to inject simultaneously up to 5 lentiviral vectors at the same time into the perivitelline space of C57BL/6N embryos. Tails from G0 pups were harvested and analysed by droplet digital PCR to determine the number of integrated copies in the founders. Up to 80 % of the pups were positive, and we detected 0.1–24 copies per genome per founder. Founders were then crossed with wild-type animals to evaluate the segregation of the transgene. We obtained a stabilized transgenic line that integrated an intact single-copy transgene using founders that contain 0.2–2 copies of the transgene per genome. Moreover, DNAseq was performed to precisely determine the insertion site in 2 G1 lines containing 1 and 5 copies of the transgene. Finally, the capacity of multiple injections in the same experiment has allowed us to generate animals with various tissue-specific inducibility.

In conclusion, our protocol allows:

(1) To establish a very cost-effective model generation technique since up to 5 different transgenic lines can be obtained from one experiment with 200 embryos injected

(2) The integration of an intact single-copy size-limited (<10 kb) transgene in the mouse genome and the establishment of stable lines, i.e. carrying single insertions, in less than 7 months

(3) To reduce the risk of transgene insertion effects by identifying the site of integration

(4) A space- and time-directed transgene expression through the use of tissue-specific promoters and/or an inducible system

Additional experiments are being performed to confirm that our protocol is highly relevant to generate tissue-specific models for knockdown, reporter, cre/creERT2 deleter, or cDNA expression in a constitutive or inducible way.

85. SCID rat via XTN^{TM} (TALEN)-mediated mutagenesis in rat spermatogonial stem cells (SSCS)

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We have successfully generated a severe combined immune-deficient (SCID) rat model with a functional deletion of *Rag2* gene expression from genetically modified SSCs. SSCs are a unique population of cells that remain in an undifferentiated, self-renewing state in cell culture but that differentiate into mature sperm when transplanted to the testis. They thus provide a useful alternative to embryonic stem cells for creating genetically modified rats carrying targeted mutations: the SSCs are modified in vitro and then transplanted into the testes of genetically sterile males, which then produce sperm derived solely from the modified SSCs. Offspring of the transplant males are then screened for the desired mutation. We targeted *Rag2* in SSCs with XTN^{TM}s and transplanted these targeted cells into sterile recipient males. Offspring were genotyped to identify potential founders and one animal with a 27 bp deletion was identified. Homozygous animals are fertile and viable, but fail to produce any detectable IgG by western blot, are athymic and fail to produce mature B or T lymphocytes as defined by surface marker analysis. This work demonstrates the utility of SSC modification as a method for the generation of genetically modified rats.

86. Use of the piggyBac transposon for high-efficiency gene trap mutagenesis

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In many fields such as neurobiology, behaviour, physiology and toxicology, the rat is a superior rodent model system to the mouse, but the mouse genome was vastly more amenable to genetic modification. However, recent technological developments make modification of the rat genome as simple as that of the mouse genome. Gene trap mutagenesis has been a valuable tool for the high-throughput generation of mutations in the mouse genome, and we report the use of a gene trap system based on the piggyBac transposon to generate a library of modified rat ES cells with high efficiency. We show the generation of germline chimeras from a clone of cells carrying 24 insertions characterized by splinkerette-PCR, and the breeding these animals to produce lines carrying mutations in the *Man2a1*, *Adsl* and *Fbn2* genes.

87. A Rapid and effective non-surgical artificial insemination protocol using the NSET^{TM} (non-surgical embryo transfer) device for sperm transfer in unanaesthetized mice

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Artificial insemination (AI) is an assisted reproductive technique that is implemented successfully in humans as a fertility treatment, performed extensively for commercial breeding of livestock, and is also successful in laboratory rodents. AI in the mouse may be especially useful for breeding of transgenic or mutant mice with fertility problems, expansion of mouse colonies, and as an alternative to in vitro fertilization. Non-surgical AI techniques for the mouse have been described previously but are not often implemented due to technical difficulties. Here, we compare various protocols for preparation of CD-1 recipient females prior to AI. Pregnancy rates were compared between naïve (in estrous), superovulated, and ovulation-induced females. Fresh C3H sperm was capacitated in HTF media at 37°C in the presence of 5 % CO_2 and transferred without the use of anaesthesia or analgesia. Sperm transfer was performed using a commercially available device originally developed for Non-Surgical Embryo Transfer in mice, the NSET^{TM} device. After sperm delivery, female recipients were immediately paired with vasectomized males. No pups were obtained using this technique with naïve CD-1 females in estrous; without the administration of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). Superovulated females were prepared by intraperitoneal (i.p.) injection with 5 IU of PMSG and hCG administered 47 h apart at various time schedules relative to the vivarium light–dark cycle. After sperm delivery, pregnancy rates of 36 % were obtained with superovulated females; however, pup mortality rates were high. To induce a more natural ovulation condition in females, the hormones were reduced to 1 IU per i.p. injection and given at the same intervals and schedules as the superovulated females. With the administration of a lower hormone dose, pregnancy rate and pup health were improved. A protocol optimized for non-surgical AI in mice is described which incorporates a convenient hormone administration schedule for female recipients and rapid, non-stressful sperm transfer without the need for anaesthesia or analgesia. Full-term pregnancy rates with fresh sperm reached 50 %. Litter size varied by sperm concentration, averaging 7–13 pups.

88. Use of blastocysts with gfp for efficient generation of chimeras from C57BL/6J- OR C57BL/6N-derived ES cells

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Gene targeting using embryonic stem (ES) cells is the standard and by far the most popular research tool for biomedical research. C57BL/6 (B6) is the most commonly used mouse strain with long research history in wide variety of fields. This trend lead to the urge to switch the ES cells genetic background for gene targeting from 129 strains to B6. Consequently, albino strains such as C57BL/6J-*Tyr*$^{c-2J}$, C57BL/6N-Tyr^{cWTSI} or BALB/c are often used as host in blastocyst injection to identify the degree of chimerism visually. Furthermore, albino B6 or wild-type B6 mice are subsequently used in germline transmission. However, most laboratories suffered significantly from the following restrictions by utilizing these strains: (1) poor breeding performance or poor response to superovulation; (2) low ES cell contribution in chimeras; (3) germline transmission cannot be identified via fur colour; or (4) heterozygosity for tyrosinase genes after germline transmission.

Here, we report a novel C57BL/JNarl spontaneous tyrosinase mutant line (C57BL/6JNarl-*Tyr*^{c}) with good breeding performance and good response in superovulation. This mutant B6 line is albino and was further engineered to express GFP specifically on the skin [C57BL/6JNarl-*Tyr*^{c} Tg(flKRT15-emGFP)] for easy visualization of the germline transmission. Such system takes full advantage of the coisogenic host strain in generating germline-competent chimeric mice when using B6 ES cells. By using C57BL/6JNarl-*Tyr*^{c} Tg(flKRT15-emGFP), we also circumvent the common drawbacks by (1) its good breeding performance and (2) its GFP expression for the visualization of germline transmission when using wild-type B6 to recover pure B6 mutant mice. Our study indicates that the use of the albino GFP B6 blastocyst from C57BL/6JNarl-*Tyr*^{c} Tg(flKRT15-emGFP) strain is very advantageous in the production of chimeras as well as the subsequent germline transmission.

89. Producing multiple-component transgene systems in rats

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The advantages of using rats in neuroscience work have been demonstrated over years of studies. Rats are especially useful for specific behavioural tests including addictive behaviour, learning and memory tasks. Transgenes have been used to alter or ablate endogenous gene expression or to introduce exogenous effector or reporter genes to make these studies even more informative.

In addition to these methods, we have used several multiple-component systems to add an additional layer of control of these transgenes. In these systems, two or more components are used to target subsets of neural cells, either for ablation or expression of a gene to subsets of neural cells at a specific time. We have produced a panel of transgenic rats that carry integrated germline-transmitted transgenes that are designed to work in multiple-component systems. This collection of animals can be generally useful for neuroscience research. These systems are designed to (1) be responsive to drug induction to induce gene expression, (2) respond to a drug to ablate a subset of neural cells, (3) induce a second transient

transgene that is delivered in a viral vector, (4) activate or inactivate specific neurons, (5) reveal the activity of neurons, or (6) genetically label a specific subsets of neurons.

Several of these lines are currently available through the Rat Resource and Research Center. Others will be made available as they are characterized.

90. Nuclease-enhanced gene modification for large genome modifications, including humanizations of mouse genes

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Gene targeting in mouse ES cells by the VelociGene® method employs large BAC-based vectors (BacVecs) with long homology arms—often with greater than 100 kb of total homology to the target—that enable the creation of very large genome modifications, such as complete deletions of large mouse genes (>100 kb) and replacement with their human homologs. To test whether engineered nucleases that catalyse targeted double-strand breaks in genomic DNA could enhance gene targeting by BacVecs, we electroporated mouse ES cells with a combination of a BacVec and two plasmids encoding the two halves of a zinc finger nuclease (ZFN) pair designed to target the same gene. The combination of BacVec and ZFN produced targeting efficiencies of 75–90 %, which represented an enhancement of two- to 16-fold compared with the BacVec alone for deletions of up to 32 kb with replacements by a variety of reporter, drug selection and conditional modules. In experiments in which we combined ZFNs with BacVecs designed to simultaneously delete a mouse gene and replace it with the human homolog, we observed ZFN enhancement of targeting ranging from 1.2- to 16-fold for deletion-humanizations up to 25 kb. Very large ($\geq$1 Mb) humanizations require multi-step sequential targeting of the same locus with mouse–human hybrid BacVecs that alternate insertion and replacement of neo^r or hyg^r selection cassette at each step. By combining mouse–human hybrid BacVecs with ZFNs directed against the neo^r or hyg^r elements, we have directed the targeting to the previously modified allele and have achieved ZFN enhancement of targeting ranging from three- to 20-fold. The significant and consistent enhancement of targeting efficiency achieved by combining a ZFN with a BacVec lowers the ES cell screening costs and increases the success rate, especially for difficultly targeted modifications, such as very large deletions, single-step humanizations of single mouse genes and large-scale megabase humanizations produced by multi-step sequential targetings. Most of our nuclease-assisted targeting has been with ZFNs, but we have recently achieved similar enhanced BacVec targeting with double strand breaks directed by CRISPR–Cas9 endonucleases.

91. Ultrasound confirmation of pregnancy in genetically modified mice reduces resources while enhancing reliability

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The mouse is widely used as a model to study embryonic development, which requires tracking conception dates through identification of a copulatory plug. Due to variability in fertility, approximately 40 % of plugged females are found not to be pregnant at the time of embryo collection. Lack of reliability of this method creates a major impact to our collaborators' experimental timelines and wastes the complex mutant mice.

The traditional way for assessment of pregnancy in mice is direct visual observation or abdominal palpation. The reliability of these methods prior to E12 depends on the skill of the technician and is dependent on litter size. To address this problem, ultrasound was implemented at our facility to confirm pregnancy in timed-pregnant females and to count embryos when required. Use of ultrasound is a non-invasive, and early and reliable means to confirm pregnancy.

The ultrasound process involves anesthetizing animals with isoflurane, chemical removal the abdominal fur, imaging the animals on a heated stage and monitoring for recovery from anaesthesia. The Vevo 770 Imaging System (120 V) with a RMV 704 scan head is used for imaging and the Stoelting Active Scavenging unit is used for personnel safety. We have determined that our capacity is approximately 15–20 scans per hour.

In summary, the use of ultrasound reduces the number of animals required for experiment and also assures an adequate number of animals for the timely completion of the experimental objectives. Non-pregnant animals can be recycled for other purposes, in accordance with the philosophy of the 3Rs.

92. Discovery of new genes that regulate CNS myelination using an ENU-based forward genetic screen in zebrafish

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Myelin is a plasma membrane extension of specialised glial cells (called oligodendrocytes and Schwann cells in the central and peripheral nervous system, respectively) that wraps around the axons of our nerve cells in order to allow rapid conduction of neural impulses and to provide metabolic and trophic support to those axons. Disruption to myelin contributes to the symptoms of numerous devastating conditions of the nervous system, including the demyelinating disease multiple sclerosis, MS. We currently have a relatively limited understanding of the molecular and cellular interactions between axons and glial

cells that specifically regulate myelination, which limits our ability to provide interventions to aid myelin repair. Zebrafish are a great model to study myelination. The small size, optical transparency, relative simplicity and rapid development of zebrafish embryos are properties that allow direct observation of biological events as they occur in living animals. The Lyons lab has developed a set of tools to visualise myelin and myelinated axons at high resolution in live zebrafish.

The aim of this project is to identify new genes that are crucial for myelination in order to understand how these cells function during myelination and remyelination. Importantly, these genes, and the proteins they encode, might be future drug targets to enhance myelin repair.

In a previous forward genetic screen in zebrafish performed in the Talbot laboratory at Stanford University, 13 mutations in 10 genes were identified that disrupted various aspects of myelination. Although very successful, the novel genes identified in that screen were shown to be required for PNS myelination, leaving a gap in our knowledge of mechanisms of CNS myelination. To identify new genes involved in CNS myelination, we are currently carrying out a new ENU-based forward genetic screen using a zebrafish transgenic mbp: EGFP reporter line that allows high-resolution analyses of CNS myelination in vivo (not possible in previous screens). Some interesting mutants have already been identified, and screening is still on going. These putative mutants are now being analysed in order to determine the exact myelination phenotype in more detail. The preliminary data will be presented and discussed.

93. Quantitation of fluorescence intensity in mice: a novel genotyping approach of fluorescently labelled transgenic mouse models

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Fluorescence proteins have been useful as genetic reporters for a wide range of applications in biomedical research. Several fluorescent markers with sufficient brightness and spectral separation are available and are frequently used for the analysis of transgene activity.

Here, we show that transgenic mice with different coat colours ubiquitously expressing a green (EGFP) or red fluorescence protein (mCherry or tdTomato) can be reliably genotyped by measurement of the fluorescence intensity. We identified the tail of the mouse as the tissue best suited for such an in vivo genotyping approach. The fluorescence intensity not only distinguishes wild-type from transgenic mice but also hemizygous or heterozygous from homozygous mutants. The results obtained by quantitation of fluorescence intensity were confirmed by standard PCR analysis or test breeding. This novel approach can be used on juvenile or adult animals and allows for instant genotyping without tissue sampling and subsequent DNA analysis. Furthermore, the feasibility of genotyping without invasive treatments to collect tissue biopsies is an important animal welfare aspect.

In summary, we demonstrate for the first time that the genotype of a transgenic mouse ubiquitously expressing a fluorescence protein can be reliably determined without tissue sampling and subsequent PCR analysis or test breeding.

94. Using *RUNX1* enhancer-reporter transgenic mouse models to dissect discrete stages of developmental hematopoiesis

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Hematopoietic stem cells (HSCs) are generated during embryonic development from a specialized subset of endothelium, the hemogenic endothelium, through an endothelial-to-hematopoietic transition (EHT). It has been difficult to obtain a mechanistic insight into EHT and HSC emergence, due to a lack of markers for hemogenic endothelium. The transcription factor Runx1 is expressed in and critically required for EHT and HSC emergence. We reasoned that distinct *cis*-elements might exist for this developmental regulator, which could be used to generate transgenic enhancer-reporter mouse models and aid the identification and isolation of cellular intermediates in the HSC lineage. Combining chromatin accessibility assays and comparative genomics, we identified several candidate hematopoietic *Runx1 cis*-regulatory elements. We previously showed that one of these, the *Runx1* +23 enhancer, marks all hemogenic endothelial cells and hematopoietic cell clusters including emerging HSCs. Using a +23 enhancer-reporter mouse line to study hemogenic endothelium, we showed that these cells undergo dynamic fate changes early in gestation and that their maturation from a competent to a specified hematopoietic stage involves an unexpected early loss of endothelial potential. Here, we characterize another *Runx1* enhancer-reporter model, based on the +205 enhancer. Interestingly, confocal imaging, flow cytometry and functional analysis of the 205GFP transgenic mouse line showed that this enhancer preferentially marks endothelium over hematopoietic cells. The 205GFP transgene partially overlapped with the +23 enhancer in the aortic endothelium, suggesting that it marks a subset of the hemogenic endothelium. Experiments are ongoing to assess this. The distinct patterns of +23 and +205 enhancer-reporter lines reveal regulatory complexity in the regulation of *Runx1* expression. In addition, these mouse models provide tools to isolate and purify discrete stages of hemogenic endothelium and to work towards a 'roadmap' of HSC emergence. Since to date HSCs cannot be expanded or generated *de novo* in culture, such a roadmap will be instrumental in advancing stem cell-based therapies and regenerative medicine for the treatment of blood-related diseases.

95. Fluorescence-tagged TALEN constructs enrich targeting outcomes

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Transcription activator-like effector nuclease (TALEN) systems are a fusion of TALEs derived from the *Xanthomonas* spp. to a restriction endonuclease FokI. By modifying the amino acid repeats in the TALEs, customized TALEN systems specifically bind target DNA and induce cleavage by the nuclease between the two distinct TALE array-binding sites. Somatic cell modification by TALENs provides not only a quality control measure for assembled TALEN constructs, but also a significant advantage by permitting the isolation of cells containing precise modifications before outlaying the expense of animal production by cloning. However, transfection efficiency of somatic cells, such as primary fibroblasts, is still low. Therefore, a strategy for enriching successfully transfected cells is desirable. Enrichment of cells by co-transfection of TALEN plasmids with an antibiotic-resistant plasmid or transposon co-selection has been previously demonstrated. In this study, we added two fluorescent protein sequences (GFP and mCherry) into backbone plasmids pCS2TAL3-DD (Addgene) and pCS2TAL3-RR (Addgene), respectively. The new backbone plasmids pCS2TAL3-DD-EGFP and pCS2TLAL3-RR-mCherry were used to generate TALENs targeting a highly conserved region of *MSTN* exon 2 using the Voytas/Bogdanove Golden Gate TALEN kit (Addgene). The MSTN-TALENs designed for inducing indels at the myostatin locus were tested in mouse ES cells. In summary, using TALENs with fluorescence tags enriched identification of targeted colonies with 14 out of 40 (35 %) colonies confirmed for mutations using high-resolution melting (HRM) analysis compared with 6 out of 70 (8.6 %) without selection. The HRM analysis results were further validated by Sanger sequencing. In conclusion, the two fluorescence-tagged plasmids enabled us to examine the transfection efficiency and facilitate the colony pickup of successfully targeted cells.

96.The role of mutant Plzf in metabolic and hemodynamic disturbances in spontaneously hypertensive rats

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The spontaneously hypertensive rat (SHR) is the most widely used model of essential hypertension and is predisposed to left ventricular hypertrophy, myocardial fibrosis and metabolic disturbances. Recently, a quantitative trait locus (QTL) influencing blood pressure, left ventricular mass and heart interstitial fibrosis was genetically isolated within 788 kb chromosome 8 segment of SHR-PD5 congenic strain that contains only 7 genes, including mutant Plzf (Promyelocytic leukaemia zinc finger protein) gene. To identify Plzf as a quantitative trait gene, we targeted Plzf in the SHR using the TALEN technique and obtained SHR-Plzftm1Ipcv line with a premature stop codon at position of amino acid 58. The Plzftm1Ipcv allele is semi-lethal since approximately 95 % of newborn homozygous animals died perinatally. All homozygous animals exhibited a caudal regression syndrome including tail anomalies and serious size reduction and deformities of long bones, and oligo- or polydactyly on the hindlimbs, as well as impaired development of the urinary tract with a vesicoureteric reflux and a hypoplastic kidney. Heterozygous rats were grossly normal and were used for metabolic and hemodynamic analyses. SHR-Plzf ± versus SHR wild-type controls exhibited reduced body weight (347 ± 9 vs. 386 ± 6 g, $P = 0.008$) and relative weight of epididymal fat (0.85 ± 0.04 vs. 1.17 ± 0.06 g/100 g BW, $P = 0.002$), lower serum triglycerides (0.43 ± 0.03 vs. 0.54 ± 0.03 mmol/L, $P = 0.03$) and liver triglycerides (7.4 ± 0.3 vs. 8.8 ± 0.5 μmol/g, $P = 0.04$), as well as lower serum cholesterol (1.13 ± 0.04 vs. 1.32 ± 0.05 mmol/L, $P = 0.009$) and liver cholesterol (5.1 ± 0.2 vs. 6.6 ± 0.3 μmol/g, $P = 0.006$). In addition, SHR-Plzf ± rats exhibited significantly increased sensitivity of adipose tissue to insulin action when compared to wild-type controls (insulin-stimulated lipogenesis 1,480 ± 88 vs. 1,064 ± 80 nmol glucose/g/2 h, $P = 0.007$). Contrary to SHR-PD5 congenic rats, SHR-Plzf ± heterozygous rats versus wild-type controls had significantly increased heart mass (0.41 ± 0.01 vs. 0.38 ± 0.01 g/100 g BW, $P = 0.002$). These results provide evidence for important role of Plzf in regulation of metabolic and hemodynamic traits in the rat.

97. Towards genome engineering in *Macrostomum lignano*

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Flatworms have been a successful model organism to study regeneration since the days of Thomas Hunt Morgan. They have deepened our understanding of the cellular and molecular processes involved in regeneration, tissue repair and stem cell biology. However, one disadvantage of commonly used flatworm model organisms is the lack of easy and readily available tools for genome engineering. *Macrostomum lignano*, a sea water free-living flatworm, shares many of the advantages of traditional flatworm model organisms but, importantly, is amenable to genome engineering. Our lab is dedicated to establishing *M. lignano* as a versatile model organism for stem cell and regeneration research and one of the most crucial technical advances to achieve this is the development of robust and reproducible transgenic techniques. Here, we present our

recent advances on establishing genome-editing tools for *M. lignano*.

98. Editing myostatin in livestock animals

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Genome editors, such as TALENs and CRISPRs, are molecules which can be designed to introduce double-strand breaks into DNA at a desired locus. Cutting of the DNA allows accurate genetic modifications to be made at the locus, either by NHEJ or by the addition of a template which is incorporated into the target DNA by HDR. The development of these new technologies means that creating livestock species with modified traits of scientific or agricultural value is now far more efficient. We have previously demonstrated successful genome editing in pigs and now aim to establish this technique in other species. Myostatin is a negative regulator of muscle growth, and removal of the functional protein can cause a double muscle phenotype where muscle mass increases by up to 20 %. As the double muscle phenotype has a high agricultural value, we designed TALENs to target the myostatin gene in cattle and sheep, with an aim to disrupt gene expression by inducing NHEJ at the target site.

Bovine and ovine embryos were injected with the myostatin TALENs and transferred into recipients. Four calves and nine lambs resulting from the injections were analysed for evidence of editing at the myostatin locus. Two calves and one lamb showed monoallelic editing while another calf had three different alleles at the locus (wild-type, an in-frame deletion and an out-of-frame deletion) demonstrating that the animal was a mosaic. This mosaic animal showed a striking double muscle phenotype when compared with its wild-type sibling. This is the first time that successful genome editing has been reported in cattle and sheep, two important livestock species.

99. Rapid generation of mutant mice using CRISPR-mediated genome editing (tips and tricks)

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Genetically modified mice are an invaluable resource for investigating gene function and identifying the molecular pathology of disease. However, generating mutant mice by modification of embryonic stem cells and subsequent breeding of chimeric mice is time-consuming and labour-intensive. Recent advances in genome-editing technology using the Clustered Regularly Interspaced Palindromic Repeat (CRISPR) system indicates that genetically modified mice can be generated by direct modification of the zygotic genome. This approach offers many advantages including rapid generation of Founder (F0) mutant mice (within 3 weeks of injection) and potential application to any mouse strain. While this exciting technology promises to revolutionise murine functional genomics, only a handful of mutants have been published.

To assess the efficacy of in vivo genome editing, we injected mouse zygotes with CRISPR mutagenesis reagents for a series of disease genes and putative enhancers and screened F0 offspring for indels at the cleavage site. Remarkably, up to 100 % of F0 embryos/mice carried a mutation. We have also used CRISPR–CAS technology to insert an epitope tag sequence into the Pcdh19 gene. Our data indicate that CRISPR mutagenesis is highly efficient and provides a rapid and cost-effective alternative to traditional gene-targeting approaches.

As the technician responsible for microinjection, I have spent a lot of time troubleshooting the finer technical issues with the technique (including issues with plasmid/oligo co-injections), and I now feel that we have now established a reliable system for generating mutant mice.

I aim to present a practical view of problems experienced and overcome in the early stages of our genome-editing adventure.

100. TALEN-mediated pronuclear microinjection-based targeted transgenesis (PITT)

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Genome-editing approaches, including ZFN, TALENs and CRISPR/Cas, are newly developed, effective and efficient for generating genome-modified mouse comparing to the traditional way using mouse embryonic stem cells though the off-target effects should be taken care carefully. Transgene expression is sometimes silenced due to high copy integration or integration of transgene into heterochromatin. Pronuclear injection-based targeted transgenesis (PITT) system has been applied to generate transgenic mice with transgene insertion into designed chromosome loci to ensure transgene expression.

In this report, we developed a TALEN-mediated PITT system to generate single-copy transgenic mice using a pair of designed TALEN targeted to the intergenic Hipp11 (H11, chromosome 11) loci which has been shown to be supportive either to ubiquitous or tissue-specific transgene expression. First, we designed a pair of TALEN against mouse H11 loci and test the genome-editing efficiency in vivo by analysing the genomic DNA isolated from ES clones established from H11-TALEN-injected embryos. Eight out of 15 (53.3 %) established ES clones are found genome-edited at H11 loci. Second, we sub-cloned upstream 1.0-kb and downstream 4.0-kb fragments to the predicted H11-TALEN site into pGEM-T easy vector with additional multiple cloning sites in between.

The resulting plasmid is served as donor backbone. Third, we sub-cloned a 1.8-kb CMV-EGFP-polyA transgene fragment into the donor backbone. The 1.0-kb and 4.0-kb genomic fragment of H11-TALEN target sites are served as homologous arms for targeted transgene integration. The Last, we co-injected the designed H11-TALEN mRNAs and the 6.8-kb EGFP donor DNA fragment into 0.5-dpc fertilized mouse zygotes. The survival embryos were in vitro culture in KSOM medium in humidified incubator at 5 % CO_2 and observed the embryos under fluorescence microscope for EGFP expression. There are 53.4 and 66.7 % of 8-cell to compact morula stages and blastocyst staged expressed EGPF, respectively. The percentage is much higher than the efficiency of traditional way of random integration for transgenic mice production.

101. Production of targeted genetic modifications in the rat using embryonic stem cell and genome editing technologies

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The rat continues to be the preferred model organism for several important fields of biomedical research, including cardiovascular and metabolic disease. Recent advances in rat embryonic stem cell (rESC) and genome editing technologies hold the promise of producing more powerful models of human disease through the precise modification of the rat genome. While still in its infancy, rESC technology has the potential to vastly expand both the size and the nature of genetic modifications available to researchers. We report here the production in rESC of precisely defined mutations in 5 genes, including several of interest to the biomedical community. Using plasmid or BAC-based vectors, we have produced a variety of mutations, including: complete coding sequence deletion, the insertion of reporter genes controlled by the endogenous promoter, and partial or complete replacement of the coding sequence with the cognate human gene (humanization). Here we describe deletions of 5.4 kbp, and an insertion of 9.0 kbp. Targeting efficiency in our rESC was comparable to mouse ESC targeting. We have achieved germline transmission for two targeted mutations and have begun phenotyping studies, including expression analysis of lacZ reporters. We also demonstrate Prm1-Cre-mediated deletion of our vectors' antibiotic resistance cassette in F1 progeny ("self-deleting cassette); incorporation of this element into our vectors eliminates the need for additional manipulations to remove the antibiotic resistance cassette. We have also explored the use of site-specific nucleases in rESC, specifically the Zinc-finger Nuclease (ZFN) and CRISPR/Cas9 systems. We have enhanced targeting efficiency at multiple loci by the introduction of double-stranded breaks in the gene of interest through the use of both these systems. By including nucleases we have also achieved biallelic targeting at several loci. Combining these two technologies allows us to carry out targeting projects with greater efficiency and versatility and may ultimately lead to the production of new human disease models.

102. Creation of citrine-tagged FANCD2 expressing mice using programmable nucleases

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FANCD2 is a gene of the Fanconi anemia (FA) pathway, involved in DNA damage response and repair. The core complex is composed of eight FANC proteins and two additional Fanconi anemia-associated proteins (FAAPs). They are particularly responsive to interstrand cross-links, which occur due to accumulation of toxic substances or during treatment with some cancer therapy drugs. The core complex activates FANCD2 and FANCI by monoubiquitination, and DNA repair machinery is recruited to repair the damage. To facilitate the analysis of FANCD2 function, we used the TALEN or CRISPR gene-editing technology to create a mouse model expressing a citrine-tagged version of the FANCD2 protein.

Citrine gene was inserted behind the start codon of the Fancd2 gene using TALENs and CRISPRs in two different approaches. The donor vector was assembled using ligation-independent cloning, and contained homology arms for the Fancd2 region surrounding the ATG site. For TALENs, the right TALEN includes the ATG site of the Fancd2 gene. For CRISPRs, we used two sgRNA combined with the nickase version of Cas9, with one of the sgRNAs including the ATG. TALENs or CRISPRs combined with the linearized donor construct were injected into mouse zygotes. Mouse embryonic fibroblasts were developed and the activity of citrine-tagged Fancd2 was examined.

103. Generation of mutant mouse lines using nuclease technologies at the ICS (The French Mouse Clinic)

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Targeted modification via homologous recombination is a powerful method but is time-consuming and requires ES cells. In the last decade, 'programmable nucleases'—ZFNs, TALENs and CRISPR–Cas system—have been shown to enable targeted genetic modifications in whole animals. These nucleases allow the induction of site-specific DNA cleavage in the genome, the repair (through endogenous mechanisms) allows high-precision genome editing. As a big transgenic facility, we are well aware that these nucleases will revolutionize the world of science and transgenesis. The use of these technologies is completely changing our organization and implies the reallocation of resources and equipment. We are by now in the evaluation of these methods for an intensive use.

We have tested with success both ZFN and TALEN technologies; we will give two relevant examples, one concern the generation of a KO mouse line (by non-homologous end

joining) and the other the introduction of a point mutation (by homologous recombination (HR)). Using the CRISPR/Cas technology, we have obtained our first HR event (the knockin of a 6.9 kb reporter) in a founder mouse (and many NHEJ events in other founders) by pronuclear injection of both a guide RNA, the Cas9 mRNA and a circular donor vector. Six other CRISPR/cas projects are being microinjected at the time of writing this abstract. One of this project aims to generate a straight knockout and 5 other aim to introduction a point mutation or LoxP sites. The data obtained at the time of the meeting will be presented.

104. Multiple locus gene targeting on rDNA combined with TALENs

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In order to solve the technical problems during obtaining stable transgenic animals and clinical application of gene therapy, our previous study has established a technique of 'multi-locus gene targeting' mediated by zinc finger nucleases (ZFN) which identified the internal transcribed spacer (ITS) of ribosome DNA (rDNA) genes in human genome and proved to be with efficient site-specific integration and stable expression of foreign genes.

Recently, transcription activator-like effecter nuclease (TALEN)-mediated genome-editing spurred interest due to the high efficiency in targeted genomic integration and its low cytotoxicity. We wonder whether the technique of multiple locus gene targeting on rDNA, which was previously established by our group, could combine with TALENs.

In the present study, by using online software to design suitable TALEN cutting the corresponding loci in human genome rDNA gene, TALENs eukaryotic expression vector was assembled with TALENs monomer and skeleton plasmid libraries. Meanwhile, the general vector of multiple locus gene targeting of human cells, which contains two homogenous recombination arms DS1 and DS2 and the marker components of protein expression, is constructed. We amplify DS1 and DS2 by PCR using human genome as a template. The fragment was inserted into pUC19 by HindIII, PstI, XbaI and EcoRI. Then, using in-fusion enzyme, the pCMV-MCS-EGFP DNA fragment amplified from the plasmid of pEGFP-N1 by PCR was connected with pUC-DS1-DS2. After enzyme identification and sequencing, results showed that the recombinant expression vector pUC-DS1-pCMV-MCS-EGFP-DS2 was successfully constructed.

The multiple locus gene targeting combined with TALENs provides the possibility for site-specific transgenic animals and human gene therapy. It is also a new attempt to improve the efficiency of gene targeting.

Keywords: Multiple locus gene targeting; TALEN; ribosome DNA

The present work was supported by NSFC (No. 81272552 and No. 81101537), National Science and Technology Major Project (No. 2014ZX08010-012B) and Guangdong Provincial High-tech Development Special Projects (No.2012A030400066).

105. Construction of multiple locus gene-targeting vector with CRISPR for Bubalus bubalis genome engineering

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Bacteria and archaea have evolved adaptive immune defences termed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems that short RNA is used to direct degradation of foreign nucleic acids. CrRNA/trancrRNA/Cas9 complex is guided to recognize and bind specific DNA sequences, to break DNA double strand and to silence foreign gene expression. Compared with the previous systems, such as Cre/LoxP, ZFN and TALEN, CRISPR/Cas9 system possesses several advantages such as easy operation, high-efficiency, low-cost and simultaneous silencing of arbitrary number of genes.

To direct Cas9 to cleave the rDNA sequences of Bubalus bubalis, crRNA–tracrRNA fusion transcripts was expressed as guiding RNAs (gRNAs) with the human U6 polymerase III promoter. Directly transcribing gRNAs allowed us to avoid reconstituting the RNA-processing machinery used by bacterial CRISPR systems. As the sequences recognized by gRNA should initiate with G and require the PAM (proto spacer-adjacent motif) sequence which means to -NGG following the 21-base pair sequences recognized by gRNA, the approach can, in principle, target any genomic site of interest. Based on those rules, three locuses in the Bubalus bubalis rDNA were found.

As gRNAs can target Bubalus bubalis rDNA sequences and guide the Cas9 to make double-strand broken (DSB) donor DNA fragment as repair donor was constructed to achieve gene targeting. Two homologous arms with the targeting locus on both sides were designed, named as homologous recombination directing sequence 1 and 2. Through digestion and ligation of restriction endonucleases, the multiple locus gene-targeting vector pUC19-DS1-DS2 was constructed. The green fluorescent protein (GFP) gene was inserted into the spacer of two homologous arms as reporter gene.

In the present work, a multiple locus gene-targeting vector for the Bubalus bubalis genome engineering (pUC19-DS1-EGFP-DS2) was obtained. As it is combined with CRISPR systems, gene targeting might become easy operated and with high efficiency in further researches.

Keywords: CRISPR; Bubalus bubalis; Multiple locus gene targeting

The present work was supported by NSFC (No. 81272552 and No. 81101537), National Science and Technology Major Project (No. 2014ZX08010-012B) and Guangdong Provincial High-tech Development Special Projects (No. 2012A030400066).

106. Interrogating the non-coding mouse genome using CRISPR–Cas9

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Cis-acting DNA elements are found in the vertebrate genome that drives gene expression in specific cell types, developmental or disease stages, and that influences the transcriptional level. In the last twenty years, the logic of gene expression has been expanded to include enhancer, silencers and insulators, coordinating gene expression in the many different cell types found throughout life and development. Some variants are also found associated with human traits and diseases. The functioning of these elements is complex and strictly dependent on the interactions that each element has with other proximal and distal sequences within the genomic locus. For this reason, targeting the endogenous locus is the tool of choice to study such elements in their original chromosomal context. Using the mouse Tyr gene as an experimental model, we interrogated the relevance of distal regulatory elements, previously described in our laboratory, located at either end of the Tyr expression domain. Using a CRISPR–Cas9 approach—but not TALENs, we efficiently generated several targeted chromosomal deletions that we previously had failed to obtain using standard ES cell-based gene-targeting strategies, due to the presence of many repetitive DNA elements. By inducing two distal double-strand breaks flanking the sequence of interest, the intervening DNA can be efficiently deleted or inverted. Founders carrying biallelic deletions of one of such regulatory elements in the murine Tyr locus were obtained and presented coat colour alterations, reproducing similar phenotypes observed with YAC-type transgenes before and indicating the relevance of this sequence for the faithful regulation of Tyr gene expression. We also proved that chromosomal inversions could be efficiently obtained in mice using the CRISPR–Cas9 system, thereby reproducing gross chromosomal rearrangement in mice. Also, deletions of different size could be obtained using the same reagents, probably generated by microhomology-driven DNA repair. A large variability of different Tyr mutations, generated as independent transgenic animal lines, could be achieved, rapidly and efficiently, in just one microinjection session.

107. TALENs-mediated gene targeting in mouse *ROSA26* locus

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Reporter gene mouse lines are routinely used for studies related to functional genomics, proteomics, cell biology or cell-based drug screenings. The preferred place for integration of reporter transgene in mouse genome is *ROSA26* locus. This well-described genomic region is commonly used for gene knockin strategies by homologous recombination (HR) in mouse embryonic stem cells. This locus provides ubiquitous transgene expression with no obvious adverse consequences on mouse viability or phenotype. In this study, we designed and tested TALEN-specific for ROSA26 locus and compared their efficiency, mutagenity and toxicity to commercially available ZFNs targeting the same sequence. We employed TALEN-assisted HR to introduce two reporter constructs encoding TurboRFP and TagBFP fluorescent proteins and reporter construct to monitor the activity of $p16^{Ink4a}$ into the first intron of the *ROSA26* gene. The $p16^{Ink4a}$-reporter mouse was generated as an ageing reporter mice model. Particulary, we prepared construct containing genes encoding for luciferase and Tomato fluorescent protein under $p16^{Ink4a}$ promoter control. After confirmation of its specific activity in senescent cells in vitro, we injected $p16^{Ink4a}$ reporter construct containing *ROSA26* homologous arms together with *ROSA26*-specific TALENs into mouse oocyte. This strategy led to successful insertion of more than 6kbp insert into *ROSA26* of mouse genome. Thus, our $TALEN^{ROSA26}$ showed to be an efficient tool for targeting construct into *ROSA26* locus using injection into mouse zygotes.

108. Generation of targeted deletions in mouse zygotes by pairs of TALENs or SGRNAS/Cas9

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Sequence-specific nucleases such as TALENs or the CRISPR/Cas-system generate site-directed double-strand breaks and enable to introduce targeted mutations into one-cell mouse embryos, facilitating the generation of disease models. Using this method, knockouts, small deletions and knockins can be generated. Our work focuses on the creation of paired, nuclease-induced double-strand breaks to create deletions, inversions or duplications to model copy number variations. Upon pronuclear microinjection of two TALEN pairs specific for sequences of the *Rab38* gene at a distance of 9.3 kb, 6 % of the mice born carried a genomic deletion. Two of these founders were homozygous mutants and exhibited a coat colour phenotype. Using the CRISPR/Cas-system, 37 % of the mice obtained showed a deletion of 3.2 kb, and in 11 % the mutation was homozygous. Due to gene disruption and impaired pigmentation, a coat colour phenotype could be readily observed. Breeding of mutant founders and genotyping of the progeny proved germline transmission of mutant alleles. In conclusion, TALENs as well as the CRISPR/Cas-system are effective tools to create targeted deletions through paired double-strand breaks.

109. Functional dissection of transcriptional silencer elements using an in vivo gene swap approach

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Characterization of transcriptional cis-acting elements is critical for understanding the regulation of individual genes and

the biological processes that they control. To accurately assess stage- and tissue-specific activity of potential cis elements requires generation of knockout mice, in which particular elements are deleted or altered in their endogenous locations. Previously, the cost and effort of gene targeting has limited feasibility of such approaches in vivo. Here, we demonstrate relatively high-throughput generation of knockin mice by using site-specific ZFNs and CRISPRs to promote homologous recombination directly in mouse oocytes. We use this approach to compare lineage- and stage-specific activity of 2 transcriptional silencers that are active in T lymphocytes, associated with the ThPOK and CD4 genes, respectively. ThPOK and CD4 silencers play critical role in suppressing expression of the ThPOK and CD4 genes, respectively, in the T killer lineage, as deletion of either element leads to dramatic defects in T cell development and function. Nevertheless, the developmental regulation of these silencers remains poorly understood. Here, we have utilized a knockin approach to assess functional conservation and/or divergence between ThPOK silencer elements from different species (mouse versus marsupial), and between ThPOK and CD4 silencers from the same species (mouse) in vivo. Our data show that the ThPOK silencer element has been functionally conserved for at least 160 million years, i.e. since separation of marsupial and placental mammalian lineages. Furthermore, we show that the ThPOK and CD4 silencers encode distinct stage-and lineage-specific activities, independent of their location in the genome. We have further utilized the ZFN-mediated gene-targeting approach to identify key motifs within the ThPOK silencer required for its activity. Our results illustrate the great utility of site-specific nucleases for studying transcriptional regulation in vivo, delineate the essential or redundant functions of silencer elements for correct T lymphocyte development, and provide insight into the upstream transacting factors that regulate them.

110. Screening and genotyping highly complex rodent models created by CRISPR–Cas9 endonuclease

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Recent advances in transgenic technology, most notably, emerging nuclease-based genome-editing procedures, are transforming the landscape in which animals are genotyped. Not only do these tools facilitate more rapid transgenic model production, but they easily allow for the creation of animals with more highly manipulated genomes. Genetic analysis laboratories need to remain responsive to these developments to cope with growing production and quality assurance needs. Here, we share some challenges derived from our experience as one of the highest throughput rodent vivaria in industry.

First, we present some potential pitfalls associated with screening and genotyping animals created from nuclease-based genome-editing technologies (ZFNs, TALENs, and Cas9/CRISPR). There exist a variety of screening tools (Sanger sequencing, pyrosequencing, PCR-amplicon size discrimination, high-resolution melting, Surveyer assay, etc.), and a single tool may be inadequate for most institutions' needs. Another consideration is the breadth of mutations presented by these technologies in simple KO model generation. Researchers previously hoping for a few founders are now presented with dozens of genetically unique founders, having to choose between a range of single-basepair mutations up to deletions of several hundred basepairs. These colonies with several unique genetic founders could require additional breeding to resolve. Moreover, we explain how certain animals will be found as compound heterozygous double-knockouts or mosaics during screening—which also demands increases in breeding. Because injections occur as the single-cell stage, mutation-harbouring cells will not be visible through coat colour scoring, requiring genetic screening for all animals. This places additional burden on the screening laboratory not present in traditional model creation workflows.

Finally, we describe highly complex genetic constructs that can prove difficult or frustrating to accurately interrogate. For instance, uncertainties arise around how to sufficiently genotype a model in which there exist multiple mutations or constructs at the same locus (such as creating a CKO construct at a given locus on one chromosome with a CKI point mutant at that same locus on the other chromosome). Complexity also rapidly inflates from crossbreeding already complex lines or using nucleases to introduce several mutations at once. As the number of modified loci per colony increases, so does the number of reactions required (per sample) to completely genotype an animal. We discuss an approach more suitable for responding to a rapidly increasing need of throughput, one utilizing acoustic-based liquid transfer rather than traditional laboratory robotics. These considerations, and others, are presented in an experience-based approach.

111. Generation of Cas9 transgenic mice and its application

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Cas9/CRISPR system is one of efficient way for KO animal production. Zinc finger and TALEN recognize specific sequences and gene targeted by Fok1 nuclease. However, Cas9/CRISPR systems recognize sequences by sgRNA and induce insertion or deletion with Cas9 complex. In mice, Cas9/CRISPR system is commonly used for KO mouse production, but they are usually null type, not conditional character.

Cas9 transgenic mouse was produced by pro-nucleus injection method with C57BL/6 background. Construct was established by CMV promoter and HA-conjugated Cas9 protein encoding gene. Finally, four founder mice were produced and they were male. Overexpression of Cas9 protein was confirmed by PCR, qPCR and western blot for HA antigen. Two founders with Cas9 protein high overexpression were selected and conducted germline transmission test. Cas9 gene integration

was shown only in male pups. Skin, GI tract, lung, liver and testis showed relatively high Cas9 expression. However, heart, kidney and brain showed lower Cas9 expression.

These Cas9 transgenic mice could be used for various purposes, such as in vivo gene targeting. With simple sgRNA injection to mice, in vivo gene targeting for specific target organ could be possible. Comparison with cre-Loxp system, Cas9 transgenic mice with sgRNA injection model has some advantages—it is more simple, is inducible type and do not need additional mating.

In conclusion, Cas9 transgenic mice were established, and overexpression and germline transmission activity were confirmed. This mouse could be used for new animal method with in vivo gene targeting.

This research was supported by the Ministry of Food and Drug Safety, Korea (KFDA2013-18075).

Keywords: animal model, Cas9, in vivo targeting

112. Evaluation of Cas9 RNA-guided nuclease for high-throughput mutant mouse production in C57BL/6N

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With the completion of the human and mouse genome sequencing projects, focus turned towards annotating the function of every gene. To do this, the International Mouse Phenotyping Consortium (IMPC), a coordinated program of 13 global mouse production and phenotyping centres, aims to produce and phenotype 5,000 knockout mouse lines by the end of 2016. The IMPC has already generated >2,000 knockout mouse lines from International Knockout Mouse Consortium (IKMC) embryonic stem (ES) cells. However, ES cell quality control failures and germline transmission rates averaging $\sim$50 % are areas where efficiencies have been sought to improve mouse line production for the remaining $\sim$15,000 genes.

The application of the Cas9 RNA-guided nuclease (Cas9-RGN) to mammalian genome editing has sparked incredible excitement in the research community. In the absence of homologous repair templates, the endogenous error-prone non-homologous end joining (NHEJ) DNA repair pathway repairs Cas9-RGN-mediated double-strand DNA breaks, introducing small insertions and deletions (indels). Targeted mutations in the mouse genome can be generated by co-injection of zygotes with synthetic guide RNAs that specify the target site and Cas9 endonuclease as a DNA expression plasmid, mRNA or protein. Homologous repair templates may also be co-injected to produce bespoke alleles. Zygote injection is faster and more economical than using ES cells. Tempering the excitement are reports of concurrent off-target mutations using Cas9-RGN. Strategies reported to reduce off-target effects include using offset single-strand nicks by a mutant Cas9 nickase (Cas9n), extending the 5' end of guide RNAs with non-templated 'GG', using Cas9 protein instead of plasmid expression vectors, and using truncated guide RNAs.

We have piloted using Cas9-RGN for mouse production in our IMPC-affiliated mouse production programs. *In vitro* transcribed guide RNAs were co-injected into the pronuclei of C57BL/6NCrl zygotes with either Cas9 or Cas9n mRNA or Cas9 protein. We produced NHEJ mutants for 11 of 12 genes and recovered an average of $\sim$40 % founders among F0 mice. We have recovered indel mutations with and without frameshifts, with both somatic and germline mosaicism in founders. We are also undertaking whole-genome and directed off-target analysis. Given the random nature of NHEJ-induced alleles, more defined alleles may be desirable for analysis in both high-throughput projects and by individual investigators undertaking hypothesis-driven disease model development. Thus, we are also testing protocols for bespoke point mutations, exon deletions and conditional alleles. We expect that the methods developed here will apply equally well to low- and medium-throughput production in transgenic core facilities.

113. Efficient PRNP deletion in bovine genome using gene-editing technologies in immortalized bovine cells

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Conformational changes of the cellular prion into the pathogenic prion (the misfolded form) can induce neuronal degenerative diseases such as bovine spongiform encephalopathy and human's Creutzfeldt-Jakob Disease. However, there are still many unknown factors on prion function in cattle to date. To know the prion gene (PRNP) function, the knockout (KO) approach of PRNP in bovine fibroblasts were used with recent genome-editing technology, transcription activator-like effector nucleases (TALENs) in this study. Ahead of KO of the PRNP gene in primary cells, bovine ear skin fibroblasts were immortalized via introduction of exogenous human telomerase reverse transcriptase

(hTERT) and B lymphoma Mo MLV insertion region 1 homolog (BMI1) in order to enhance the efficiency of DNA delivery in vitro. These immortalized bovine fibroblasts maintained population without cellular senescence and chromosomal abnormality until passage 60 (180 days of culture) and showed improved colony-forming capacity originated from one single cell. Lower expression level of the main tumour suppressor gene, p53, and higher activity of telomerase compared with primary fibroblasts were detected in the immortalized cells. Furthermore, there was no significant difference in development competence of somatic cell nuclear transferred (SCNT) embryos according to the different donor cells, primary and immortalized cells. After establishing and analysing the bovine immortalized cell line, the cells were electroporated with the mixture of DNA vectors, the TALEN pair for PRNP sequence-specific KO and its reporter for magnetic activated cell sorting (MACS; Kim et al. 2012). The MACS-positive cells were seeded at a density of 100 cells in 100 mm culture dish, and after 10 days, a number of single colonies, intact and round, were isolated. After analysing the genomic DNA from each single colony with T7 endonuclease I, 66 % of isolated colonies were T7E1 positive, mutated with large or small deletion. Among them, two clones with 4 and 104 bp deletion, respectively, were used as a donor cell for SCNT, and the nucleotide sequence of the KO target region in the all cloned blastocysts was the same with the donor cell's. In summary, it was demonstrated that bovine fibroblasts with exogenous genes were immortalized and PRNP-KO via TALEN on those cells were developed into pre-implantational stage without loss of development competence. In the future, PRNP mutated cells or embryos will provide fundamental understanding of its function in vitro. This work was supported by IPET (#109023-05-5-CG000 and 111078-03-3-CG000) and Biogreen (PJ0090962012).

Keywords: prion, immortalized cells, TALEN, Knockout, SCNT

114. A simple method that supports high-throughput assembly of TALEN plasmids

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TALEN has been used as a powerful tool for precise engineering of the genome. A major challenge in construction of TALEN plasmids is the assembly of the customized DNA-binding domains, which consist of a battery of TALE repeats in a specific order. Currently, the two-step Golden Gate method is the most commonly used approach for TALEN DNA-binding domain assembly. In this method, the first step of reaction requires ligation of up to 10 units of TALE repeats with one plasmid vector in a specific order. The large number of DNA fragments in the reaction, however, often leads to low efficiency production and errors in the assembly. Here, we present an alternative approach for TALEN plasmid assembly using a more balanced two-step Golden Gate reaction. In this approach, each reaction contains no more than five DNA fragments, which allows for efficient production of TALEN plasmids with high fidelity. To simplify the procedure, we have generated a tetramer TALE repeat library, with which generation of TALEN plasmid requires only one step of Golden Gate reaction. Importantly, this tetramer library supports high-throughput assembly of TALEN plasmids, by which one person can easily carry out assembly of one 96-well plate (48 pairs of TALEN) to sequencing-verified TALEN plasmids in only three working days. In line with assembly procedure, we will present a cell-based method for high-throughput testing TALEN activity. Furthermore, we show that TALEN assembled by this method can efficiently modify the target genomic loci in cells, and in mouse and rat when injected into the one-cell embryos.

115. Production of an immunodeficient common marmoset by ZFN-based mutagenesis

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Non-human primate (NHP) experimental animals are important for our understanding of human diseases, physiology and genetics, due to their high level of similarity to humans. The common marmoset (*Callithrix jacchus*) is a species often used in biomedical research because of its prolificacy and ease of handling. Previously, transgenic marmosets have been generated using lentiviral vectors. However, in many species, including the marmoset, targeted gene knockout (KO)/knockin (KI) animals cannot be produced through traditional gene targeting of embryonic stem cells (ESCs) as they lack the ability to contribute to the germline, although this is not the case in rodents.

On the other hand, the recent development of innovative genome-editing technologies, such as ZFN, TALEN and CRISPR/Cas9, can resolve this issue, and it is now possible to generate target gene KO or KI animals without the use of ESCs.

The interleukin receptor 2 gamma (Il2rg) gene is one of the genes responsible for X-linked severe combined immunodeficiency (X-SCID) in patients and the immunodeficient mouse. These immunodeficient mice are used widely in research. Immunodeficient NHP models for use in biomedical science would constitute an excellent animal model. Here, we report our attempt to generate an immunodeficient marmoset by knocking out the Il2rg using two versions of ZFN (HiFi-ZFN and eHiFi-ZFN).

In our results, four of 16 neonates showed mutagenesis in the Il2rg. Two (one HiFi-ZFN and one eHIFi-ZFN) of the four neonates survived for six and nine days, and the remaining two eHiFi-ZFN neonates continue to survive (over 70 days). Detailed analyses of post-mortem tissues showed that the deceased animals harboured the mutated Il2rg in all tissue samples analysed. One of these animals had a 1 bp deletion in

the Il2rg, and flow cytometric analysis of blood and spleen samples showed that the numbers of CD4−/CD8+ T cells, CD4+/CD8− T cells and CD2−/NKp46+ NK cells were dramatically decreased. Moreover, the anatomical dissection indicated a lack of a thymus in the animal. In the live animals, one neonate was homologous and the other was heterogeneous for a KO in the Il2rg. Flow cytometric analysis of umbilical cord blood samples from the homologous gene KO animal showed a remarkable reduction of CD4−/CD8+ T cells, CD4+/CD8− T cells and CD2−/NKp46+ NK cells.

This study indicates that ZFN-mediated targeting of the marmoset Il2rg has the potential to produce an X-SCID marmoset model that is more similar to humans and would contribute greatly to research in the very near future.

116. Attempts to generate knockin marmosets using the CRISPR/Cas9 system

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The common marmoset (*Callithrix jacchus*) is a useful experimental non-human primate because of its similarity to humans, high reproductive efficiency and easy handling. In marmosets, embryonic stem cells have been established; however, germline transmission ability has not been confirmed. In many species, this situation limits production of target gene knockout (KO) or knockin (KI) animals that would be useful models in various studies. Recently developed innovative genome-editing technologies may resolve this situation. Furthermore, recent studies suggest that genome-editing technologies could be used to produce target gene KO/KI animals. In this study, we used clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9), a modern genome-editing technology, in an attempt to generate the world's first KI marmosets.

The *c-kit* gene is known to play important roles in the proliferation and differentiation of melanocytes, mast cells, interstitial cells and hematopoietic stem cells. In previous reports, *c-kit* mutagenesis in mice caused anaemia and white spotting on the coat. Therefore, if *c-kit* mutant marmosets could be produced, the phenotype could easily be confirmed by coat colour.

In this study, to modify the marmoset c-*kit* gene using CRISPR/Cas9, we designed several U6-driven guide RNA vectors. Then, we co-transfected guide RNA vectors and a humanised-Cas9 expression vector into marmoset fibroblasts and carried out a SURVEYOR nuclease assay to detect modifications in the target sequence. From this assay, insertion and deletion modifications were observed in the c-*kit* gene with frequencies of 2–32 %. Sequence analyses identified frameshift deletions (2–25 bp) and insertions (1 bp) in the *c-kit* gene that yielded non-functional proteins. This result suggests that CRISPR/Cas9 is a new technology that could be used to produce target gene KO marmosets.

117. Generation of Nrf2 point mutant mice by CRISPR/Cas9 system

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Nrf2 is a key molecule regulating anti-oxidative response and xenobiotic-metabolizing enzyme genes. Small Maf (sMaf) proteins (MafF, MafG and MafK) make a heterodimer with Nrf2, and regulate transactivation activity of Nrf2. Nrf2–sMaf heterodimer activates the transcription of many cytoprotective genes through antioxidant response element (ARE). Nrf2–MafG heterodimer and MafG homodimer bind to the consensus Maf recognition element (MARE) with high affinity, but bind differentially to the suboptimal binding sequences degenerated from the consensus MARE. Alanine 502 residue in the basic region of Nrf2 and a tyrosine reside of MafG in the position corresponding to alanine 502 in Nrf2 are essential to generate specificity of recognition sequences. Heterodimer of mutant Nrf2(A502Y)-MafG displayed a binding specificity similar to that of MafG homodimer. To investigate in vivo function of mutant Nrf2(A502Y), we generated point mutation of Nrf2 mice using novel genome-editing technology CRISPR/Cas9 system. The mutant mice will be a good model to explain the biological significance of differential binding by Maf homodimer and CNC-Maf heterodimer.

118. A Report of applying TALEN and CRISPR/Cas9 genome-editing technologies to five genes on mouse zygotes

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Using animals to simulate human diseases has provided a means to study pathologies and explore therapeutic agents. Genetically tractable diseases have mainly been studied using genetically modified mouse models such as knockout (KO) and knockin (KI) mice through embryonic stem (ES) cell-based approach. Recent advances using genome-editing technologies, such as Zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) systems, have provided the possibility of producing genetically modified animals by modifying genomes directly on zygotes. Here, we showed the results of applying TALEN and CRISPR/Cas9 for genetic engineering on five individual

genes. In two cases in which TALEN RNA was injected into cytoplasm of zygotes then implanted to fosters, the respective indel rates were 37.5 % (6/16) and 60 % (3/5) in live pups. With respect to CRISPR/Cas9 system, three genes were tested. In two cases, injection of Cas9 plasmid together with sgRNA into the nucleus of zygotes yielded respective indel rates of 0 % (0/8) and 12.5 % (1/8) in live pups. In one case, the indel rate was increased to 20 % (1/5) when Cas9 RNA instead of Cas9 plasmid was co-injected with sgRNA into the nucleus. We analysed the preserved embryos (E3.5) from the plasmid ($n = 9$) and RNA ($n = 10$) injection experiments. The successful indel rates were 55.6 % (5/9) and 80 % (8/10), respectively. These results further indicate that RNAs (Cas9 and sgRNAs) are better choice for injection into nucleus. In conclusion, we successfully induced indel on target genes by applying TALEN or CRISPR/Cas9 on mouse zygotes. Optimizations of procedures, such as adjusting RNA concentrations and injection into cytoplasm instead of nucleus, are needed to enhance efficiency and reduce toxicity, which are currently under investigation.

119. Use of TALEN for a double gene extinction on two closely linked loci

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The proteins of the SIBLING family ('Small Integrin Binding ligand N-linked Glycoproteins') are components of the bone extracellular matrix which plays a major role in skeletal biology and more precisely in the biomechanical competence. They are encoded by genes grouped into a 'cluster' on mouse chromosome 5. Among them, Ibsp, encoding the bone sialoprotein (BSP) and Spp1, and encoding osteopontin (OPN), are highly expressed by bone cells. Knocking out of the two genes independently induces different bone phenotypes, suggesting separate functions. However, our previous work has highlighted many possibilities for compensations/functional substitutions between BSP and OPN (Bouleftour and al. JFBTM 2012 and Boulefour and al. Plos one 2014), which complicate the understanding of their respective roles in bone physiology. We generated mice devoid of both BSP and OPN. The tight linkage between Ibsp and Spp1 excludes the possibility to generate double-mutant mice by crossing mice. We generated Spp1 mutations in mice already carrying a Ibsp mutation using TALEN ('transcription activator-like effector nucleases').

We fertilized in vitro wild-type oocytes with sperm taken from Ibsp−/− males. The eggs were microinjected with mRNA encoding a pair of TALEN targeting Spp1, then implanted in pseudopregnant females; 43 % (9/21) of the Ibsp ± offspring had mutations in the Spp1 gene. Several mutations were often present within the same individual (19 mutations in total for 9 mice), reflecting the possibility that TALEN continue to be active in the embryo after the first cell division. We crossed these founder mice with wild-type mice and identified Spp1+/mut Ibsp ± offsprings. We identified founders carrying the OPN and BSP mutations on the same chromosome. Spp1−/mut Ibsp−/− mice were interbred to obtain double homozygous mice. Most Spp1 mutations (deletions and/or substitutions of bases or nucleotides) did not block the mRNA translation. Nevertheless, an ELISA dosage of the circulating OPN protein allowed us to identify three homozygous strains in which OPN is virtually extinct. These Spp1−/− Ibsp−/− mice reach adulthood. The double extinction is not lethal. They have a normal morphology, but a lower weight than wild-type mice, as Ibsp−/− mice do (Malaval and al, J Exp Med, 2008.).

With TALEN approach, we obtained strains of mice with mutations on two genes closely linked in the SIBLING cluster. Comparing phenotypes of Spp1−/− mice, Ibsp−/− mice and mice homozygous for both mutations will permit to more precisely analyse the respective functions of BSP and OPN in bone physiology.

120. Generation of mutant model for fragile X mental retardation syndrome 1 NEIGHBOR (FMR1 NB) USING NUCLEASE TECHNOLOGY: - IS FMR1NB INVOLVED IN MEIOTIC DIFFERENTIATION OF MALE GERM CELLS?

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Hybrid sterility (HS) represents a biological phenomenon safeguarding newly originated species in statu nascendi. This natural mechanism is widely spread among animal and plant species although majority of identified genes and chromosome loci responsible for HS came from studies on Drosophila species. Laboratory of J. Forejt developed a mammalian model for genetic analysis of HS, with the mouse subspecies Mus m. musculus (PWD) and Mus m. domesticus (B6) allowing investigation of molecular mechanisms and genetic networks underlying HS. In this model, incompatibilities between subspecific alleles of two major hybrid sterility loci, PRDM9 on chromosome 17 and Hstx2 on chromosome X, constitute the major components of genetic control of HS (Bhattacharrya et al. Proc Natl Acad Sci U S A. 2013 Feb 5;110(6):E468–E477. doi: 10.1073/pnas.1219126110). Based on the testis-specific gene expression and polymorphisms between PWD and B6 alleles, it was found that the Hstx2 locus contains eight HS candidate genes. One of them, Fragile X mental retardation syndrome 1 neighbour gene (Fmr1nb) showed expression culminating in late meiotic prophase I. To characterize the function of Fmr1nb, we prepared a set of targeted deletion mutants by TALEN

technology. The established Fmr1nb deletion mutant mouse lines have been studied to address the gene-specific function in key meiotic stages of male germ cell differentiation. We found that Fmr1nb is most likely not responsible for HS as the mutant mouse lines did not exhibit defects in spermatogenesis, especially in synapsis and recombination of homologous chromosomes during meiotic prophase I. However, we noticed that mutation in Fmr1nb leads to an increased risk of maternal death during partition. Currently, efforts are being made to identify the underlying cause at this maternal death.

121. Generation of kallikrein 5 and kallikrein 7 double-deficient mice employing-specific programmable nucleases

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The kallikrein-related peptidases (KLKs) are a family of 15 serine proteases that create a large gene cluster on chromosome 19 in human and chromosome 7 in mice. Although KLKs appear to be involved in many physiological processes, their roles in vivo are still incompletely understood partially due to unavailability of suitable mouse models. Klk5 and Klk7 have been identified as participants in tightly regulated proteolytic pathways that are crucial for epidermal homeostasis. Although knockout mouse models for Klk5 and Klk7 have been generated, there was no obvious phenotype in KO animals with deficiency in these proteases. This may be caused by redundancy and/or compensatory mechanisms, in which one protease could partially substitute the function of the other. However, generation of mouse model lacking both proteases by conventional strategies is highly time-consuming and expensive since both genes are located in the same locus, i.e. in the close proximity. In this study, we show generation of Klk5/7-deficient mouse by microinjection of TALEN mRNA.

122. TALEN-mediated site-directed mutagenesis of bhlh proteins Id4 and Lyl1 to study their role in zebrafish development

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bHLH proteins are transcriptional regulators that is important in development process. Here, we describe the function of two different bHLH proteins Id4 and Lyl1 in neural progenitor cell proliferation/differentiation and haematopoiesis, respectively. Using translation-blocking morpholino for Id4, we show that knockdown of Id4 in zebrafish resulted in severely malformed brains lacking distinct boundaries. Id4 as well as Id4/p53 morphants exhibited precocious neuronal differentiation with reduced proliferation and increased apoptosis albeit to different extend. Inhibition of p38MAPK resulted in complete rescue of Id4/p53 morphants and partial rescue of Id4 morphants. Overexpression of Lyl1 mRNA resulted in increased production of haematopoietic but not endothelial precursors at both the primitive and definitive waves of haematopoiesis. Together these data suggest that Id4 negatively regulates p38MAPK (and possibly p53) activity, both of which have been shown to block proliferation and stimulate apoptosis. Also the increased production of haematopoietic precursors in the Lyl1 ectopically overexpressed embryos shows Lyl1 is important in both the waves of haematopoiesis. To further dissect the molecular mechanisms of Id4 function and study the role of Lyl1 in haematopoiesis, we have established Id4 mutant and Lyl1 zebrafish using TALENs.

123. Survey of mutant mice generation using TALEN or CRISPR/Cas9 technology in the czech centre for phenogenomics

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TALE nucleases and the CRISPR/Cas9 system have revolutionized the production of genetically modified animals. These technologies allow easy programming for targeting a specific locus in the genome to introduce DNA double-strand breaks. Repair of this DNA break by NHEJ leads to modifications of the targeted locus causing insertions or deletions (Indels), which often lead to frame-shifts. Since such frame-shifts result in non-functional genes/proteins, TALENs or CRISPR/Cas9 can be used for the generation of knockout animals. However, these nucleases are not limited to production of knockouts. Targeting two sites simultaneously allows specific deletions within the genome. Also, once a DNA double-strand break was introduced, it is possible to introduce dsDNA-constructs or ssOligonucleotides via homolog recombination. With these new tools modifications of any kind in the gene of interest (including mutation of a few basepairs, tagging of proteins in vivo or creating conditional knockouts) are possible in a short period of time. In our lab, we are employing TALEN and CRISPR/Cas9 technology for our own projects as well as in collaboration with groups from outside. This enables us to evaluate the efficiency of these two technologies for generation of genetically modified mice. Here, we present our results from 27 finished and 30 ongoing projects.

124. In vivo comparative analysis of 50 sgRNA for mouse knockin models: efficacy and off-target activities

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Generation of genetically modified animals can be a long and laborious process involving sophisticated molecular biology

work and stem cells skills. In the past decade, nucleases have emerged as a major breakthrough in the transgenic field, paving the way for quicker, cheaper and straightforward genetically modified model development. During the past 10 years, genoway has generated more than 1,500 animal models and has accumulated a strong experience in nucleases-based gene modifications, particularly using ZFNs and TALENs.

Even though off-target activity due to nuclease activity is commonly observed and still not properly evaluated, the use of CRISPRs/Cas9 nuclease for constitutive knockout is nowadays largely widespread. Moreover, generation of knockin models to introduce point mutations using CRISPRs/Cas9 still needs to be optimized and refined.

In this study, we evaluated RNA-guided Cas9 nuclease for its in vivo efficiency but also its limits for the development of C57BL/6 knockin mouse models.

Thus, a set of 50 different sgRNA targeting a large number of loci including *Rosa26* locus were designed and cloned for in vitro transcription or for DNA expression. sgRNA were tested in presence of Cas9 mRNA or Cas9 expression vector for in vivo NHEJ/homologous recombination efficiency.

Sequencing analysis allowed us to determine and compare the nature and frequency of genomic Cas9-induced mutations depending on experimental conditions: pronuclear versus cytoplasmic injection, sgRNA or DNA expression vector. The impact of the targeted sequence features on Cas9 nuclease-specific activity, and its off-target effect were evaluated.

As a conclusion, out of 50 targeted sequences analysed, more than 80 % of the designed sgRNA led to DNA mutation events at the targeted genomic locus. NHEJ event varied from 13 to 100 % from one locus to another, whereas Cas9-mediated homologous recombination efficiency reached 13–89 %.

We are currently investigating further to identify the sgRNA design parameters that could lead to the highest in vivo activity and lowest off effects.

125. CRISPR/Cas9 editing of mouse THRA gene by pronuclear microinjection of a single plasmid into fertilized oocytes

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Five cases of a new human disease due to mutations of THRA, a gene encoding thyroid hormone receptor a1 (TRa1), were recently described. Affected patients present symptoms resembling hypothyroidism but without any important change in circulating levels of thyroid hormone. Finally, mutations of TRa1 were discovered for all of them, mostly in the terminal helix 12 of the receptor.

In order to create mouse model of this genetic disease, we decided to test the RNA-guided CRISPR–Cas nuclease system. We chose to use non-homologous end joining (NHEJ) repair mechanism. Indeed, among four patients, different mutations were observed in the last helix of TRa1 receptor. So we aimed at obtaining different random mutations located in this helix.

Our first experiment was done in vitro using C17.2 neural cell line, in order to test CRISPR technology and validate our single guide RNA (sgRNA). Sequence allowing targeting helix 12 of TRa1 was designed and cloned into pX459 plasmid (Addgene id: 48139). Once transfected into cells, this plasmid allows expression of both Cas9 nuclease and sgRNA. It also enables selection of transfected cells with puromycin antibiotic. Resistant cell clones were amplified and genomic DNA analysed by PCR amplification and sequencing of the targeted region. Among the resistant clones, 65 % were shown to carry mutation in at least one allele, 25 % were homozygous for a single nucleotide insertion and 8 % had large indels (100 bp and more).

We then decided to perform in vivo experiments. The same sgRNA sequence was cloned into a similar vector, without puromycin resistance gene (pX330, Addgene id: 42230). This new plasmid was injected into fertilized mice oocytes. Out of 160 injected embryos, 81 survived injection and 65 reached the two-cells stage and were transferred into foster mothers. Out of these transferred embryos, 32 pups were born. Among 14 analysed pups, three were mutated at least on one allele and seem to be mosaic. The last 18 pups are currently analysed for mutations. Mutated mice will be mated with wild-type animals so as to permit alleles segregation and allow precise characterization of the different mutations.

The overall protocol is thus suitable to generate germline mutations in mice within few days without using in vitro transcription.

126. A genome-editing custom service by tale nucleases for zebrafish model

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Knockout animal models are important for the scientific community to study and understand gene of interest function in vivo. Transcription activator-like effector (TALE) nucleases are able to induce DNA double-strand breaks at a specific locus in genomic DNA. This double-strand break is then repaired by NHEJ (non-homologous end joining) and homologous recombination (HR) mechanisms, leading to insertion–deletion mutations in the targeted gene. The TALE nucleases present the advantages to be easier to design than ZFN and meganucleases and can target any locus in the genome.

We are currently investigating the relative efficiency of the TALE nucleases injected as mRNA or protein. TALE nucleases protein or mRNA directed against Green Fluorescent Protein (GFP) is injected in zebrafish transgenic lines expressing GFP. TALE nucleases efficiency is assessed both by residual GFP expression in the larvae and genomic sequencing.

The two academic platforms AMAGEN and TACGENE provide already a genome-editing integrated service for model fish. TACGENE design the genome-editing tools according to user's needs while AMAGEN realize fish eggs microinjection, F0 founder isolation and F1 animals rearing. F1 progeny can be provided either as unscreened egg batch from positive F0 founders or as genotyped adult animals.

The poster will present the results of the mRNA/protein comparison as well as a detailed workflow of the knockout service offered by AMAGEN and TACGENE.

127. Highly efficient biallelic knockout of the porcine *GGTA1* locus by using CRISPR/Cas9

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Genetically modified pigs lacking a functional α1,3-galactosyltransferase gene (*GGTA1*-KO) prevent the hyperacute rejection associated with pig-to-non-human primate xenotransplantation. In the past, we successfully generated pigs with a biallelic *GGTA1*-KO by using zinc finger nucleases. With CRISPR/Cas9, a new promising tool for genetic engineering is now available. We tested the efficacy of CRISPR/Cas9 to generate a biallelic knockout of the porcine *GGTA1*-locus. Simultaneously, we transfected cells with a CRISPR directed to target the porcine cytidine monophosphate-N-acetylneuraminic acid hydroxylase-locus (*CMAH*) to determine the capability of CRISPR/Cas to target several loci in the porcine genome at once. About 3 million day 25 foetal fibroblasts were transfected with two CRISPR/Cas9 constructs (px330, Addgene plasmid 42230). The first one encoded for a guide RNA targeting exon 9 of *GGTA1* (5′-CACCGCTGACGAGTTCACCTACGAG-3′) and the second one targeted exon 10 of *CMAH* (5′-CACCGCCTGAACTACAAGGCTCGGC-3′). Transfected cells were cultured for 5–7 days until they reach confluency in a 75-cm2 culture flask. Subsequently, cells were counter-selected for Gal epitopes by using biotin-conjugated Griffonia simplicifolia IB4 lectin and Streptavidin-coated magnetic beads. Cells not being attracted by the magnetic field remained in the supernatant and were seeded onto 96-wells. Cells were further analysed by FACS staining for Gal epitopes and surveyor nuclease assay (Cel-I) to determine mutations at the *GGTA1* and *CMAH* locus. All cells did not express Gal epitopes on their surface and showed indel formation at the GGTA1 and CMAH locus. As the mutations at the GGTA1 locus induced a biallelic knockout of the gene proven by the complete absence of Gal epitopes, this still has to be determined for the CMAH locus. Cells served as donor cells for somatic cell nuclear transfer to generate foetuses for further analyses and to generate living offspring. At the time of writing this abstract, it was still too early to check the two recipients for pregnancy. The CRISPR/Cas9 has successfully proven its efficacy in generating a biallelic knockout of a porcine endogenous gene comparable to zinc-finger nucleases. Off-target analysis will be an essential part of the foetus analysis.

This project was funded by DFG TR CRC 127.

128. Syndrome of apparent mineralocorticoid excess: rat model

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The Syndrome of Apparent Mineralocorticoid Excess (SAME) is caused by a deficiency of 11b-hydroxysteroid dehydrogenase type 2, which converts corticosterone (A) to its inactive metabolite, 11 dehydrocorticosterone (B) and renders the mineralocorticoid receptor aldosterone specific.

We generated multiple rat knockout strains, through pronuclear microinjection of targeted zinc finger nuclease mRNAs. The Hsd11b2 gene was targeted in exon 2 and one founder carried multiple targeted alleles with 1, 4, 16 and 123 bp deletions. These were transmitted independently to progeny, and one strain, carrying a 123 bp deletion, spanning the 3′ end of exon 2 and part of the following intron, was characterized in detail.

Plasma ratios of glucocorticoid (A) and its metabolite (B) suggested a complete lack of functional Hsd11b2 protein. Absence of full-length protein was confirmed both by western blot and immunohistochemistry. Homozygous animals were 13 % smaller than wild-type littermates, but they had enlarged kidneys, adrenals and heart. Mesenteric (omental) fat pads were significantly smaller than those of controls. Homozygous knockout rats were polydypsic and polyuric, with an increased haematocrit, suggesting volume depletion.

On standard 0.3 % Na diet, their urinary Na/K ratio was slightly higher than controls, but they were in salt balance. Glucocorticoid levels showed normal circadian variation, (low at 7am; high at 7 pm) and were not significantly different from control levels on a 0.3 % or a 0.03 % salt diet. Plasma renin was significantly lower in homozygous animals on normal salt diet and remained suppressed, even on a low salt diet.

Homozygous animals showed similar response to controls on a glucose tolerance challenge, but insulin production was very significantly suppressed, suggesting an increased insulin sensitivity. By telemetry, they were shown to have a mean arterial blood pressure of $\sim$ 180 mmHg (compared to control levels of $\sim$110 mmHg).

This rat model recapitulates many symptoms of SAME and will help unravel the links between sodium handling, hypertension and metabolic complications.

129. Using CRISPR/Cas9 where conventional breeding fails: the construction of a mouse strain deficient in IL1R, IL18R and IL33R

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The interleukin-1 (IL-1) receptor family belongs, together with the Toll-like receptors, to a superfamily of receptors which are characterized by extracellular immunoglobulin domains and intracellular Toll/IL-1R resistance domains (TIR). In this project, we are focusing on IL-1R, IL-18R and IL-33R (ST2), which signal through MyD88. The interleukins associated with

named receptors regulate the functions of T cells subsets. While IL-1 acts on T cells in a general manner, IL-18 and IL-33 act more specifically on Th1 and Th2 cells, respectively. IL-18 induces IFN-γ secretion from Th1 cells. In compliance with this finding, the IL-18 receptor is a stable marker for Th1 cells that produce IFN-γ. IL-18 can also promote IL-17 production of Th17 cells. IL-33 and ST2 show similar features as IL-18 and IL-18R but oriented towards Th2 cells. ST2 is selectively expressed on Th2 cells but not Th1 cells. Analogue to IL-18 for Th1 cells, IL-33 promotes the secretion of Th2 cell effector cytokines. In addition, it has been shown that IL-1 signalling is essential for the development of Th17 cells. To understand the role of the interplay of these ILs, it would be interesting to analyse mice deficient for two or all three receptors. A common procedure for the creation of multiple knockouts in mice is breeding homozygous single knockout mice with each other. The likelihood of crossover events depends on the distance between the genes of interest and can be expressed in the unit centimorgan (cM) which is defined as the distance between genes for which the recombination frequency in a single generation is 1 %. The distance between the IL-1r1 locus and the IL1rl1 (ST2) locus is roughly 125 kb, which corresponds to 0.065 cM. This means that if one would try to achieve this double knockout by breeding 0.065 % of first-generation offspring would carry the double knockout. For the IL1rl1 and IL18R double knockout, it is practically impossible to achieve a double knockout by breeding, the distance here is 0.14 kb. Due to these extremely low recombination frequencies, the use of modern genome-editing techniques is necessary for generating multiple knockouts of the IL-1 receptor family. Therefore, we used the CRISPR/Cas9 system and generated gRNA specific for IL-18R and IL-33R, which were subsequently injected into IL-1R deficient or wild-type oocytes. A deep analysis of these mice will improve our knowledge of the interplay of the IL-1R family.

130. Interspecific recombination between orthologous human and mouse BAC clones in *E. coli*: exploring scalable humanization of cancer-relevant genes in the mouse genome

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Our interests lie in humanizing specific regions of the mouse genome using large segments of human DNA with extents of 10–100 s of kilobase pairs. To that end, we have undertaken a series of projects to transfer large segments of human DNA, from human BAC clones, into the orthologous region of corresponding mouse BAC clones using—(1) traditional recombineering methods; (2) recombinase-mediated cassette exchange (RCME); and (3) a novel technology that we call *trans*- or trimolecular-recombineering. In each instance, the goal is to place human DNA within the larger context of homologous BAC-derived mouse arms in *E. coli*.

Completed constructs will be introduced into the mouse genome in several ways including—(1) homologous recombination into mouse embryonic stem (ES) cells with positive selection imparted at each end of the transferred human segment; (2) homologous recombination into wide double-strand breaks created in zygotes with two Cas9/sgRNAs; and (3) obligate ligation-gated recombination (*ObLiGaRe*) into wide double-strand breaks created in zygotes with two *Cas9*/sgRNA-guided obligate heterodimeric *Fok*I nuclease domains.

Properly targeted ES cells/zygotes will be used to develop viable mouse strains carrying the humanized segments. Any selection cassettes used will be removed by crossing to mouse strains with germline expression of the appropriate recombinases. All experiments have been designed to maintain a fixed genetic background from community-standard C57BL/6 strains. Our poster presentation details the specifics of our projects and describes progress to date.

This research is supported by National Cancer Institute Grant Number 5P30CA034196-28.

131. One-step generation of mice carrying gene-edited alleles by the CRISPR/Cas-mediated genome engineering with high efficiency

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With genomes from different human populations being extensively sequenced, the urgency is to establish a genotype/phenotype correlation by recreating these variant alleles in model organisms. The type II bacterial CRISPR/Cas system is a newly emerging genome engineering technology with the promise of multiplexed gene targeting and a short generation timeline. We previously reported the creation of reporter and conditional mutant mice by coinjection of zygotes with Cas9 mRNA combined with single guide RNAs (sgRNAs) targeting different genomic loci and DNA donor vectors bearing different homology sequences (Cell, 2013). In current study, homology-directed repair (HDR) and homologous recombination (HR) mediated by CRISPR/Cas are being further examined and optimized using a 4-day embryo culture system, where mouse zygotes are injected at the pronuclear stage, cultured to blastocysts and analysed for reporter expression at the Nanog and Oct4 loci. Combining a new single guide RNA scaffold and/or CRISPR/dual nickase, we were able to achieve over 40 % targeting efficiency inserting the mCherry reporter before the Nanog stop codon and over 50 % targeting efficiency inserting the EGFP reporter into the Oct4 3′ UTR region, on the hybrid B6D2 F2 zygotes (F2 offspring produced from the cross between C57BL/6J female x DBA/2J male). When the two sgRNAs targeting the Nanog stop codon and the Oct4 3′ UTR regions are combined with the Cas9 mRNA and their respective donor DNA plasmids, over 10 % targeting efficiency were achieved for insertion of both the mCherry gene at the Nanog and EGFP reporter at the Oct4 loci. Further study is in progress testing the CRISPR/Cas system in inbred strains of mice and for various genome engineering needs, including point mutation incorporation, reporter insertion and gene swapping. Our study showed that the CRISPR/Cas-mediated technology could be employed for genome engineering with an efficiency that is practical and a timeline unprecedented.

132. Nuclease-mediated gene knockout in an allele refractory to gene targeting in ES cells

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Muscle phosphofructokinase is an essential enzyme for glucose homeostasis. Patients with phosphofructokinase deficiency present with Tarui disease and exercise intolerance in the clinic. In order to generate a mouse model for this disease, exon 3 in the mouse *Pfkm* gene was selected for gene targeting in mouse embryonic stem (ES) cells. Mutation of exon 3 is expected to disrupt both isoforms of *Pfkm* by nonsense-mediated decay of mRNA encoding a premature termination codon. A gene-targeting vector designed to replace exon 3 with a sequence flanked by LoxP sites was introduced into mouse ES cells. Genetic screening of 480 drug-resistant ES cell clones showed that none of them had undergone homologous recombination with the targeting vector. Subsequent efforts to generate a mutant mouse model turned to the direct manipulation of the mouse genome in fertilized eggs by the microinjection of nucleases targeted to exon 3. Four transcription activator-like effector nucleases (TALENs), one zinc finger nuclease (ZFN) and one CRISPR-associated Cas9 nuclease were designed to target *Pfkm* exon 3 and prepared for microinjection. Nucleases were obtained from commercial vendors and also prepared in-house from publicly available resources. Microinjection of plasmid vectors expressing TALENs targeted to exon 3 did not produce mutant mice although this method has been reported to be effective for other genes. The microinjection of mRNA coding for TALENs also failed to generate mouse mutants, independent of the origin of the TALENs reagents. The use of ZFN mRNA for microinjection produced multiple mouse mutants. Co-injection of Cas9 mRNA and guide RNA produced numerous mice that are undergoing genetic analysis. Factors affecting the efficiency and success of nuclease-mediated gene targeting include the activity of nucleases on target sequences and toxicity of microinjection preparations.

133. Single-step megabase deletion in the mouse Ig locus: efficient and precise gene editing using zinc finger nucleases

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Deletion or rearrangement of large genes or gene complexes has traditionally been a lengthy and risky venture: multi-step gene-targeting events are typically followed by recombinase-mediated gene rearrangement, but the targeting events can be difficult to maintain on the same chromosome, and frequently loss of pluripotency accompanies this multi-step process. If one wishes to further manipulate and/or compliment the resultant cells, further losses in pluripotency frequently result. The goal of these studies was to develop a process of simplified targeting and gene excision for large genetic intervals, while maintaining pluripotency and the ability to further manipulate the resultant cells. Using two pairs of zinc finger nucleases whose recognition sites were separated by $\sim$2.6 Mb in concert with a $\sim$3.5 kb targeting vector, precise targeting and deletion were achieved in mouse ES cells in one step, without the need for recombinases or subsequent manipulation. The modified ES cells were then used to generate chimeric mice via standard blastocyst injection, followed by efficient germline transmission. The resultant mice when bred to homozygosity revealed the expected immune-deficient phenotype. Furthermore, the originally modified 1-step ES cells could be transfected, modified at the junctional site of Ig gene deletion and used again for chimera generation. The process described here represents a generally applicable approach for other gene-editing tools, other candidate genes, while providing a massive savings in the manpower and expense that is typical for such programs.

134. Targeted RMCE-live piglets generated by SCNT following sequential double site-specific gene modifications of a porcine EGFP line

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Site-specific nucleases (ZFN, Tal effector nucleases and CRISPRs) boosted the genome editing of different species, and EGFP-specific ZFNs were successfully used in rat (Geurtz et al. 2010) and in pig (Watanabe et al. 2010, Whyte et al. 2010). Previously (Brunetti et al., 2008) we generated an EGFP transgenic porcine line (V2G) characterized by a single integration of pCAGGS-EGFP cassette, high ubiquitous EGFP expression, Mendelian transgene transmission and expression in F1. The aim of this work was to generate live cloned piglets using modified cells after two site-specific modifications (ZFNs and RMCE) on the transcriptionally active GFP-locus (V2G), avoiding any cell rejuvenation by SCNT. Homology arms for promoterless targeting vector were derived from pCAGGS-EGFP vector (promoter fragment = Left-Homology-Arm = LHA; polyA sequence = Right-Homology-Arm = RHA). Cloning floxed (lox2272/lox5171) puromycin and hygromycin resistance coding sequences between LHA and RHA sequences, and we generated the targeting/RMCE vectors (pB5′3′Puro-PL and pB5′3′Hygro-PL) and theirs positive controls (C+) for PCR set-up (100–1,000 plasmid copies). V2G fibroblasts cultured in DMEM + M199(1:1) +10 %FCS, bFGF in 5 %CO_2, 5 %O_2, were transfected using Nucleofector

(V-024 program). In ZFN-mediated gene targeting, 2 μg of each ZFNs coding vectors (Sigma-CompoZr®) and 2 μg of pB5′3′Puro-PL/KpnI vector, were used to 'nucleofect' 0.9×10^6 Verro2GFP fibroblasts. Transfected cells were cultured under puromycin selection (2 μg/ml) for 8 days, and 0.45×10^6 cells were used for the Cre-mediated cassette exchange (1 μg pB5′3′Hygro-PL/KpnI + 2 μg pCAG:CreE-GFP). Transfected cells were plated in 10 Petri dishes (Ø = 150 mm) and cultured under hygromycin selection (200 μg/ml) for 12 days, picked up and expanded in 24 multi-well plates for SCNT. Thirty-one colonies were PCR screened for hygromycin and 16 (51.6 %) colonies were positive for Cre-mediated targeted insertion. Four colonies were used in zona-free SCNT experiments. On Day6, 172 compacted morulae/blastocysts were transferred into two synchronized sows, one became pregnant and went to term delivering seven live and four stillborn piglets. Cre-mediated hygromycin cassette insertion into the V2G locus was PCR-detected in all the animals that resulted also negative for insertions of puromycin and EGFP cassettes. Combining ZFN-mediated targeting with Cre-mediated cassette exchange, we demonstrated that two sequential site-specific modifications could be easily done with high efficiency producing live cloned animals. Moreover, we have validated the V2G locus as a feasible SCNT-tested platform for further site-specific gene modifications. This work is supported by European FP7 grants Translink (no. 603049) and Xenoislet (no. 601827).

135. Conditional gene targeting in mice using paired Cas9 nickase and single DNA template

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Genome editing using CRISPR/Cas9 technology continues to gain widespread acceptance across the biomedical research community. One of the most sought after goals of this technology is the ability to derive conditional alleles in mice. While there has been some success using Cas9 to insert *LoxP* sites that flanking a critical exon to create a conditional allele of a target gene, the approaches used to date have relied on multiple simultaneous targeting events. We sought to simplify the approach to generating a conditional allele by using one DNA template to elicit a single targeting event using paired Cas9n (nickase) and corresponding gRNAs instead of Cas9 nuclease. Into the pronucleus and cytoplasm of 1 cell stage C57BL/6n mouse zygotes, we microinjected Cas9n RNA and sgRNAs together with a single DNA template encoding two *LoxP* sites flanking exon 2 of the isoprenoid synthase containing domain (*Ispd*). In the first litter born, we genotypically confirmed one F0 mouse expressing a conditional allele. Further, we showed that this flox'd allele could be successfully transmitted to F1 progeny through the germline and was functionally recombined using Cre recombinase. Our study demonstrates for the first time that zygote injection of paired Cas9n and a single DNA template of a flox'd allele can be used to successfully derive conditional alleles in mice.

136. Applying the CRISPR/Cas system to enhance the C57BL6/N genetic background: desired modifications, deletions, duplications and mosiacism

Joffrey Mianne, Gemma Codner, Adam Caulder, Martin Fray, Debora Bogani, Microinjection Team, Sara Wells, Lydia Teboul

Mary Lyon Centre, MRC Harwell, Oxford, UK

The recent description of the CRISPR/Cas system as a genome engineering tool has brought new perspectives in the generation of mouse models of human disease by enabling the introduction of targeted point mutations and subtle modifications into the mouse genome in relatively short time frames. Initial applications of the CRISPR/Cas system at the MRC Harwell were focused on the repair of two mutations leading to age-related defects found in the C57BL/6N mouse strain, in order to enhance the genetic background of this strain for long-term studies.

Here, we report the outcome of CRISPR/Cas pronuclear injections into mouse zygotes using the Cas9 D10A nickase (double nicking system). The analysis includes data from 9 injections resulting in the birth of over 200 pups, with F0 animals showing a range of mutations from legitimate repairs/desired modifications, deletions, duplications and insertions. We also include the data from investigating transmission of the repair to the next generation with the analysis of the F1 populations for each repair modification. Transmission of the repair to the next generation was demonstrated in a largely Mendelian fashion, but also revealed mosaicism previously undetected by the analysis of ear clip-derived DNA from the F0 population. Further investigation of off-target effects on the genome as a result of CRISPR/Cas activity in F0 population using Next Generation sequencing (NGS) technologies is underway.

Since these pilot studies, we have expanded the study to include a greater number of targets (24 targets) and greater complexity in the modifications introduced, LoxP site insertions, codon changes and floxing of exons. The outcome of these microinjections is presented in a parallel poster.

137. Optmisation of a production pipeline for CRISPR/Cas-mediated mouse mutants at MRC Harwell

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Traditionally, gene targeting has been used to generate mouse models for human disease research. More recently, the CRISPR/Cas system for genome modification has been introduced which enables a range of modifications from tailored point mutations to targeted floxing of exons in a time-efficient, cost-effective manner. As with any new system, a series of production parameters need to be optimised before a robust pipeline can be established.

We are investigating the following aspects:

- Altering the design parameters i.e. offset between single guide RNAs (sgRNAs) for a particular target
- Increasing the complexity of the modification i.e. generating indels, point mutation, floxing exons
- Use of the Cas9 system versus the Cas9 (D10A) nickase
- Altering the ratio/concentrations of injection components i.e. sgRNAs, donor oligos, Cas9 nuclease/nickase RNA
- Altering the nature of donors (single stranded donor oligo versus plasmid)
- Altering the volume injected and location of injection.

We have so far attempted pronuclear injection of 12 designs and analysed 7, all of which produced sequence changes in the genome. We have sequenced the target locus and found evidence for homologous recombination, illegitimate repair or non-homologous end joining in both F0 and F1. We will present the experience we have accumulated to optimise the parameters for mutant production, both in terms of efficiency and mutant quality.

As part of the transgenics services at MRC Harwell, we are able to offer tailored CRISPR/Cas mutants (sequence dependent) from the design stage to the establishment of genotyped F1 mouse colonies.

138. Targeted insertion of short sequences in the mouse *ROSA 26* locus using CRISPR/Cas9 system

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Mouse ROSA 26 locus has been one of the most highly used sites to insert transgenes such as reporters to achieve stable and reliable transgene expression. The available methodologies, for targeted insertion of sequences, however, constitute extensive and time taking steps that involve the use of embryonic stem cells. A recent approach called pronuclear injection-based targeted transgenesis (PITT) that uses recombination-mediated cassette exchange (RMCE) mechanism enabled direct insertion of exogenous sequences at predetermined loci including *ROSA 26*. PITT technique is performed on fertilized eggs collected from so called 'seed mice' containing the RMCE landing pad that are developed using conventional ES cell-based approaches. Here, we demonstrate that seed mice for PITT can be developed rapidly using the latest gene-editing tool CRISPR/Cas9 system. Notably, the RMCE landing pad sequences were precisely inserted at the original provirus integration site in the *ROSA 26* locus and the new seed mouse developed can enable insertion of larger cassettes at this site. This approach can also be used to rapidly insert landing pad sequences at other loci to develop larger array of PITT tools.

139. Feasibility of a combined system with IMPC vectors and genome-editing nucleases to achieve homologous recombination in ES cells and embryos

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One of the major tools for understanding the mouse genome developed in the past 25 years has been the systematic gene targeting in mouse embryonic stem cells by international consortia. Recently, strategies based on editing nucleases have demonstrated to be powerful tools to modify the genome with high precision. Here, we try to combine both efforts of the scientific community in order to achieve increased efficiency in the generation of mouse models for elucidating the function of genes.

We will present the use of different strategies based on genome-editing nucleases in failed projects with IMPC-targeted cells. Efficiencies between the use of IMPC-targeting vectors in our embryonic stem cells (ES cells), with and without editing nucleases, and co-injecting nucleases in embryos with IMPC vectors to increase homologous recombination will be discussed.

140. CRISPR/Cas9 nuclease-mediated targeting in marmosets, rats and mice

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Modifying the genome of several species of research animals has been feasible for decades. The methods for producing these transgenic manipulations have been refined over those years, but for some species, these methods have been less useful. Some of the difficulties are due to limitations in the number of oocytes or embryos available for manipulation. Lengthy generation time for gestation and development to sexual maturity may limit the utility of some species. For this reason, ES cell-mediated gene targeting may be prohibitively long. In addition, germline-competent embryonic stem cells are difficult to generate. For these reasons, nuclease-mediated genome modification has been shown to be useful. Application of these methods to those species that have been

intractable to genetic modification will be especially important.

We have targeted genes in three species: marmosets, rats and mice, using a range of methods in order to optimize this process.

141. Genome-editing technology in modelling human diseases

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Genome-editing technologies offer a rapid, cost-effective approach to engineering specific mutations in the mouse genome to model human diseases. Genome editing enables insertion, replacement or deletion of DNA from the genome, using engineered nucleases to create specific double-strand breaks at directed sites in the genome. The cell's endogenous repair mechanism resolves these breaks by one of two or more pathways, non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ can result in insertions or deletions (indels) at a specific genomic site to disrupt the translation of coding sequence, while HDR can be used to generate a point mutation or insert precise genomic modifications using donor sequence via homologous recombination. The strength of this technology is the rapid and precise genome sequence modification without selectable markers or residual sequence elements (e.g. LoxP sites). This approach allows us to expeditiously generate mutations to model human disease with greater accuracy and relevance, serving as a platform to develop novel therapeutics to combat the disease process. We utilize zinc finger nuclease (ZFN) and CRISPR/Cas9 systems in our genome engineering efforts at GRS, examples highlighted below.

Previous work with a spontaneous mutant that models Duchenne Muscular Dystrophy (DMD) showed that genetic background plays a significant role in the severity of the disease phenotype. The challenge is to create a model that more closely resembles the clinical symptoms of DMD and to generate this model in multiple genetic backgrounds to elucidate the genetic modifiers of the phenotype. To accomplish this, we used ZFN technology to create knockout (KO) alleles in the dystrophin gene in multiple inbred backgrounds eliminating the need for deriving new embryonic stem (ES) cell lines or for extensive backcrossing. We have successfully targeted seven inbred backgrounds and identified multiple mutations at this site.

We implemented a high-throughput platform and workflow to accommodate a Knockout Mouse Project2 (KOMP2) CRISPR pilot project and JAX researchers and collaborators requests for genome editing using the CRISPR/Cas9 technology. We have to date generated over 34 targeted genes. Using sequence analysis, we identified founders and F1 carriers for 30 of these alleles, with >88 % success rate. Though much of our focus has been on KO alleles for a variety of genes, we have also undertaken several knockin projects to engineer specific point mutations found in humans, for studying the correlation with disease phenotype in cancer, Alzheimer's Disease, and rare and orphan diseases using mouse models.

142. Knockin (KI) in rat zygotes with TALENs and CRISPR/Cas9 systems as protein forms

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Genome editing using ZFNs, TALENs and CRISPRs/Cas9 have been successfully applied to generate gene knockout animals in several species, including the rat, but KI is still less robust. We have recently described the use of TALEN mRNA for rat Rosa26 to KI a GFP expression cassette with frequencies of 1.67–2.2 % of the microinjected and transferred embryos (1). We asked whether KI efficiency could be improved if TALENs or Cas9 are provided as proteins versus mRNA since this would accelerate genomic cleavage and the KI process.

To this end, we used as proteins the same pair of rat Rosa26 TALENs used previously as mRNA to perform KI. We analysed the KI efficiency of Cas9 protein and sgRNA recognizing the same Rosa26 sequence as TALENs. The donor DNA was the same previously used with TALENs mRNA and contained a CAG-EGFP expression cassette flanked by 0.8 and 1 kb homology arms, and it was used at 2 ng/ml. Analyses of GFP expression and genotyping were performed in day 15 foetuses.

TALENs proteins were mixed at final concentrations of 2, 4 and 10 mM together with donor DNA and microinjected in the pronuclei and cytoplasm of 165, 138 and 144 rat zygotes, respectively, which were then transferred into pseudopregnant mothers. In day 15 foetuses, GFP expression was detected in 1 embryo (0.72 %) from the 4 mM group.

Cas9 protein at 0.14 and 0.28 mM were complexed with Rosa26 sgRNA at room temperature and microinjected along with donor DNA in the pronuclei and cytoplasm of 94 and 108 rat zygotes, respectively. In day 15 foetuses, GFP expression was detected in 2 embryos (1.85 %) from the 0.28 mM group. Microinjection of Cas9 as mRNA with the same sgRNA and donor DNA are underway to compare efficiencies with the Cas9 protein KI rates.

Embryo development was not affected at any concentration of neither TALENs nor Cas9 proteins.

In conclusion, TALEN and Cas9 used as proteins to obtain KI are feasible, but at least for TALENs the efficiency of the method was not superior to the use of mRNA.

- (1) Remy S et al., 2014, Genome Res. in press.

143. Optimizing the procedure for generating gene-modified mice by the CRISPR/Cas system

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The CRISPR/Cas system has emerged as a powerful tool for creating genetically modified mice. It allows for the rapid production of mice that carry multiple genetic alterations, including targeted deletions, insertions and substitutions, in a single procedure. Here, we present a further optimized protocol that enables the consistent and efficient generation of genetically modified mice via the CRISPR/Cas system. We, as a core facility, have undertaken a wide variety of projects and have optimized each step in production: from guide RNA design to founder generation. We found that $\sim$25 % of guide RNAs tested had genome-editing activity levels below our criteria for acceptance; it is therefore crucial to validate the activity of guide RNAs before injection into one-cell embryos. We also identified that piezo-driven cytoplasmic injection is the best method to deliver the CRISPR/Cas system into embryos. Furthermore, we compared genetic modification efficiencies in mice between normal (20 nt) and truncated guide RNA, single and pooled guide RNAs, Cas9 mRNA and Cas9 protein, and so forth. To better identify mice carrying genetic modifications, we developed a sensitive T7E1 cleavage assay that can reliably detect a single nucleotide change. Thus, the presented protocol provides a general guideline for the utilization of the CRISPR/Cas system to generate mice carrying desired genetic alterations.

144. Functional characterization of a novel G protein using CRISPR/Cas-mediated knockout

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Genome editing is a method in which DNA is inserted, replaced or removed from a genome. Targeting specific loci in the genome for mutagenesis would greatly speed up analysis of gene function and behaviour. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system has derived from bacteria and archaea adaptive defence mechanism, which together with CRISPR-associated (Cas) proteins provides an acquired resistance to invading viruses and plasmids. The system can be adapted to create guide RNAs (gRNAs) capable of directing site-specific DNA cleavage by the Cas9 nuclease. In this study, we have used the CRISPR/Cas9 technology, a genome-editing approach, to knockout the gnav1 gene in zebrafish. Recent work demonstrated that the gna gene family in zebrafish was analysed, and a novel Gα protein, which was named Gv, for class V was identified. In zebrafish, the localization of Gv with antibodies in the ear suggests a role in mechanotransduction.

Using the CRISPR/Cas9 technology, we have effectively performed genome editing in the region of interest in the zebrafish genome. The knockout was done by co-injection of Cas9 mRNA and guide RNA (gRNA) into 1-cell stage zebrafish embryos. Mutants were confirmed by single colony sequencing. Our preliminarily sequencing data for F0 generation of zebrafish embryos show the knockout of the gnav1 gene. The further work will be to detect whether the mutation is in the germline. Furthermore, we would like to perform behavioural experiments to compare the mutant with the wild-type fishes to identify physiological changes. Knockout of the gnav1 gene in zebrafish will help us to understand the developmental and behavioural roles of this gene.

145. Myc-induced B-cell malignancy in transgenic zebrafish

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Myc is a pleiotropic transcription factor with fundamental and far-reaching effects on cellular activities including proliferation, metabolism, apoptosis and angiogenesis. Its activation is estimated to be associated with 20 % of all human cancers. Transgenic zebrafish have been used to study cancer since 2003, and many transgenic models of cancer are generated including blood tumours and solid tumours. These tumours share striking histological similarities with human cancers and resemble their human counterparts at both the genetic and molecular levels. Although some T cell lymphoma or leukaemia models have been generated by expressing a frequently mutated oncogene of Myc or NOTCH1 under the promoter of Rag2, attempts to study B cell lymphoma in zebrafish have yet to be successful. The only B cell malignancy so far modelled in zebrafish is a B cell leukaemia established by expressing a specific human oncogene TEL-AML1.

A recent study by D. M. Page and his colleges described a B cell reporter zebrafish model that demonstrated the evolutionary conservation of B cell development, highlighting the potential of zebrafish for modelling human B cell malignancy. The first part of our study aims to establish an inducible B cell malignancy model using the Cre-ER^{T2}/LoxP system and a B cell-specific promoter (IgM1). Transgenic founders of Tg (IgM1: Cre-ER^{T2}) and Tg (IgM1: LoxP-dsRed-LoxP-cmyc-EGFP) have been generated using a multi-site gateway technique. Founder embryos will be raised to adulthood and crossed with wild-type/casper zebrafish for transgenic screening. By pairing founders, Tg (IgM1: Cre-ER^{T2}; IgM1: LoxP-dsRed-LoxP-cmyc-EGFP) will be generated, and this model will allow us to induce Myc expression at any stage of B cell development by adding tamoxifen. By modulating c-Myc expression, it should be possible to induce various B cell malignancies including both leukaemias and lymphomas. The Myc-EGFP fusion protein will allow us to observe tumour cell initiation, progression and matastasis in real time.

This model may have broad potential for studying various aspects in B cell oncogenesis by crossing with other transgenic zebrafish. For example, the role of host immunity can be

studied by crossing with zebrafish having fluorescent-tagged immune cells.

146. Development of transgenic zebrafish as a predictive model for assessment of drug-induced hepatotoxicity

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Drug discovery is a complex process, laborious, very expensive and time-consuming studies. Developing a new drug to the average costs a pharmaceutical or biotechnology company about $400 to $900 million and takes an average of 10–15 years. Of all the compounds entering the R&D project less than 1 % will be launched to the market. Drug-induced hepatotoxicity is one of the most important causes of drug withdrawal in the process of drug discovery. In the early stages of drug development, the low correlation of the in vitro cytotoxicity studies with human hepatotoxicity makes difficult to predict.

Zebrafish shows a great potential to be used in early stages of discovery, benefits to its properties that include transparency, short development cycle [embryogenesis is complete by 72 h post fertilization (hpf)], easiness to treat with compounds, small amounts of drug required, high genetic homology with humans (over 85 %), inexpensive to maintain and possibility to generate transgenic lines targeting specific organs. Researchers over the past 10 years have characterized many of the mechanisms that occur during the process of development, leading to an important knowledge about the signalling pathways and physiological function of zebrafish liver. Therefore, we are developing an in vivo assay for assessing drug effects on hepatotoxicity in zebrafish liver. For this purpose, the following aspects have been studied: (1) Development of test methods for assessing the hepatotoxicity of a reference compound in zebrafish. (2) These methods will be validating by testing various drugs. (3) The results will be compared with those obtained in mammals.

147. Generation and characterization of alpha-1 type VI collagen zebrafish mutant line

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Mutations in the triple helical collagenous domain of alpha-1 type VI collagen have been demonstrated to be linked to the onset of muscle diseases such as Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) in patients. Possible targets for treatment have been identified thanks to experiments performed on a mouse line and zebrafish morphants both deficient in collagen VI. However, until now, both disorders remain without cure, emphasizing the need for supplementary data in order to better understand their aetiology and facilitate the process of finding a treatment. In our study, we opted for creating a zebrafish line expressing a modified form of the collagen VI protein with altered functionality. We used a transcription activator-like effector nuclease (TALEN^{TM}) to mutagenize an essential splice donor site within intron 16 of the *col6a1* gene (zebrafish genome assembly Zv9) and provoke skipping of exon 16 in the processed mRNA. The induced mutation was situated in the N-terminal portion of the collagenous domain and was homologous to a dominant mutation observed in patients with Bethlem myopathy. To characterize a potential phenotypic manifestation of the modified collagen VI, we performed behavioural and morphological analysis on zebrafish heterozygous or homozygous for the isolated mutation. Mutant zebrafish embryos and larvae did not show significant alterations in touch-evoked response or birefringence measurements as compared to wild-type siblings, whereas fluorescent phalloidin staining of muscle as well as histopathological and ultrastructural analysis revealed mild disorganization of muscle tissue in mutants. One advantage of the mutant fish line we established is the possibility to analyse the progression of putative muscle degeneration in time as this has been described for BM patients. The mild phenotype we observed should thus be confirmed in adult fish. In addition, the potential implication of collagen VI in zebrafish tissues other than muscle may be further studied and possible related defects characterized. On the whole, by using TALEN^{TM}-mediated targeted mutagenesis, we obtained for the first time a zebrafish mutant line with altered alpha-1 type VI collagen, which can serve as an animal model to study the progression in time of a degenerative phenotype related to the synthesis of this protein with reduced functionality.

148. CRISPR/Cas-mediated gene targeting in porcine embryos

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Gene targeting whereby intended gene sequences can be targeted for deletion, modification or incorporation of exogenous DNA is achieved by homologous recombination (HR) with site-specific targeting vectors. Even though HR-based gene-targeting events are extremely rare, the efficiencies can be improved by several orders of magnitude (>1,000-fold) by introducing a double-strand break (DSB) at the target locus. Following DSB, either a single stranded oligo or a double-stranded targeting vector with homology to the ends flanking the DSB can generate targeted genomic alterations. In our laboratory, methodologies for CRISPR/Cas-mediated HR and gene targeting were successfully established by direct injections into the porcine zygotes bypassing the need for

performing gene targeting in an intermediate cell type, typically foetal fibroblasts, and perform nuclear transfer/cloning to generate modified animals. As a proof of concept, *PRNP* locus has been selected as a safe harbour locus for genomic alterations. Co-injection of in vitro transcribed mRNA expressing *Streptococcus pyogenes* Cas9 nuclease alongside a chimeric single guide RNA targeting *PRNP* resulted in the generation of homozygous knockouts. Additionally, by co-injection of CRIPSRs alongside a single-stranded oligo bearing sequences for LoxP site or a DNA-targeting vector consisting of GFP expression cassette, the *PRNP* locus was successfully targeted for incorporation of exogenous LoxP site and GFP expression cassettes, respectively. The efficiencies of gene targeting were close to 100 % with all of the blastocysts screened showing accurate targeting in embryos with no evidence of mosaicism. Additionally, using this methodology, up to two loci could be simultaneously targeted in one round of injections into the embryos. In summary, we have developed efficient means to generate desired edited animals for agricultural and biomedical applications, and help usher animal biotechnology into the functional genomics era.

149. An egg metalloprotease plays a key role during fertilization in mammals

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Molecular mechanisms of fertilization by which a competent acrosome-reacted sperm bind to the oolemma remain uncharacterized. To identify oolemmal-binding partner(s) for sperm acrosomal ligands, affinity panning was performed with mouse oocyte lysate using sperm acrosomal protein, SLLP1 as a target. An oocyte-specific membrane metalloproteinase, SAS1B (Sperm Acrosomal SLLP1 Binding), was identified as a SLLP1-binding partner. Mouse ovarian cDNA cloning revealed six SAS1B splice variants, each contains a metalloprotease signature of zinc-binding active site and a putative transmembrane domain, with signal peptides in three variants. SAS1B transcripts were found to be ovary specific. The precise temporal and spatial onset of SAS1B expression was first detected in early secondary follicles and intensified with oocyte growth. The restricted expression of SAS1B protein in growing oocytes was found to be conserved in seven eutherian species, including non-human primates. Immunofluorescence localized SAS1B to the microvillar region of oolemma in mouse M2 oocytes. After fertilization, SAS1B decreased on the oolemma and became virtually undetectable in blastocysts. In transfected CHO-K1 cells, SAS1B localized to the polarized surface of un-permeabilized cells. Native proteins of both the germ cells as well as its binding recombinant protein were colocalized to the microvillar domain of ovulated M2 oocytes and to the acrosomes of acrosome-reacted sperm. Molecular interactions between mouse SLLP1 and SAS1B were demonstrated by surface plasmon resonance, far-western, yeast two-hybrid, recombinant- and native-co-IP analyses. SAS1B was observed to bind with SLLP1 through high affinity. Further, recombinant SAS1B was found to be proteolytically active and significantly inhibited in vitro fertilization. SAS1B knockout female mice showed 34 % reduction in fertility. The study thus identified SAS1B-SLLP1 as a pair of novel sperm–egg-binding partners involving the oolemma and intraacrosomal compartment during fertilization.

150. Presence/absence of a marker gene in artificial microrna expression constructs affects knockdown efficiencies in a targeted transgenic system

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RNAi-mediated gene silencing has proven to be very effective for loss-of-function analysis of a target gene and for hypomorphic analyses both in vitro and in vivo. However, knockdown mice generated by conventional random integration-based transgenesis often show variation in their knockdown efficiency. In this study, we attempted to reduce such a variation by employing a pronuclear injection-based targeted transgenesis (PITT) method, which had been originally developed by us and permits single-copy transgene integration into the *Rosa26* locus. When artificial microRNA (amiR) expression mice, in which an amiR-EGFP cassette had been placed in the 3′UTR region of a tandem dimer Tomato (tdTomato) reporter gene expression cassette (+RG), were produced by this technology, double transgenic (Tg) mice harbouring both amiR-EGFP and EGFP transgenes showed highly reproducible and consistent knockdown efficiencies. Production of other knockdown mice harbouring amiR-Tyr (tyrosinase) in the 3′UTR region of the EGFP reporter gene expression cassette (+RG) confirmed the above result: they exhibited light coat colour in contrast with that of the wild-type mice. This finding suggests the utility of PITT for knockdown of the expression of a target endogenous gene. However, the knockdown level was unsatisfactory for another amiR. In an attempt to increase the knockdown efficiency leading to intense hypomorphic phenotypes, we constructed a series of amiR-EGFP expression constructs. Embryonic stem cells constitutively expressing the EGFP gene were tested for their knockdown potential. As a result, the simplest expression cassette lacking a reporter gene (−RG) showed a higher degree of knockdown efficiency than other reporter gene-containing cassettes (+RG). Similar results were obtained when a reporter gene was replaced by a neomycin resistance gene. Next, we generated amiR-EGFP expression mice lacking a reporter gene (−RG). Unfortunately and unexpectedly, we failed to obtain offspring of such transgenic lines (−RG): all of the resulting offspring harbouring the amiR-EGFP expression cassette (−RG) died just after birth. This finding suggests that reporterless expression cassettes (−RG) produce higher amounts of amiR in vivo as well as in vitro, impairing murine development. This hypothesis is supported by the following observations: (1) it was difficult to obtain homozygotes for both amiR-EGFP (+RG) mice and amiR-Tyr (+RG) mice and (2) mosaic mice containing

cells from both 'amiR-EGFP (−RG)' and 'amiR-EGFP low-expression cell' tended to show growth retardation and cataract.

151. Trophectoderm complementation assay to iPS cells: a proposal to the cattle

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To date, mouse tetraploid complementation assay (TCA) is a powerful tool to produce individuals only from embryonic stem cells (ESCs) since they fail to differentiate on chorion. The complementation of ESC with cells committed to the chorion lineage (i.e. tetraploid cells) allows the chimeric embryo to establish pregnancy and deliver healthy offspring. For unclear reasons, attempts to mimic TCA in cattle were not successful. We aimed to propose a model to mimic TCA in cattle through the complementation of induced pluripotent stem cells (iPSC) with trophectoderm (TE). *In vitro* produced bovine blastocysts were used to obtain TE fragments. They were obtained by sectioning of expanded/hatched blastocyst tangentially to the inner cell mass (ICM). The resulting fragments contained either ICM (and some residual TE) or only TE. The sectioning was performed with a microblade and micromanipulators. The bovine iPSC were produced by transduction of Oct4, Sox2, c-Myc and Klf4 genes in bovine foetal fibroblasts (Bressan et al., 2013; Anim. Reprod., v.10, n.3, p.603, Jul./Sept.). Aggregation pairs (TE fragment and a clump of approximately 15 iPSC in close contact each other) were incubated in microwells system (*Well of the Well*), with SOF medium covered by mineral oil and under 24 h, 38.3 °C, 5 % CO_2 and saturated humidity. Replacement of iPSC by bovine foetal fibroblasts (bFF) in the aggregation pairs was used as control. After culture, the presence of a normal blastocyst (morphologically assessed, i.e. with ICM, blastocoel and a monolayer of TE cells) was considered successful. Attempts (two replicates) to produce the chimeric blastocysts were successful on 78.6 % (22/28). There were 42.9 % (12/28) of partial and 35.7 % (10/28) of full aggregation of the iPSC with TE fragment. There was no aggregation in bFF control since cultured TE fragment was assessed as a trophoblastic vesicle without ICM. The obtaining of morphologically normal blastocysts (in a quality degree compared to IVF embryos that establish pregnancies) was effective and could support a subsequent transfer of the embryo to recipients. Although still a prototype, the technique has proved feasible to reconstruct blastocysts from iPSC and TE fragments derived from IVF embryos. A parallel exists between TCA and our model in which pluripotent cells (ESC and iPSC, respectively) were complemented with chorion-committed cells (tetraploid and trophectoderm, respectively). Currently we are engaged to establish full-term pregnancies. Acknowledgements to FAPESP for funding (06/06491-2) and fellowships of IPE (09/10679-5), FFB (09/11631-6) and EMR (12/23409-9).

152. Cloned embryonic development using porcine fibroblasts via BMI1 gene

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Immortalized cells are effective for evaluation of gene-targeting tools or molecular cell pathway because of relatively longer lifespan than normal somatic cells. The BMI1 gene, oncogene belonging to the mouse polycomb group, has been used to immortalize the human and murine somatic cells, but there was no available data in porcine primary cells to date. In this study, we investigated the effect of BMI1 expression on the cellular lifespan in porcine primary fibroblasts and cloned embryonic development using those cells. The minipig skin fibroblasts of passage 3 after primary culture were trypsinized and one half was transfected with pCAG-BMI1-T2A-RFP plasmids using electroporation method. After plating of cells in 100-mm dish at 100 cells/dish, well-formed colonies were picked up after 2 weeks and expanded until they were fully confluent in 100-mm dish (designated to 'passage 0'). And then, the cell were maintained in DMEM with 20 % FBS. Doubling time and cell size were checked at passage 5, 10 and 15 by calculation of cells that had been plated in 12-well plates at 4×10^4 cells/well. The p16 gene expression was determined by qRT-PCR at early (passage 5) and late (passage 19) stage. And SCNT embryos using primary and BMI1-immortalized cells were evaluated in terms of reprogramming efficiency. Data were analysed by t test using GraphPad Prism version 5.01. While the mean doubling time was progressively increased (from 127.9 h to 189.5 h) in primary cells that of BMI1 cells was maintained under than 30 h (mean 23.7 h). Mean cell size of BMI1 cells (16.9 μm) was significantly smaller than that of primary cells (24.8 μm). Expression of p16 was significantly lower in BMI1 cells than the primary cells at early and late stage. While the rates of SCNT blastocysts derived from early-stage cells were not different between primary cell and BMI1-immortalized group (12.4 and 11.6 %, respectively), the rate of blastocyst using late-stage cells was decreased in primary cell group (5.2 %) compared to BMI1-immortalized group (16.3 %; $p < 0.05$). In summary, BMI1 increased cell lifespan in porcine skin fibroblasts by downregulating the p16 expression. And overexpression of BMI1 had no detrimental effect on pre-implantational development of SCNT embryos. In future, longer cell lifespan by BMI1 in porcine fibroblasts can be used for gene targeting like homologous recombination and provide in vitro embryonic developmental model for genetic understanding.

This study was supported by NRF-2013R1A1A2010766, Biogreen (PJ0090962012) and IPET (311011-05-2-SB020).

153. Generation of a PDGF-C-CREERT2 mouse allele to trace PDGF-C expressing cells during mouse development

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PDGF-C is a newly identified ligand in the family of platelet-derived growth factor (PDGF). It has been demonstrated to signal through PDGF receptor-alpha required for multiple developmental processes. To further understand the developmental role of PDGF-C, we have generated a mouse allele to trace the fate of PDGF-C expressing cells in mice. In this mouse allele, termed Pdgf-c-CreERT2, an inducible Cre (CreERT2) expression cassette was knocked into mouse Pdgf-c locus, so that the expression of CreERT2 can be tightly controlled by the Pdgf-c endogenous regulatory elements. When Pdgf-c-CreERT2 mice were crossed with the ROSA-26-LacZ or ROSA26-YFP reporter mice, PDGF-C-expressing cells were permanently labelled after an administration of tamoxifen. Using this system, we found that some of PDGF-C expressing cells in mouse brain can function as neural stem cells required for the development of cerebellum. This is the first genetic evidence indicating that PDGF-C signalling could play a role in the regulation of cerebellar stem cells. We are currently using this established mouse allele to trace PDGF-C expressing cells in other organ systems.

154. Analysis of TN5 and sleeping beauty transposon-mediated transgenesis in in vitro bovine embryos

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Transposable elements are DNA segments with the unique ability to move about in the genome. Although transposons have great potential for animal transgenesis; they have been tested only in mice and swine. The high costs associated to long pregnancy periods turn it difficult to test new transgenesis techniques in large mammals. In this work, Tn5 and sleeping beauty transposon systems were analysed in in vitro bovine embryos. The Tn5 transposition system relies on the commercial Tn5 transposase to generate in vitro the transposome complex with the transposon. On the other hand, sleeping beauty transposition requires two plasmids, coding for transposase and transposon. Both transposition systems were cytoplasmically injected into IVF presumptive zygotes. The integration status of transgene was determined in blastocysts by Southern blot (eSBlot). To this aim, bovine COCs were IVM and subjected to IVF in standard conditions. Afterwards, presumptive zygotes were cytoplasmically microinjected with (1) complex Tn5:*egfp* transposon (Tn5:*egfp*, 20 ng/ul in Mg^{+2} free medium) or (2) two plasmids, coding for sleeping beauty (5 fg/pl) and pCAGVenus (10 fg/pl) flanked by inverted terminal repeats recognized by SB (SB:Venus). E*gfp* expression was evaluated and controls lacking transposase included. *Egfp* blastocysts obtained by Tn5:*egfp* or SB:Venus ($n = 4$ each), by transposon alone injection ($n = 4$) and a control IVF blastocyst ($n = 1$), were subjected to whole-genome amplification. Afterwards, 10 ug DNA were digested with *Bam*H1, *Cla*I and *Sal*I and used for the eSBlot. As probe, CAG promoter labelled by nick translation with gamma-32P]CTP was used. Two positive controls (1 ng and 10 ng unlabelled probe) were included. Data were statistically analysed by Fisher's test. *In vitro* transgene expression rates in blastocysts did not differ among Tn5:*egfp*, SB:Venus and the transposon alone control (50/90, 55.5 %; 14/32, 43.7 %; 7/27, 25.9 %, respectively). The eSblot revealed clear bands compatible with integration in the 4 blastocysts produced by injection with Tn5:*egfp*. For SB:Venus group, the high amount of Venus plasmid present in blastocysts made it difficult to distinguish among integration and episomal persistence of transgenes. In the transposon alone controls, defined bands corresponding to the size of the transposon digested or not were obtained, indicating episomal transgenes. The results suggest that both systems could be efficient for inducing transgene integration in bovine by simple cytoplasmic injection into IVF zygotes. eSblot provides relevant information regarding integration of transgenes, cheap and useful as a start point to application of these techniques to domestic species.

155. Anti-'Flu RNA interference in the chicken: using precision genome engineering to hitchhike on mirna expression

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RNA interference (RNAi) has emerged as a powerful experimental tool and an opportunity for the control of important traits in production animals. MicroRNAs (miRNAs) are endogenous small-hairpin-like molecules that regulate mRNA translation. This system can be exploited by introducing miRNA mimics—short hairpin RNAs (shRNAs). These are often expressed by integrating a complex transgene into the genome using transposons or integrating viruses. Typically transgenes incorporate broad constitutive promoters or tightly controlled tissue-specific promoters, coding sequences, splicing sites, polyadenylation signals and transcription terminators. For RNAi, this may be reduced to promoters (usually RNA polymerase III) and terminators.

The advent of precision genome engineering has provided tools to allow us to approach RNAi transgenesis by a different method that can enable tissue/timing-specific expression. Our aim has been to insert shRNA sequences adjacent to natural miRNA sequences to achieve parallel processing of these RNA hairpin structure to effectively hitchhike a ride with the microRNA expression, achieving RNAi without exogenous regulatory sequences (promoter and terminators). We have used a zinc finger nuclease to incorporate anti-viral hairpins into chicken cells against avian influenza virus. We targeted a genome location adjacent to a constitutively highly expressed miRNA (miR-107), residing in the 5th intron of the pantothenate-kinase (PANK-1) gene. PANK-1/miR-107 was chosen to provide the widest range of tissue expression of the anti-viral shRNA. By homologous recombination DNA repair, we have introduced coding sequence for a single anti-viral shRNA hairpin into one PANK-1 allele and established a pure cell line for this event, in the chicken fibroblast cell DF-1. We have then introduced a second different anti-viral hairpin into the other PANK-1 allele. For both of these cell lines, we have confirmed the precision placement of the very small transgene, and assessed appropriate expression of appropriately spliced PANK-1 mRNA, the native miR-107 and the two individual hairpins. We have assessed the RNAi activity of the hairpins, with luciferase reporter constructs. We are currently validating a new DF-1 cell line with a single allele triple anti-viral shRNA precision insertion. Once confirmed, all cell lines will be challenged with the avian influenza virus. Our initial work is focused on the production of virus-resistant chicken cells. We are also working on the technical steps required to facilitate and screen for these precision insertion events in primordial germ cells (PGCs). Ultimately, we hope to work towards establishing a line of live chickens with the multiple shRNA hitchhiking construct.

156. Generating a porcine bladder cancer model

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Here, we report the mouse Uroplakin-II promoter's ability to drive transgene expression in the pig. Three different transposons sizing 4.7, 11.2 and 11.9 kb were simultaneously transpositioned into primary fibroblasts, subsequently used for somatic cell nuclear transfer to create various transgenic pigs harbouring all three transposons. The pigs were made from different transgenic fibroblast colonies and contained different copy numbers of each transposon. The two largest transposons contain the Uroplakin-II promoter driving an array of genes including either the EGFP or RFP reporter gene linked to different oncogenes and the rtTR-KRAB-2SM2 (KRAB-off) element. The pig with the highest copy number contained eighteen of these large transposons, whereas the pig with the lowest copy number contained three. Expression from the mouse Uroplakin-II promoter was relatively week but enough to promote dysplasia in the bladder of three-month-old female and male pigs. Some of the pigs are now 4 months old and will be analysed further using cystoscopy.

157. Examination of a regulatory SNP of bovine RCAN2 gene in transgenic mouse models

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There are several single nucleotide polymorphisms (SNPs) in the different genes of farm animals, which can call forth advantages and disadvantages in the economically important traits. The aim of our research was to detect regulatory SNPs (rSNPs) in the promoter region of bovine genes and to prove the differences in the gene expression in transgenic mouse models.

After bioinformatical analysis of the bovine genome and the association surveys of genotypes/gene expressions, 13 pair rSNPs were selected by our colleagues, which probably can regulate gene expression. Vector plasmids with reporter genes were constructed to examine the role of rSNPs. Two different allele variants of these rSNPs were tested in vitro in transfected cell lines, then the linearised gene constructs were used to produce transgenic animal models through embryo microinjection.

One of the examined genes was the bovine RCAN2 (regulator of calcineurin), which is particularly expresses in brain, heart and skeletal muscle. The RCAN2 protein has important role in the calcineurin regulation. This protein has influence to the attachment of transcription factors in the promoter region of calcineurin sensible genes. RCAN2 protein controls the food intake and body weight, hereby it plays an important role in age- and diet-induced obesity. Thus, it can be responsible for the regulation of economically relevant traits.

We created two–two transgenic mouse lines with both alleles of the RCAN2 promoter. After genotyping progenies, we could identify transgenic offspring. We compared the expression level of marker genes by qPCR, and we confirmed that there is an expression difference between the examined alleles in vivo. We verified the expression differences between the mouse lines at protein level by confocal microscopy. Consequently, our study shows that rSNP-dependent gene expression can be relevant in animal breeding.

158. In vitro and in vivo assessment of cryopreserved two-cell rat embryos from five strains of rats

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The genetically engineered rat is becoming more readily available in the research community through the use of genetic

modification technologies, such as the transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFN) and clustered regularly interspaced short palindromic repeats (CRISPR). Once these genetically modified rats have been characterized, the need to efficiently preserve them becomes important. The cryopreservation and recovery of rat sperm are still challenging processes. Using cryopreserved embryos provides a more efficient and effective alternative to frozen sperm. The advantage of using morulae/blastocyst-stage embryos is that they generally survive the freezing process well. One of the disadvantages is the recovery of blastocyst from various strains is lower than the recovery of pre-implantation embryos.

In our facility, we have been cryopreserving blastocyst-stage embryos for over a decade. In this study, we wanted to see the in vitro and in vivo affect using cryopreserved two-cell embryos. Two-cell embryos were recovered from Crl:CD(SD), Crl:SD, Crl:WI, F344/NCrl and LEW/Crl. These embryos were cryopreserved in ethylene glycol using a slow-rate freezing procedure. The live recovery rate of these embryos was >95 %. The embryos were cultured in R2ECM and assessed for morulae and blastocyst development. The in vitro development was 39, 37, 29, 34 and 34 %, respectively. Bilateral embryo transfers were then performed using 20 embryos per female. The in vivo rate was 34, 34, 26, 38 and 35 %, respectively. The data shows that two-cell rat embryos can be successfully cryopreserved and recovered.

159. Generation of a novel inducible neuronal-specific C-fos knockout mouse model for memory formation study

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C-fos is well known as a molecular marker of neurons activated during the learning process. So far very little is known about the molecular mechanisms downstream to c-fos-related neuronal plasticity. The c-fos mouse models derived so far were either a constitutive deletion or conditional, but not inducible, knockout. Consequently, the c-fos gene was deleted on very early stages of development what caused severe abnormalities leading to approximately 60 % loss of homozygous mutants at birth. Our aim is to obtain a novel inducible mouse model where, to overcome developmental disorders, c-fos gene will be removed in adult animals.

The LoxP sequences were introduced to BAC-based targeting vector to flank mouse c-fos gene. Verified construct will be subsequently introduced into W4 embryonic stem cells and transferred into blastocysts derived from C57 mouse strain. The obtained c-fos floxed mouse line will be further on crossed with mice expressing inducible variant of Cre recombinase, controlled by CamKII promoter (CaMKCreERT2). As the deletion of c-fos gene will occur in animals with completed brain development, the model will facilitate the investigation of specific c-fos function in memory formation.

We hypothesize that c-fos activation is a crucial process, necessary for the regulation of expression of proteins required for memory formation. We aim to show that the lack of c-fos protein will result in diminished cognitive function.

160. Novel transgenic mouse for studying effects of PML gene manipulation in neural system

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The PML protein name derives from promyelocytic leukaemia, a disease that is caused by the specific t(15;17) chromosome translocation. PML protein is the crucial component and organizer of the PML nuclear bodies. In the nervous system, a physiological function of PML protein/PML nuclear bodies is a maintenance of neural stem cells and progenitor cells during development of the nervous system; PML disappears from the brain after birth. Nevertheless, there have been several reports indicating PML bodies activity in the adult brain, where it can contribute to the pathogenesis of age-related neurodegenerative disorders. In contrast to the hitherto prevailing view, in our laboratory, we demonstrated that PML protein is widely expressed in the adult mouse brain. Although the PML protein is involved in many processes and appears on crossroads of multiple signalling pathways, still its role in the nervous system has not been comprehensively investigated.

The aim of our studies is to elucidate the physiological function of PML protein/PML nuclear bodies in the brain based on experiments in *de novo*-created transgenic animals with (1) inducible neuron-specific PML knockout using modified embryonic stem cells (ESC), (2) inducible neuron-specific PML overexpression through microinjection of DNA into the pronuclei and (3) inducible neuron-specific PML overexpression—through direct injection of adeno-associated virus (AAV) into the brain.

We have chosen the Cre/LoxP expression system inducible by tamoxifen. The PML gene was cloned into the AAV plasmid—pAAV-DIO-EF1a-PML with Cre-dependent activation of EF1a promoter. This plasmid will be used for creating both transgenic overexpression models (2, 3). To obtain inducible neuron-specific PML overexpression, we will apply the method of microinjection of linear DNA into the pronuclei of C57BL/6J mice. Transgenic offspring will be cross-bred with CaMKCreERT2 line mice, and overexpression of PML gene in double transgenic mice will be induced by injection of tamoxifen. For creating inducible, neuron-specific PML knockout, we will use the mutant ES cell clones (containing

floxed PML gene) with conditional potential. We will use the C57BL/6J line mouse for microinjecting ES cell clones into the blastocyst. Transgenic offspring will be cross-bred to the CaMKCreERT2 line mice and knockout of PML gene in double transgenic mice will be induced by injection of tamoxifen.

161. Slow freezing versus vitrification of transgenic mouse embryos obtained by in vitro fertilization (IVF) intended for rederivation use

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Rederivation of cryopreserved embryos is an effective strategy for managing the introduction of mice into a barrier facility, avoiding pathogen entry. Furthermore, the use of IVF could produce supernumerary embryos in a single cycle using few animals, making the whole process more efficient. The aim of this study was to evaluate the effect of vitrification and slow freezing in the viability, in vitro and in vivo development of transgenic embryos produced by IVF for rederivation. A transgenic mouse line was subjected to IVF (Takeo and Nakagata 2011). Twenty-four hours later, two-cell embryos were incubated in M16 (37 °C, 5 % CO_2) until morula or blastocyst stage; 160 embryos were rederived to SPF facility into pseudopregnant females (0.5 or 2.5dpc; fresh group). Additionally, 941 morulae were subjected to slow freezing (SF group, $n = 474$) using propylene glycol (Renard and Babinet 1984) or vitrification (V group, $n = 467$) using a home-made spatula (Tsang and Chow 2009). Two-hundred and sixty-two and 266 morulae from SF and V group, respectively, were thawed and immediately transferred or incubated 12 h, evaluated for in vitro development and then transferred in pseudopregnant females in blastocyst stage. Embryo recovery (recovered/cryopreserved embryos) and viability (viable/recovered embryos), blastocyst development (blastocysts/viable embryos) and pregnancy rates (pregnant/transferred females) were determined. Statistical differences were evaluated using chi-square test. IVF overall cleavage rate was 75.5 % (1,134/1,502) and in vitro development to morulae was 73.3 % (1,101/1,502). After embryo thawing, recovery rate resulted in 95.8 % (251/262) and 94.4 % (251/266) for SF and V group (p = NS), and viability rate was 84.9 % (213/251) and 98.8 % (248/251) for SF and V group ($p < 0.05$). Development of thawed embryos from morula to blastocyst resulted in 89 % (72/81) and 92 % (69/75) for SF and V group, respectively (p = NS). Pregnancy rates were 67 % (6/9), 40 % (4/10) and 42 % (5/12) for fresh, SF and V group, respectively (p = NS). In conclusion, using these methods we were able to recover more than 90 % of the cryopreserved embryos, but vitrification was more efficient than slow freezing regarding viability rates. Thawed morulae from SF and V group developed to blastocyst in an efficient manner. Pregnancy rates of thawed transgenic IVF embryos were similar using both methods. Finally, IVF technique resulted in an efficient method to produce a large number of transgenic embryos which could be cryopreserved to be introduced into the SPF facility.

162. The crescendo mouse drug discovery platform: a transgenic mouse generating high affinity fully human antibody fragments for therapeutic use

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Crescendo Biologics is a young drug discovery company, founded in late 2008. Underpinning its drug discovery pipeline is the Crescendo Mouse: a series of complex genetically manipulated mouse lines with three non-functional (knockout) loci and one transgene, all maintained at homozygosity. Here, we describe the making of Crescendo Mouse using yeast artificial chromosome (YAC) transgenics and fast breeding onto a 'triple knockout' background incapable of making native mouse immunoglobulin heavy or light chains.

The Crescendo Mouse has normal spleen size and architecture, normal B cell development, normal levels of mature B cells in the spleen and physiological levels of antibody in the serum. It responds robustly to a range of antigens, producing a diverse repertoire of fully human antibody fragments which have matured in the absence of any contaminating light chain.

The antigen-binding region (VH) of the Crescendo Mouse antibodies is derived from fully human V, D & J immunoglobulin heavy chain genes located within the transgene and uses the mouse's antibody recombination machinery to produce a large diverse repertoire of heavy chain only antibodies. We harvest VH domains from immunised mice and screen them in vitro to identify potent candidate therapeutic drugs for use in patients.

The Crescendo Mouse harnesses the power of in vivo maturation (somatic hypermutation and clonal selection) to increase affinity, stability and solubility of the VH domains, providing a faster route to high-quality candidate drugs. We describe the use of our mouse in our drug discovery pipeline and its benefits over conventional antibody drug discovery routes.

163. Germ cell differentiation of bovine pluripotent stem cells (BIPSC)

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The use of pluripotent stem cells in bovine transgenesis, as well as their differentiation towards primordial germ cells (PGC), represents conceptually feasible alternatives to reach high economic impact goals such as generating transgenic animals through germ cell transplantation. The goal of this study was to

establish conditions for the in vitro germ cell specification of bovine-induced pluripotent stem cells (biPSCs). In order to do so; two (XY) biPSCs cell lines were establish as we described before [1]. These cell lines displayed comprehensive differentiation potential, both in vitro and in vivo. Next, using previously described [2] candidate regulatory sequences for the PGCs-specific lineage marker bovine VASA homologue (DDX4), we generated biPSC$^{Tg(bvh\text{-}EGFP)}$ cell lines (VASA-GFP reporter). We then evaluated the potential of the inducers: retinoic acid (RA) and bone morphogenetic protein 4 (BMP4), to influence the rate of PGC differentiation and upregulation of key genes ($n = 4$) of the reporter cell lines either individually or in combination. The use of BMP4, RA or a combination of the two increased the number of GFP-positive cells. Cells without the stimulus (control) differentiated at a very low rate (0.15 ± 0.03 %). However, following treatment with RA, BMP4 or both there was an increase to 1.40 ± 0.1, 0.97 ± 0.2 and 1.33 ± 0.2 respectively. Although there was a statistically significant difference between treatment groups and control ($p = 0.0016$), corresponding to nearly a ten-fold increase in the rate of induction, there were no differences between the three treatment groups: RA, BP4 and BMP4 + RA ($p = 0.2779$). Finally, we compared the expression level of key genes of the germ cell differentiation lineage by qPCR. VASA, Oct4, Sox2, FRAGILIS, DAZL and SMAD5 were detected, and though the rate of differentiation was significantly increased by the induction with both BMP4 and RA, there was no relative change in the level of expression of the genes evaluated compared with the cells differentiating spontaneously ($p > 0.05$ in all cases). Our results suggest that both RA and BMP4 alone or in combination are enough to increase PGCs differentiation in biPSCS. This study was funded by The Dairy Futures Cooperative Research Centre (CRC), Australia.

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164. Evaluation of different protocols for the cryopreservation of mouse ovaries

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Introduction: Cryopreservation of gametes and embryos is a routine approach for the long-term storage of genetic material. Cryopreservation of ovarian tissue has been developed as an additional strategy for archiving genetically valuable mouse lines. In the last decades, different protocols have been described for the cryopreservation of mouse ovaries. In the presented study, we compared these protocols and examined such factors as time requirement until conception, pregnancy rate of transplanted females and differences in litter size.

Methods: Ovaries from 25 C57BL/6N 5-month-old females were collected and bisected. Tissues were allocated to the following four freezing protocols and stored in LN2:
Gr. I (Migishima et al.): vitrification in cryo vials with 2 M DMSO, 1 M acetamid and 3 M propylene glycol.
Gr. II (Wang et al.): solid surface vitrification in cryo vials with 2.55 M ethylene glycol and 2.55 M DMSO.
Gr. III (Wang et al.): controlled freezing in straws with 1.5 M DMSO.
Gr. IV (Liu et al.): controlled freezing in cryo vials with 1.5 M DMSO.
Gr. V: Control, no cryopreservation.
Thawed ovary halves were transplanted bilaterally to 8-week-old C57BL/6N ovariectomized recipients (5 per group). After recovery, recipients were mated. Pregnancies and litter sizes were recorded. After weaning, ovaries with surrounding tissues were dissected for histology.
Results: Differences in the pregnancy rate (40–100 %) were not statistically different in any of the protocols including the control group. There was also no significant effect on the time interval until pregnancy. However, three mice out of the four pregnant mice from group III were already pregnant after 4 weeks, even better than the control group with freshly transferred ovaries. The average litter size in group III and the control group was significantly higher than in group I (6.5 and 6.3 vs. 1.8 pups). Histological analyses of dissected tissues are still ongoing.
Conclusions: Ovarian cryopreservation is an easy but valuable additional tool to archive mouse strains. All tested protocols used in this study did not significantly differ from the pregnancy rate of recipients after transplantation of unpreserved ovaries. Ovarian tissue generally survived well the aforementioned preservation techniques and resumed ovarian cycle after transplantation. Controlled freezing in straws with DMSO as cryoprotectant (group III) resulted in the best performance considering the fast resumption of reproduction and a generally higher litter size.

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165. Variation of stress hormone metabolites over the lifespan of two commonly used mouse strains

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Introduction: The non-invasive measurement of stress hormone metabolites in the faeces of animals is an approved refinement for the assessment of pain and distress. Although

frequently used to evaluate the burden of specific treatments, there is no data available on the characteristics of stress hormones during the lifespan of untreated animals.
Here, we present the results of a long-term study to determine the concentrations of faecal corticosterone metabolites (FCM) in two commonly used mouse strains over a period of 26 months.
Methods: FCM was measured in 10 C57BL/6N and 10 ICR(CD-1) females at the age of 1 month, 2 months and continued at every 2 months until the end of the study. For each faecal collection session, the animals were separated for 5 days. To include the circadian progress of FCM, faeces were collected every 4 h over a period of 24 h on the 5th day of separation. Between each faecal collection session, the animals were housed in groups. Frozen faecal samples were extracted with methanol and analysed using an enzyme immunoassay. Data were evaluated by IBM SPSS v19, using linear regression analysis for each strain.
Results: Daily mean values of FCM increased over the lifespan of ICR mice from 80 to 200 ng/0.05 g faeces to 130–500 ng/0.05 g faeces. In contrast, for B6 N mice mean values of FCM were nearly constant over their lifespan in the range of 60–250 ng/0.05 g faeces. Although we generally observed a strong individual variation for the measured concentrations of stress hormone metabolites, we could still confirm its diurnal periodicity in almost all animals. This circadian oscillation decreased continuously with the advancement of age in both strains.
Conclusions: The assessment of burden in animal experiments by non-invasive stress measurement is usually restricted to defined time points or periods. The results of our lifespan study revealed that the age of the animals have significant impact on the level and on the circadian rhythm of stress hormones in untreated animals. Moreover, we observed strain-specific differences in the mean values and for the development of FCM levels over the lifespan. Therefore, strain and age of the animals should be taken into account as possible modifying factors for the measurement of FCM to improve comparability of FCM data in animal experiments.

166. Administration of analgesia with the food: a step forward in refining surgery in embryo transfer

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Surgical transfer of mouse embryos (embryonic transfer, ET) is a standard procedure in producing transgenic mouse models for research, and it is also used to reestablish pathogen-free stocks of mice. The ideal analgesia regimen for this technique would safely manage pain in the female without adversely affecting the quality or number of offspring from implanted embryos. Studies to date have found no effect on buprenorphine, AINEs or multi-modal analgesia in number of embryos surviving after ET in single postsurgical subcutaneous injection (Parker, 2011). However, most of those drugs have a maximum effect of 8–12 h, and repeated injections are needed to maintain effective therapeutic levels. The current study aimed to explore whether a recently described method of administering buprenorphine with the diet (Molina-Cimadevila, *in press*) offers a potential treatment option in complementing the pain management of embryo-transferred females.

To assess drug's ability to provide postoperative analgesia, hot-plate test was conducted in 30 Hsd:ICR (CD-1®) mice divided in 5 experimental groups with 6 mice each. Group 1 received diet with glucosaline, group 2 received buprenorphine SC 0.1 mg/kg, group 3 oral buprenorphine in diet 0.03 mg/pellet, group 4 oral buprenorphine in diet 0.15 mg/pellet and group 5 buprenorphine SC 0.1 mg/kg plus oral buprenorphine in diet 0.03 mg/pellet. Relative increase from baseline latency (% maximal possible effect) in hot-plate test at 20 h of administration was significantly higher for oral buprenorphine in diet 0.03 mg/pellet (groups 3 and 5), and 0.15 mg/pellet, compared with placebo, and no differences were found between those oral administrations and buprenorphine SC 0.1 mg/kg measured 3 h later.

To assess effects on embryo survival, 24 0.5 dpc pseudopregnant Hsd:ICR (CD-1®) mice were transferred unilaterally with 13 embryos of C57BL/6J strain in 1.5 or 2.5 stages. Mice were randomly assigned to one of the following groups (6 mice each): group A ketamine-xilacine and buprenorphine SC 0.1 mg/kg after surgery plus oral buprenorphine in diet 0.03 mg/pellet; group B ketamine-xilacine and buprenorphine SC 0.1 mg/kg plus placebo pellet with glucosaline; group C avertine and same analgesia as group A; group D: avertine and same analgesia as group B. Neither the analgesic regimen nor the anaesthesia significantly affected the number of pups born.

In conclusion, diet with buprenorphine can easily be added after a standard anaesthetic, and analgesic procedures in embryo transfer does not affect birth rates and improves analgesia of embryo-transferred females and thus wellbeing.

167. A protocol for non-surgical embryo transfer with improved pregnancy and birth rates over surgical implantation

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Non-surgical embryo transfer (NSET) of blastocysts to pseudopregnant female recipients provides many benefits over surgical implantation with less distress for the mice, no anaesthesia or analgesia required and a considerable reduction in implantation time per mouse. Although many transgenic facilities have considerable interest in the procedure, only few have implemented it in their SOPs, most likely because the traditional surgical implantation is efficient and inexpensive. The obvious benefit to the animal and the gain time per implantation convinced us to switch to the NSET method in 2011. For that we use the disposable

NSET device from ParaTechs. Initially, we observed a considerable drop in live born pups. However, with some fine-tuning to the original protocol, we managed to improve both the pregnancy rate and the birth rate to even surpass that of the surgical implantation procedure (Bin Ali et al., Transgenic Research 2014). Furthermore, the repeated use of the same NSET catheter for multiple implantations reduces the cost of the procedure to a reasonable level. Currently, almost all of our embryo transfers are performed by NSET, with only few exceptions.

168. Refinements for implant surgery: the effects of different anaesthetic agents on pregnancy and pup survival

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The most critical aspect of generating genetically engineered mice is the ability to produce live animals for analysis after the appropriate injection procedure. Animals are produced by implantation of manipulated embryos into pseudopregnant females for gestation, parturition and subsequent growth to the weaning stage. Animal loss can occur during any of these stages, which results in repeated procedures and increased animal usage. One might predict that the anaesthesia used during implant surgery could affect the number of pups produced. Anaesthetic agents commonly used in the United States for implant surgery include avertin (a tri-bromoethanol, tert-amyl alcohol mix) delivered by IP injection, ketamine:xylazine (100 mg/kg:5 mg/kg) delivered by IP injection, and inhaled isoflurane (2.5 % in oxygen). To determine whether the type of anaesthesia used would affect the number of animals produced, we tested each type in implant surgeries and assessed the numbers of pups produced. Sufficient numbers of embryos and implants were used to ensure an appropriately powered study. The results of this analysis will be presented.

169. Numerical assessment of animal reduction by proper transgenic colony management

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The workshop held in The Netherlands by the end of 2013 (1) highlighted the proportion of unemployed animals in transgenic colonies. It supported the need to convince research team and animal facility managers to properly manage their colonies in order to reduce the number of unused animals produced. To reach this goal but also to value this effort front to authorities, it's important to provide simple-to-use tools and numerical reduction data.

This study shows simple formula to evaluate number of breeding cages needed to reach a production goal. But, it also calculates animal savings made by using all colony management tools as backcross (2) or cryopreservation (3):

- Use of cryopreservation: up to 86 % reduction of animals used compare to breeding over a period of 2 years
- Use of congenic or co-isogenic lines: up to 75 % reduction of animals produced by using homozygote breeding compare to heterozygote breeding
- Calculation of needed breeding cages: up to 40 % reduction of animals produced over a 1-year period compare to empirical breeding.

In the current economic situation, it's also important to keep in mind the fact that reducing the number of unused animals is also a matter of reducing workload and costs allowing to reallocate valuable resources to research.

1. Workshop: 'Animals bred, but not used in experiments', October 18–20, 2013, Santpoort, the Netherlands

2. The Jackson Laboratory Handbook on Genetically Standardized Mice 6th Edition

3. Strategies for managing an ever increasing mutant mouse repository, M.T. Davisson, R.A. Taft Brain Research. 2006;1091:255–257

170. Generation OF genetically modified mouse models: the role of the international society for transgenic technologies in refinement and reduction

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After the development of technologies to efficiently manipulate the murine genome during the 1980s, the generation and use of model organisms in many areas of basic and biomedical research is without alternative at present. With the proliferation of technologies, networks have formed that have provided for an exchange among each other. From these networks emerged in 2006, the International Society for Transgenic Technologies (ISTT). The ISTT is a meeting place for all those who generate and use genetically modified animals—currently still mainly mice and rats. The aim of the society is to disseminate techniques and their improvements and to facilitate the optimal use of these techniques in all laboratories employing those technologies.

The ISTT promotes Refinement through the dissemination of improved surgical techniques and non-surgical alternatives. These techniques focus on embryo transfer and vasectomy and help to reduce pain and trauma. This is achieved by optimizing surgical routes as well as anaesthetic and analgesic regimen.

The ISTT promotes Reduction through the dissemination of state-of-the-art technologies. New genome-editing technologies show highly increased efficiency compared to conventional technologies and need fewer animals to generate a targeted mutation. Improvements of standard operating procedures reduce animal numbers by increasing embryos yields needed for manipulation or rederivation and by increasing pup yields from embryo transfer surgery. Robust cryopreservation protocols lead to a reduction of underutilized strains by abolishing the need to maintain live mice.

The ISTT, in addition to its interactions with its members, engages in a number of affiliations, cooperations and sponsorships to reach its aims.

171. Implementation of directive 2010/63/UE in a transgenic core facility: a practical example

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Impact of Directive 63/2010 UE is starting to show up in Europe. Meanwhile in some countries, the gap between the old and the new directive has been lessened by national regulations, and others had to face a complete change of procedures. We will present how our facility has adapted to the new regulations. How welfare assessment has been proposed and how license requests have been organized in an attempt to facilitate the accessibility of researchers to services provided. We will discuss major changes in internal procedures and liability management as well as the impact of this directive in the number of animals reported as to be used in experimental procedures according to the new statistical tables.

172. Use of permethrin treatment on genetically modified mice housed in isolators infested by fur mites (*Myobia musculi*)

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Fur mite infestations are common in many rodent facilities. Obtaining complete eradication of a fur mite infestation is often difficult to accomplish. However, it is imperative to eradicate because of the overall impact on animal welfare and the potential impact on research results. Fur mite outbreaks are often related not to new infestations but to recrudescence of past infestation not eradicated by past treatments, despite the high number of documented treatments available. Therefore, we investigated the effect of a permethrin treatment on a natural infestation by *Myobia musculi* of 24 different genetically modified mouse colonies housed in positive pressure plastic film isolators.

The contamination was detected in one isolator during routine health monitoring. Two dirty bedding sentinel mice for every 50 cages were tested via direct microscopic pelt examination after euthanasia. Due to the unique breeding requirements and the movement of active breeders, we suspected possible contamination of another 8 isolators and immediately quarantined them. Subsequent contamination was confirmed using the scotch tape impression test.

It had been previously documented that permethrin is effective to eradicate mite infestations in combination with an environmental decontamination (permethrin indeed is not ovicidal). In isolators, the environmental decontamination by washing and autoclaving of cages and equipment is unpractical. We assessed therefore the treatment of a large colony containing 2,250 genetically modified mice housed in nine plastic film isolators by using only cotton balls bedding impregnated with 7.4 % permethrin solution. A total of three cotton balls per adult rodent and one cotton ball for each pup were placed in all cages as nesting material and replaced weekly at cage cleaning. After 6 weeks treatment, a period of 2 weeks without treatment was provided. This was then followed by and additional administration period of 3 weeks. At the end of the treatment, health monitoring was conducted on both original animals and dirty bedding sentinels. All animals remained mite-free to date (11 months, $N = 518$ tested mice).

Our results show that permethrin-soaked cotton balls as nesting material is an effective means for mite treatment when respecting the correct length of treatment. To our knowledge this study is the first analysing the efficacy and the feasibility of a treatment against mites on mice housed in isolators. In our experience, this treatment is free from adverse effects, reliable and can be considered as a valid option for eradication of fur mites in environments difficult to decontaminate.

173. Refining timed matings in a high-throughput pipelines environment

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Timed matings are widely used in the generation and phenotyping of transgenic mice. The Wellcome Trust Sanger Institute (WTSI) pairs an estimated 500 timed matings a week, across a number of wild-type and transgenic B6N strains. The implementation of a mouse-tracking database 5 years ago has allowed the institute to monitor and refine a range of parameters including stud male age and the effects of seeding females prior to oestrus selection. Here is an outline of those refinements and their positive effect on plugging and pregnancy rates.

174. Generation of blastocysts by superovulation and natural matings: a comparison of key production parameters in ES cell knockout mouse lines

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The MGP programme at the Wellcome Trust Sanger Institute is at the forefront of mouse production for large international consortia such as the IKMC (International Mouse Knockout Consortium) and the IMPC (International Mouse Phenotyping Consortium), producing over 250 knockout-first mouse lines every year for a first line primary phenotyping screen and

making these lines available to the wider scientific community for further study. For this, we require blastocyst donors for ES cell injection that are currently derived from natural matings. In an effort to reduce the number of animals needed, but without compromising the quantity, quality of the blastocysts and efficiency of the results, we set up a comparative trial using blastocysts produced via superovulation and natural matings and the effects these had on key production parameters such as viable embryos obtained, numbers of injectable blastocysts, chimera quality, birth rates, sex ratios, germline transmission rates and ratios of heterozygote to wild-type animals in litters from Chimera crosses. Both groups were injected on the same day with the same ES cell clone. Here, we present the analysis of the results comparing these different production parameters.

175. What does good practice look like in the *transgenic technology* field?

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Research Integrity and *Openness and Transparency* are broad terms that have become hot topics within the public science arena. Some scientific disciplines have already initiated community-led efforts to improve aspects such as experimental design, research reporting, resource and data sharing. This presentation will give an overview of research integrity issues that have scientific, ethical and animal welfare consequences in terms of validity, reproducibility and translatability. It will then take a look at what the transgenic technology community currently does well and which practises present challenges for the future.

176. Effective and efficient zebrafish sperm archiving

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In recent years, the zebrafish (Danio rerio) has emerged as one of the leading vertebrate model organisms for functional genomics and developmental studies. The increasing popularity of the zebrafish model combined with the development of more efficient genome-editing techniques and the quick developmental time span of the zebrafish has led to the generation of thousands of transgenic, mutant and wild-type lines. There has been steady increase in the number of zebrafish housed at each individual Institute, with Home Office Returns reporting a 34 % increase in the use of zebrafish from 2001 to 2011. On the whole, it is becoming a struggle for the research community to maintain these large numbers of live animals; therefore, development of reliable, high-throughput and safe long-term storage of these fish lines is of paramount importance. Cryopreservation allows an archive of genetic material, reducing the costs associated with the maintenance of live zebrafish lines, and it encourages collaboration by enabling frozen samples to be shared around the world without the need for shipping live animals, and it creates a backup to protect against the loss of important research lines.

At the National Institute for Medical Research, we are currently developing our cryopreservation protocol in order to create a backup of archived stock in preparation for our oncoming move to The Francis Crick Institute. We have reviewed current protocols in order to develop a method which is successful at the NIMR. We have overcome problems such as poor egg quality and poor sperm yield; developed a database and storage system to record and achieve the samples; and liaised with scientists to generate a list of priority lines.

The Dooley C. M. et al., Methods. 15, 197–206 (2013) cryopreservation method gave us the best results, but were still inconsistent. However, we made several adaptations to the protocol to give us a more controlled rate of freezing and thawing and to lower variability. With our methodology, we have started archiving our stock of lines, in excess of 500, and the quality control system we have instigated gives us the confidence that we can recover all the lines with the same success rate as Dooley et al.

177. Blastocyst Genotyping: refining cryopreservation quality control

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Genotyping blastocyst-stage embryos instead of live mice, to confirm the zygosity of cryopreserved sperm will dramatically reduce the number of mice required to archive and distribute GA mouse lines.

In the last year, at the Wellcome Trust Sanger Institute ('WTSI'), over 400 GA mouse lines have been cryopreserved

via sperm freezing, and the vast majority of these will be distributed to repositories and made available to the scientific community. Prior to sustainable repositories accepting cryopreserved material, a quality control ('QC') process is carried out by the depositor. To pass QC, cryopreserved sperm must be used in an in vitro fertilisation ('IVF') where each sample must achieve a predetermined fertilisation percentage and the expected zygosity must be confirmed. To confirm the expected zygosity, embryos are transferred into pseudopregnant embryo recipients and live pups genotyped.

The WTSI has never failed to recover live pups from an IVF once embryos have been generated. It was therefore proposed that instead of carrying out embryo transfers and genotyping live pups to confirm the expected genotype, it would be possible to culture IVF-generated embryos to blastocyst stage, which infers viability, and is a point at which the embryos can be tested to confirm the correct zygosity.

Due to new genotyping protocols developed between EMMA/Infrafrontier nodes, blastocyst genotyping has now become much more robust. In light of this, Infrafrontier have recently changed their level of accepted QC from genotyping live born pups, to genotyping blastocyst-stage embryos for the EUCOMM/KOMP Knockout first alleles. This has already saved many hundreds of mice across the partner countries.

Genotyping blastocyst-stage embryos offers many ethical benefits and will significantly decrease the number of embryo transfer recipients required to pass QC. Crucially no live born pups are produced solely for the purpose of genotyping, resulting in fewer regulated procedures.

Genotyping blastocysts also gives more material to genotype, >90 % of 2 cell embryos develop to blastocyst while only 30 % of transferred embryos are recovered as live pups. This significantly decreases the likelihood of requiring more than one IVF to pass QC. It also means that the number of donor females required for each IVF can be reduced.

178. Environmental enrichment effects on reproductive performance of foster females used in transgenesis and rederivation techniques

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Based on the 3R's concept, environmental enrichment (EE) has become an effective tool to improve animal welfare in breeding and experimental animal facilities. In recent years, the differences in behavioural profiles induced by social, cognitive and physical enrichment have been a matter of research. In this study, we analysed the EE effects on foster female performance used for transgenesis and rederivation techniques. The EE plan was applied during four months to Swiss Webster breeding colony and their female offspring used for embryo transfer, until weaning of the pups. In the experimental group, breeders and transferred females ($n = 29$) were provided with materials for social and physical enrichment (environment complexity, nesting material, sensory and nutritional enhancement) in different combinations and alternation during the weekly cage change. Control breeding group and control transferred females ($n = 40$) did not received enrichment materials (only wood shavings and paper towels). Pup number and weight at weaning were evaluated on the breeding colony. Moreover, pregnancy rate (pregnant/transferred females) and birth rate (pups born/transferred embryos) were registered on transferred females. Statistical analysis was performed using chi-square test. Breeding colony pup number per litter size was 6.5 (72/11) and 5.6 (74/13) and pup weight average resulted in 14.4 and 16.0 g for experimental and control group, respectively. Pregnancy rate of transferred females resulted in 62.1 % (18/29) and 52.5 % (21/40) for experimental and control group, respectively. Birth rate was 6.9 % (39/567) and 6.8 % (49/724) for both groups. No statistical differences between values comparing the experimental and control groups in breeders and transferred females ($p > 0.05$) were found. In conclusion, environmental enrichment did not significantly impact on the performance in Swiss Webster colony or in foster females. However, enriched foster females showed best performance if we consider that fewer animals resulted on the same pregnancy and birth rates than those obtained from the control group. Further studies need to be performed in order to evaluate long-term data.

179. An overview of the collaborative programme to rederive and amalgamate mouse stocks in advance of the opening of the Francis Crick Institute

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2015 sees the opening of the Francis Crick Institute in London UK. An interdisciplinary medical research institute, formed by a consortium of the Medical Research Council (MRC), Cancer Research UK (CRUK), the Wellcome Trust and Colleges of London University—Imperial (ICL), Kings (KCL) and University College (UCL).

Two renowned institutes, the National Institute for Medical research (NIMR) and the London Research Institute (LRI) will be closing, and scientists and animals will be transferring to the completed new building, sited at Brill Place, Kings Cross, London, where they will be joined by researchers now based within London University Colleges.

Both institutes have a considerable and developed mouse genetics program. Health status will directly influence experimental variability. This is recognized by science journals now requiring evidence of health status. Healthy pathogen-free animals are key to producing reliable, reproducible science using minimum numbers as well as ensuring optimal animal health and welfare. It was therefore determined that existing mouse strains should be rederived into the new facility. A facility that is mainly based in central London but which also encompasses an animal facility based at Clare Hall, Hertfordshire, currently part of the LRI. The cost to rederive financially and in terms of time are considerable, but the existing flora, while acceptable for either institute, in combination was such that rederivation was the only way to provide the microbiological quality of laboratory animals required.

Having determined the need to rederive, NIMR and LRI together have identified in excess of 1,350 mouse strains to be transferred to the new Crick Institute, each of which will undergo rederivation in the period June 2014–November 2015.

This is a large rederivation project calling for collaboration of animal care staff, transgenic and laboratory staff. Here, we detail the rederivation process happening across 3 sites in and around London which aims to amalgamate stocks from multiple animal units, initially into one barrier facility prior to their subsequent transfer to the Frances Crick Institute.

180. A Proactive, customer-partnered process for genetically altered model management

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The production of genetically altered rodents results in the development of phenotypes that can affect animal welfare. In order to meet our regulatory and ethical obligations, we created a customer communication process for the development of a proactive colony management plan for the genetically altered rodent colonies in our care. This process focuses on enhanced communication with the customer that (1)pre-identifies humane endpoints, (2)makes specific husbandry and enrichment recommendations with the specific phenotype's limitations and supportive care needs in mind, and (3) makes severity level recommendations prior to colony receipt. We developed enhanced communication tools allowing us to partner with our customers to adopt or adapt established humane endpoints, and supportive care needs through the comparison of the proposed model to known phenotypes of similar models. Where such previous information does not exist, a team with professional expertise in the management of genetically altered animals is consulted for recommendations. The ultimate colony management plan then undergoes careful review and recommendations of a team of experts. This team is composed of scientists, project managers and veterinary staff with experience in genetic model development in a highly interactive partnership with our customers. In order to keep up with best practices, the program includes an ongoing education process for the communication of advances in the support and management of the welfare of genetically altered models.

In the development of this process, we incorporated the most current recommendations and guidelines from the BVAAWF/FRAME/RSPCA/UFAW Joint working group, FELASA, the EU Directive recommendations on severity assessment, and other current literature on the best practices for the management of genetically altered rodents to create our proactive colony management plan.

As projects commence, a robust veterinary care program captures unexpected phenotypes, characterizes them, and documents the resolution. By proactively setting up the expectations of the phenotype and what the humane endpoints will be, veterinary intervention can be expedited and immediate notification and reaction can occur if/when an unexpected phenotype occurs. Thus, we avoid unnecessary delays in responding to conditions that may affect animal welfare. Through the ongoing collection of phenotypic information, we streamlined the onboarding of future projects and have a documented reference for making recommendations for the management of future models This proactive customer partnership meets or exceeds global regulation expectations, while supporting exemplary animal welfare for genetically altered models in our care.

181. Is SAC insufficiency responsible for recurrent pregnancy loss in human?

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Chromosome aberrations (aneuploidies mostly) are the cause of the majority of spontaneous abortions in humans. However, little is known about defects in the underlying molecular mechanisms resulting in chromosome aberrations and following failure of pre-implantation embryo development, initiation of implantation and post-implantation pregnancy loss. We suggest that defects of the spindle assembly checkpoint (SAC) are responsible for aneuploidy and the following abortions. To develop our hypothesis, we modelled this process in the mouse after inactivation of protein BUBR1, one of the key players of SAC.

We found that soon after implantation, more than 50 % of cells of *BubR1*$^{-/-}$ embryos were aneuploid and had an increased level of premature sister chromatid separation (PSCS). Aneuploid cells do not have a predominant gain or loss of some specific chromosomes, but they have mosaic variegated aneuploidy (MVA), which is characterised by random mixture of different chromosomes. MVA leads to growth retardation, stochastic massive apoptosis, disruption of bilateral symmetry and embryo death between embryonic days 7.5 to 13.5. Analysis published human data revealed that human recurrent pregnancy loss (RPL) embryos and rare infant patients carrying *BubR1* mutations that have been described so far have the PSCS and MVA as in BUBR1-deficient/BUBR1-insufficient mice. Based on this data, we predict that deficiency/insufficiency of BUBR1 and other components of the SAC in human are responsible for a significant fraction of both early and late RPLs.

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182. Characterisation of a human amylin transgenic mouse model of insulin resistance

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Introduction: Type 2 diabetes Mellitus (T2DM) is a growing problem worldwide. Pronounced changes in human behaviour, lifestyle and ageing populations have resulted in a large increase in patients diagnosed with diabetes. Diabetes is characterised by insulin resistance and β-cell dysfunction, which with time leads to death through macrovascular and microvascular complications. Human amylin (hA) is a 37 amino acid protein that is cosecreted with insulin from the pancreatic islet β-cells upon stimulation with glucose or other chemicals. Human amylin aggregation has been linked to β-cell degeneration in type 2 diabetes. We have report here characterisation of a mouse model carrying a non-aggregating version of human amylin[25,28,29], triprolyl human amylin which shows insulin resistance and transient hyperglycaemia.

Study Protocol: Male animals were housed from weaning (21 days) in environmentally controlled conditions with a 12-h light/day cycle and were fed standard rodent chow and water ad libitum. Hemizygous [25,28,29] triprolyl hA transgenic mice and their non-transgenic littermates were used in the study. Body weight, blood glucose and food intake were measured weekly from weaning to death. Longitudinal tail serum samples were taken from each mouse at five different stages in their life for hormone analysis. Serum was analysed for [25,28,29] triprolyl hA, insulin, leptin and adiponectin levels.

Results and discussion: The transgenic mice overexpress [25,28,29] triprolyl hA in the pancreas which leads to a phenotype characterised by hyperphagia, weight gain, hyperglycaemia and corresponding insulin resistance. Food intake increased rapidly in the hemizygous mice at around 100 days of age. Weights of the two groups diverge from just after 100 days of age, with the hemizygous animals showing significantly higher body weight by 125 days of age. Blood glucose levels showed significant elevation in the hemizygous animals, post 100 days, but eventually returned to normoglycaemia by around 300 days of age. Serum insulin, leptin and [25,28,29] triprolyl human amylin were all increased in the hemizygous animals at 100 days of age and before the onset hyperphagia, weight gain and hyperglycaemia.

Conclusions: We have identified important physiological and phenotypic changes associated with the onset of insulin resistance, and we show that these changes are driven by the overexpression of [25,28,29] triprolyl human amylin. Our aim now is to determine the cell signalling mechanisms occurring in the different tissues which are associated with the physiological changes leading to the development of insulin resistance observed in these animals.

183. Infrafrontier research infrastructure

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INFRAFRONTIER is the European Research Infrastructure for phenotyping and archiving of model mammalian genomes. The INFRAFRONTIER Research Infrastructure provides access to first-class tools and data for biomedical research, and thereby contributes to improving the understanding of gene function in human health and disease using the mouse model. INFRAFRONTIER was among the six research infrastructure initiatives in the Biological and Medical Sciences (BMS) that were included in the first ESFRI Roadmap in 2006.

The core services of INFRAFRONTIER comprise the systemic phenotyping of mouse mutants in the participating mouse clinics, and the archiving and distribution of mouse mutant lines by the European Mouse Mutant Archive (EMMA). In addition, INFRAFRONTIER provides specialised services such as the generation of germ-free mice (axenic service) and training in state-of-the-art cryopreservation and phenotyping technologies.

Key INFRAFRONTIER objectives are:

- Providing access to mouse models, data, and scientific platforms and services to study the functional role of the genome in human health and disease
- Archiving and distribution of scientifically valuable mouse strains through the European Mouse Mutant Archive (EMMA), one of the world's leading mouse repositories
- Providing access to a whole-organism, systemic analysis of genotype–phenotype interactions using cutting-edge analytical and diagnostic methodology in the INFRAFRONTIER mouse clinics
- Providing bottom-up access for individual scientists and research groups and top-down capacities for large-scale international initiatives such as the International Mouse Phenotyping Consortium (IMPC)

Access to all resources and services of the INFRAFRONTIER Research Infrastructure as well as background information on its organisation and on the ESFRI process is available via the web portal at https://www.infrafrontier.eu/.

The INFRAFRONTIER GmbH, based at the Helmholtz Zentrum München, coordinates the transnational activities of the national partners that together form the European INFRAFRONTIER Research Infrastructure. The INFRAFRONTIER network currently comprises 23 partners, among them are mouse clinics from Europe and Canada, EMMA archiving and distributions nodes as well as the European Bioinformatics Institute (EMBL-EBI). The network is currently engaged in a number of EC-funded projects such as INFRAFRONTIER-I3, InfraCoMP and Biomedbridges and contributes to the International Mouse Phenotyping Consortium (IMPC).

184. Disruption of STAT3 signalling promotes *KRAS*-induced lung tumourigenesis

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STAT3 is considered to play an oncogenic role in several malignancies including lung cancer; consequently, targeting STAT3 using small-molecule inhibitors has been proposed as therapeutic intervention. We found that STAT3 plays an unexpected tumour-suppressive role in *KRAS*-mutant non-small-cell lung cancer (NSCLC). In mice, lung tissue-specific inactivation of STAT3 resulted in increased $Kras^{G12D}$-driven NSCLC initiation and malignant progression leading to markedly reduced survival. Clinically, the transcriptional profile of *STAT3*-deleted murine tumours strongly correlates with advanced and poor prognosis in human NSCLC. Furthermore, low *STAT3* expression levels correlate with poor survival in human lung adenocarcinoma patients with smoking history, which are prone to harbour *KRAS* mutations. Consistently, we found that *KRAS*-mutant human lung tumours showed reduced STAT3 levels. Mechanistically, we showed that STAT3 represses NFκB-induced expression of the chemoattractant cytokine Chemokine (C-X-C motif) ligand 1 (*Cxcl1*, murine ortholog to human *IL-8*) by sequestering NFκB in the cytoplasm and therefore reducing NFκB transcriptional activity. Consequently, deletion of STAT3 results in increased NFκB-dependent IL-8 expression, which augments myeloid tumour infiltration and tumour vascularization thereby promoting tumour progression. These results identify a novel STAT3-NFκB-IL-8 axis in *KRAS*-mutant NSCLC with therapeutic and prognostic relevance.

185. The Akt multi-hit mouse model to study oncogenic cooperativity

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Cancer formation is a multi-step process, which proceeds in a Darwinian fashion. The establishment of genetic models to test the cooperativity of different mutations is essential to elucidate the mechanisms of tumour development.

The Akt/kinase pathway mediates various intracellular processes including survival, growth and proliferation. The family is composed of three members: *Akt1*, *Akt2* and *Akt3*. Both, gene amplification or over activation of each respective member has been suspected to play a role in ovarian, gastric, colorectal, prostate and breast tumours formation. However, to date the exact role of each Akt isoform and how the combinations of deregulated Akt isoforms contribute to tumour formation has remained largely unknown. To address these issues we have generated transgenic Akt^{MH} mice based on the 'Multi-hit' technology. This technology allows for the expression of each member of the Akt family in an inducible, tissue-specific and stochastic fashion, thus mimicking the tumour Darwinian selection.

In order to investigate the overall impact of Akt isoforms in tumour formation, we have crossed transgenic Akt^{MH} mice with the $Rosa26^{CreERt2}$ and MxCre lines. Upon Cre activation, double transgenic Akt^{MH}:Rosa26 and Akt^{MH}:MxCre mice showed diverse malignancies, including breast tumours

Although deregulation of Akt is common in breast cancer, the Akt^{MH} mouse is the only Akt-based overexpression mouse model that results in spontaneous breast tumours. Analysis of breast tumours derived from Akt^{MHH}:Rosa26 and Akt^{MH}:MxCre mice revealed that *Akt3* was activated in all the tumours (positively selected), while *Akt2* was never expressed (contra-selected). *Akt1* was expressed in 50 % of the tumours. These results show that the breast tumours observed in our model are mainly reliant on *Akt3*, thus *Akt3* behaves as an oncogene. Conversely, tumours are contra-select for the expression of *Akt2*, suggesting a tumour suppressor role for this isoform. These results are in agreement with recent findings showing that *Akt3* is amplified and *Akt2* is deleted in human breast tumours. Consequently, these findings in human breast tumours validate the applicability of our mouse model to study the role of the Akt family in mammary gland tumourigenesis.

186. Avoiding genetic artefacts- isogenic C57BL/6J Prnp knockout mice generated with TALENs help discount spurious phenotypes

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Genome editing with programmable nucleases such as ZFNs, TALENs and Cas9/CRISPR allows the rapid mutation of virtually any chromosomal target sequence. In a marked contrast to gene targeting in ES cells, the nuclease-based genome-editing facilitates gene disruption in preimplantation stage embryos of most laboratory animals. This important advance has paved the way for creating truly isogenic mouse and rat models.

We have used TALEN-mediated genome editing in fertilized mouse oocytes to rapidly create the Zurich-III (ZrchIII) Prion Protein (Prnp) knockout mouse on a pure C57BL/6J genetic background. Phenotypic analysis of ZrchIII mice and isogenic C57BL/6J mice confirmed earlier findings that ablation of PrPC results in a late-onset peripheral polyneuropathy but does not alter the phagocytosis of apoptotic cells, which was implied in all other Prnp−/− mouse models that were generated using 129S6/SvE ES cells. ZrchIII mice circumvent genetic confounders present in other Prnp−/− lines, which make them an important resource for dissecting the physiological functions of PrPC. Taken in a broader context, our findings illustrate an intrinsic limitation to interpreting the phenotypes of congenic knockout mice and suggest that isogenic knockouts generated using designer nuclease may be a more reliable and rapid alternative.

187. Bmp2 patterns prospective valve tissue and regulates EMT and mesenchyme proliferation and morphogenesis

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The specification and patterning of chamber versus non-chamber (valve) territories is a crucial event during cardiac development. Atrio-ventricular canal (AVC)-restricted *Bmp2* expression in the myocardium is a key signal for the specification of the cardiac valve territory and the promotion of the epithelial-mesenchyme transition (EMT) that gives rise to the mesenchyme that will form the cardiac valve primordium. We have generated two different Bmp2 transgenic lines targeted into the R26 locus tat affect cardiac development at a different extent. *R26-CAG-Bmp2* transgenic mice show valve and chamber dysmorphogenesis. This abnormal phenotype includes interventricular septal defect, dilated ventricles and thickened valves and trabeculae leading to foetal death at E14.5. In *Bmp2* overexpressing embryos, both outflow tract (OFT) and AVC valves remain primitive and show increased proliferation and mesenchymal expansion in vivo. In the second line, *R26-Bmp2*, we observe a more dramatic effect due to stronger expression of *Bmp2*, and the embryos die at E10.5 AVC, and OFT present mesenchymal expansion in vivo, and the ventricles show features of AVC, with poorly developed trabeculae and mesenchymal cells colonizing the extracellular matrix that separates ventricular endocardium from myocrdium. Furthermore, endocardial cells of ventricular tissue explanted onto collagen gells undergo EMT and behave as AVC explants, indicating that Bmp2 alone is an instructive signal for the specification of the cardiac valve tissue. Expression analyses reveal that various valve and chamber markers are dysregulated in both transgenic Bmp2 lines. Embryoid bodies (EBs) constitutive expressing *Bmp2* (CAG line) do not mature and proliferate more than control EBs, transcribe early mesodermal and cardiac specification markers, but do not progress into cardiomyogenesis. These data demonstrate that Bmp2 is a tigthly spatio-temporally regulated patterning signal for cardiac valve formation, allowing for the maintenance of this tissue in a primitive, proliferative and immature state, which in turn enables cardiac cells to respond to further morphogenetic cues. Our data also suggest that pathological reactivation of *Bmp2* expression in the adult may lead to valve disease.

188. Design and generation of a glutaminase *Gls2* conditional knockout mice

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Mammalian glutaminase (GA; EC 3.5.1.2) is the main enzyme involved in brain generation of glutamate (Glu). This amino acid acts as an excitatory neurotransmitter within the CNS, and it is also implicated in behavioural sensitization through the mesolimbic pathway. Two different GA genes have been described: *Gls* that encodes the isozymes KGA and GAC; and *Gls2*, which encodes GAB and LGA isozymes. *Gls* and *Gls2* isoforms are co-expressed in different brain regions and cells. Of note, location of *Gls2*-encoded isoforms in neuronal nuclei suggests a novel role in the regulation of gene expression. The co-expression of different GA isoforms in mammalian brain is so far unexplained. Our objective is to study the cerebral function of *Gls2*; for this purpose, we develop a conditional *knockout* (KO) mouse model to silence GAB and LGA expression in brain.

A vector carrying the *Gls2* gene from exon 1 to 12 (obtained from the EUCOMM consortium) was transfected by electroporation into B6D2F1 murine embryonic stem cells (ES). These ES were selected by geneticin and PCR-genotyped before their microinjection in 8-cell stage embryos (Swiss strain). Embryo implantation was performed in pseudopregnant state mice, which leads to chimeric pups. This vector targets chromosome 10 and will yield a conditional KO mouse model, since exons 2–7 are included between *LoxP* sites. The chimeric pups carrying this modification within their germline were used to generate the homozygous *Gls2* (−/−) mice. After

integration of the vector in both alleles, the mice will be mated with mutant *Cre* mice, which express this recombinase enzyme under control of the synapsin specific promoter. This will result in a deletion of the exons 2 to 7 giving rise to null *Gls2* mutants mainly in the following brain areas: cortex, hippocampus, amygdala and cerebellum, which are essential for glutamatergic transmission and related to the mesolimbic pathway.

189. Characterization of a novel dwarf and obese mouse line generated by the gene trapping method

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The hypothalamus is the primary site in the central nervous system for controlling hormonal balance, energy balance, as well as many other important functions such as reproduction, thirst, temperature and biological rhythms. The hypothalamic arcuate nucleus is not completely insulated from the blood–brain barrier and thus is particularly important for sensing peripheral metabolic signals, and it also represents the final integrated neuronal output from the brain to regulate the endocrine functions of the pituitary gland. Dissecting the physiological function of the hypothalamus through genetic perturbation experiments is fundamentally important for understanding the regulation of growth and energy metabolism. In this study, we generated a novel dwarf and obese mouse line using the gene trapping method. The homozygous mutant mice were born smaller, and at weaning age, they weigh about one half as much as their wild-type and heterozygous littermates. The anterior lobe, and to a lesser degree the intermediate lobe, of the pituitary gland are thinner and often abnormally shaped in the mutant mice, while the posterior lobe was much less affected. At 6 weeks of age, these mutant mice gradually become obese, although they remained shorter in length. Immunohistological and molecular analyses have indicated that there is a severe shortage of GABAergic neurons in the arcuate nucleus of the hypothalamus, while the glutamatergic neurons are not much affected. It appears that the hypothalamic neurons are correctly differentiated in young mutants, but they failed to mature and survive beyond weaning. These neuronal defects result in reduced numbers of GHRH, POMC and NPY neurons, which are critical for central regulation of somatic growth and energy metabolism, and consequently caused the dwarf and obese phenotypes. The mutant mouse line generated here may provide new leads for understanding the signalling mechanism of ARC neuronal maturation and neural circuit development, and it is also a useful model for studying growth retardation and obesity.

190. Mouse Tafazzin is required for male germ cell meiosis and spermatogenesis

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Barth syndrome is an X-linked mitochondrial disease, symptoms of which include neutropenia and cardiac myopathy. These symptoms are the most significant clinical consequences of a disease, which is increasingly recognised to have a variable presentation. Mutation in the Taz gene in Xq28 is thought to be responsible for the condition, by altering mitochondrial lipid content and mitochondrial function. Male chimeras carrying a targeted mutation of Taz on their X chromosome were infertile. Testes from the Taz knockout chimeras were smaller than their control counterparts, and this was associated with a disruption of the progression of spermatocytes through meiosis to spermiogenesis. Taz knockout ES cells also showed a defect when differentiated to germ cells in vitro. Mutant spermatocytes failed to progress past the pachytene stage of meiosis and had higher levels of DNA double-strand damage and increased levels of endogenous retrotransposon activity. Altogether these data revealed a novel role for Taz in maintaining genome integrity in meiosis and facilitating germ cell differentiation. We have unravelled a novel function for the Taz protein, which should contribute to an understanding of how a disruption of the Taz gene results in the complex symptoms underlying Barth Syndrome.

191. Dissecting neuronal pathways involved in pain transduction

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Application of the Cre-LoxP method to specifically ablate mouse genes in a specific tissue and/or particular developmental stage has been instrumental in understanding the role of individual molecules and specific cell types in pain transduction. The method relies on controlling the expression of Cre recombinase spatially and temporally and the presence of LoxP sites that flank critical exons. We have produced a range of mouse lines that express either Cre recombinase under the Advillin promoter (i.e. pan-dorsal root ganglia (DRG) neuronal expression) or under the Nav1.7 promoter (i.e. a subset of DRG neurons). Crossing these lines with the lines containing genes flanked with LoxP sites effectively produces knockouts of these genes either in all DRG neurons or in Nav1.7 expressing neurons. I will describe why these mouse lines are a useful tool to study not only the role of individual genes in pain transduction but also the role of individual classes of neurons.

192. A mouse model for neuronal-specific expression of wild-type and Rett syndrome causing mutated MeCP2

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Expression of MeCP2 in the brain occurs in both glial and neuronal cells. Neurons express approximately six- to sevenfold more MeCP2 protein than glia, and therefore, the abundance of MeCP2 in neurons is comparable to the histone octamer. It has been demonstrated that MeCP2 binds to methylated DNA and recruits the NCoR complex to chromatin. In humans, mutations in either the methylated DNA-binding

domain (MBD) or the NCoR interaction domain (NID) cause Rett syndrome. This demonstrates that both the MBD and the NID are crucial for MeCP2 function, but what MeCP2 really does in neurons is still unclear. To gain a better understanding into the neuronal function of MeCP2, we have generated mice expressing several versions of MeCP2 from a neuronal-specific promoter. We used either wild-type MeCP2 or MeCP2 with a mutation in the NID or the MBD. Mice were generated by blastocyst injection of genetically modified ES cells. The locus was engineered to allow removal of neuronal-specific expression at different time points during the life of the animal. We are currently analysing these novel transgenic lines using a phenotyping regime and behavioural tests. We are also conducting biochemical analysis of MeCP2 localisation and interaction partners in the brains of these mice.

193. Behavioural phenotyping of mouse models for cognitive disabilities

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GENCODYS is a translational program aiming to identify new genes involved in cognitive dysfunctions, to elucidate molecular mechanisms underlying cognitive deficits, and to screen for potential therapeutics. In this regard, one of the objectives of GENCODYS consists of neurological and behavioural phenotypic analysis of mouse models for mental retardation, focusing particularly on evaluation of cognitive dysfunction. Among syndromes with intellectual disabilities, the Kleefstra syndrome is caused by heterozygous mutations in the EHMT1 gene, which is located within the region of chromosome 9q34.3, either by deletion or nonsense or frame-shift mutations leading to a non-functional EHMT1 product. Common features in patients are severe mental retardation, hypotonia, delayed motor function, autistic features, epileptic seizures, brachy(micro)cephaly, flat face with hypertelorism, synophrys, anteverted nares, everted lower lip, carp mouth with macroglossia and heart defects. Haploinsufficiency of the EHMT1 gene is responsible for the main phenotypic features. Other genes are reported to be involved in the Kleefstra syndrome including EHMT2, MBD5 and NR1I3.

In the present study, mice carrying mutations in EHMT1, EHMT2 or NR1I3 were submitted to behavioural tests designed to evaluate a wide range of functions or their pathologies, including circadian activity, neurological reflexes and specific motor abilities, anxiety-related behaviour, sensorimotor gating or learning and memory processes. EHMT1 and EHMT2 mutants displayed reduced spontaneous activity and exploration, reproducing thus the human phenotype. EHMT1 mutants also showed altered sensorimotor gating and EHMT2 decreased motor performance. On the other hand, NR1I3 mutants tended to have altered fear conditioning. These data show heterogeneity of phenotypes between the mutation types, recapitulating some of the human features and suggesting thus the relevance of these mutants for better understanding human syndromes.

194. Precisely engineered rodent models of Parkinson's with CRISPR technology

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α-Synuclein (αSyn) was the first gene identified to cause Parkinson's when mutated. Several point mutations in the αSyn gene, as well as duplication and triplication of the wild-type gene, have been identified in families with autosomal dominant inheritance of this condition. A novel mutation in αSyn, 'G51D', was recently identified in familial cases exhibiting very early onset of Parkinson's. Functional studies showed that G51D αSyn has anomalous interactions with lipid bilayers, and it is more toxic to cells than the wild-type protein. The aim of this project is to generate precisely engineered rodent model of Parkinson's by (1) introducing G51D mutations into the endogenous rat and mouse αSyn genes and (2) to replace the rat αSyn gene with a triplicated and mutant (G51D) human version. For one-step generation of G51D αSyn mutant mice and rats, the mRNA for Cas9, sgRNA and a 90-bp donor oligo DNA were co-injected into mouse and rat zygotes. G51D mouse and rats were successfully generated by CRISPR/Cas9 technology. Three out of 26 founder mice analysed had the G51D mutation and two of 11 rat founders carried the desired mutation. To generate knockin rats with replacement of the rat αSyn gene with a triplicated and mutant (G51D) human version, CRISPR technology will also be applied. The donor plasmid has been generated and CRISPRs are being tested. The donor plasmid will also be used to replace the rat αSyn gene in rat embryonic stem cells (ESCs). The correctly targeted ESC clones will be used as a model for cytotoxicity studies after differentiation into cortical neuronal cell types. CRISPR technology to generate specific point mutations in mice and rats is very efficient. Progress towards gene replacement is ongoing.

195. Development of the ASBT-Cre mouse for the study of biliary pathologies

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Background: Bile duct pathologies such primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC) and cholangiocarcinoma (CC) account for a significant percentage of patients with end-stage liver disease requiring liver transplantation. To date, the study of genes which play a major role in

these pathologies has been hampered by the fact that no cholangiocellular Cre recombinase (Cre) mouse system has been developed to systematically knock out genes in a cell-specific manner within the bile ducts of the liver. Such a system would provide a powerful tool in unearthing the exact role of cholangiocytes to these aforementioned diseases. To this end, we chose the ASBT promoter to drive cholangiocellular Cre expression as ASBT is a highly expressed protein in cholangiocytes and functions as a 348 amino acid protein localized on the cholangiocyte apical membrane which functions to transport-conjugated and transport-unconjugated bile acids in the biliary tract.

Aims: To create a cholangiocellular-specific Cre expressed solely within the bile ducts of the mouse liver.

Methods: A transgenic construct containing a 3.2-kbp mouse ASBT/promoter-modified Cre recombinase was incorporated into the RP24-396J19 bac clone containing the ASBT genomic region. The recombinant bac was subsequently injected into C57B6N pronuclei.

Results: The strain lacks any disease or metabolic phenotype related to the transgenic allele. When crossed with a Tom-red/GFP reporter mouse, ASBT-Cre expression leads to recombination within over 90 % of cholangiocytes indicating Cre expression specific for bile duct epithelial cells in the liver. Expression of the Cre was also observed in the small intestine colon and kidney, respectively. Additionally it was observed that 0.01 % of hepatocytes expressed Cre recombinase, possibly as a result of biliary precursor to hepatocyte differentiation near the portal field.

Conclusion: ASBT promoter-driven Cre expression results in over 90 % recombination within cholangiocytes of the liver with minimal expression found in other tissues, highlighting this Cre expressing mouse model as a possible tool for further study of biliary diseases.

197. Perinatal induction of Cre recombination with tamoxifen

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Temporal control of site-specific recombination is commonly achieved by using a tamoxifen-inducible form of Cre or Flp recombinases. In this system, the recombinase coding region is fused with the ligand-binding domain of the oestrogen receptor (ER), that has been mutated (ERT2) to bind synthetic steroids, either tamoxifen or 4-hydroxytamoxifen. Current induction procedures involved the injection of 1 mg Tam or OHT for 5 consecutive days in double transgenic adult mice aged 8–10 weeks (1). This procedure has been proven to be efficient on a majority of floxed loci without obvious toxicity. Powerful protocols of induction have also been developed for gene inactivation during embryonic development (e.g. 2). However, induction of recombination at late gestational or early postnatal stages is more difficult to achieve, due to the interference of tamoxifen with delivery and to difficulty of administration to pups, respectively. In this context, using the ubiquitous CMV-CreERT2 transgenic mice (2), we have tested and validated two procedures to achieved recombination just before birth (embryonic days E16.5-17.5) and just after birth (postnatal days PN1-3). The efficacy of recombination was evaluated in the brain, which is known to be more difficult to target, at least at adult stages. For the late gestation treatment of tamoxifen, different protocols of complementary administration of oestrogen were tested. However, delayed delivery and/or mortality of pups due to difficult delivery were always observed. To circumvent this problem, pups were extracted from tamoxifen-treated pregnant dams by caesarian at E18.5 and bred to foster mother. For postnatal treatment, different dosages of tamoxifen were administered by intragastric injection to the pups during 2 or 3 days after birth. The efficiency of these treatments were first analysed at PN7 using the reporter line reporter R26tdTomato (3). They were next validated on the Hoxa5 floxed locus (4). In conclusion, we have developed efficient procedures that allow to achieve efficient recombination of floxed loci at perinatal stages. These protocols will allow to investigate the late/adult functions of many developmental genes, whose characterization has been so far restricted to embryonic development.

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198. A newly engineered high-resolution mapping panel to identify dosage-sensitive genes in Down syndrome-associated motor defects

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Down syndrome (DS) is caused by trisomy of chromosome 21 (Hsa21). This increased gene dosage leads to a number of disorders such as cardiac defects, Alzheimer's disease, learning and memory deficits and motor coordination problems.

In order to identify dosage-sensitive genes that when present in three copies, cause specific phenotypes in DS, we have generated a high-resolution mapping panel of 15 new mouse strains with partial trisomies and monosomies for regions of mouse chromosome 16, orthologous to Hsa21. These have duplications and deletions of DNA segments ranging in length from 1.7 to 22.9 Mb.

Individuals with DS have problems with fine motor control and are delayed in their motor development; however, the precise genetic or mechanistic causes of these defects remain unclear. In order to map this phenotype to the causative genes, we have assayed motor co-ordination and learning in DS mouse models using a rotorod. We have seen that a large duplication encompassing a 22.9-Mb region of Mmu16, containing around 179 genes orthologous to Hsa21, results in defects in this assay. We have now used our high-resolution mapping panel to map a dosage-sensitive gene that could be responsible for this defect and are currently analysing the requirement for this gene within the motor system.

199. A newly engineered high-resolution mapping panel to identify dosage-sensitive genes in Down syndrome-associated heart defects

Eva Lana-Elola[1], Sheona Watson-Scales[1], Amy Slender[1], Dorota Abucewicz[1], Alexandrine Martineau[1], Charlotte Douglas[1], Timothy Mohun[1], Elizabeth Fisher[2], Victor Tybulewicz[1]

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Down syndrome (DS) is caused by trisomy of chromosome 21 (Hsa21). This increased gene dosage leads to a number of disorders such as cardiac defects, Alzheimer's disease, learning and memory deficits and motor coordination problems.

In order to identify dosage-sensitive genes that when present in three copies cause CHD in DS, we have generated a high-resolution mapping panel of 15 new mouse strains with partial trisomies and monosomies for regions of mouse chromosome 16, orthologous to Hsa21. These have duplications and deletions of DNA segments ranging in length from 1.7 to 22.9 Mb.

DS is the most common genetic cause of congenital heart defects (CHD), particularly atrio-ventricular septal defects (AVSD). However, the precise genetic or mechanistic causes of these defects remain unclear

Using high-resolution episcopic microscopy (HREM) in embryonic hearts (E14.5), we have established that a large duplication encompassing 22.9 Mb region of Mmu16, containing around 179 genes orthologous to Hsa21, results in CHDs whose typology closely resembles those observed in individuals with DS, including the AV septal defects. Moreover, analysis of embryonic hearts from a number of other strains with shorter duplications has allowed us to narrow down the critical genomic region for DS-CHD and demonstrate that DS-associated AVSDs are caused by an additional copy of at least 2 different loci/genes.

200. The impact of gut microbiota on phenotype following rederivation

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The phenotype of transgenic rodents arises from a combination of both genetic and environmental influences that include their gut microbiota. It has become increasingly evident in multiple species that this colonizing microbiota plays a profound role in host physiology. The impact of the dynamic relationship between host and gut microbiota has yet to be explored in the context of rederivation. Rederivation has rapidly become commonplace in rodent research facilities as a means to remove pathogens from colonies and for resuscitation of valuable and unique cryopreserved rodent models. During rederivation, the embryos are recovered into a pseudopregnant recipient dam chosen for high fecundity and superior maternal care, typically an outbred stock. It is suspected that resulting pups will be colonized with the microbiota of this recipient strain/stock. To study the inheritance of maternal microbiota and the effect of rederivation on disease phenotype, genetically identical embryos from a rat model of colorectal cancer (Pirc: Polyposis in the rat colon) were transferred into recipients of three different strains (F344/NHsd, LEW/SsNHsd and Crl:SD). Tumour burden in the pups was tracked longitudinally via colonoscopy, and end-stage tumours were counted and graded histologically. The gut microbiota was characterized in the recipient dams pre-partum and in Pirc rat offspring prior to and during disease progression via next-generation sequencing of the bacterial 16 s rRNA gene to identify associations between disease phenotype and the gut microbiota. We confirmed that microbiome does vary between rat strains, with LEW/SsNHsd having a unique enterotype featuring a high percentage of the bacteria *Prevotella copri*. Pups microbiomes did in fact mimic the enterotypes of their dams, indicating that the dam is the main influence on the developing microbiome. Male Pirc rats had no difference in tumour number between enterotypes; however, tumour counts were lower than what is observed for this strain on the F344/Tac background. Female Pirc rats with the Crl:SD microbiome had significantly more colonic tumours than those with the Lewis/SsNHsd microbiome. There was also a difference in tumour location within the colon, with localization of tumours to the distal colon in the Crl:SD group. In conclusion, the microbiome varies between strains of rats, and rats produced via embryo transfer have a similar microbiome to their recipient dam rather than the strain of origin. Lastly, this variation in microbiome was associated with a change in phenotype of the Pirc rat model of colon cancer.

201. First transgenic rat model for Alzheimer's disease neurodegeneration

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Alzheimer's disease (AD) is progressive neurodegenerative disease that affects higher structures of the brain. Prominent neuropathologic features of AD are neurofibrillary tangles, senile plaques, neuroinflammation, synaptic and neuronal loss. AD is a leading cause of dementia worldwide. Currently, there is no disease-modifying drug for AD treatment available. In order to identify novel disease pathways, drug targets and diagnostic biomarkers, several transgenic rodent models have been generated.

We developed the first transgenic rat model of human AD based on expression of disease-modified protein tau—Alzheimer's tau. Three transgenic rats were generated by pronuclear

injection of 1-day old SHR rat embryos. Transgenic lines have stably expressed human Alzheimer tau for more than 30 generations. The rat model recapitulates several feature of human Alzheimer's disease including development of neurofibrillary pathology, synaptic impairment, oxidative stress and neuroinflammation. Extensive neurofibrillary degeneration in the rat brain fulfills all criteria for human AD neurofibrillary pathology including phospho-tau immunopositivity, argyrophilia, Thioflavin S positivity and Congo red birefringence. Rat neurofibrillary degeneration was characterized by extensive formation of sarkosyl insoluble tau protein complexes. These pathological changes led to the progressive decline of sensorimotor functions and the impairment of several reflexes. It is important to note that the larger size of transgenic rats compared to mice permits serial sampling of the CSF and monitoring of the longitudinal changes in CSF biomarkers. We found that increased levels of phospho-tau in the CSF well correlated with neurobehavioural impairment.

The transgenic rats represent unique animal model for AD that fully recapitulates neurofibrillary pathology in the brain. The rat model demonstrates stable expression of transgenic protein and high reproducibility of the phenotype. Availability of CSF make this model suitable for identification of novel biomarkers for AD.

202. RNAI mouse models: revolutionizing drug discovery in vivo

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An estimated $1.8 billion dollars is spent for each new successful drug developed, primarily in failed clinical trials due to lack of efficacy and safety. New approaches for rapid identification and early preclinical validation of novel therapeutic targets are crucial to make important 'go/no-go' decisions and curb the cost of drug development. Genetically engineered mouse models (GEMMs) have provided a powerful platform to study disease initiation and maintenance, the microenvironment and the responsiveness of disease to known or novel therapeutics; the long lead times and high costs required to develop, intercross and maintain models with various disease predisposing gene combinations have limited their practical utility in the drug discovery process. RNA interference (RNAi), a mechanism that controls gene expression, is a rapid and cost-effective alternative to gene deletion that can be exploited experimentally to reversibly silence nearly any gene target not only in vitro but also in live mice. Previously, the main hurdle in generating effective RNAi mice was the identification of potent RNAi triggers, or short hairpin RNAs (shRNAs), that would induce stable and regulated gene silencing with limited off-target effects. To overcome this hurdle, we created a 'Sensor assay' to biologically validate effective shRNAs at large scale, and we engineered a new miRNA scaffold, miR-E, that is more efficiently processed and thus produces more potent knockdown of target genes than other RNAi systems. By combining our sensored, tetracycline-regulated miR-E-based shRNAs with high-efficiency ES cell targeting, we have developed a fast, scalable pipeline for the production of shRNA transgenic mice with reversible gene silencing. Recently, with the advent of new genome-editing technologies, we are able to introduce additional sensitizing lesions to induce disease pathogenesis in combination with RNAi technology, enabling the generation of sophisticated GEMMs without extensive intercrossing. We demonstrate that RNAi can cause sufficient knockdown to recapitulate the phenotypes of knockout mice. Using this robust system, we generated a number of inducible RNAi transgenic lines and demonstrate how this approach can identify predicted phenotypes and also unknown functions for well-studied genes. Through RNAi-regulated gene silencing, we are able to mimic drug therapy in mice, allowing us to determine the therapeutic value and/or reversible and irreversible toxicities associated with specific gene inhibition. This system provides a cost-effective and scalable platform for the production of RNAi transgenic mice targeting any mammalian gene—mice with enormous predictive power that will shape our development of better tolerated therapies.

203. State-of-art humanized mouse models and the CRISPR/Cas9 TECHNOLOGY AT TaconicArtemis

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Quality, speed and innovation have been the driving forces for TaconicArtemis to develop custom-tailored genetically engineered mouse (GEM) models for fast and efficient in vivo drug target validation. We are offering a wide range of mouse models that include constitutive/conditional knockout, constitutive/conditional knockin, humanizations, targeted transgenesis and inducible gene knockdown by RNA interference with high-quality design retaining competitive timelines.

Especially humanized mouse models, in which mouse genes are replaced with their human counterparts, bridge the gap between mice and men and allow for a more predictive compound testing in pre-clinical studies as well as the modelling of human diseases. This feature makes humanized mice a highly valuable model for the analysis of drug metabolism and disposition, immune and hematopoietic systems, infectious disease, regenerative medicine and cancer biology.

While large humanizations are realized via homologous recombination in mouse embryonic stem cells, we have

recently implemented the CRISPR/Cas9 technology in our production pipeline for an accelerated generation of knockout and knockin as well as humanization models, allowing for example the rapid generation of an allelic series for a gene. The straightforwardness of the method by omitting the cell culture part, combined with the high efficiency of the CRISPR/Cas9 system, facilitates the generation of founder animals with reduced costs and effort, providing an attractive alternative to homologous recombination approaches.

204. Retinal abnormalities found in common albino outbred mice

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During the course of some electroretinography phenotyping studies, we detected abnormal cone-derived electrical signals in some individuals of common albino outbred mice obtained commercially from various vendors. This functional retinal abnormality was confirmed at the structural level by standard histology and immunofluorescence analyses, using both optic and electron microscopy. These observed retinal alterations were similar to abnormalities reported in some human retinopathies, including age-related macular degeneration and some cone distrophies, such as acromatopsia.

We decided to identify the gene responsible and the associated mutation (which we named as coneless) for the observed photoreceptor cone deficit segregating in common albino outbred stocks commercially available. Various pedigrees were established and individuals from three consecutive generations were obtained and eventually phenotyped and genotyped using the Illumina MD panel, spanning the entire mouse genome. This initial genetic linkage analysis indicated that the studied trait was monogenic, autosomal and recessive, mapping within a region of one of the mouse chromosomes. However, about two hundred genes with annotated expression in the retina were found in the identified region. We tried to reduce the size of the candidate region using microsatellites (SSLPs), but we could not find suitable polymorphic markers in the pedigree, thus implying that the region was inherited as a haplotype (identical by descent). Some of these genes were selected, according to the expected function, and directly explored by standard DNA sequencing and/or qRT-PCR analyses with negative results. Finally, we decided to apply a next generation sequencing (NGS) approach in order to cover all those genes and the rest of the mouse genome. Complete mouse exomes from representative mouse individuals from all genotypes were analysed and after careful bioinformatic analyses, in combination with the previous genetic linkage information, we could eventually identify the gene and the mutation causing the observed phenotype. We have detected a substantial proportion of animals carrying this mutation in most stocks of common outbred albino mice. Therefore, these results will be of interest for anyone using common albino outbred mice for research involving retina, vision or behaviour studies.

205. Rescue of the ocular coloboma phenotype of the *FOXG1*$^{-/-}$ null mutants in *FOXG1*$^{-/-}$;*WNT8B*$^{-/-}$ double mutants

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Foxg1 is a transcription factor which is expressed in the telencephalon and in the developing retina. *Foxg1*$^{-/-}$ null mutant mice with no functional Foxg1 protein have small telencephalic lobes and microphthalmic eyes. Although the role of Foxg1 in the telencephalon has been examined thoroughly, its role in eye development is less understood. One major defect in the development of the *Foxg1*$^{-/-}$ eyes is the absence of a normal optic stalk, resulting in an aberrant joining of the eye to the hypothalamus. Another important defect in these mutant eyes is the fact that as development progresses, the *Foxg1*$^{-/-}$ eye cup does not close along the optic fissure, at the bottom of the eye, leading to a large ventral coloboma.

It has previously been shown that in the telencephalon Foxg1 represses the signalling molecule Wnt8b by direct binding and this repression is crucial for dorso-ventral telencephalic patterning to take place. Using a genetics approach, the effect on the eyes of the loss of Wnt8b in the *Foxg1*$^{-/-}$ mutant background was studied by generating double *Foxg1*$^{-/-}$;*Wnt8b*$^{-/-}$ mutants. These double mutants show a remarkable recovery of the eye phenotype of the single *Foxg1*$^{-/-}$ mutant. Specifically, both the optic stalk and optic fissure closure defects are substantially rescued.

When expression of Wnt8b was examined at embryonic day (E) 12.5 in the developing forebrain, there was specific expression in the optic recess and optic stalk. In the *Foxg1*$^{-/-}$ mutant, *Wnt8b* expression was found upregulated in these regions. A similar upregulation was observed in the expression of molecules of the Wnt/beta-catenin signalling pathway. These results indicate that normally Wnt/beta-catenin signalling needs to be repressed for optic stalk formation and proper optic fissure closure to take place and implicate abnormal Wnt/

beta-catenin signalling in the formation of ocular coloboma. Foxg1 may repress directly or indirectly this pathway.

Wnt8b expression in the *Foxg1*$^{-/-}$ mutant is currently being studied at earlier stages of development to establish when and where Wnt8b upregulation is first observed in the developing optic vesicle and neighbouring region. In parallel, the phenotypes of the developing eye of the single (*Foxg1*$^{-/-}$) and double (*Foxg1*$^{-/-}$;*Wnt8b*$^{-/-}$) mutants are being investigated by means of patterning, cell proliferation and cell death and are compared to controls to establish which cellular process underlines the effect of Wnt/beta-catenin signalling on optic stalk and optic fissure formation.

206. Modelling and remodelling: the use of CRISPR/Cas9 and TALENs to create and modify mouse models of human disease

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The direct introduction of targeted nucleases, including ZFN, TALEN and CRISPR/Cas9 into fertilized mouse oocytes, allows the creation and further modification of established inbred mouse strains. Further, by using these approaches with inbred mouse strains, each with its own defined and reproducible genetic background, any gene modification can be examined coherently and without the confounding effects of undefined (outbred) genetic influences. However, compared to outbred or hybrid strains (F1), inbred strains can present unique technical challenges, e.g. superovulation conditions, oocyte/embryo survival post genetic manipulation, lower genetic modification frequencies. Here, we will present our experience using nuclease directed knockouts (KO) and knockins (KI) of multiple strains including: C57BL/6J, C57BL/6N, FVB, NOD and MRL. Genetic modifications have included KOs and KIs to more than 20 genes using a single nuclease to modify single or multiple loci, and multiple sgRNAs to cause larger gene deletions. Additionally, we will show that established genetically modified animals, e.g. NRG, NSG and Tg(FCGRT)32, can be further modified as oocytes, allowing rapid sequential refinement and the building upon previous data using established models. Here, we present our general strategy enabling the rapid development of sgRNAs from design to injection and review some of the successes and challenges to the approach in the context of the inbred mouse embryo.

As an example, we will review our gene correction of the Retinal Degeneration mutation RD8 in the Crumbs Homolog 1 (Crb1) gene of C57BL6/NJ strain by HDR. This used a ssDNA donor oligo microinjected with TALEN mRNA to correct a single base deletion and resulted in $\sim$20 % of founders carrying the corrected allele. C57BL6/NJ is the basic background being used for The International Knockout Mouse Consortium (IKMC) as the standard for phenotypic analysis of thousands of newly generated KO lines. This gene correction offers the possibility of its use where a retinal degeneration mutation may confound other precision modifications.

TT2014 Late Abstracts

207. A large-scale mouse phenotyping screen for the discovery of novel biotech targets in oncology and angiogenesis and other therapeutic areas

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The recent availability of large numbers of gene-targeted mice is now allowing new large-scale phenotyping efforts to systematically evaluate gene function on an unprecedented scale. Following on our previous gene-targeting efforts funded by the NIH KOMP (Knockout Mouse Project), we have established a novel high-volume, broad phenotyping platform, or Tier1 screen, as a primary approach to evaluate secreted and transmembrane genes as potential biotechnology therapeutic targets. Targets of interest identified in the Tier1 screen can be rapidly followed up in therapeutic-area-specific secondary assays. In this Tier1 screen, we subject large F2 cohorts from 100 projects per year to an analysis including: (1) high-resolution reporter gene expression imaging and analysis in embryos and adults to reveal sites of potential gene function in situ; (2) Evaluation of mendelian inheritance to assess viability; (3) Evaluation of timing and causation of embryonic lethality to discover genes critical in embryonic development (Embryo morphology is analysed by optical projection tomography (OPT) and soft tissue-enhanced micro computed tomography (μCT)); (4) clinical serum chemistry and haematology analysis to broadly evaluate health; (5) μCT-based analysis of body composition and bone to elucidate metabolic and skeletal phenotypes; (6) sequencing-based transcriptome profiling of key tissues to reveal expression-based phenotypes; (7) FACS-based profiling of hematopoietic cell populations in key immune organs, to reveal immune phenotypes, and uniquely, and (8) Our screen also includes a novel challenge of tumour engraftment to evaluate the host response to cancer. All phenotype data are collected in a custom image and phenotype database. We mine the resulting data to help to understand biological functions of genes of interest as well as to evaluate potential therapeutic targets for our human antibody-based therapeutic pipeline. We have analysed over 400 genes through this screen and have successfully identified targets that have progressed through our development pipeline in a variety of therapeutic areas. The novel tumour challenge screen has been particularly fruitful in identifying vascular and immune genes in cancer biology and will be a focus of this talk.

208. Speed backcrossing through the female germline

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Normal development and physiology vary significantly between inbred mouse strains, complicating the analysis of mutant mice of heterogeneous genetic background, as subtle effects caused by the mutation and effects due to other genes within the background of the mutant strain cannot be distinguished. To allow for a direct comparison, the mutant mouse strain should only differ from a particular inbred strain at a single locus, such that a phenotypic difference between these strains must be a consequence of the mutant allele. Such strains have been classically obtained by repeated backcrosses to an inbred (background) strain, with selection for the desired mutation donor strain. Mouse strains developed by this method are considered congenic after a minimum of 10 backcross generations to the background strain. With each generation requiring 8–10 weeks, conventional backcrossing is a lengthy process that may last up to 3 years. More recently, speed backcrossing procedures have been developed that incorporate mouse strain genotyping panels such that at each generation, offspring can be selected that not only retain the desired chromosomal fragment, but that also 'lose' the maximum amount of background genetic information from the donor strain. With these approaches, congenic mice can be obtained in as few as 5 backcross generations.

Here, we evaluated a potentially more rapid approach of creating congenic mice via speed backcrossing (SB) using the female germline. Traditionally, speed backcrossing is done using the male germline, i.e. selected males with the donor mutation are mated to females of the background strain. This approach requires at least eight weeks per generation for a male to be viable to mate. By using superovulation of female mice at 3 weeks of age, a congenic mouse of a desired background can be tentatively be generated approximately six months sooner. This poster describes the actual outcome and feasibility of this approach to rapidly change the background strain from a transgenic strain with an undefined C57BL/6 background to a defined congenic C57BL/6J (Tg). The outcome was the production of candidate animals after 10 months of superovulation aided backcrossing. Critical factors influencing production time were (1) obtaining background genotyping results within a 7-day window and (2) producing sufficient candidate animals per generation. Shorter time and associated reduced housing and animal husbandry costs support the viability of this approach for producing congenic mice.