

Program and Abstracts of the 8th Transgenic Technology Meeting (TT2008)

27–29 October 2008, Toronto, Canada, University of Toronto Chestnut Conference Centre, 89 Chestnut Street, Toronto

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TT2008 PROGRAM

Monday, 27th October 2008

9:00–11:00 Registration. Pickup name badges and conference materials

11:00–11:30 Welcome address

11:30–14:00 **Session I: Stem cells and early embryos**

Functional exploration of the mouse genome

Janet Rossant

The Hospital for Sick Children Research Institute, Toronto, Canada

Gene expression during the mammalian oocyte to embryo transition

	<p>Davor Solter The Jackson Laboratory, Main, USA and IMB-A STAR, Singapore</p> <p><i>Mouse chimeras—past and present</i></p> <p>Andrzej Tarkowski University of Warsaw, Poland</p> <p><i>Using transgenics to discover the in vivo source and migration of somatic stem cells</i></p> <p>Derek van der Kooy University of Toronto, Canada</p>		<p><i>The Transgenic Core and Specialty Resources at the Toronto Centre for Phenogenomics</i></p> <p>Marina Gertsenstein Toronto Centre for Phenogenomics, Toronto, Canada</p> <p><i>Running a transgenic unit</i></p> <p>Elizabeth Williams University of Queensland, Brisbane, Australia</p> <p>Roundtable discussions</p>
		12:30–13:30	Lunch
14:00–14:45	Coffee break	13:30–15:30	Session V: High-throughput production of mutant mice
14:45–15:45	<p>Session II: The ISTT Prize on Transgenic Technologies</p> <p><i>Presentation of the ISTT prize winner</i></p> <p>Lluis Montoliu National Centre of Biotechnology, Madrid, Spain</p> <p><i>Lung development and repair: insights from transgenic mice</i></p> <p>Brigid Hogan, ISTT prize winner Duke University, Durham, NC, USA</p>		<p><i>NorCOMM: High throughput mammalian functional analysis for the discovery of novel determinants of human disease</i></p> <p>Geoff Hicks Manitoba Institute of Cell Biology, Winnipeg, Canada</p> <p><i>Using ENU mutagenesis and high-throughput phenotyping methodologies to create mouse models of human disease</i></p> <p>Ann Flenniken Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, Canada</p> <p><i>Pleiades Promoter Project: new tools for promoter and expression analysis employing knock-ins at Hprt1</i></p> <p>Elizabeth Simpson University of British Columbia, Vancouver, Canada</p> <p>Selected poster—oral presentation</p>
15:45–17:00	Social get-together with snacks		
17:00–19:00	Poster session		
19:00	Free time to discover Toronto by night		
Tuesday, 28th October 2008			
8:00–9:00	Breakfast		
9:00–10:30	<p>Session III: Basic techniques</p> <p><i>Murine embryo transfer, vasectomy and ceasarean</i></p> <p>Elizabeth Williams University of Queensland, Brisbane, Australia</p> <p>ES cell culture and derivation</p> <p>Sagrario Ortega Spanish National Centre for Oncologic Research (CNIO). Madrid, Spain</p> <p>Pronuclear and blastocyst injection</p> <p>Johannes Wilberts Karolinska Institute, Stockholm, Sweden</p> <p>Artificial chromosome-type transgenes</p> <p>Lluis Montoliu National Centre of Biotechnology, Madrid, Spain</p>	15:30–16:00	Coffee break
		16:00–18:30	Session VI: Cutting edge transgenic technologies
10:30–11:00	Coffee Break		<p><i>Transgenic technology and stem cell research</i></p> <p>Robin Lovell-Badge National Institute for Medical Research, London, UK</p> <p><i>PDGF signaling controls multiple steroid producing lineages</i></p> <p>Philippe Soriano Fred Hutchinson Cancer Research Center, Seattle, USA</p> <p>Mount Sinai Hospital School of Medicine, New York, USA</p>
11:00–12:30	<p>Session IV: Running a transgenic unit</p> <p><i>The operation of the Gene Targeting and Transgenic Facility at HHMI, Janelia Farm Research Campus</i></p> <p>Caiying Guo Howard Hughes Medical Institute, Janelia Farm Research Campus, USA</p>		<p><i>Lineage specific differentiation of embryonic stem cells</i></p> <p>Gordon Keller McEwen Centre for Regenerative Medicine, Toronto, Canada</p> <p><i>Obtaining full control of transgene activation and inactivation</i></p>

Andras Nagy
Mount Sinai Hospital, Samuel Lunenfeld
Research Institute, Toronto, Canada

18:30–19:30 ISTT General Assembly
ISTT members

19:30–22:00 Conference Gala dinner

Wednesday, 29th October 2008

8:00–9:00 Breakfast

9:00–11:00 **Session VII: Phenotyping**

Imaging for mouse phenotyping

Mark Henkelman
The Hospital for Sick Children, Toronto,
Canada
Toronto Centre for Phenogenomics, Toronto,
Canada

Evaluation of cardiovascular phenotypes in mice

Lee Adamson
Mount Sinai Hospital, The Samuel Lunenfeld
Research Institute, Toronto, Canada

*The Toronto Centre for Phenogenomics:
Innovation in facility design, equipment,
technologies and research services to enable
the best transgenic science*

Colin McKerlie
Toronto Centre for Phenogenomics, Toronto,
Canada
Selected poster—oral presentation

11:00–11:30 Coffee Break

11:30–12:30 **Closing Session**
Introduction of TT2010
TBA
Poster award and closing remarks
TT2008 Organizers

12:30–13:30 Lunch

13:30–15:00 **TCA course introduction**
Tetraploid complementation assay—the theory
Andras Nagy
Mount Sinai Hospital, Samuel Lunenfeld
Research Institute, Toronto, Canada

16:00–18:00 **MGI bioinformatics workshop**
Only for specifically registered workshop
participants

Thursday and Friday, October 30–November 1, 2008

Full day **Practical Course in the tetraploid
complementation assay**
Only for specifically registered course
participants

TT2008 Invited Speaker Abstracts

1. Functional exploration of the mouse genome

Janet Rossant

Hospital for Sick Children, Toronto & the Department
of Molecular Genetics, University of Toronto

The tools of mouse genetics allow extremely sophisticated dissection of the function of individual genes and chromosomal regions. Still, for the average biologist, the expense, the long timelines and the technical complexities of making transgenic or targeted mutations make this approach daunting. However, this is changing as community resources of targeted or trapped mutant ES cells, targeting vectors and RNAi libraries come on-line. I will describe the current status of gene trapping resources with particular emphasis on the NorCOMM polyA trap resource. Generation of mice directly from ES cells is also having an impact on the speed and effectiveness of functional genomics. I will describe the results of a rapid screen for X-linked embryonic lethal mutations in the mouse using the gene trap resource and ES-derived embryos. Finally, standard transgenic methods using DNA injection into zygotes are not very efficient, making this unsuited for a genome-wide, high-throughput approach. Lentivirus infection of early embryos is very efficient and is less subject to the silencing of gene expression seen after standard retroviral infection. I will describe how lentiviruses can be used to efficiently achieve gene expression, conditional gene ablation and RNAi knock-down in specific lineages of the preimplantation embryo.

2. Gene expression during the mammalian oocyte to embryo transition

Mimi De Vries, Joel Graber, Barbara B. Knowles,
Davor Solter*

The Jackson Laboratory, Bar Harbor,
Maine and IMB-A*STAR, Singapore*

In normal development the nuclei of the gametes are reprogrammed to totipotency in the ooplasm during transcriptional quiescence. The protein products required to accomplish this task are controlled by protein and mRNA lability, and by translation of stored, stable maternal mRNA. This process follows a strict timetable that commences with oocyte maturation, continues through fertilization and ends with activation of the embryonic genome. In turn these processes are dependent on trans-acting proteins that interact with a set of cis-acting RNA sequences. Our laboratories are focused on identifying the control of this molecular process. By examining the 3' UTRs of maternal mRNAs we have identified transcripts with sequence motifs that regulate mRNA stability.

3. Mouse chimeras—past and present

Andrzej K. Tarkowski

Department of Embryology, Faculty of Biology,
University of Warsaw

This article describes the first attempts to produce chimeric mice that were undertaken half a century ago, and summarizes

the evolution of techniques and various sources (embryos, EC, ES cells) used for obtaining chimeric animals. It also describes other applications, such as interspecific chimeras, ‘rescuing’ diploid ↔ diploid chimeras, and diploid ↔ polyploid chimeras. Recently, we have used the aggregation technique to produce diploid ↔ triploid mice and to evaluate the developmental potential of single blastomeres from cleaving embryos by supporting them with diploid or tetraploid blastomeres. We present some of the results from these experiments.

4. Using transgenics to discover the *in vivo* source of somatic stem cells

D. Van der Kooy, S. Smuckler

University of Toronto, Canada

The search for putative precursor cells within the pancreas has been the focus of extensive research. Adult mouse Pancreas-derived Multipotent Precursor (PMP) cells, possessing the intriguing capacity to generate cross-germ layer progeny in the pancreatic and neural lineages, have been identified. Here, genetic lineage-labelling experiments excluded the neural crest as the developmental source of PMPs. It is also demonstrated that the PMP cell expresses insulin *in vivo*, providing reconciliation with reports that new adult cells are formed exclusively by self-replication, and shown that insulin + cells contribute to multiple pancreatic and neural cell populations *in vivo*. Further, PMP cells were shown to exist within adult human islet tissue, each capable of extensive proliferation, self-renewal, and generation of multiple differentiated pancreatic and neural cell types. Finally, the newly generated human cell progeny were found to display regulated insulin secretion. These findings demonstrate that the adult mammalian pancreas contains a population of insulin + multipotent stem cells, capable of contributing to the neural and pancreatic lineages.

5. Lung development and repair: insights from transgenic mice

Brigid L. M. Hogan

Department of Cell Biology, Duke University Medical Center, Durham, NC 27705

The lung is a complex and dynamic organ, designed to function in gas exchange throughout life. It is made up of four interdependent branched systems—the airways and alveoli, the pulmonary vasculature, the lymphatics, and nerves. A major challenge for developmental biologists is to understand how the growth and differentiation of these different components are co-ordinated during embryogenesis. The lung initially forms as two small buds composed of endodermal epithelium surrounded by a jacket of undifferentiated mesenchyme. As development proceeds the buds grow out and undergo a process known as branching morphogenesis, giving rise to more and more, progressively narrower branches and terminal buds. Thanks to the comparative analysis of normal and transgenic and mutant mice, and we now know a great deal about the fundamental mechanisms driving the branching morphogenesis of the lung, and how these compare with similar mechanisms essential for the development of other branched

organ systems such as the kidney and salivary gland. We are also beginning to understand how the growth and development of the vasculature of the lung is co-ordinated with the branching of the endoderm. Unexpectedly, recent studies from our laboratory have shown that the development of the pulmonary vasculature involves a previously underappreciated population of cells—the mesothelium—covering the surface of the embryonic lung. Lineage labeling experiments show that mesothelial cells on the surface of the lung undergo epithelial–mesenchymal transition and move deep into the interior of the lung and contribute to the smooth muscle cell layer around the developing blood vessels. Again, transgenic mice have played an essential role in uncovering this process, which is also seen in other organs within the coelomic cavity, including the heart and gut. Once it has formed and the newborn animal has taken its first breath, the lung has to keep functioning throughout life. Unlike other organs such as the stomach and intestine, where the turnover of the lining epithelial cells is very rapid, the epithelial cells of the normal lung do not proliferate very actively and the whole organ is relatively quiescent. However, if epithelial cells are killed, for example by certain toxic agents in the air, then the surviving cells can leap into action, proliferate rapidly and restore the damaged layer. We are using a series of transgenic mice to understand how this repair process is carried out and what cells can function as epithelial stem cells in the adult lung. In conclusion, transgenic and gene targeted mice have given powerful insights into processes such as the organogenesis of the lung, and they continue to be essential for our further understanding of disease and repair.

6. Murine embryo transfer, vasectomy and caesarean

Elizabeth Williams

Transgenic Animal Service of Queensland (TASQ),
University of Queensland

With the ever increasing demand for Murine mouse models by the scientific community, transgenic facilities are faced with the constant pressure of producing transgenic and knock-out lines. Once these lines are established and used in research projects, they may be cryopreserved or shipped around the world to and for collaboration within the research community. Animal houses also are faced with the constant threat of pathogen outbreaks, with the resuscitation into a pathogen free vivarium, the only solution to keeping the strain and research projects viable.

The most fundamental principle to any transgenic/knock-out production or rederivation project is the ability to successfully transfer a viable embryo/fetus to a pseudopregnant/foster mother. No matter how efficient a microinjection session may be; how high the percent of fertilization for IVF is or how excellent the survival rate for an embryo thaw is, if the resulting embryo/fetus does not result in a live birth, then all the work has been done to no avail.

In this talk I will briefly cover the basic techniques/protocols for producing vasectomised males (scrotal vs. abdominal), embryos transfer (0.5 vs. 2.5 dpc; unilateral vs. bilateral), and caesareans. I will then examine the rationale and success in the use of these two protocols for rederivation projects. The main focus of my talk will be on discussing the technical variations that have developed over time within each of these procedures and their rationale behind the divergence. To conclude I will compare success or lack of, for each these variations.

7. ES cell culture and derivation

Carmen Gómez, Verónica Domínguez, Javier Martín, Marta Riffo, Jaime Muñoz, Sagarrio Ortega

Transgenic Mice Unit, Biotechnology Program, Spanish National Centre for Oncologic Research (CNIO), Madrid, Spain

Embryonic stem (ES) cells derive from a transient group of cells that constitute the epiblast in the preimplantation embryo. This group of cells contains the founders of all the cellular lineages in the embryo contributing also to part of the extra embryonic structures. Murine ES cell lines were first established from mouse blastocysts in 1981. In the last 20 years, they have been extensively used as a tool to introduce targeted mutations in the germ line of the mouse since these cells, kept in the proper culture conditions, behave as permanent lines that retain the pluripotential character of their embryonic counterparts, being able to contribute to all cell lineages when re-introduced into a host embryo (chimera formation). Traditionally mouse ES cells have been derived and cultured in DMEM medium containing fetal calf serum (FCS) and LIF (leukemia inhibitory factor), with or without feeder cells, to preserve their characteristics of pluripotency and self-renewal and to prevent differentiation. The variability in the quality and composition among different batches of FCS is a critical parameter that affects the success rate in the culture and derivation of mouse ES cells. Progress in the understanding of ES cell biology has more recently led to the development of alternative culture conditions for ES cells, in which FCS is replaced by defined media supplements that support ES cell growth and prevent differentiation with the same efficiency or better than FCS, and are less susceptible to batch to batch variations. We have used defined serum-free media to establish ES cells from different genetically modified mouse lines, with good efficiencies. The advantages and disadvantages of the use of serum-free conditions will be discussed. For many years the only known source of ES cells has been the preimplantation embryo. However, the striking finding that a somatic differentiated cell, from both mouse and human origin, can be reprogrammed in vitro by retroviral expression of a reduced (no more than four) number of genes to become a cell that shares all the phenotypic characteristics of a *bona fide* ES cell (pluripotency, self-renewal and chimera formation) has revolutionised the way we think about ES cell derivation, bypassing the embryo as the unique source of pluripotent ES cells, and opens exciting new venues in stem cell biology, genome reprogramming and therapeutics.

8. Artificial chromosome-type transgenes

Lluís Montoliu^{1,2}

Centro Nacional de Biotecnología (CNB-CSIC)

¹Department of Molecular and Cellular Biology, Campus de Cantoblanco, Darwin 3, 28049 Madrid, Spain; ²Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain

The proper design of transgenes has been always compromised by the amount of available information on the genomic locus whose expression patterns was to be used to drive the expression of the

transgenic construct. In particular, in those cases whose expression domain has not been completely documented or studied, their inclusion in transgenes usually results in an unpredictable expression pattern and suboptimal performance in transgenic animals. Using genomic comparative approaches highlights evolutionary conserved homologous sequences in 5' and 3' neighbouring regions that are normally associated with crucial regulatory elements most relevant for the proper behaviour of the expression domain. Therefore, the inclusion of these conserved regulatory elements in transgenic constructs would result in optimal expression levels of transgenes in genetically modified animals. In order to achieve the inclusion of these important regulatory elements, that can be located far away from the body of the gene, transgenic constructs can be prepared using artificial-chromosome type vectors (bacterial artificial chromosomes, BACs; yeast artificial chromosomes, YACs; or, P1-derived artificial chromosomes, PACs). The size of their genomic inserts (ranging from 100 to 1,000 kb, depending on the vector type) usually ensures the inclusion of the evolutionary preserved regulatory elements that are required for the faithful expression of the gene. Further, both BACs/PACs and YACs can be efficiently modified at will using homologous recombination techniques operating in bacterial or yeast cells, respectively. There are many types of regulatory elements that can modify the expression pattern of a locus. Among them, the insulators or boundaries are most relevant since they are in charge of shielding the position effects and any type of external transcriptional interferences while maintaining the proper endogenous expression pattern of the locus. Therefore, the generation of transgenic animals with artificial chromosome-type (genomic-type) constructs is the recommended approach in order to achieve optimal transgene expression, in agreement with the expected pattern of the corresponding endogenous locus.

9. The operation of the Gene Targeting and Transgenic Facility at HHMI, Janelia Farm Research Campus

Caiying Guo

Gene Targeting and Transgenic Facility at HHMI, Janelia Farm Research Campus, Ashburn, VA

The Gene Targeting and Transgenic Facility at HHMI, Janelia Farm Research Campus was established recently. The facility contains a molecular lab for making gene targeting constructs, a cell culture room for ES cell manipulation, a procedure room for generation of genetically modified animals located in a barrier area. The facility has started with three technicians: one makes gene targeting constructs, one manipulates ES cells and one for transgenics. The capacity of the facility is projected as 30 complete gene targeting projects per year and other projects as requested, such as DNA pronuclear microinjections, cryopreservation, IVF, rederivation and derivation of new ES cell lines. We are using a Filemaker database to manage the facility. The functions of the software include: recording the detail information of all the procedures, mouse colony management, management of lab and IACUC protocol, and cage counting and billing.

The users are exclusively the scientists at JFRC who are on their missions to take on some challenging projects in neuroscience. Therefore most of the targeting projects they have requested

are more complicated than conventional or conditional knockouts. The fees for a gene targeting project, from construct to F1 mice, range from \$16,000 to \$19,000.

In the past 2 months we have completed six gene targeting constructs and are ready to start ES cell targeting. We expect a fully functional facility within 6 months.

10. The Transgenic Core and Specialty Resources at TCP

Marina Gertsenstein

Toronto Centre for Phenogenomics, Toronto, Canada

Toronto Centre for Phenogenomics (TCP) is a collaboration of four founding Member Research Hospitals: Mount Sinai Hospital, The Hospital for Sick Children, University Health Network and St. Michael's Hospital. TCP opened its doors in October 2007 in Toronto's Discovery District. TCP Transgenic Core and Specialty Resources is an amalgamation of Transgenic Facilities of Mount Sinai Hospital and the Hospital for Sick Children. These facilities have pioneered such technologies as production of chimeras by aggregation method as well the Tetraploid Complementation Assay (TCA) developed by Dr. Andras Nagy. TCA course following the conference will take place in TCP Transgenic Core Laboratory. TCP Transgenic Core provides services to investigators of the four member hospitals, the University of Toronto and external academic, not-for-profit and for-profit institutions. The Core facility structure, workflow and services will be described during the session 'Running a Transgenic Unit'..

11. Running a transgenic unit

Elizabeth Williams

Transgenic Animal Service of Queensland (TASQ),
University of Queensland

The Transgenic Animal Service of Queensland (TASQ) was established in 1999 as a joint venture between the Faculty of Science (BACS) and the Institute for Molecular Bioscience (IMB) to provide researchers at The University of Queensland with the latest in transgenic/knock-out and associated technologies. TASQ is run as a cost-recovery service. TASQ services are also offered to non-University of Queensland research groups.

Services first offered to researchers were the production of transgenics and knock-outs via PN injection and morula aggregation, respectively. As the need for additional services increased TASQ soon offered ES cell injection into blastocyst and embryo freezing.

The full list of services now available to researchers includes: transgenic production; knock-out production; embryo and sperm freezing; colony management; genotyping (either or all-tissue sampling, DNA isolation and PCR); IVF; rederivation of mouse strains; training; mouse/frozen gamete importation and exportation; AEC and grant reviewing; microinjection and embryology training and project consultations.

12. NorCOMM: high throughput mammalian functional analysis for the discovery of novel determinants of human disease

Geoffrey G. Hicks¹, Carolyn Ashley¹, Tania M. Bubela², Edna F. Einsiedel³, Colin McKerlie⁴, Lauryl Nutter⁴, Andras Nagy⁵, Derrick E. Rancourt³, William L. Stanford⁶, Janet Rossant⁴

¹Manitoba Institute of Cell Biology, Winnipeg, Canada;

²University of Alberta, Edmonton, Canada; ³University of Calgary, Canada; ⁴Toronto Centre for Phenogenomics, Toronto, Canada;

⁵Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada;

⁶University of Toronto, Canada; ⁷Hospital for Sick Children, Toronto, Canada

NorCOMM (North American Conditional Mouse Mutagenesis project) NorCOMM is a large-scale research initiative to develop and distribute a resource of mouse embryonic stem (ES) cell lines carrying single conditional knockout mutations across the mouse genome. We are creating a publicly accessible library of ES cells suitable for drug discovery, target discovery and validation, and investigating mouse models of human diseases. Development of the knockout mouse resource itself is the major activity using a combination of high throughput random gene trap mutagenesis and systematic high-throughput gene targeting. NorCOMM vectors are designed to generate expression and functional information for the gene targeted in each mutant ES clone. To ensure the widest utility of the NorCOMM mouse knockout resource, we have developed a universal docking site strategy with a tool box of exchangeable cassettes. This design will ensure that each mutation can be used to create a conditional knockout allele and a variety of functional replacement alleles. Since the project began on April 1, 2006 NorCOMM's three major gene trap centres have contributed more than 50,000 mutant ES cell lines to the resource. This large and growing archive of ES cells is publicly available on a cost-recovery basis and access to clones is unrestricted and nonexclusive. Complementing the trapping approach, over the next 2 years NorCOMM will create up to 2,000 targeted cell lines by specifically targeting genes not mutated in the gene trap resource. Many of the genes targeted will be those specifically requested by Canadian academic researchers and biotechnology companies. NorCOMM continues to invite input from the scientific community on which genes would have the greatest impact if a knockout mouse ES cell line were available. We have established an online Gene Submission Form on our website www.NorCOMM.org. Researchers can request genes of interest and the information provided becomes part of the NorCOMM gene targeting prioritization process. Submissions through this form are confidential and secure, Resource archiving and distribution of all mutant ES cells, vectors and other associated resources generated by NorCOMM are available to the scientific community at large through the Canadian Mouse Mutant Repository. NorCOMM is also exploring the economic impact of Public-Private sector partnerships and the implications of the intellectual property and public databases generated by the NorCOMM project.

NorCOMM is founding member of International Knockout Mouse Consortium and its partner projects EuCOMM, KOMP and TIGM. Together, the projects hope to accomplish complete coverage of the mouse genome.

NorCOMM is a Genome Canada—Genome Prairie project.

13. Using ENU mutagenesis and high-throughput phenotyping methodologies to create mouse models of human disease

Ann M. Flenniken

Centre for Modeling Human Disease, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada

The aim of the Centre for Modeling Human Disease is to discover novel genes and mutations relevant to human disease processes and biological pathways.

Mutant mice are created by injection of male mice with the potent chemical mutagen, ethylnitrosourea (ENU), which randomly introduces point mutations in their spermatogonial stem cells, so that on average, new point mutations in any gene can be induced in one out of every 500–1,000 offspring.

The offspring of the mutagenized males are subjected to high-throughput screening protocols to identify clinically relevant phenotypes in all major organ systems. We screen for in-life defects in: morphology and general behaviour (external defects, vision, hearing); cardiac function (ECG, heart rate, blood pressure, blood flow velocity); bone and soft tissue composition and skeletal structure; hematological profile (quantity of RBC, WBC, and platelets); renal function (urinalysis); and neurobehavioural/neurophysiological function (learning and memory, motor coordination); complemented by post-mortem histology and pathology analysis.

Using this methodology we have been successful in generating a wide range of exciting new mouse models for human conditions including oculodentodigital dysplasia, polydactyly, osteogenesis imperfecta, osteopetrosis, osteoporosis, aortic valve stenosis, cardiac arrhythmia, thrombocytopenia, thrombocytopenia, polycythemia, glomerulocystic kidney disease, ataxia, and alopecia.

14. Pleiades Promoter Project: new tools for promoter and expression analysis employing knock-ins at *Hprt1*

E. M. Simpson^{1,2,3}, K. G. Banks¹, R. J. Bonaguro¹, C. N. De Leeuw^{1,2}, J.-F. Schmouth^{1,2}, D. J. Swanson¹, G. S. Yang⁴, M. Amirabasi¹, N. Babyak¹, S. F. Black¹, T. Candido¹, J. Chen¹, Y. Chen¹, L. Dreolini⁴, G. Wilson⁴, K. Hatakka¹, T. Hearty¹, S. Khorasan-Zadeh¹, I. Komljenovic¹, S. Laprise¹, F. Liu¹, L. Liu⁵, J. Mis¹, B. Palma¹, J. L. Turner¹, S. H. Wong¹, A. R. Ypsilanti¹, S. J. Jones^{2,4}, W. W. Wasserman^{1,2}, D. Goldowitz^{1,2,5}, R. A. Holt^{3,4}

¹Centre for Molecular Medicine and Therapeutics, Child & Family Research Institute, University of British Columbia, Vancouver, BC, Canada, V5Z 4H4; ²Department of Medical Genetics, University of British Columbia, Vancouver, Canada; ³Department of Psychiatry, University of British Columbia, Vancouver, Canada; ⁴Canada's Michael Smith Genome Sciences Centre, British Columbia Cancer Agency, Vancouver, BC, Canada, V5S 4Z6; ⁵Department of Anatomy and Neurobiology, The University of Tennessee Health Science Center, Memphis, TN 38163, USA

The Pleiades Promoter Project is working towards the generation of 160 new knock-in mouse strains with defined brain-

region and cell-type gene expression patterns. These strains will be valuable tools for a wide variety of brain research applications, and especially in the development of gene therapy approaches for Alzheimer, Parkinson, Huntington, depression, autism and brain cancer. We employ a site specific single copy knock-in approach at the *Hprt1* locus to deliver small promoter sequences that will drive gene expression in defined brain regions of therapeutic interest. We maintain a high throughput mouse production pipeline resulting in the weekly production of over 40 targeted Embryonic Stem Cell (ESC) lines, the microinjection of approximately 400 blastocysts, to produce four new germline mouse strains per week for extensive neurophenotyping analysis; all of which requires over 2,000 mouse cages that house over 10,000 mice. Presently, the Pleiades Promoter Project has generated 38 new germline mouse strains, and imaging data is being compiled into a neuroimage database which is taking form at www.pleiades.org. We have specifically designed delivery vectors with reporter genes EGFP or EGFP-Cre and assays for the *Hprt1* loci. We have generated a number of new untargeted ESC lines with a high germline competency, as well as a comprehensive analysis of different strategies for successful chimeric mice production. We have applied a range of different techniques including blastocyst microinjection, morula microinjection, co-culture and tetraploid complementation; along with a number of different genetic backgrounds of host embryos to determine the most efficient method for generating large numbers of chimeric offspring able to pass the modified ESC lines efficiently through their germline. The Pleiades Promoter Project is making all of this information and related tools publicly available to promote research on brain development and disorders.

15. Transgenic technology and stem cell research

Robin Lovell-Badge

Division of Stem Cell Biology and Developmental Genetics, MRC National Institute for Medical Research

Transgenic technology is central to our ability to understand the biology of stem cells and their niches in mice. We have used targeted null and conditional mutations and conditional gain of function transgenes to study the role of *Sox1* genes, notably *Sox2* and *Sox3*, and the *SoxE* genes, *Sox9* and *Sox10*, in stem cell populations in the developing and adult CNS and pituitary. Standard reporter genes have been used to identify stem cells populations and for short-term lineage analysis while cell culture systems and conditional reporters *in vivo* are used to trace the longer-term fate of cells derived from these stem cell populations. We have also made use of direct electroporation of DNA constructs into both mouse and chick embryos as a more rapid test of gene function. Using these methods we have shown that SOX2 and SOX9 are required together to establish and maintain the potential of stem cells in the developing CNS to generate both neuronal and glial cell types. Similar findings have been made in the pituitary, although in this case both genes are required in transit amplifying cells that have the ability to give all hormone producing cell types in the adult gland, whereas largely quiescent stem cells are SOX2-positive, SOX9-negative.

Expression of SOX2 and SOX9 themselves or of reporters driven by their gene regulatory sequences can help define the precise location of the stem cells with respect to their niches in vivo, and can be used to monitor the effects of manipulations designed to mobilise the stem cell populations; for example, the induction of a stroke or altering the demand for a specific pituitary hormone. Recent progress on this work will be presented. In addition, approaches involving conditional and inducible ‘cell death’ genes will be discussed. These are being used to ask what are the consequences of loss of specific stem cells or their derivatives, both to activity within the niche and to the phenotype of the animal. Finally, we are also using transgenic technology to explore the roles of *Sox2* and *Sox9* as ‘stem cell genes’ in the biology of specific cancers.

16. PDGF signaling controls multiple steroid producing lineages

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The PDGF signaling pathway is known to regulate numerous lineages of mesenchymal cell origin during development and in the adult, but the targets of this pathway in specific tissues still need to be identified. Recently, 11 genes identified as PDGF transcriptional targets were mutated and shown to have essential roles in multiple PDGF dependent tissues. Mutations in five different PDGF target genes lead to male and/or female sterility, in some cases depending on a *Pdgfra*^{+/-} background. All infertile mutations of PDGF target genes resulted in reduced hormone production and defects in the steroid producing cells of the testis and/or ovary, suggesting that the PDGF pathway plays an important role in these cells in both sexes. Conditional mutations of both PDGF receptors in steroidogenic cells indicated an important role for this pathway in the development of these cells in the testis and ovary as well as in the adrenal cortex, a major site of steroid hormone production. Thus the PDGF pathway and its targets are required for the normal development of steroidogenic cells in different organs, and may constitute a common mechanism in the control of steroidogenic lineages.

17. Lineage specific differentiation of embryonic stem cells

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The hematopoietic and cardiovascular lineages are derived from mesoderm, one of the primary germ layers formed during gastrulation. Lineage tracing studies in the mouse have

provided evidence suggesting that subpopulations of mesoderm are induced in the gastrulating embryo in a defined temporal pattern with hematopoietic mesoderm preceding the formation of cardiac mesoderm. Studies with the mouse embryonic stem cell (mESC) differentiation system showed that lineage commitment follows a similar pattern in this in vitro model as hemangioblasts, representing the earliest hematopoietic progenitors develop before cardiovascular progenitors. The hemangioblasts and cardiovascular progenitors both express the VEGF receptor Flk-1+ and are multipotent, displaying the capacity to generate either hematopoietic or cardiac progeny together with cells of the endothelial and vascular smooth muscle lineages. To determine if the human hematopoietic and cardiac lineages also develop from multipotent progenitors, we analyzed human embryonic stem cell (hESC)-derived embryoid bodies (EBs) for the presence of comparable populations. When differentiated under conditions optimized to promote mesoderm development, hESCs generate a KDR+ (Flk-1+) population within 4 days of differentiation. Analysis of this population revealed that it contains hemangioblasts that are able to generate both hematopoietic and vascular progeny. By day 6 of differentiation, a second KDR+ population appeared within the hESC-derived EBs. In contrast to the earlier KDR+ cells, this day 6 population displayed robust cardiovascular potential, but showed little capacity to generate hematopoietic cells. Clonal analysis revealed that this population contains cardiovascular progenitors able to generate cardiomyocytes, endothelial cells and vascular smooth muscle cells. Following 8 days of culture on a gelatinized substrate in serum-free media, the day 6 KDR+ cells differentiate to generate a population consisting of greater than 50% cardiomyocytes. Taken together these studies demonstrate that differentiation in hESC cultures parallels that observed in the mouse cultures and accurately reflects the patterns of lineage development in the early embryo.

18. Obtaining full control of (trans)gene activation and inactivation

Andras Nagy

Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Canada

The ability to modify the mouse genome by utilizing ES cell-based technologies has had an unprecedented impact on our understanding of developmental and disease processes. However, the random nature of transgene insertions as well as the “fixed structure” of targeted or gene trap mutations, hinders us from utilizing the full potential of these genetic approaches.

Here I present a generalized system based on novel integrases and the well known Flp recombinase to obtain a clean replacement of a specifically designed genomic site (docking site) with any desired transgenic element. A combination of positive and negative selection systems assist the replacement procedure, eliminating cells which have not undergone the designed genomic insertion. Furthermore, we are combining this technology with the tetracycline inducible system to obtain an ultimate specific and temporal control of

gene expression. Cre, the most powerful recombinase, was intentionally left out from these genetic tools to allow additional utilization of this enzyme. Different combinations of these novel tools provide us with a new arsenal of sophisticated genetic approaches to facilitate the functional annotation of the mouse genome.

19. Imaging for mouse phenotyping

R. Mark Henkelman

Mouse Imaging Centre (MICe), Hospital for Sick Children, University of Toronto

Imaging has a major role to play in understanding the relationships between genotype and phenotype. As in clinical imaging, a number of imaging techniques need to be used in concert to obtain optimal information. Furthermore in mouse imaging, sophisticated computer techniques are also needed to obtain statistically meaningful information.

This talk will describe MR, Ultrasound, CT, and OPT at the Mouse Imaging Centre (MICe) in Toronto. It will illustrate the use of these imaging modalities for phenotyping with a number of examples drawn from models of human disease and developmental biology.

20. Evaluation of cardiovascular phenotypes in mice

S. Lee Adamson

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Our lab uses *in vivo* and post mortem methods to evaluate cardiovascular phenotypes during pregnancy in mice. Echocardiography using micro-ultrasound is used to evaluate cardiac function of the mother during pregnancy, and throughout prenatal and postnatal development in the offspring. An *in vivo* indication of the vascularity of the placenta is obtained using Doppler ultrasound to examine the pulsatility of the umbilical and uterine arterial blood velocity waveforms. We used these methods to evaluate cardiovascular changes in the mother and in the placenta during pregnancy in normal and mutant mice. More detailed information on the vascularity of organs can be obtained by filling vessels with methylmethacrylate then digesting tissue to obtain corrosion casts of the vasculature. Scanning electron microscopy can then be used to obtain a 3-D visualization of the vascularity of the organ. An exciting recent development is the use of X-ray opaque silicone to fill the vasculature followed by imaging using micro-computed tomography. Dr. John Sled (Mouse Imaging Centre, Toronto) has generated 3-D images of vessels down to $\sim 50 \mu\text{m}$ and has developed software to obtain quantitative structural information on vascular branching patterns. We have collaborated to show significant changes in branching patterns in the placenta during development and between strains in the placenta. In summary, *in vivo* and post mortem methods are available to permit detailed evaluation of

cardiovascular phenotypes in mice at all stages of development. Funding from the Canadian Institutes of Health Research, and the Heart and Stroke Foundation of Ontario is gratefully acknowledged.

21. The Toronto Centre for Phenogenomics: innovation in facility design, equipment, technologies and research services to enable the best Transgenic science

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Background Systematic identification of the function of all genes in the mammalian genome is one of the major scientific challenges for the 21st century and the mouse is the model system of choice. Large, state-of-the-art, and comprehensive facilities that are designed and equipped with the latest innovations in facility equipment and are programmed to provide the widest range of research-enabling expertise and services are required to accomplish this task. **Materials and methods** The Toronto Centre for Phenogenomics (TCP) is an innovative, scientific collaboration between four major research hospitals to operate a centralized, research-enabling mouse facility. The Centre differentiates itself in mouse-based research and discovery in two fundamental ways. First, the TCP with capacity for 36,000 individually vented cages is the largest Centre of its kind in Canada. The building is approximately 12,000 gross sq metres of space, supported by robotic cage processing and ergonomic equipment innovations for cage change management. Secondly, the TCP is functionally programmed to pool scientific expertise and resources under one-roof so the Centre can enable the latest science supported by state-of-the-art infrastructure. The TCP has been designed and constructed specifically to conduct and support genetic research involving generation of mutant mice, physiological and cardiovascular phenotyping, neurobiology and behavioral analysis, imaging, pathology, and cryopreservation for storage and distribution. Three hospital-based research programs are located at the TCP including the Mouse Imaging Centre (MICe), the mutagenesis and high-throughput phenotyping program of the Centre for Modeling Human Disease (CMHD) that supports a comprehensive phenotyping and gene-discovery platform and has demonstrated success by generating new models of human syndromes such as glomerulosclerosis, osteoporosis, platelet disease, and alopecia. Finally, the Canadian Mouse Mutant Repository (CMMR) provides cryopreservation, *in vitro* fertilization, and multiple format tissue archiving. Examples of representative data and research technologies will be presented. **Results and Conclusions** The effort to advance the genome from a simple sequence-based annotation to one that identifies and characterizes gene function is underway. Innovation in facility design, equipment, technologies, and research services at the TCP are required to enable investigators in Toronto and their collaborators to contribute effectively to this international initiative.

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22. Next generation tools for high-throughput promoter and expression analysis employing single-copy knock-ins at the *Hprt1* locus

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We have engineered a comprehensive set of tools that facilitate targeted single copy knock-in (KI) at the hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) locus. We employed fine scale mapping to delineate the precise breakpoint location at the *Hprt1b-m3* locus allowing allele specific PCR assays to be established. Our suite of tools contains four targeting expression vectors and a complementing series of embryonic stem cell lines. Two of these vectors encode enhanced green fluorescent protein (EGFP) driven by the human cytomegalovirus immediate-early enhancer/modified chicken beta-actin (CAG) promoter, whereas the other two permit flexible combinations of a chosen promoter combined with a reporter and/or gene of choice. We have validated our tools as part of the Pleiades Promoter Project (<http://www.pleiades.org>), with the generation of brain-specific EGFP positive germline mouse strains.

(* = Authors contributed equally to this work)

23. Expression pattern and function analysis of Emilin genes in zebrafish

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Emilins constitute a family of glycoproteins of the extracellular matrix with common structural organization and containing a peculiar N-terminal cysteine-rich domain. The prototype of this family, Emilin-1, is found in human and murine organs in association with elastic fibers, and four other emilins were recently described in mammals (Braghetta et al. 2002, 2004). The function of all the members of the family is under investigation as knockout mice for these genes have been produced in our lab. At the moment only the phenotype of Emilin-1 deficient mice have been partially characterized (Zacchigna et al. 2006). Considering the complex patterns of expression of the emilins in mammals and the resulting possible

difficulties in unravelling the phenotype of the mutated mice, we decided to study the distribution and the function of these proteins in lower vertebrates and we chose the fish *Danio rerio* as animal model.

Using sequence similarity tools, we identified eight members of this family in zebrafish. Each emilin gene has two paralogs in zebrafish, showing conserved structure and syntenicity with the human ortholog. Whole-mount *in situ* hybridization revealed that expression of zebrafish emilin genes is regulated in a spatio-temporal manner during embryonic development, with overlapping and site-specific patterns mostly including mesenchymal structure (Milanetto et al. 2008).

Among all, the most distinctive pattern is the one of Emilin-3, the only component of the family lacking the C-terminal gC1q domain, which is expressed at early stages in the notochord and in the floor plate, indicating that Emilin-3 may play key roles during early development. To understand the function *in vivo*, we started functional studies in zebrafish. Emilin-3 expression does not appear to be regulated by either Hedgehog, FGF or Notch signalling, as indicated by ISH of embryos in which these molecular pathways were blocked by drug treatments. Additionally, expression of the two Emilin-3 genes appears normal in embryos mutant for Chordin (*dino*) or for BMP2 (*swirl*).

The microinjection of mRNA or morpholino oligonucleotides in fertilized oocytes has led to preliminary observations which suggest that *myoD* expression is increased in embryos overexpressing Emilin-3, while morphant embryos with down-regulated Emilin-3 mRNAs have an apparently altered development of somites.

1. Braghetta P et al (2002) *Matrix Biol* 21:603–609
2. Braghetta P et al (2004) *Matrix Biol* 22:549–556
3. Zacchigna L et al (2006) *Cell* 124:929–942
4. Milanetto M et al (2008) *Dev Dyn* 237(1):222–232

24. Isolation and characterization of porcine Gal KO fibroblasts expressing hCD55-hCD39 and hEPCR-hTPA

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Xenotransplantation will benefit from genetic modification of the pig genome to reduce immunogenicity; the first obstacle in

pig to human xenotransplantation was represented by hyperacute rejection (HAR), which has been overcome by genetic ablation of $\alpha 1,3$ galactosyltransferase and by expression of the hDAF. The second obstacle is represented by acute vascular rejection (AVR) that is characterised by vascular thrombosis, blood extravasation and edema. Expression of molecules involved in the coagulation cascade (ePCR and tPA) and in inflammatory-apoptotic events (CD39) are potential strategies to bypass AVR. The aim of this work is the production of transgenic cell lines to use in SCNT for the production of pigs for xenotransplantation studies.

Two neonatal pig Gal KO fibroblasts lines cultured in DMEM/M199 1:1 + 10% FCS + 5 ng/ml bFGF were co-transfected by nucleofection with ubiquitous expression vectors pMG5'3'/MARHyg2272-CXhDAF and pCXhCD39-3'/MAR or pMG5'3'/MARPuro5171-CXhEPCR and pJC131-CXhTPA.

After nucleofection, cells were plated in Petri dishes and selected for 8 days with Hygromycin: 150 μ g/ml or Puromycin: 1 μ g/ml. Drug resistant colonies were isolated and expanded for transgene expression analysis. We used immunohistochemistry (IHC) to detect the expression of the protein. For hDAF we used IA10 (BD Pharmingen) and for hCD39 BU61 (Ancell). For hEPCR and hTPA we screened a battery of antibodies without detecting a clear specific signal. Therefore we used RT-PCR to detect the presence of the transcripts. Cells from DAF-CD39 colonies were serum starved for 24 h before being fused to enucleated oocytes. Following electric activation, embryos were grown *in vitro* to the blastocyst stage.

Thirty-nine colonies derived from a cell line transfected with hDAF-hCD39 were analysed by IHC: 48.7% of the colonies expressed both hCD55-hCD39; 28.2% expressed only CD55; 15.3% expressed only CD39; 5.1% showed mosaic expression. IHC findings were confirmed also by RT PCR.

We analysed nine selected colonies from hEPCR-hTPA by RT PCR; six colonies (66.6%) showed the presence of both transcripts, two colonies (22.2%) showed only TPA transcript. Gal KO cell colonies co-expressing DAF-CD39 were used in SCNT experiments obtaining 32.4% compacted blastocyst development ($n = 1,446$). We obtained 40.7% ($n = 2,583$) blastocyst development using only Gal KO cells.

In this experiment was demonstrated that this system is very efficient to produce DAF-CD39 cloned pig blastocysts using GAL KO cells. Moreover, Gal KO TPA/EPCR cell lines were established for the first time.

This study was supported by EU grant no. LSHB-CT-2006-037377 and Fondazione Banca Popolare di Cremona.

25. Distinguishing between homozygous and hemizygous transgenic mice

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Usually, the phenotype of a given transgenic mouse line is established by analysing hemizygous transgenic individuals.

These are mice carrying one copy of the transgene, or the transgene tandem, inserted somewhere in the mouse genome. Some of these transgenic mouse lines might become very useful and therefore are kept alive in the animal house and constantly bred to generate young individuals that are used and analysed. As outlined, one of the major drawbacks of this strategy is the genotyping requirement for each litter being produced, since crosses are normally established between transgenic hemizygous animals with its corresponding wild-type partners. Assuming a stable Mendelian pattern of inheritance, about half of individuals from each litter will eventually be detected as hemizygous transgenic mice.

An alternative approach to consider for some transgenic mouse lines is the generation of the corresponding homozygous line, with several individuals, so that they can be bred among themselves and be maintained without the need of systematic genotyping efforts. In this case, all animals born from each litter will indeed be homozygous transgenic mice. For this purpose, it should be possible to differentiate hemizygous and homozygous using a suitable and reproducible molecular biology method.

We have compared the results of three different experimental approaches to assess the identification of hemizygous and homozygous individuals in selected transgenic mouse lines. The three methods that have been considered are: (1) Southern blot analysis using transgene-internal and unrelated-external probes corresponding to a single copy endogenous locus, followed by quantification and comparison of individual ratios of the hybridising signal at both locations, (2) a similar strategy using dot blot analysis, in which dot blot replicates are hybridized with the transgene-internal and unrelated-external probes, and (3) using SYBR Green real-time quantitative PCR, as described by Hauroné et al. (2007) *Transgenic Research* 16: 127–131.

The results obtained will be presented and discussed, highlighting the advantages and disadvantages of each experimental approach.

26. Pleiades Promoter Project: optimizing the generation of knock-in mice by blastocyst injection

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The main objective of the Pleiades Promoter Project (www.pleiades.org) is to generate a set of tools, including 205 new strains of knock-in mice, that will allow for brain region and cell-specific gene delivery therapies, which may aid in the treatment of brain associated diseases such as Alzheimer, Parkinson, Huntington, Amyotrophic lateral sclerosis, Multiple sclerosis, Spinocerebellar ataxia, depression, autism, and cancer. Injections of Embryonic Stem (ES) cells into blastocyst hosts have proved to be the most successful mode of generating transgenic mice to date. Here, we address the

optimization of techniques required for the production of chimeric mice.

We evaluated the rate of chimerism in chimeras produced from four different genetic backgrounds; C57BL/6J (abbreviated here B6, JAX Stock#000664), B6(Cg)-Tyrc-2/JJ (abbreviated here B6-Alb, JAX Stock#000058), CrI:CD1 (ICR) (abbreviated here ICR, Charles River, Strain Cod 022), and ICRBAF1 (first generation from ICR crossed to B6-Alb). Direct comparisons between the strains were performed using a one-way ANOVA test. The data revealed that B6 and B6-Alb blastocysts produce, on average, higher scoring chimeras than ICR and ICRBAF1 blastocysts. However, a statistically significant difference could only be demonstrated between chimeras produced from B6-Alb and ICRBAF1 blastocysts. Comparison between the remaining strains showed no statistically significant difference. Furthermore, we investigated the optimal number of ES cells injected into each blastocyst to generate the greatest number of chimeras with the highest chimeric scores. We compared 5–10 ES cells injected/blast with 15–20 ES cells injected/blast. Our results indicate that 15–20 ES cells injected/blastocyst produce the highest number of chimeras with a higher degree of chimerism compared to 5–10 ES cells/blastocyst. Finally, we tested whether different approaches to implantation of injected embryos had an effect on the size of the litter born. Injected embryos were implanted either into the right, left or both uterine horns of recipient mice. Our statistical analysis shows that the different locations of uterine horn implantation showed no significant effect on the number of offspring produced. Overall, these studies we have successfully developed specific parameters that have allowed us to maximize the efficacy of transgenic mice production for the Pleiades Promoter Project.

27. Efficient production of chimeras by microinjection of ES cells into eight-cell embryos

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Current transgenic technology has proved enormously important for investigating fundamental mechanisms of mammalian development and disease. Modification of the mouse genome by gene targeting through homologous recombination, and by gene trapping approaches have provided a powerful tool to give new insights into human disease models. However production of chimeric mice from manipulated embryonic stem cells is still a laborious and time consuming endeavor. Widely used blastocyst injection procedures suffer from the fact that the germline transmitted line needs to be established through several rounds of backcrosses. Alternatively the tetraploid embryo complementation method avoids the need of breeding chimeras and has been very successful, but highly technical and of low efficiency. Methods for developing efficient germ line transmitted mutant mice using simple injection techniques without the use of additional expensive equipment such as a laser or Piezo system will become more desirable in the mouse transgenic technology research.

Eight-cell stage embryo injection method has been reported to produce high viable germline transmitted chimeras. Here, we report preliminary data employing this procedure without the assistance of either high cost laser or Piezo drill equipment; we have successfully produced 100% agouti pups directly from control ES cells and modified KO ES cells.

Control ES cell line PRX 129/S6 and one selected KO ES cell line were tested. A simple modified ES cell injection needle was used to make an opening in the zona and to deliver the ES cells. Injected 8-cell stage embryos were transferred into the oviduct of 0.5 dpc pseudo-pregnant females. For non-targeted ES cells we injected 40 eight-cell embryos with PRX 129/S6 ES cells, 10 pups were born of which four were 100% agouti male pups and one was a chimeric female. Using targeted ES cells, 20 8-cell embryos were injected, five live pups were born of which three were 100% agouti, one was a high contributing chimeric male and one was a non-agouti female. We are in processing of genotyping and testing the contributions from 129/S6, and checking the frequency of germline transmission. Theoretically, using this method of making a hole in the zona and delivering the ES cells in one step should result in the same 100% contribution to the founder mouse as with using either a laser system or the Piezo drill.

28. Novel ES cell lines from strains C57BL/6J and B6-albino reveal robust germline transmission properties

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To explore the possibility for improving the utility to generate gene targeted mice on both the inbred backgrounds C57BL/6J and C57BL/6-albino, we set out to derive novel ES cell lines from these strains. In the process we initiated two independent derivation experiments and, in effect, obtained three novel cell lines from B6/J, termed 2.1, 10.1 and 5.3, as well as one from B6-albino, termed 4.3. All these novel lines comprised an XY male karyotype that was either uniformly diploid or showed a minor degree of trisomy 8 or 11. During the course of characterizing these lines, chimeras were generated by blastocyst injection or by deposition of ES cells under the zona pellucida in 8-cell precompaction embryos.

Preliminary evaluations for chimera formation, using B6/J ES cells lines 2.1, 10.1, and 5.3 by deposition into 8-cell precompaction embryos from strain CD-1 or B6-albino showed an average chimera frequency of 46.2%. The corresponding ratio of male/female chimeras was 11:1, suggesting that ES cells caused sex conversion in female host embryos. Chimeras that showed a uniformly black coat and extensive ES cell contribution were tested for germ line transmission; it revealed that among the predominantly black chimeras that were evaluated, all fertile ones transmitted the B6 genotype to offspring. In contrast, chimera development using all three B6/J ES cell lines by blastocyst

injection assays yielded a frequency of a mere 18.3%, although the male/female sex ratio remained distorted at 9:2.

The B6-albino line 4.3 was evaluated for chimera development in B6/J host embryos both using ES cell deposition under the zona of 8-cell precompaction embryos as well as in blastocyst injection. The chimera frequency obtained from blastocyst injection was 42.9%. However, while deposition these ES cells in 8-cell precompaction embryos only yielded 16.7% chimeric offspring, germline transmission has thus far only been achieved with this approach. Germline testing of chimeras obtained from blastocyst injections is in progress.

Gene targeting experiments with the C57BL/6J and C57BL/6 albino ES cell lines described here have been performed, and germline transmission of targeted clones is anticipated.

29. Cryopreserved eight-cell embryos can be used to efficiently generate fully ES cell-derived mice

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There are several methods for generating mutant mice with targeted embryonic stem cells (ESC). Methods include embryo-microinjection or embryo-ESC aggregation (co-culture of ESC with tetraploid embryos). In the past, we have used conventional injections of ESC into blastocyst (3.5 day embryo) hosts to produce F0 generation chimeras. This traditional method requires a large number of mice to obtain sufficient number of embryos for daily microinjections. In order to reduce the number of mice, we have improved our method by microinjecting earlier stage embryos (8-cell stage/2.5 day embryo) that have been previously cryopreserved by a standard slow rate freezing procedure. Eight-cell embryos can be microinjected immediately after thawing to generate fully ES cell-derived mice. This new procedure provides a flexible microinjection schedule and reduces our donor animal requirement by 60–80%. Our result show that germ-line transmitting mice from various ESC lines (129S6/SvEv, 129/Ola and C57BL/6J) can be generated by microinjection into corresponding 8-cell embryo donors (C57BL/6J, Balb/c and CD-1). Additionally, having fully ESC derived F0 generation mice allows for a higher efficiency of germ-line transmission and immediate phenotypic analysis.

30. Generation of knockdown transgenic mice via pronuclei microinjection

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Double stranded short interfering RNA (siRNA) has recently been identified as a powerful tool to study gene function(s)

via gene silencing. This phenomenon has been extensively studied in plants, worms and flies, which often referred as RNA interference (RNAi). In mammalian cells, successful RNAi strategy can efficiently down-regulate specific gene expression by 60–90%. Thus far, *In vivo* transgenic siRNA knockdown can be achieved by Lentiviral transduction, ES transfection, or by pronuclei microinjection. Among these approaches, ES transfection has been reported as a preferred methodology for numerous advantageous over others. However, pros and cons in regards to the use of pronuclei microinjection approach may be overlooked. The potential usages of pronuclei microinjection for knockdown will be discussed.

31. Design of vectors expressing an interfering RNA for the inhibition of IE (immediate early) gene from pseudorabies virus in transgenic mice

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Small interfering RNAs (siRNAs) are still rarely used in transgenic vertebrates to knockdown gene expression. Indeed, the *in vitro* highly efficient expression vectors for siRNA requiring polymerase III promoters as U6 or H1 gene promoters are often silenced *in vivo*. In the present study, various shRNA expressing vectors have been used in cultured cells and in transgenic mice to inhibit the IE (Immediate Early gene) mRNA from the pig pseudo-rabies virus. The amount and the sequence of produced siRNA were studied by qPCR. In transfected CHO cells, vectors containing the U6 gene promoter (U6-shRNA) were by far the most efficient to inhibit IE mRNA, mainly due to the very high level of produced α siRNA. Besides, two constructs encompassing a RNA polymerase II promoter (EF-1 upstream of either the miR30 skeleton containing IE sequence or the IE sequence bordered by 5T induced a significant but moderate inhibition. This could be related to the low siRNA production by these two constructs. Notably, despite the fact that the sequence of the siRNA was shorter than expected with the miR30 construct, this did not affect its efficiency. The three constructs were then introduced in transgenic mice. Transgenic mice harbouring an intact transgene were obtained only with the miR30 construct. The absence of U6-shRNA or 5T transgenic mice could result from a potent off-targeting effect of the siRNA sequence produced with these two constructs, that was not the case with the miR30 construct producing shorter siRNA. Our data suggest that (1) the efficiency of a siRNA producing construct is linked both to the sequence and to the amount of the siRNA, (2) a potent RNAi effect may occur even at low concentration of siRNA when it is well targeted on mRNA (3) the off-

targeting activity of siRNA may occur even at low concentration. Mice are now currently infected by the virus to test for resistance induced by miRNA production.

32. In vivo and in vitro multipotency of transgenic rat embryonic stem like (ES-like) cells: another step closer to knockout rats?

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The rat is a model of major importance to biomedical research. Genuine embryonic stem (ES) cells in the rat are highly anticipated and desirable as they would allow gene-targeting technology in this species and permit further elucidation of gene function. At present, this technology is only available in mice. In fact, derivation of rat ES cells has long remained elusive. However, recently we have demonstrated that rat ES-like cells could be efficiently derived and cultured in vitro for extended periods while maintaining pluripotent cell markers. When transfected with a reporter gene, injected into diploid host blastocysts, and transferred to pseudopregnant females, these rat ES-like cells contributed to multiple developing extraembryonic tissues of both midgestation and term fetuses. However, their in vivo contribution appeared limited only to the extraembryonic tissues. We hypothesized that rat ES-like cells could contribute to a wider range of tissues if we tested their developmental potential using (1) host embryos at an earlier developmental stage than the blastocyst, and (2) in vitro differentiation. To this end, we adapted an assay frequently used in mice to generate embryonic chimeras, i.e. diploid (2n) and tetraploid (4n) aggregation. The results obtained showed efficient development of 2n and 4n embryo aggregates to the morula/blastocyst stage, indicating an efficient culture system. No difference was observed in the rates of development to the morula/blastocyst stage between controls, 2n ↔ ES, and 4n ↔ ES embryo aggregations. The implantation rates were similar in all groups, suggesting that the presence of rat ES-like cells in aggregates does not adversely affect implantation following embryo transfer. Confirming the results from blastocyst injections, the rat ES-like cells were found to have contributed in vivo to multiple extraembryonic tissues at midgestation following aggregation. In vitro differentiation was then carried out to evaluate the capacity of these cells to generate tissues of all three primary germ layers of the embryo. Most interestingly, in addition to their in vivo multipotency, the rat ES-like cells were also able to differentiate into derivatives of ectoderm, endoderm and mesoderm in vitro, as demonstrated by flow cytometry, immunofluorescence and confocal microscopy, and RT-PCR. In conclusion, for the first time, we have shown that rat ES-like cells are multipotent not only in vivo but also in vitro. This research therefore constitutes another important step forward in the search for genuine rat ES cells. Supported by an NSERC of Canada Industrial Postgraduate Scholarship to S.-P. Demers and by Clonagen Inc.

33. The European Mouse Mutant Archive

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The European Mouse Mutant Archive (EMMA; www.emmanet.org) is a non-profit repository for the collection, archiving (via cryopreservation of mouse embryos and sperm) and distribution of relevant mutant strains essential for basic biomedical research. The EMMA network is a partnership of several laboratories and other institutions throughout Europe. The current membership includes the CNR Istituto di Biologia Cellulare in Monterotondo, Italy (core structure), the CNRS Centre de Distribution, de Typage et d'Archivage animal in Orleans, France, the MRC Mammalian Genetics Unit in Harwell, UK, the KI Karolinska Institutet in Stockholm, Sweden, the FCG Instituto Gulbenkian de Ciência in Oeiras, Portugal, the HMGU Institute of Experimental Genetics in Munich, Germany, the EMBL European Bioinformatics Institute in Hinxton, UK, the GIE-CERBM Institut Clinique de la Souris, Illkirch, France, the Wellcome Trust Sanger Institute in Hinxton, UK and the CSIC Centro Nacional de Biotecnología in Madrid, Spain. The EMMA network is directed by Professor Martin Hrabé de Angelis who also heads the HMGU/IEG in Munich. EMMA is supported by the partner institutions and by the European Commission's FP6 Research Infrastructures Programme. EMMA is also a founder member of the Federation of International Mouse Resources (FIMRe).

The laboratory mouse is the most important mammalian model for studying genetic and multi-factorial diseases in man. Thus the work of EMMA will play a crucial role in exploiting the tremendous potential benefits to human health presented by the current research in mammalian genetics. EMMA's primary objective is to establish and manage a unified repository for maintaining mouse mutants and making them available to the scientific community. EMMA also hosts courses in cryopreservation, to promote the use and dissemination of frozen embryos and spermatozoa. Up to 2008,

EMMA has processed 1,450 mouse line submissions and distributed mice or frozen embryos or spermatozoa included in 1,031 requests.

34. Results of a Large-Scale Rederivation Program

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UC-Irvine

In September, 2007, we launched a campus-wide program to eliminate mouse parvovirus. This pathogen has been detected, at various times, in 23% of all mouse rooms on campus, affecting about 170 different strains. We present the results of this effort in the hope that this will help other institutions struggling with this elusive and persistent threat.

35. Advances in rat transgenesis

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The rat is an important animal model for cardiovascular, cancer, and pharmacological research. Anecdotal evidence suggests that the production of transgenic rats is less efficient than transgenic mouse production. To compare transgenic efficiency between rats and mice we first optimized procedures for rat superovulation and preparation of pseudopregnant egg recipients. To this end, we compared five different superovulation treatments and four methods of pseudopregnancy induction. The most effective superovulation treatment was 30 IU PMSG followed by 50 IU HCG 48 h later. The preferred method for pseudopregnant recipient preparation combined estrus synchronization with LHRH agonist treatment and mating with vasectomized males. These procedures reduced the number of rats used for egg donors and the number of rats used for pseudopregnant recipient production. We generated 225 transgenic rat founders from 21 transgenes. This includes 65 transgenic founders produced for six BAC transgenes. The average transgenic founder production for both small plasmid and large BAC transgenes were the same (10.7 and 10.8 founders per transgene). Technical factors, which increased success rates, included modified microinjection needles and post-surgical treatment of egg recipients with antibiotics. The efficiency of SD transgenesis was one transgenic founder per four egg donors or 1.9% of injected eggs developed into transgenic founders. We found that the transgenic efficiency with outbred Sprague–Dawley rats was more efficient than transgenesis with inbred C57BL/6J mice. Transgenic facilities experienced in the use of inbred mouse lines for transgenic founders may wish to explore the feasibility of transgenic rat production. Identification of rat lines with desirable superovulation characteristics and birth rates may further improve the efficiency of rat transgenesis in the future.

36. Ubiquitous expression of the monomeric mCherry fluorescent protein in the mouse and its use as a fluorescent marker in tetracycline-inducible lentiviral vectors

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The use of the green fluorescent protein to track gene expression in animal models such as mice evolved to a powerful tool. Transgenic animals, co-expressing the gene of interest and a green fluorescent marker transcribed of an internal ribosomal entry site (IRES) or as a fusion protein are widely used. Recently, a new generation of red fluorescent proteins (RFP) were derived from the mRFP1 such as mCherry. mRFP1 is less toxic compared to its ancestor DsRed, derived from the red discosoma sp. reef coral. mCherry is a true monomer *in vivo* as is mRFP1, but is spectrally further red-shifted and exhibits brighter fluorescence in fusion proteins due to codon optimization. Here, we report the successful generation of mCherry transgenic mice. We show that ubiquitous expression of mCherry does not effect normal development, general physiology, or reproduction. It is highly expressed in various tissues including the skin and peripheral blood cells. mCherry on the far red side of the spectrum is genetically and spectrally distinct from eGFP and serves as a distinguished fluorescent marker beside eGFP. Furthermore, we cloned a tetracycline inducible lentiviral vector containing a tetracycline-inducible promoter and the tTR-KRAB (tetracycline transrepressor fused to the KRAB domain of human Kox1) or the rtTA3 (reverse tetracycline transactivator 3) on a single construct utilizing mCherry or eGFP as a reporter gene. Founder mice positive for the inducible vector containing the tTR-KRAB are currently under investigation. Altogether, we show the production of tetracycline-inducible lentiviral transgenic animals with eGFP or mCherry as fluorescent markers and for the first time that mCherry is an excellent red fluorescent marker in transgenic mouse models.

37. Elimination of mycoplasma contamination in murine embryonic stem cell culture and subsequent germline transmission

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The bacteria *Mycoplasma* is on the exclusion list at virtually every embryonic stem (ES) cell and animal facility in the world. *Mycoplasmas* are different from many other bacteria in that they have no cell wall which makes them antibiotic resistant. Several *Mycoplasma* species are pathogenic to both humans and mice, depending upon their species origin. *Mycoplasma pulmonis* infection in mice is highly contagious and causes respiratory disease. *Mycoplasmas* from human,

murine, and bovine origins are often contaminants in cell cultures and may induce cellular changes, including chromosome aberrations, changes in metabolism and cell growth. Since infected ES cells cannot always be discarded, many complicated methods have been suggested for the elimination of the *Mycoplasma*. Our lab purchased ES cell line E047D03 from The German Genetrap Consortium (GGTC). A sample was sent to Charles River for testing prior to its use and the cells tested positive for *Mycoplasma arginini*. To try to eliminate it, we first treated the infected cells with *Mycoplasma* Removal Agent (MRA) while culturing them on feeder cells for 7 days. Subsequent testing revealed that the cells were still positive for *Mycoplasma*. Next, we attempted a second type of treatment that involved the use of Plasmocin (InvivoGen). The ES cells were cultured on feeder cells and treated with Plasmocin for 14 days. Subsequent testing indicated that the infection had been cleared. Karyotyping indicated that only 30–40% of the cells were normal. To determine whether these clean ES cells were efficient in generating chimeric mice, the cell line was subcloned and 30 more clones were karyotyped. Six clones with a chromosome count/karyotype of 80% diploid or higher were chosen and grown as a mixed culture. The cells were then aggregated with diploid CD1 embryos to create chimeras. We were able to generate 18 chimeric mice (14 males and four females) and from them four male chimeras were chosen to breed with CD1 females to test for germline transmission. The resulting offspring were genotyped by PCR and were found to carry the desired genotype. Together this suggests that Plasmocin treatment of *Mycoplasma arginini* infected ES cells is sufficient to clear the cells of infection and the resulting cells were extremely capable of generating chimeric mice.

38. Transgenic mice expressing iCre in the brain from a BAC containing the vesicular GABA-transporter VGAT

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Transgenic mice created by injection of bacterial artificial chromosomes (BACs) are becoming more and more popular. Transgenes based on genomic clones such as BACs are often large enough to contain complete driver genes with all their endogenous regulatory sequences. This makes them useful for expression of exogenous proteins in defined subpopulations of cells within the transgenic animal. Here we used a BAC that contains the vesicular GABA transporter gene (VGAT or *Slc32a1*) to drive expression of iCre in GABAergic and Glycinergic neurons of the brain. The iCre recombinase is a codon-optimized version of the original phage Cre that catalyzes excision of genomic regions flanked by loxP sites. The cDNA for iCre was introduced into the VGAT genomic locus of the BAC by recombineering in *E. coli*. Purified, linearized, recombined BACs were injected into zygotes and offspring were screened by PCR of tail DNA. We obtained two positive founder animals in one injection session and characterized F1 generations of both of them for iCre activity. In order to assess the utility of our VGAT-iCre transgenic line, we

bred animals to the Rosa26-lacZ reporter strain. LacZ expression was unblocked in GABAergic and Glycinergic neurons that contain VGAT and therefore allow transcription of the VGAT-iCre transgene. We visualized individual neurons expressing LacZ in brain sections of double transgenic animals (VGAT-iCre × Rosa26) by staining for LacZ activity and also by immunohistochemistry with an antibody against beta-galactosidase. In order to prove iCre expression in a large proportion of GABAergic interneurons we performed double immunostaining with antibodies against beta-galactosidase and GAD67, a GABAergic marker. We found complete overlap of signals from both antibodies which shows that the BAC transgene drives iCre expression in all GABAergic neurons. The VGAT-iCre transgenic lines that we generated can therefore be used to genetically modify neurons that belong to the GABAergic subpopulation. These neurons have been implicated to play important roles in neurological diseases such as epilepsies, neuropathic pain, Alzheimer's disease, Parkinson's disease and also in brain development. Thus, our VGAT-iCre transgenic animals can be used to study these diseases in an animal model that enables researchers to modify specific genes in GABAergic neurons and link their function to these neurological disorders.

39. Examination of microRNA mmu-miR-290 expression level alteration in mouse embryonic stem cells with increasing passages number

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Embryonic stem (ES) cells are widely used for introducing targeted mutations and other genetic alterations into the mouse germ line. Unfortunately, chromosomal abnormalities that provide cell proliferation advantages frequently accumulate in ES cell clones. These abnormalities generally result in reduced or diminished germ cell forming ability. It would be of great practical value for gene targeting experiments to optimize the rate of success without relying on elaborate tests such as karyotyping individual clones prior to blastocyst injection. The aim of our experiments was to find an alternative strategy to identify clones with high probability of contributing to the germ line.

The chimera forming ability of transgenic subclones derived from the R1 or R1/E ES cell lines were compared. 67% of newborns were chimeras using transgenic subclones derived from R1/E ES cell line, while only 41% created with R1 subclones. Notably, the germ line transmission was restricted to R1/E subclones. The ratio of aneuploid cells was high in the R1 ES cell line already at early passages and the chromosomal anomalies dramatically increased after 10 passages both in R1 and R1/E cells and along with it, several autosomal trisomies (Chr 2, 3, 5, 7) were identified.

Immunostaining and RT-PCR were used to analyze the expression pattern of pluripotency-related genes at different passage numbers, but we could not detect significant differences between the analyzed cell lines. However, qRT-PCR analysis revealed a significant difference in the expression

level of the mouse embryonic stem cell specific microRNA mmu-miR-290 in R1 and R1/E cells. In R1/E cells, the mmu-miR290 expression level increased with passage number, while in R1 cells mmu-miR-290 expression was elevated even in early passages. In parallel the expression levels of some transcription factors (Pou5f1, Zfp42, Nanog, Kdr and Brachyury) were analyzed. Only Brachyury expression showed significant difference between the R1/E and R1 ES cells.

We suggest that high mmu-miR-290 expression indicates a fast-growing population of ES cells with altered ability of differentiation that might lead to reduce capacity in chimera formation.

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40. Non-Surgical Embryo Transfer (NSET) device for the transcervical transfer of genetically modified mouse embryos into pseudopregnant female mice

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The ability to manipulate the mouse germline via gene transfer, gene knock-out, and targeted gene modification has dramatically advanced the use of mice in biomedical research. Despite the excitement associated with these advances, aspects of mouse research are becoming more challenging as per diem charges and regulatory guidelines continue to increase. In regards to the genetic modification of mice, particular challenges are associated with the surgical transfer of embryos. These include the pain and distress that occurs with this surgical procedure, potential problems associated with anesthesia and post-operative recovery, including possible complications such as post-surgical infection and the time spent to monitor animals, specialized surgical equipment and the facilities to carry out sterile procedures, and the substantial training that is needed to become proficient in uterine and oviduct surgery.

To address these problems listed above, we have developed a device called NSET (Non-Surgical Embryo Transfer) that can be used for the rapid and efficient transcervical transfer of embryos into pseudopregnant female mice. The NSET device can be used with a standard p2 pipetman, requires little training, is rapid, and does not require anesthesia (or the amount of post-operative care) for recipient female mice. For transfer of embryos microinjected with DNA, fertilized eggs are cultured in vitro for 3.5 days prior to transcervical transfer. Our data indicates that NSET-mediated transfer is equally efficient as standard oviduct transfer for the production of transgenic mice via microinjection. We have also successfully used this technology to transfer blastocysts generated by the aggregation of morulas with gene-targeted R1 ES cells. Germ-line transmission of the targeted allele was achieved from the resulting chimeric mice that were born after NSET transfer. Finally, we have successfully transferred cryopreserved embryos using the NSET device.

We believe that the NSET device provides a promising alternative to surgical procedures that are currently required for the production of genetically modified mice, and will have

wide applicability in the field of mouse genetics. Current efforts are underway to further refine this technology and to manufacture NSET devices for distribution.

41. A FileMaker database for transgenic facility and mouse colony management

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A FileMaker database has been developed for the management of transgenic facility and mouse colony. The database is designed to minimize data entry in order to prevent human error and improve operation efficiency. The information in the database is propagated automatically during set up breeding units and wean litters.

The database includes five portions. Each contains:

1. Gene targeting procedures: construct, ES cell manipulation, primers, PCR and derivation of ES cell lines.
2. Transgenic procedures: microinjection, aggregation, rederivation, cryopreservation and IVF.
3. Mouse colony management: tracking mice, breeding units, litters and cages.
4. Lab management: lab order, protocol, storage, lab memo...
5. Vivarium: IACUC protocols, animal user information, animal orders.

The information flow starts from service request. Client's contact and service information will be entered into the database. The information will be passed to the procedure database then to mice database. One needs only to click a few buttons to setup breeding units, wean litters, setup cages, print cage cards, remove mice or retire a breeding unit. Other functions of the software include auto-email notification, reports/counts on cages/mice/breeding units, billing and counting mice used in an IACUC protocol.

The database can handle a large amount of information. An earlier version used at UConn Health Center contains more than 80,000 entries. The database is located in a FileMaker server. The end users can login for data entry or view only according to the privileges assigned. The database provides a template for those who would like to have a custom-made database to manage their facility and mouse colony.

42. A new recombineering-base method for generation of mutations in large constructs

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Generation of subtle mutations in a large gene targeting construct can be tricky. We describe a recombineering-base method to introduce mutations anywhere in large constructs without using a selection marker.

We first created a unique restriction site(s) at the location where the mutation will be introduced in a large construct using recombinering vector PL452.¹ The mutant fragment was generated by PCRs with the mutation in the middle flanked by 200–300 bp homologous sequences. The large construct with the unique restriction site and the PCR fragment containing the mutation were co-electroporated into EL350 allowing homologous recombination to take place. The electroporated bacterial cells were cultured in 5 ml LB overnight. One micro liter of the plasmid prep from the culture was digested with the unique restriction enzyme and treated with CIP. The wildtype vectors will be linearized while the vectors containing the mutation remain circular. Four microliters of the reaction mix were then transformed to DH10b. Typically three quarters of the clones formed would carry the mutation.

Using this method we have generated different mutations, such as three point mutations at different locations in one targeting construct, 8 point mutations clustered in a small region, in-frame insertion and deletion. The method can be used to create any mutations anywhere in a large construct without a selection marker. The method is quick and efficient. It enhances our ability in the generation of complex gene targeting constructs.

1. Liu P, Jenkins NA, Copeland NG (2003) A highly efficient recombinering-based method for generating conditional knockout mutations. *Genome Res* 13:476–484

43. ES cell targeting efficiency with constructs made by the recombinering method

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Gene targeting is a powerful tool in bioscience research but the targeting efficiency in ES cells remains a challenge. The Gene Targeting & Transgenic Facility at the University of Connecticut Health Center adopted the recombinering method described by Liu¹ in 2004. During the period of 2004 to the end of 2007 we produced 85 gene targeting constructs including 62 conditional knockouts (CKO), 10 conventional knockouts (KO), 10 subtle mutations (Mut), 2 knockins (KI) and one CKO plus mutation.

The genomic DNA fragments were derived from the C57Bl/6 BAC clones provided by the BAC/PAC Resource, CHORI. The ES cell lines were derived from 129S6/C57Bl/6j F1 hybrid blastocyst at our facility. We used nested PCR for ES cell screening which is efficient and reliable in our hands since all the mouse lines generated were verified by homozygosity testing for the targeted allele.

The average length of total homologous arms for these constructs is 11.81 kb which includes long arm, short arm and floxed fragment in CKO. The average size of floxed fragment is 4.75 kb (range 1.9–11.5) for CKO and the deletion size for KO is 3.05 kb (range 68 bp to 8.15 kb). The mutations include 1–27 nucleotides mutations and 2–21 bp deletions.

We typically picked two 96-well plates and screened one plate first. The screening would be stopped if we obtained five positive clones before completing the whole plate. The second plate would be screened only if there were fewer than five positive clones in the first plate. The average targeting rate is 10.6% (range 1–66.7%) for CKO, 15% (2–42%) for KO, 10.5% (0.05–40%) for Mut and 17.5% (6–29%) for KI. The chimeric mice were generated by aggregation. We routinely aggregated three ES cell lines with 200–300 CD-1 embryos in one session and achieved germline transmission for all the constructs except one in which the mutation caused embryonic lethality.

The ES cell targeting rates with these constructs are higher than that reported by Valenzuela² in which the BACs were used as targeting vectors. The recombinering method allows one to choose the region and length of the homologous arms with great ease. Combined with good ES cell targeting practice, gene targeting is not that intricate.

1. Liu P, Jenkins NA, Copeland NG (2003) A highly efficient recombinering-based method for generating conditional knockout mutations. *Genome Res* 13:476–484
2. Valenzuela DM et al (2003) High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. *Nat Biotech.* 21:652

44. Construction of hair follicle cells with specific expression of IGF1 and production of transgenic Cashmere goat red fluorescent embryos by somatic cell nuclear transfer

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The inner Mongolian Cashmere goat is a fine local breed with high productivity for both undercoat and meat. A healthy adult yields 380–450 g of cashmere wool of high quality (less than 16 microns) annually. However, Cashmere goats currently bred in Inner Mongolia, China, face the problem of declining yield and low rate of selective breeding within herds. A possible improvement relies on alteration of the level of growth factors such as insulin like growth factor 1 (IGF1) which is known as one of the vital factors in growth cycle of mammalian hair follicle cells. Somatic cell nuclear transfer (SCNT) provides a possibility to generate transgenic animals with higher efficiency than by classical microinjection.

We constructed an overexpression vector that transcribes IGF1 cDNA driven by the caprine keratin associated protein 6.1 (KAP6.1) promoter, a gene previously reported to be specifically expressed in hair follicle cells. The red fluorescent protein DsRed2 was used as a transgenic marker and neomycin resistance for screening the transgenic somatic cells. These two markers were both expressed in a non-tissue-specific manner. Caprine fetal fibroblast cells (CFFCs) were derived from a 45-day-old Cashmere goat fetus and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO₂ and a humidified atmosphere. The linearized expression vector was introduced into the cells using lipofectamine 2000 at a ratio of 4 µg DNA: 8 µl lipid when growing cells reached 80% confluence. Twenty-three transgenic cell

colonies were obtained by selection with G418 at a concentration of 800 µg/ml for 12 days and 300 µg/ml for 3 days.

After SCNT with the transgenic cells 15.3% (31/203) of the reconstructed embryos developed to the blastocysts stage, in which 17 (54.8%) embryos strongly expressed red fluorescence. Two of them were chosen randomly to confirm the transgene identity by PCR, and both of them were found positive. These results showed that (i) RFP and Neo gene could be expressed correctly which lead to the establishment of the transgenic cell lines and production of positive transgenic embryos; (ii) although the donor cells were transgenic positive not all of the SCNT blastocysts expressed red fluorescence, and (iii) the production of the embryos expressing red fluorescence and containing IGF1 expressional cassette could be used to further investigate the regulation of IGF1 in hair follicle cells of transgenic cashmere goat.

45. A comparison of analgesic regimes used during embryo transfer surgery in mice

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There is little published regarding optimum analgesic regimes for embryo transfer surgery in mice. Commonly a single analgesic is used, but in larger species and man multi-modal analgesia is the preference. We have compared carprofen alone, against a combination of carprofen with buprenorphine.

To assess whether the pregnancy rates are affected we have collected data from over 300 mice that have undergone embryo transfer surgery. We show that there is no significant detrimental effect to the pregnancy or birth rate when buprenorphine and carprofen are combined to produce a multi-modal analgesic regime.

46. Dimerizable Cre (DiCre) as a tool for conditional transgenesis: a first evaluation and comparison with Cre2ERT2, a tamoxifene-inducible form of Cre

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Cre recombinase is extensively used to engineer the genome of experimental animals. However, its usefulness is still limited by the lack of an efficient temporal control over its activity. To overcome this, we have developed DiCre, a regulatable fragment complementation system for Cre. The enzyme was split into two moieties that were fused to FKBP12 (FK506-binding protein) and FRB (binding domain of the FKBP12-rapamycin associated protein), respectively. These latter can be

efficiently hetero-dimerized by rapamycin. After testing several variants, we have been able to show, using in vitro approaches, that ligand-induced dimerization is an efficient way to regulate Cre activity, and presents a low background activity together with a high efficiency of recombination following dimerization. We have knocked-in the best pair into the ROSA26 locus of mice. To evaluate the performance of this system, mice have been mated with indicator mice (Z/EG or R26R) and Cre-induced recombination was examined following activation of DiCre by rapamycin during embryonic development or after birth of progenies. While embryonic treatment with the dimerizer led to no or only very low level recombination, postnatal treatment induced recombination in several (liver, heart, kidney, muscle), but far from all, tissues of animals. Moreover, this recombination was also highly mosaic within any given tissue. It was also less important than that observed using another conditional Cre-deleter mouse line that we have created for comparison, and that had the tamoxifene-regulated ERT2-iCre-ERT2 (Cre2ERT2) construct expressed from the ROSA26 locus. Note that this latter construct was also highly efficient during embryonic development. Interestingly, however, the order of efficacy between different tissues was the same for these two forms of regulatable Cre. This observation, as well as the lack of correlation between the level of recombination and that of Cre expression, as measured by RT-qPCR, suggest that part of the apparent limitation of the recombination might be linked to the reporter system rather than to the used Cre system itself. In conclusion, we have documented that DiCre has indeed the potentiality to be used to establish conditional Cre-deleter mice, however, its performance should be ameliorated before its use could be generalized.

47. Generating tools to modify the *Mus spretus* genome

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Mus spretus diverged from the *Mus musculus* complex of house mouse subspecies about 1.5 million years ago. Several inbred strains, as for instance SPRET/Ei, have been derived from this species. Recently, we have described that SPRET/Ei mice show a remarkable resistance in models of acute inflammation, e.g. triggered by cytokines or by bacterial components. 1, 2 The etiology of this trait is complex, and multiple genetic factors seem to be involved. Therefore, the genetic manipulation and functional research of the SPRET/Ei genome is fundamental in order to identify the causative genes. We have recently derived original ES cell lines from hybrid embryos resulting from a cross between C57BL/6 and SPRET/Ei.3. These (BxS)F1 ES cells will be used to mutate the SPRET/Ei genome as all of the phenotypes found so far are dominant in nature. Such F1 ES cells were shown to be able to form chimeric mice using SWISS blastocysts. Moreover, the ES cells are able to generate fully ES derived mice after tetraploid aggregation. Manipulation of the SPRET genome of these ES cells will be possible using commercially available SPRET/Ei BAC libraries, and a custom made full-length cDNA library derived from SPRET/Ei macrophages.

48. Gene-targeting in C57BL/6 ES cell lines: effect of genetic variation and genetic instability

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We assessed genetic variation in six C57BL/6 mouse ES cell lines with 275 SSLP markers and compared the results to C57BL/6J reference DNA. The Bruce4 and CMTI-2 ES cell lines were the most divergent from C57BL/6J DNA, differing at 34 SSLP markers and showing significant regions of heterozygosity on chromosomes 3, 7, and 9. The BL/6#3 and Dale1 ES cell lines were the most similar to C57BL/6J, differing only at three SSLP markers. The C-2 and WB6d ES cell lines differed from C57BL/6J at six SSLP markers. No C57BL/6 ES cell line was genetically identical to C57BL/6J. One factor reducing gene targeted mouse production efficiency is chromosomal instability. We counted chromosome spreads from 820 gene targeted ES cell clones and subclones and found the Bruce4 C57BL/6 ES cell clones were more likely to be aneuploid and unsuitable for ES cell-mouse chimera production than 129 mouse derived ES cell clones. Transfer of mutations engineered in Bruce4 ES cells to a defined C57BL/6J background, despite segregating alleles, is more rapid than transferring changes engineered in 129 mouse ES cells. The benefits of analyzing mutant phenotypes in a defined genetic background can offset the inefficiency of gene targeting in C57BL/6 ES cells.

49. WEB version of a simple database to monitor transgenic mouse colonies

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A few years ago, we described three independent FileMaker databases (*transgenics*, *mymouse* and *cages*) that were developed to track the generation of transgenic mice, the organisation of transgenic mouse colonies and the distribution of mice in cages (Montoliu 2003, Simple databases to monitor the generation and organisation of transgenic mouse colonies, *Transgenic Research* 12: 251–253). These databases, have been freely distributed among academic researchers and have been proven useful and robust over the past years in many laboratories and transgenic facilities, specially *mymouse*. The initial database clones were developed using FileMaker Pro v.5, a user-friendly program that does not require extensive

computer expertise and whose subsequent available updated versions have largely maintained compatibility (not always 100%) with previous ones.

At present, current laboratories and transgenic facilities can use a variety of operating systems and computer platforms, including Windows-PC, Apple-Mac or Linux, plus, often, different platforms might even coexist in one place. In addition, we thought these databases would be much useful if data could be easily accessed through the WEB, using everyone's favourite WEB browser, from any computer, rendering them therefore independent from a local computer or particular program. This could allow entry, modification and search of mouse data contained in the server database from any computer in the world, through a dedicated URL address, following an organized scheme of users with their corresponding usernames and passwords, to respect the adequate privacy associated with each project.

In this regard we have prepared an updated WEB version of *mymouse*, to efficiently monitor transgenic mouse colonies from any computer. This new system relies on a WEB server, where the actual mouse database is stored and maintained (as MySQL database format). This new database system will allow more efficient and rapid process of large amounts of records. All the routines and functions implemented, along with the interactions with the MySQL database have been written in PHP language. The resulting resource, including a configuration procedure to customize everyone's requirements, will be made eventually available to interested academic researchers.

50. A comparison of new sperm cryopreservation methods

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MRC-NIMR, NC3Rs

This poster describes the approach to, and results from, the use of sperm cryopreservation as a means to archive genetically altered strains of mice, rats, *Xenopus* spp. and zebrafish. It presents results from historical and emerging protocols analysing their success as measured by motility and fertilisation capacity. In addition it provides a critique on the appropriate choice of protocol and ease of use of the methods described.

51. Evaluation of the thrombotic risk of an engineered coagulation factor IX with augmented clotting activity in mouse models

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Current treatment of patients with hemophilia B mainly relies on intravenous infusion of plasma-derived or recombinant factor IX (FIX). Both forms of FIX are available yet complications of using either product still exist despite the safety profiles of factor concentrates have improved. Gene replacement therapy although is an attractive approach, the human studies as well as the others using mice and dogs have revealed complications of the risk of a neutralizing immune response to the therapeutic gene product and delivering vehicles when used in large quantity. To circumvent these obstacles, FIX molecule with high clotting activity has been attractive because lower amount of protein or delivering vectors will be necessary for replacement therapy and for gene therapy, respectively. We previously generated a variant FIX (Variant-IX) molecule with three point mutations in the epidermal growth factor-like domains and the catalytic domain, exhibiting 10–12 times higher clotting activity than that of wild-type FIX (WT-IX). To dissect the relationships of FIX with enhanced clotting function and thrombosis, a mouse model expressing human WT-IX or Variant-IX was generated by the knock-in (KI) technology. Briefly, the human FIX gene was targeted into the murine FIX locus so the endogenous murine FIX was deleted, and the human FIX was expressed under the control of the murine FIX promoter and regulators. By the activated partial thromboplastin time, we have detected 8-times more FIX enzymatic activity in the plasma of Variant-IX KI mice than in that of WT-IX KI mice with the same amount of human FIX in the circulation. The haemostatic efficacy of Variant-IX KI mice was further analyzed by multiple functional assays. That is, Variant-IX KI mice exhibited significant decrease in both R and K values, and increase in alpha angle on the parameters of the thromboelastography. Bleeding time of Variant-IX KI mice was ameliorated in contrast to that of IX-WT KI mice. We then investigated the thrombotic relationships among these KI mice by ferric chloride (FeCl₃)-induced carotid artery and vena cava thrombosis models. We identified, nevertheless, equal thrombotic tendency among the KI mice treated with FeCl₃ up to 10% in both arterial and venous models. Moreover, when the Variant-IX gene was delivered into C57BL/6J mice, there was also no obviously deleterious or thrombogenic effect on the virally-transduced mice. All these results indicate that Variant-IX has significantly enhanced clotting activity which is not more thrombotic and can be a potential substitute for WT-IX.

52. Use of anesthesia and analgesia in the production of genetically modified mice

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In the production of genetically modified animals welfare and pain management has not always been a major issue in the past but is obviously important from an animal ethics point of view

and for animal welfare reasons. Regarding anesthesia, Avertin (tribromoethanol) was a commonly used drug in transgenic production earlier, but has always been questioned because of reportedly adverse effects such as local irritation and even deaths in animals often within 24 h after administration due to accumulation of toxic byproducts in the solution.¹ Therefore, we started to use Isoflurane as anesthesia in 2007. The mice that were given Isoflurane (Baxter) rapidly achieved a surgical depth of anesthesia and experienced a rapid postoperative recovery (2–3 min). We use two Univentor 400 Anesthesia units as anesthesia appliance, one for initiation of anesthesia (4% Isoflurane) and one for maintenance (1.5–2.5% Isoflurane).

As pre-surgical analgesia we are using Vetalgin (Metamizole Dipyron/Intervet AB, Sweden) as described earlier² but in a changed concentration of 200 mg/kg BW.

Some commonly post-operative analgesics used for lab animals might have negative effects on pregnancy, mainly during implantation. Besides this, they have to be injected several times per day to achieve proper analgesic effect causing stress on foster mothers that can result in reduced pregnancy rates, therefore oral administration of analgesic drugs is preferable.³ We started to use Paracetamol (acetaminophen; Alvedon, AstraZeneca, Sweden) post-operative, diluted in the drinking water with a concentration of 3.5 mg/ml for 48 h post surgery.

In summary, the use of Vetalgin as pre-operative analgesia, Paracetamol as post-operative analgesia and replacing Avertin with Isoflurane showed no negative effects on pregnancy and birth rate in the production of transgenic mice. On the contrary, this is beneficial for the foster mothers with regard to pain management and thus animal welfare.

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53. Establishment of stable EGFP-transgenic BALB/c mouse strain by lentiviral transgenesis

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Due to its improved efficiency and relative technical ease, lentiviral technology has become the method of choice for the creation of stable transgenic small laboratory animals. However, according to literature uncertainties remained whether strong ubiquitous promoters resist long-term silencing.

We have used HIV-1 based lentiviral vectors to create stable transgenic BALB/c mice. The utilized pWPTS is an HIV-1 based second generation self-inactivating lentiviral backbone that encodes EGFP driven by the human EF1a promoter. The construct also contained a cPPT sequence for easier nuclear translocation and the WPRE sequence for prolonged EGFP mRNA half-life. The original viral envelope

protein was pseudotyped by VSV-G protein. For injection viral particles were harvested and concentrated from the supernatant of 293T virus producer cells, at biological viral titers usually exceeding 108 TU/ml.

The perivitelline space of BALB/c zygotes was injected with concentrated lentiviral vector. Altogether 20 injected zygotes were implanted into recipient females for gestation. After the regular developmental period 12 mice were born. According to PCR analysis seven pups expressed EGFP, of which four were macroscopically positive by EGFP fluorescence. The transgenic animals were analyzed for EGFP expression by PCR, Western-blot, histology and flow-cytometry, and showed even distribution of EGFP expression without the traits of mosaicism. In addition, tissue-dependent differences of EGFP expression pattern due to the context-dependent activity of the EF1a promoter could also be observed.

F0 founder EGFP+ BALB/c mice were crossed with wild-type BALB/c mice to establish the F1 generations. Macroscopically EGFP-positive F1 offsprings were mated to determine the stability and transmission of lentiviral transgene. Up to the sixth generation only one newborn showed EGFP inactivation by macroscopic inspection, whereas the EGFP-positive progeny bred and developed normally (with the litter size of 4–7 pups). The long-term follow up of the stable EGFP-transgenic BALB/c mouse strain highlights the stability of lentiviral transgenesis even with the use of a strong, ubiquitous EF1a promoter sequence.

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54. Function of miR-290-295 in mouse embryonic stem cell self-renewal and early differentiation

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The RNAi machinery has been implicated in maintaining stem cell characteristics in both plants and animals. The development of mouse embryos, in which the miRNA genesis pathway has been rendered non-functional, was arrested at early stage of embryogenesis. Similarly, Dgcr8 knockout ES cells expressed ES cell-specific markers but accumulated in the G1 phase of the cell cycle, showing that miRNAs are required for normal ES cell proliferation. In the present study, we focused on the physiological function of miR-290-295 microRNA cluster in mouse ES cell self-renewal and early differentiation. miR-290-295 cluster overexpressing ES cell lines were established. Comparison of miR-290-295 overexpressing ES lines with control lines, revealed faster proliferation and higher colony formation ability of the genetically modified clones.

The TaqMan[®] Mouse Stem Cell Pluripotency Array analysis revealed, that expression of several early differentiation marker genes was decreased in the miR-290-295 overexpressing ES lines. However, only a minute decrease in the expression of the stemness markers Pou5f1, Sox2 and Fgf4 was detected. Other pluripotency markers did not show any alteration between the miR-290-295 overexpressing and control samples.

Upon serum deprivation in miR-290-295 overexpressing ES cell lines, substantial increase was detected in the ratio of S phase cells, while cell numbers in G0/G1 phase were slightly increased and the G2/M ratio decreased.

PicTar and miRBase programs were used to in silico identify candidates for direct targets of the mmu-miR-290-295 cluster. The conserved target sites between human, rat and mouse were aligned and several cell cycle inhibitors were predicted as potential targets. In vitro validation by dual-luciferase assay was performed with Fbx14 and Wee1, which's 3' UTRs decreased the luciferase activity to about 50%. A further decrease of reporter activity was observed when the constructs were transfected to the miR-290-295 overexpressing ES lines, indicating that the effect is specific for this miRNA cluster.

55. Overexpression of hepsin in vascular endothelial cells regulates LPS-induced toxemia and tumor metastasis

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Hepsin, a type II transmembrane serine protease is an important regulator of controlling cell proliferation, blood coagulation and embryonic development. Although hepsin homozygous deficient studies have revealed viable and fertile mice and no significant difference in normal hemostasis, the exact in vivo function and mechanism remain unclear. To dissect the relationship of hepsin and vascular endothelial cells, we generated Tie2 promoter/enhancer–hepsin transgenic mice. The transgenic construct including human hepsin cDNA and a reporter IRES-Lac Z sequence was under the control of an endothelium specific promoter (Tie-2) and enhancer. The transgenic construct was verified in bovine endothelial cells by transient transfection experiments and then used for microinjection of the zygotes of C57BL/6 mice. These different transgenic mice lines did not reveal any abnormalities in growth, biochemical screening and without spontaneous thrombus formation in circulation. But they exhibited enhanced sensitivity to LPS-induced toxemia and inhibitory effect to tumor metastasis to lung. Whether these phenotypes involved the role of hepsin in immune or in metastasis especially in activating endothelial cell function, the regulation of hepsin is under investigation.

56. Knock-out mouse model for the study of human ASPM gene

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ASPM (abnormal spindle-like microcephaly associated) gene is the human orthologue of the *Drosophila* abnormal spindle (*asp*) and the most commonly mutated gene of autosomal recessive primary microcephaly (MCPH). The homozygous semi-lethal mutation of *Drosophila asp* causes abnormal spindles, frequent polyploidy cells and cytokinesis failure, leading to arrest of neuroblasts in metaphase and larval-pupal lethality. In human, the ASPM protein has been implicated in the determination of human cerebral cortical size and the defective neurogenesis caused by homozygous mutation of ASPM leads to microcephaly and mental retardation. Recently, ASPM is shown to express in nearly all transformed human cell lines and in multiple fetal tissues. These findings suggest ASPM play an important role in cell cycle progression and cell proliferation in embryonic development and tumorigenesis. Previous studies in mouse suggest that ASPM is widely expressed in the developing brain, fetal and adult tissues and upregulated in malignant cells. However, mouse ASPM functions need to be further verified. Using differential display analysis of gene expression profile of HCC, we found frequent overexpression of ASPM, which is located at chromosome 1q31, a region with frequent gain in HCC. In our study, we showed that ASPM was often overexpressed in human HCC and associated with high grade (poor differentiation) and high stage (vascular invasion) tumor, early tumor recurrence (ETR) and poor prognosis. Our observations provide in vivo evidence that ASPM expression plays important role in the cell proliferation and differentiation in embryogenesis and its overexpression correlates with enhanced metastatic potential of HCC. Consistent with these findings, knockdown of ASPM by RNA interference (RNAi) treatment led to a reduced anchorage-independent growth and tumor cell invasion capability in HCC36 and HuH-7 cells. In the animal model, we study the effect of loss of ASPM in the ASPM knockout mice. Targeted disrupted of mouse ASPM Exon 2 and 3 by the recombineering in mouse embryonic stem cells which caused out of frame deletion. The homozygous F2 knockout mice have obtained. Further studies include the continued analyzing the phenotypes, characterization of the difference between ASPM knockout and wild-type mice and studying the role of ASPM in effecting the phenotypes.

57. Hormone concentration and timing of its administration, individual males used to mating and vaginal plug presence as predictive factors on mouse embryo production

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In mouse, some transgenic techniques demand a large number of oocytes (SMGT), ova (pronuclear microinjection) or embryos (TCA) in order to produce the transgenic offspring. However, ethical concerning should be considered to minimize the number of sacrificed females. Differently to other species, mouse

depends on the reliability of the superstimulation protocol variables to produce embryos. The aim of this work was to evaluate hormone concentration and the timing of its administration, the individual male used to mating donors and vaginal plug presence as predictive factors on the mouse embryo production. Forty eight female mice (Swiss strain, 30–45 days old and under the same conditions of housing), were randomly allocated in one of the four groups ($n = 12/\text{group}$). Superstimulation treatment was given at 1300 h (G13) or at 1700 h (G17) on randomly days of the estrous cycle. It was administered—via i.p. in 0.1 mL—5 (G13/5 and G17/5) or 10 IU (G13/10 or G17/10) of eCG (Folligon[®], Intervet) and, after 48 h, of hCG (according the same given eCG concentration; Chorulon[®], Intervet). After hCG administration, the females were put to mating with identified Swiss males (1:1) until next morning (0700 h; D0). Every male was selected to mate with females from all groups. The mating was confirmed by visual presence of vaginal plug. Embryo recovery was performed on D3.5 to D5.5 by flushing the oviducts and uterine horns of all females with DMPBS. Total structures recovered (TSR) were classified to detect viable embryos (VE) according IETS parameters. The percentage of VE related to TSR was considered viability rate (VR). The results were analyzed by *t* test, Mann–Whitney, Kruskal–Wallis or Fisher exact test, depending analyzed variable and normality of data (significance was considered when $P < 0.05$). There were no differences among groups on the median (percentiles 25th–75th) of the TSR, VE and VR, respectively, for G13/5 [12.5(4.5–19.5); 5.0(0–10.5) and 73.9%(20–90)], G13/10 [13.5(6.5–23.5); 12.0(2–19) and 90.4%(75–100)], G17/5 [11.5(1.5–17.5); 6.5(0–13) and 61.1%(0–97.1)] and G17/10 [3.5(0–16); 0.0(0–6) and 44.4%(8.3–91.6)]. There was difference between the mean (\pm SEM) of VR from animals with (76.3% \pm 7.2) or without (40.7% \pm 9.6) vaginal plug. The time of hormone administration, independently of its concentration, was significant to vaginal plug absence (29.2 and 66.7%, respectively, for 1300 and 1700 h). There was difference between VR from the three worst versus three best male VR (35.8% \pm 12.9 and 80.0% \pm 7.8, respectively), although there was no difference on vaginal plug presence from both groups. Twenty five percent of the males produced no plugs on all mated females, whereas 25 and 50% of them produced 20–50 and $\geq 60\%$ of plugs, respectively. There was no difference between VR of males with low (60.9%) and high (63.9%) percentage of plug presence, respectively for \leq and $>50\%$. Males #24 and 28 represent extreme variation, since there was a discrepancy between % of plug presence (20 and 75%, respectively) and VR (74 and 18.6%, respectively). It was concluded that neither the hormone concentration nor timing of its administration, or their association, was significant on TSR, VE and VR variables. Moreover, the presence of vaginal plug was generally related to higher VR, while some males appeared to produce higher VR independently of vaginal plug presence.

58. Chimera production through inverted diploid aggregation

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Generation of chimera is typically achieved by combining a host wildtype embryo with genetically dissimilar, or modified, pluripotent cells in a model that recapitulates normal development.¹ Here, an alternative diploid aggregation approach was established through use of transgenic morula to provide a compromised environment in which the performance of wildtype embryonic stem cells outside of the normal milieu could be assessed. The stress intolerant *KCNJ11* gene knockout^{2,3} produced a low yield of quality morula, and was used as a prototype to generate mosaic embryos using competent wildtype embryonic stem cells labeled with a constitutively expressed *B-galactosidase (lacZ)* gene.⁴ Proper cavitation and inner cell mass formation in aggregated knockout–wildtype blastocysts were achieved with a 4–6 h delay compared to wildtype–wildtype counterparts. Chimera demonstrated robust and maintained engraftment of wildtype embryonic stem cell progeny at this pre-implantation stage, and upon intrauterine transplantation differentiated into morphologically-normal, age-appropriate embryos. Staining for *lacZ*, traced stem cell incorporation throughout developing tissues including the heart, brain, somites, pharyngeal arches, and primordial liver at 9.5 dpc. The achieved functional integration justified adult chimera generation. To this end, a high-throughput protocol was required to produce chimeric blastocysts in sufficient numbers due to stress intolerance imposed by the *KCNJ11* deficient background. Full-term embryonic development in pseudopregnant surrogates produced live offspring with various degrees of chimerism. Vulnerability was however realized as the majority of born pups were destroyed, peri-natally, by surrogate mothers. This suggests a selection process that identifies unfit offspring, and eliminates progeny according to perceived maladaptive behaviors. Of note, chimeric pups were apparently larger than non-chimeric littermates suggesting advantage conferred by stress-tolerant wildtype embryonic stem cells during the stressful processes of diploid aggregation and embryogenesis. This study thus transforms a vulnerable knockout morula into a mosaic adult incorporating a competent stem cell load. Engineered knockout–wildtype chimeras provide thereby a previously unavailable tool to examine the contribution of genetic variance in stress tolerance, and anticipate the fitness of the adaptive response during prenatal and postnatal development.

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59. Molecular evolution of a novel hyperactive sleeping beauty transposase enables robust stable gene transfer in vertebrates

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DNA-based transposons are natural gene delivery vehicles. Similarly to retroviruses, these elements integrate into the chromosomes of host cells, but their life-cycle does not involve reverse transcription and they are not infectious. The vertebrate-specific transposon Sleeping Beauty (SB) has opened new opportunities in genome manipulation, including transgenesis, insertional mutagenesis and human gene therapy. Transposons have several advantageous features compared to viral vectors; however, the efficacy of gene delivery has been the bottleneck of their utilization. Since transposons co-exist with their hosts, transposition activity is down-regulated in order to avoid insertional inactivation of essential genes. Thus, transposons are excellent candidates for *in vitro* directed evolution, aiming at the generation of hyperactive mutants.

Recently, using a novel strategy for *in vitro* evolution, we have conducted a large-scale genetic screen to develop hyperactive SB transposases. We derived new versions of the transposase that are significantly (>100-fold) more active in mammalian cells than the prototype. Our best hyperactive transposase, SB100X, has the potential to power up technologies where transposons have already been established as research tools and open up new avenues of applications. It offers an easy and efficient solution for the creation of transgenic animals carrying single copy transgenes, clearly preferred by certain applications. Therefore we optimized the injection conditions, using circular plasmid template and *in vitro* synthesized SB100X mRNA, for BDF hybrid and C57BL6 fertilized mouse oocytes, in an *in vitro* embryo culturing and monitoring arrangement. At the optimal condition we achieved ~45% transgenic embryos on average. Using these conditions the rate of transgenic animals born was similarly high. In general, our transgenic animals showed no apparent sign of mosaicism and carried 1–2 copies of the transgene. Accordingly, we have proven that utilizing a simple method for the preparation of the injected material, SB100X is an extremely effective and safe gene delivery vector for animal transgenesis.

60. Utrophin up-regulation mediated by an artificial transcription factor ameliorate the dystrophic symptoms in *mdx* mice

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Duchenne Muscular Dystrophy (DMD) is a severe muscle degenerative disease, due to the absence of functional dystrophin, for which there is not yet an effective treatment. A promising cure for DMD is based on increasing levels of utrophin, a cytoskeletal protein similar to dystrophin and able to compensate for its absence. Many approaches have been proposed to increase utrophin levels in muscle but the size of the gene (one of the largest known) restrict its use in pre-clinical studies based on gene therapy. The development of small molecules able to up-regulate utrophin *in vivo* is then crucial. To this end we designed a synthetic three zinc finger

based transcription factors (TF), named “Jazz”, which has been demonstrated to drive *in vitro* the transcription of a test gene from the utrophin promoter¹ and then we realized a transgenic mice expressing “Jazz” under the control of a muscle specific promoter. We demonstrated by Chromatin Immunoprecipitation assay (ChIP) that Jazz selectively binds its DNA target sequence on the utrophin promoter and is able to up-regulate endogenous utrophin gene expression in the skeletal muscle as demonstrated by real-time PCR, immunohistochemistry and western blot analyses.²

The transgenic mouse expressing Jazz was then crossbred to the mdx (murine dystrophy X-linked) model of dystrophy and several experiments were performed to check the effects of Jazz-induced utrophin up-regulation on the progression of the disease. Histological and immunochemical analysis of muscles from mdx-Jazz mice demonstrated a reduced degree of necrosis and inflammatory infiltration, together with lower serum levels of creatine kinases (a specific marker of muscle destruction), as compared with the dystrophic mdx mice. Moreover, we observed an enhancement of muscular strength in Jazz-mdx mice, as demonstrated both *in vitro* by electrophysiological analysis and *in vivo* by exercises performance tests (tread mill).

To our knowledge, this is the first example of a transgenic mouse expressing an artificial gene coding for a zinc finger based transcription factor and the results obtained expressing the Jazz-TF in a dystrophic background, indicate that the strategy of transcriptional targeting of endogenous genes could represent a useful tool for the treatment of genetic disease, which still lack a cure.

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61. New approaches to develop mouse model systems toward detection of radiation-induced somatic and germ line mutations

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For the detection of radiation- or environmental carcinogen-induced mutations occurring in somatic or germ cells *in vivo*, specific locus tests, or transgenic mouse systems carrying exogenous marker gene have been adopted. However, in the specific locus tests, detection of mutants has been limited in the tissues where the genes are actively transcribed, while ectopic expression of exogenous marker gene set limits of the detectable size of deletion mutations. To improve such limitations, we plan to develop new assay systems that utilize GFP gene expression upon mutation induction in a specific locus in a living mouse tissue. The principle of the approaches is to make the cells fluorescent when mutation occurred at specified gene loci in the genome. For example, co-expression of two vectors, one produces GFP constitutively and the other produces repressor protein to silence the expression of GFP

gene. In this system, any kinds of forward mutation in the repressor gene allow GFP gene transcription from null state, which should make the mutant cells to glow. Another approach consists of a simple tandem duplication of the structural gene, where the one segment of the duplicate has a GFP gene tag. Once a deletion occurs at one of the duplicated segments, then the active gene containing GFP tag recovers, thereby producing fluorescent protein. We have evaluated the systems in cultured cells and have been generating knock-in mice.

62. Intraoviductal insemination with frozen C57BL/6J sperm increases fertility rate compared to standard IVF

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In vitro fertilization (IVF) with C57BL/6J frozen sperm has shown in general limited success. Some approaches report a significant improvement by selecting motile population prior to freezing (Szczygiel et al. 2002, BOR 67:287–292) or when certain components are added to the freezing solution (Takeo et al. 2008, BOR 78:546–551). The aim of our work was to study if *in vivo* intraoviductal insemination with frozen C57BL/6 sperm can improve fertility rates compared to IVF.

Best conditions for intraoviductal insemination procedure were defined in a first experiment testing six treatments: two insemination volumes, 2.5 and 5 mcl, and three different quantities of spermatozoa placed in each oviduct: 4,000, 20,000 and 100,000. In each one of the five replicates performed, six superovulated females were used, one for each treatment. AI was performed bilaterally under anaesthesia. The following day, 2-cell embryos were collected and fertility was assessed by culture to the blastocyst stage. No significant differences were found among volumes or total number of spermatozoa used. Values ranged between 15% fertility (5 mcl with 100,000) and 49% with 20,000 spermatozoa in 5 mcl.

Frozen sperm from C57Bl/6 and B6CBAF1 in a raffinose skim milk extender as described (Sztejn et al. 2000 BOR 63:1774–1780) was thawed and diluted to achieve 20,000 spermatozoa in 5 mcl and used to inseminate superovulated B6CBA F1 females 13.5–14.5 h after hCG injection. Twenty-four hours later, the number of oocytes and two-cell embryos was recorded. Fertility was further assessed by culture to blastocysts in KSOMaa. *In vitro* fertility rates were assessed through IVF with frozen sperm. Meanwhile IVF with frozen B6 sperm provided 5% fertilization rates (Eight two-cell embryos out of 130 inseminated oocytes), 29% (28 out of 96) of two-cell embryos were collected from intraoviductal inseminated females. In contrast, frozen B6CBAF1 sperm provided 36% fertility (Forty two-cell embryos out of 96 oocytes) compared to 71% fertility (Eighty nine two-cell embryos out of 124 oocytes) by IVF. These results suggest that oviductal insemination may be another method for the selection of viable spermatozoa when IVF fertility rates are reduced. Intraoviductal insemination provides an alternative to the standard IVF-ET to be used with B6 frozen sperm.

63. Genetic deficiency in the renal expression of CD36 increases blood pressure in the spontaneously hypertensive rat

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To search for quantitative trait loci (QTL) involved in the renal pathogenesis of essential hypertension, we combined genome wide expression QTL and quantitative trait transcripts (QTT) analyses of the kidney transcriptome in recombinant inbred strains derived from the spontaneously hypertensive rat (SHR/N). This strategy identified inherited variation in the renal expression of Cd36 encoding fatty acid translocase as a possible determinant of inherited variation in the risk for hypertension. In renal cross transplantation studies in SHR progenitor, transgenic, and congenic strains, selective genetic deficiency of wild type Cd36 in the kidney increased blood pressure. In addition, renal and urine levels of the nitric oxide second messenger cGMP were significantly reduced in the SHR/N progenitor with mutant Cd36 compared to the SHR congenic strain with wild type Cd36. Transgenic expression of wild type Cd36 in the kidney restored both renal and urine cGMP to normal levels and decreased blood pressure. These data provide evidence that a functional deficit exists in the renal nitric oxide system in the SHR/N strain and that this deficit can be repaired by transgenic or congenic expression of wild type Cd36 thereby implicating Cd36 in a novel renal gene pathway influencing blood pressure and the risk for spontaneous hypertension.

64. Heterologous promoter interference affects the expression of transgenes delivered by a bicistronic lentiviral vector

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For the production of transgenic livestock, it is highly desirable the selection of transgenic embryos prior to their transference to a foster mother, avoiding the transference of non-transgenic embryos. This strategy has been successfully accomplished by using lentiviral vectors containing the green fluorescent protein (GFP) gene. However, there are no reports on the use of lentiviral vectors carrying another gene in addition to GFP as a selection marker for such purposes. In this work we constructed two lentiviral vectors, one encoding the GFP under the control of the ubiquitous early SV40 promoter and the other one containing an additional transcriptional unit for the expression of E2 glycoprotein from classical swine fever virus (CSFV), driven by a mammary gland-specific promoter. Direct observation under

fluorescence microscope and flow cytometric analysis showed that the GFP expression was markedly reduced in cell cultures transduced with the bicistronic lentiviral vector. The microinjection of single-cell mouse embryos with the monocistronic lentiviral vector containing the GFP expression cassette rendered GFP positive embryos beginning at the two-cell stage. Of the 31 born mice, 23 (74%) mice carried the transgene DNA and 19 (82.6%) of these mice expressed the foreign protein. In contrast, the microinjection of the bicistronic lentiviral vector did not rendered GFP positive embryos. However, of the 28 born mice, 24 (85%) carried the transgene DNA. Of these, three of the six female assayed expressed E2 in the milk at levels detectable by Western blotting. Previous works informed about the interference among promoters in the context of lentiviral vectors. However, in all of these reports the interference takes place among ubiquitous promoters which presumably compete for the transcriptional factors or their transcriptional activity induce negative or positive supercoiling ahead of polymerases. Our results demonstrate that, in the context of a late-generation lentiviral vectors, a tissue specific expression cassette is able to interfere with the expression of an ubiquitous transcriptional unit. In addition, we can not exclude a possible down-regulation of the mammary specific expression cassette caused by the ubiquitous downstream promoter. These results highlight the importance of designing better lentiviral vectors in order to minimize interferences among promoters.

65. The effects of recipient status on transgenic mouse production

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Pseudo-pregnant female mice (recipients) serve as surrogate mothers to carry micro-injected embryos to term. Yet the potential impact of the interval between the pairing of females with vasectomized males and subsequent embryo transfer on transgenic mouse production has generally received little attention. Therefore, a retrospective study was conducted to assess the effects of this interval on pregnancy rate and efficiency of producing transgenic pups using ICR mice. Data were collected over a five year period at the Ohio State University Transgenic Mouse Core Facility. We compared transgenic mouse production using females paired with vasectomized males on the morning of embryo transfer surgery (0 dpc) with females paired the day prior to surgery (0.5 dpc). Conception frequency was approximately 85% in both cases (1,197/1,428 = 83.8% for 0.5 dpc; 28/33 = 84.8% for 0 dpc). Similarly, the proportion of transgenic pups within litters was not affected by the timing at which mating had occurred (24/151 (16%) vs. 709/5363(13%) for same-day vs. previous-day matings, respectively). Thus, the efficiency of producing pregnancies as well as transgenic offspring does not appear to depend on whether potential recipients are paired and plugged on the day of transfer as opposed to the more traditional method of placing them with males on the preceding

day. In contrast, when vaginal plugs were seen in the morning but could no longer be detected by the time of embryo transfer (mostly late afternoon), the pregnancy rate decreased to 54.2% (13/24) with a concomitant reduction in the appearance of transgenic pups (1/36 (2.8%)). The principal conclusion from this study is that the pseudo-pregnant recipients obtained by pairing of females with vasectomized males on the morning prior to embryo transfer surgery can be used without reservation, while the recipients lost the vaginal plugs should be used with caution.

66. Vitrification of mouse embryos is a useful tool in a mouse transgenic facility

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Introduction The production of transgenic mice is a complex process, requiring the coordinated production of a large number of donor embryos and the availability of appropriately timed pseudo-pregnant recipients. We tested vitrification of mouse embryos as a means of storing excess embryos from donor mice for later use with the hope that these vitrified-warmed (V-W) embryos could be utilized in the event of a donor embryo shortfall. **Methods** Mouse embryos in different developmental stages were equilibrated in cryovials containing 1 M DMSO for 5 min, following equilibration the cryoprotectant DAP213 was added and the cryovials were plunged directly into liquid nitrogen. After 1 week to 10 months, the vitrified embryos were warmed in 0.25 M sucrose/M2 or FHM solution, and cultured 1–2 h in KSOM at 37°C in 5% CO₂ in air. **Results** 98% (350/357) of C57BL6, and BALB/c blastocysts survived V-W. The V-W blastocysts underwent ES cell microinjection utilizing standard techniques and 34.4% (86/250) of injected blastocysts produced live pups with 43% (37/86) of these resulting in chimeras. These results were comparable to control experiments utilizing fresh blastocysts and the same ES-cell clone which generated 34.3% (58/169) live pups, with 46.6% (27/58) of these pups being chimeras. With regard to pronuclear injection, 94% (311/331) of C57BL6 zygotes survived V-W. V-W zygotes were injected with construct DNA by standard methods and cultured overnight and the resulting 138 two-cell embryos were implanted into recipient mothers producing seven live pups (5.1%). These results compare favorably to injection of fresh zygotes with the same construct which produced 18 live pups from 274 implanted (6.5%). **Discussion** Our results demonstrate that preimplantation mouse embryos can be cryopreserved and recovered by vitrification and warming and these recovered embryos are suitable for use in both pronuclear and ES-cell injection. In summary, vitrification is a very simple, fast and reliable method to cryopreserve mouse embryos. In our facility, vitrification has aided in standardizing the number of embryos we inject for investigators and eliminated the number of wasted embryos from unavailable recipients or days when too many embryos are harvested.

67. Conditional expression of calcium channel transgenes in murine smooth muscle

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The TRPC subfamily of mammalian TRP channels forms heteromultimeric complexes consisting of several subunits that form a non-selective cation permeable channel. Of particular interest are channels containing TRPC1/4/5 subunits which may play an important physiological role in vascular disease through their observed sensitivity to lipid and redox status, and causal link to a modulated, non excitable state of vascular smooth muscle cells. Previous in vitro data have shown these channels to have important functional role in smooth muscle cells. These channels have been identified in cardiovascular system and there is substantial evidence for abnormal intracellular calcium levels as a factor in cardiovascular disease.

To investigate these mechanisms in vivo, we are generating a dominant negative TRPC5 (DN-TRPC5) to inhibit the TrpC1/4/5 channel function and a wildtype TRPC5 to increase it. The expression of these genes will be under the control of the Tet-system. Transgenic progeny will be crossed with mice expressing the rTta element under the control of the SM-22 promoter to drive expression in smooth muscle cells. With the addition of doxycycline to the drinking water the expression of the DN-TRPC5 is expected to inhibit TRPC1/4/5 channel function, as shown previously in vitro. The inhibition is expected to be partial, which may reduce risk of compensatory reactions or lethal effects. Expression of the wildtype TRPC5 transgene will provide confirmation for the DN-TRPC5 data and also provide a useful model to study any potential therapeutic value of activators of these channels. The expression of both of these transgenes will be studied on the C57Blk6 and ApoE^{-/-} backgrounds.

Physiological and biochemical analysis of the transgenic mice will dissect the relationships between the smooth muscle cellular phenotype and its function. We will look closely at cell functions that are already linked to TRPC channels, such as migration, invasiveness, proliferation and the role of these channels in the progression of atherosclerotic plaques. Alongside in vitro work in tet-inducible cell lines to identify endogenous activators and modulators of these channels, we hope to gain a better understanding of the role these particular channels play in the cardiovascular system.

68. Pregnancy on demand

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Summary Traditionally, there have been three methods of breeding mice used—Monogamous, Polygamous and Harem. All three are unpredictable regarding timing and colony management. In addition, Monogamous require precise weaning of pups at 21 days to avoid missing the next, postpartum

oestrus, breeding window. It is also, stressful on the females with resultant underweight pups and limited numbers of pups. And Polygamous and Harem will not give precise information on when the pups were born, and, to which mother.

Pregnancy on demand—Every transgenic facility has stud males which are used maximum of 2× per week. Each facility is buying females from vendor at an additional cost. To reduce the cost of buying females, the stud males could be used for a “breeding” colony.

Females are mated with stud males—after injection of 5 I.U.s of PMS and 5 I.U.s of hCG. After mating, they are checked for the presence of plugs and, those that are, are housed four per cage for 2 weeks. These females will be the starters for the new breeding colony “Pregnancy on Demand” You will know—in advance—the due date and also, the litter you obtain from these females will be larger than that produced by natural mating.

A few days before the due date, the mice are separated two per cage, to avoid competition for nesting territory. The mice are kept on a PICO Lab diet 5058. To avoid distocia, they are given Hydrogel (Transgel, Aquagel) along with additional treats like transgenic dough and/or treats from BioServ to avoid cannibalism.

Advantages of pregnancy on demand

Increased number of pups/mother—up to 15 per female.

Reducing the number of cages required—by up to 60%.

Increased precision of DOB of pups—avoids the need for weekend checks.

Increased predictability of colony maintenance schedule.

Moms can be rebred for the second term, one month after parturition.

In house colony eliminates stresses due to transportation.

Savings in costs of females—up to 30% of typical monthly cost from vendor.

69. Derivation and characterization of a C57BL/6Ncrl embryonic stem cell line

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The use of embryonic stem cell (ESC) lines from the inbred strain C57BL/6 as a tool for gene targeting has become increasingly utilized since the availability of such cell lines. The main advantage is the possibility of avoiding a mixed 129/B6 genetic background, which is traditionally obtained in standard gene targeting experiments using 129-derived ESC lines. Here, we report on the derivation of a new germline-competent ESC line (KCTT-9E2) from the C57BL/6Ncrl inbred strain using standard methods. The ESC line is XY with a normal euploid karyotype. For functional testing, we injected ESC of this line into FVB/N blastocysts that were subsequently implanted into pseudopregnant foster mothers. The resulting male chimeras were mated with FVB/N females, which produced agouti offspring indicating germline competence of the ESC. In ongoing gene targeting experiments we are testing the germline capability of targeted clones of the ESC line after standard

electroporation with B6 targeting constructs. This new ES cell line should be a useful complementary tool to the existing C57BL/6 ESC lines for gene targeting and other experiments, particularly those that require the C57BL/6N substrain.

70. Comprehensive production of bi-allelically mutated ES cell bank

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Genetically modified mice can be generated from genetically modified ES cell lines. Since most genetically modified ES cell lines are only mono-allelically mutated, researchers need to make bi-allelically mutated (homozygous) mice with mating to analyze gene functions. However mating scheme is a time consuming procedure. To overcome this difficulty, bi-allelically mutated mice can be directly generated from bi-allelically mutated ES cell lines using tetraploid (4 N) complementation technique.

To date, there is no bi-allelically mutated ES cell bank available. Here, we report a strategy for comprehensive production of bi-allelically mutated ES cell bank. Deficiency of Bloom syndrome gene (Blm) results in high frequency of chromosomal crossing over in the genome. We previously reported that tetracycline-regulatable Blm allele in ES (ES tet/tet) cells allowed high frequency of generation of bi-allelically mutated ES cells. With treatment of doxycycline (an analogue of tetracycline) in ES tet/tet cells bearing mono-allelic mutation, approximately 1 of 4,000 ES tet/tet cells became bi-allelically mutated. To fish out bi-allelically mutated ES cell clones, a retroviral gene-trap vector consisting of splice acceptor (SA)-hygro, lox2272-Neo-Puro-lox2272 (Puro and the second lox2272 were in reverse orientation to Neo and the first lox2272) has been newly constructed. Additionally, 4-hydroxytamoxifen regulatable Cre (ERT2-iCre-ERT2) was inserted into the ROSA26 locus.

Double selections with G418 and puromycin after transient activation of Cre with 4-hydroxytamoxifen resulted in further enrichment of bi-allelically mutated ES cells (~1 of 20 cells). Analyses of double resistant colonies are streamlined to achieve rapid identification of bi-allelically mutated ES cells. Using this protocol, we have started production of bi-allelically mutated ES cell bank.

71. Number of copies, germ line transmission, and expression level of a GFP transgene in mice generated by lentiviral vectors

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Transgenic mice are often obtained by pronuclear microinjection of DNA. However, an alternative method based on the use of lentiviral vector is increasingly used providing a better efficiency of transgenesis. The aim of this work is to characterize genetically the founders and their progeny, obtained by this new method. Also, we correlated the expression between the number of integrated copies and the GFP expression level from three ubiquitous promoters—cytomegalovirus immediate-early enhancer/chicken beta-actin hybrid (CAG), spleen focus-forming virus (SFFV) promoter and ubiquitin promoter—in blood circulated lymphocytes.

Lentiviral vectors carrying a GFP transgene were injected into the perivitelline space of fertilized oocytes, resulting in 52–80% of founder animals carrying the transgene, depending on the titre of lentivirus vector preparations. All founders transmitted the transgene to the germline. We used quantitative PCR to determine the number of copies of integrated transgene. The number of copies varies between 0.1 and 50, and is directly correlated with the titer of the lentivirus vectors. In the progeny of founder animals having less than 1 copy (0.1 to 1 copy) mice possess 1 copy, demonstrating that these founders are mosaics, and that integration occurs probably between two and four cells stages. In a more general manner, some F1 mice present a higher number of integrated copies than their founder suggesting that all founders obtained by this lentiviral transduction method, even with many copies, are mosaics. We determined GFP expression by flow cytometry, in lymphocytes presents in the blood circulation of founders, 90% of mice showed GFP expression. This expression does not depend on the number of copies. Expression of GFP seems to be stable during generations. Nevertheless, we noticed that in certain lines, the expression faded during the generation.

In conclusion, lentiviral transduction allows to obtain very quickly a large number of transgenic mice, widely different in regard of the transgene integration. Associating this method with RNAi, should allow obtaining animals with variable expression levels and thus interesting knock down models.

72. Targeting of Cre activity to subpopulations by use of two promoters and trans-splicing

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The Cre/loxP system has provided researchers with the possibility to investigate the function of genes of interest in specific tissues or cells by overexpression or deletion of genes *in vivo*. However, the more this system is exploited, the more its limitations become obvious. Cell specificity in the Cre/loxP system is achieved by placing the Cre recombinase coding sequence under the control of a cell specific promoter. Hence, only cell populations which can be defined by the activity of one promoter can be targeted by this approach. We therefore sought to broaden the applicability of the Cre/loxP system by controlling functional activity of the Cre recombinase by two

independent promoters. This can be achieved by segmental trans-splicing. This technology makes use of the fact that separate pre-mRNAs can be spliced to one mature mRNA if brought into spatial proximity by use of a hybridisation domain. We created artificial exons of the Cre coding sequence and cloned these into expression plasmids together with intronic sequences providing the proper splice consensus sites, as well as a hybridisation domain. Four pairs of plasmids were constructed, varying in their intronic sequence and the site of the exon 1/exon 2 division. The respective plasmids were tested *in vitro* to assess their efficiency to mediate deletion of loxP flanked sequences. We identified one combination of Cre-exon 1/2 plasmids with high activity in our reporter assays.

73. Olfactory dysfunction in alpha-synuclein transgenic rat: on the way to Parkinson's disease

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Parkinson's disease (PD) is a neurodegenerative disorder that leads to severe motor impairment as well as others earlier troubles such as olfactory dysfunction. The pathology is neuropathologically characterized by the presence of Lewy bodies, whose major component is alpha-synuclein (a-syn). a-syn is known as key protein in PD pathogenesis but its function and its implication in the development of brain disorder remain unclear. To understand a-syn functions and to get a rat model of PD, we developed transgenic animals expressing the double mutated form of human a-syn (ha-synmut) under the control of tyrosine hydroxylase promoter. Protein and mRNA analyses indicate an expression of the ha-synmut in the substantia nigra (SN), the locus coeruleus (LC) and the glomerular layer of olfactory bulbs (OB). The level of expression was lower in the SN as compared to the OB but it increased as the transgenic rats were getting older. The transgenic protein colocalized with tyrosine hydroxylase in the brain and dopaminergic neurons exhibited ha-synmut accumulation. Thioflavin labelling shows a-syn aggregates in the SN, the LC and to a lesser extend, in the OB of aged rat (24 months). Since olfactory dysfunction precedes often motor impairment in PD, olfactory functions was tested in control and transgenic rats at different ages. At birth or young, neither wild-type nor transgenic rats have olfactory dysfunction but deficits were observed in transgenic rats from the age of 6 months up to 2 years old. As a weak neuronal loss was observed in the SN at this time, this new ha-synmut transgenic rat seems to be an attractive model of presymptomatic Parkinson's disease.

74. Integration, expression, and efficiency in mouse bacterial artificial chromosome transgenesis

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Transgenic mice are widely used in biomedical research to study gene expression, developmental biology and gene therapy models. Bacterial artificial chromosome (BAC) transgenes are used to generate animal models that express at physiological levels with same developmental timing and expression pattern as endogenous genes. BACs contain long segments of genomic DNA which often contain regulatory information necessary to provide desired expression patterns. We generated 277 transgenic founders from 55 BAC transgenes prepared by three different methods: ion exchange chromatography, CsCl gradients, and size-exclusion chromatography. Transgenesis efficiency was the same for all BAC DNA purification methods. Polyamine microinjection buffer was crucial for successful transgenesis of intact BACs. Standard injection buffers used with plasmid DNA transgenes resulted in integration of small BAC fragments. We found that the transgenic efficiency of linearized BAC DNA and circular BAC DNA molecules were the same. We found similar transgenic rates, birth rates, and transgenic efficiency when we compared BACs of different sizes (70 Kb to 350 Kb), size did not affect the efficiency of BAC transgenesis. A narrow DNA concentration range generated the highest transgenic efficiency. Too high DNA concentrations reduced birth rates while too low concentrations resulted in higher birth rates and lower transgenic efficiency. Genotyping with multiple markers showed that 65% of transgenic founders carried BAC fragments instead of intact BAC DNA molecules. Expression from BAC transgenes occurred in 43 of 52 transgenes tested. Consistent and reproducible success in BAC transgenesis requires the combination of careful DNA purification, polyamine buffer, pulsed field gel analysis, and sensitive genotyping assays.

75. Conditional inactivation of the TRPV6 channel in mice

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TRPV6 is a member of the superfamily of transient receptor potential (TRP) channels which are involved in a variety of important physiological functions ranging from phototransduction, olfaction, nociception, and heat and cold sensation to epithelial calcium transport. The mouse TRPV6 gene is localized on chromosome 6 flanked by EPH-6 gene and the TRPV5 gene and extends over 15.66 kb. The encoded TRPV6 protein comprises 727 amino acid residues with a calculated relative molecular mass of 83,210 Da. Mouse TRPV6 transcripts are found in pancreas, kidney and placenta. Recently, it could be shown that overexpression of TRPV6 cDNAs in eukaryotic cell lines induces formation of constitutively active channels highly selective for Ca^{2+} . However, such currents

have not been detected in primary cell types so far. To investigate the special role of the TRPV6 gene in its native environment, we used the Cre/loxP recombination system to inactivate the mouse TRPV6 gene both ubiquitously and in a time dependent or tissue-specific manner. Accordingly, we used homologous recombination in embryonic stem cells (R1) to generate mice carrying an allele in which exon 13–15 were flanked by loxP sequences (L3F2). These exons contain the sequence contributing to the selectivity filter of the channel and upon removal of these exons the ion conducting pore of the TRPV6 channel will be deleted. Chimeric males were obtained that transmitted the mutant allele to their progeny. These heterozygous F1 offspring (TRPV6+/L3F2) were crossed to Cre deleter mice (CMV-Cre) to generate TRPV6+/- mice. These TRPV6+/- mice were intercrossed to produce F2 homozygous mice, as confirmed by Southern blot analysis. The mutation was transmitted at Mendelian ratio, suggesting normal fetal and embryonic development of homozygous mutant mice. The inactivation of the TRPV6 gene was confirmed by Northern blot analysis. No transcripts could be detected in poly(A)+ RNA prepared from TRPV6-/- placenta (embryonic day 15.5 and 16.5) using a probe covering the targeted exons 13–15. Intercrosses of TRPV6-/- animals exhibit reduced fertility which might be due to impaired Ca^{2+} transport.

76. A novel human chromosomal vector for mouse transgenesis: study of increased gene dosage in X-linked mental retardation

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It is now generally accepted that microdeletions and microduplications can underlie a wide spectrum of human diseases. In our lab we use a chromosome X-specific tiling BAC array in combination with qPCR, to identify causal copy number variations in patients with X-linked mental retardation (XLMR). In this way, we detected several novel microaberrations harboring only a few genes of which some have been proven to be novel MR genes while others are regarded as MR-candidate genes.^{1–3} Especially duplications of genes seem to contribute to diseases more often than previously anticipated. To validate the genotype–phenotype correlation of duplicated candidate genes we will generate mouse models with the aim to mimic the increased gene dosage. Immunohistological analyses followed by behavioral tests will allow us to study the contribution of the duplicated gene(s) in our transgenic lines.

To generate mouse models that modestly overexpress the gene(s) to the same levels as observed in patients, usually a two-fold overexpression, we are performing pronucleus injection of BAC DNA containing the genes of interest flanked by their regulatory sequences. Selection of several lines with different levels of overexpression could help to dissect the functional importance of increased protein content on cognition. Simultaneously, we are optimizing a human chromosomal vector (CV) as a potential new valuable tool for mouse transgenesis.³ This 4 Mb large circular CV is episomally

maintained within the cell and thus eliminates any adverse interaction with the host genome. Furthermore, the CV stably segregates in vitro as well as in vivo, is successfully transmitted through the germline and allows the site-specific insertion and expression of new genes. Moreover, we showed a human-specific expression pattern of the F3 gene, present on the CV, for which the expression levels were proportional to the average CV copy number per cell. For further optimization we linearized the vector using Cre-loxP-mediated telomere association and demonstrated that this linear CV was 150-times more stable in vitro as compared to its circular counterpart. The presence of functional centromere and telomeres was shown by FISH. Finally, transgenic mice carrying the linear CV clearly demonstrated that the main characteristics of the CV were maintained in vivo as well. We are currently adding an RMCE cassette for efficient introduction of BAC DNA and subsequent validation of our CV as a novel tool for transgenesis with the aim to study gene dosage effects in disease.

1. Van Esch et al AJHG 2005
2. Froyen et al Hum Mut 2007
3. Froyen et al AJHG 2008
4. Voet et al Gen Res 2001

77. Gene targeting in *Xenopus laevis* via site-specific modification of sperm DNA using mobile group II introns (“targetrons”)

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We are developing methods for gene targeting in the frog *Xenopus laevis* by using mobile group II introns (“targetrons”) for site-specific DNA modification in isolated decondensed sperm nuclei, followed by in vitro fertilization to generate genetically modified animals. Mobile group II introns consist

of a catalytically active intron RNA (“ribozyme”) and an intron-encoded reverse transcriptase, which act together in a ribonucleoprotein particle (RNP) to promote site-specific DNA integration. The latter occurs by a remarkable mechanism in which the intron RNA inserts directly into a DNA strand and is reverse transcribed by the intron-encoded protein. Because the DNA target sequence is recognized largely by base pairing of the intron RNA, group II introns can be retargeted to insert into desired sites simply by modifying the base-pairing sequences in the intron RNA. This feature enabled us to develop mobile group II introns into highly efficient bacterial gene targeting vectors with programmable target specificity. To use targetrons for sperm DNA modification, we are adapting the standard *X. laevis* transgenesis protocol in which permeabilized, decondensed sperm nuclei are mixed with a linear donor DNA, then partially digested with a restriction enzyme to make strand breaks, and injected into eggs for in vitro fertilization. This procedure generates hundreds of transgenic embryos per day, but has been limited to random integration. We constructed targetrons that insert site-specifically into three *X. laevis* target genes—Tx1, a transposon present at ~ 150 copies/genome, and two protein coding genes, MitF (microphthalmia-associated transcription factor) and Tyr (tyrosinase)—and developed relatively mild RNP incubation conditions that enable detection of targeted integrations in as few as 50 sperm nuclei for Tx1 and several hundred sperm nuclei for the protein-encoding genes. For Tx1, we used the method to obtain healthy tadpoles with targeted integrations detected in DNA from tail clippings by PCR and confirmed by sequencing. To facilitate screening larger numbers of embryos for targeting protein-encoding genes, we incorporated a GFP-RAM (Retrotransposition-Activated Marker), which is expressed only after DNA integration, and showed that it could be used to detect targeted integration into Tx1. Additionally, we used this marker with a targetron library to obtain embryos with integrations at multiple genomic sites. The methods we are developing for targeted sperm DNA modification in *X. laevis* may be generally applicable to other animals that are amenable to in vitro manipulation of sperm DNA followed by in vitro fertilization.