

## Brinster Spermatogonial Stem Cell Transplantation 25<sup>th</sup> Anniversary Symposium

Philadelphia, August 14, 2019

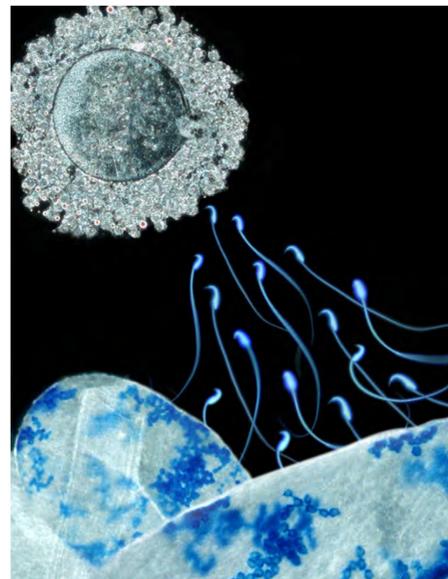
**ABSTRACT** The Symposium, co-sponsored by the Institute of Regenerative Medicine, the University Research Foundation, the Center for Research on Reproduction and Women's Health, the Penn Center for the Study of Epigenetics in Reproduction, and Penn Vet at the University of Pennsylvania, commemorated the 25<sup>th</sup> anniversary of the first publications describing spermatogonial stem cell (SSC) transplantation in mice. This transformative approach has propelled advances in our understanding of germ cell biology, has been translated to a variety of vertebrate species, and holds translational potential for fertility restoration in patients. The symposium opened with a lecture by Dr. Brinster reflecting on the origin of the work, as well as advances over the 25 years up to present ongoing studies. Following Dr. Brinster's remarks, 10 lectures were presented by distinguished scientists, including several of Dr. Brinster's former trainees and colleagues. The symposium closed with a keynote lecture by Dr. David Page. Topics ranged from aspects of basic SSC biology to applications in large animal models and potential translation to treating human male infertility. Many of the studies presented directly resulted from SSC transplantation technology highlighting its tremendous impact in advancing the field. The Symposium program and the lectures can be found at <https://spark.adobe.com/page/jS0cDLzLHvOiJ>

**KEY WORDS:** *spermatogonial stem cell, transplantation, animal model, fertility preservation*

### Introduction

A day long symposium was held at the School of Veterinary Medicine at the University of Pennsylvania to commemorate the 25<sup>th</sup> anniversary of the publication of two landmark papers by Dr. Brinster and colleagues describing for the first time that transplantation of spermatogonial stem cells (SSCs) from a fertile donor mouse to the testes of an infertile recipient mouse can restore fertility and result in donor-derived offspring. This work established SSC transplantation as the only conclusive bioassay for SSCs and it laid the foundation for an ever increasing body of work from insights in the biology of SSCs, SSC fate decisions, and the stem cell niche in the testis, to applications in animal

### IRM/Penn Vet: Frontiers of Germ Cell Research Brinster SSC Transplantation 25th Anniversary Symposium



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Hill Pavilion, 380 S. University Avenue  
School of Veterinary Medicine  
University of Pennsylvania  
Philadelphia, PA 19104

reproduction, germline gene editing, and as an option for fertility preservation in survivors of childhood cancer. The symposium was conceived and organized by Dr. Brinster's former trainees and his colleagues at Penn to recognize these landmark publications and their impact in the field. As Dr. Jeremy Wang pointed out in his welcoming remarks, at the date of the symposium the two 1994 papers had received 1527 and 1100 citations, respectively! Since the publication of these papers, approximately 20 students, while

\*Address correspondence to: Ina Dobrinski. Dept. of Comparative Biology & Experimental Medicine, University of Calgary, Calgary, AB, Canada.  
Fax: +1-403 210 8821. e-mail: [idobrins@ucalgary.ca](mailto:idobrins@ucalgary.ca) - web: <https://vet.ucalgary.ca/idlab/> -  <https://orcid.org/0000-0002-0695-2606>

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in Dr. Brinster's laboratory, published 85 papers with an H index of 55, representing progressive development of the original method. Showcasing the broad impact of SSC transplantation in basic and translational research, the ten invited speakers – several of whom had trained with Ralph Brinster and are now leading research groups in Canada, the US and Japan- illustrated how SSC transplantation has influenced the trajectory of their research in areas ranging from animal agriculture to medicine.

## Synopsis of presentations

### Opening address

#### **Spermatogonial stem cell overview**

by Ralph Brinster (University of Pennsylvania)

Introduced by Dean Andrew Hoffman, Penn Vet, Dr. Brinster reviewed the work that led to the development of SSC transplantation and the milestones of the technology following its initial report. In his trademark understated style, he told the audience that he developed SSC transplantation because he “didn't know much about spermatogenesis”. Needless to say, the audience knew otherwise. Dr. Brinster then briefly reviewed subsequent studies that have established many characteristics of SSCs, notably their ability to be easily cryopreserved, which makes individual males biologically immortal. His group also demonstrated that these stem cells could survive for months *in vitro*, which eventually led to culture techniques and the ability to modify the germline. He then touched on the applications to preserve fertility in prepubertal boys receiving treatment for cancer that will make them sub-fertile or infertile. After the cancer is eradicated, the cryopreserved cells could be grown in culture to increase their number and to remove any malignant cells, and then transplanted back into the patient's testes to restore fertility. Extending the scope beyond SSCs harvested from testicular biopsies he also highlighted studies to reprogram somatic cells into germline cells, particularly to SSCs, that have been initiated by several laboratories. As spermatogonial stem cell modification is applicable to many species, future development of *in vitro* germ cell differentiation and spermatogenesis will present an extraordinary opportunity for genetic alterations in a wide range of species. The ability to harvest, culture, freeze and transplant spermatogonial stem cells will not only allow sophisticated genetic modification but will make individual males biologically immortal. Ongoing work in the Brinster laboratory is exploring transdifferentiation of SSCs from somatic stem cells which would negate the necessity of reprogramming to the pluripotent state for potential applications. In closing, Dr. Brinster acknowledged the contributions of his students, colleagues and collaborators expressing that he feels fortunate and honored to have worked with them. At the end of his presentation and throughout the symposium, it was very apparent that this feeling is mutual.

### **Session 1 - Foundation and applications of SSC transplantation**

Chaired by Jeremy Wang

#### **Germ cell transplantation and beyond: applications in large animal models**

by Ina Dobrinski (University of Calgary)

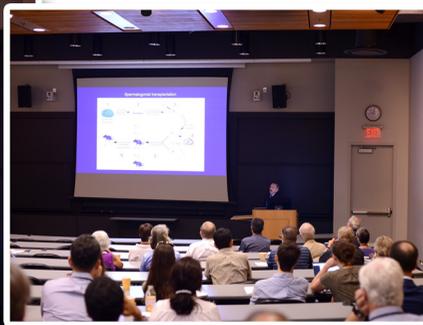
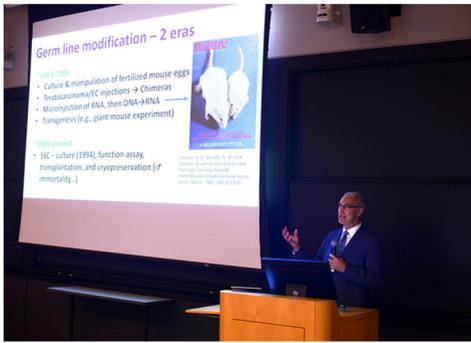
Dr. Dobrinski trained in the Brinster lab before establishing her

own research group first at Penn and now at the University of Calgary. She related that her own fascination with SSC transplantation dated back exactly 25 years to the publication of the 1994 PNAS papers that she discovered when she was looking for a ‘cool’ paper for journal club. Needless to say, she didn't hesitate when offered a position in the Brinster lab three years later and this experience has shaped her independent work ever since. She focused her presentation on applications and advances of SSC transplantation technology in large animal models. Since transplantation of genetically modified germ cells provides a strategy for germline genetic modification, transmission of genetic material has become the main application for germ cell transplantation in large animal models. Following on the first report of xenogeneic spermatogonial transplantation by Brinster's group in 1996, her group developed several platforms to facilitate study of germ cell biology and testis function in mammalian species other than rodents. Grafting of testis tissue to autologous or heterologous hosts recapitulates complete donor spermatogenesis and can serve for fertility preservation. Grafting of isolated cells provides accessibility for cell-type specific manipulation or analysis followed by testicular morphogenesis and reconstitution of functional spermatogenesis. This morphogenic capacity of isolated testis cells has more recently been harnessed to form testicular organoids for the study of cell-cell interactions and spermatogenesis *in vitro*. These approaches ranging from SSC transplantation to testicular organoids represent a powerful toolbox for germline transgenesis, study of testis function and fertility preservation.

#### **Surrogate sires: germline transplantation to enhance livestock production**

by Jon Oatley (Washington State University)

Dr. Oatley was a postdoctoral fellow in the laboratory of Dr. Ralph Brinster at the University of Pennsylvania, and began as an independent investigator in 2007. His research focuses on deciphering the mechanisms that regulate formation of the germline stem cell pool in mammalian testes during development and maintenance of the population in adulthood. Dr. Oatley prefaced his presentation by outlining the growth necessary in animal production to ensure global food security. While artificial insemination has allowed vastly accelerated genetic progress in dairy cattle, alternative approaches may be needed for beef and pigs. He then described a paradigm in which SSCs are removed from a male with desirable genetics and transplanted into the testes of a battery of recipient males as an advanced reproductive technology for enhancing genetic gain in commercial beef cattle and pig production. Realizing this potential requires the generation of surrogate recipient males with testes that completely lack endogenous germline but still possess normal somatic support cell function. To achieve this, he described targeting the NANOS2 gene in mice, pigs, and cattle with CRISPR-Cas9 based editing. Nanos2 knockout mice males are germline ablated but attain natural fertility following transplant with wild-type SSCs. To translate this approach to livestock, they targeted NANOS2 in porcine and bovine embryos. For pigs, NANOS2 knockout boars were found to be sterile due to germline ablation but similar to mice, the testes are capable of supporting regeneration of donor-derived spermatogenesis following transplantation of wild-type SSCs. They are also currently in the process of creating NANOS2 knockout cattle and goats. Dr. Oatley emphasized that these achievements represent key steps in the development of a ‘surrogate sires’ concept



in livestock that will be an invaluable breeding tool for impacting how meat, milk, and fiber are produced to feed an expanding global human population over the coming century.

### **Spermatogonial stem cells, piRNA and monospermic fertilization**

by Jurrien Dean (NIH/NIDDK)

Dr. Dean is an NIH Distinguished Investigator and Chief of the Laboratory of Cellular and Developmental Biology in NIDDK. His investigations are focused on gametogenesis, monospermic fertilization and maternal effects in pre-implantation development. Dr. Dean began his talk by pointing out that hallmarks of spermatogenesis include: 1) maintenance of germline stem cells in adults; and 2) spermiogenesis in which round haploid spermatids transmogry into spermatozoa. He then described two proteins his lab identified to be major players. First, a germ-cell specific cytoplasmic protein, PRAMEF12, which is required for maintenance of stem cells as its genetic ablation restricts spermatogenesis to a single wave after birth. Single cell RNA-seq showed an expression pattern similar to PLZF. Adult males are infertile and mutant mice lacking germ cells provide a model of the human Sertoli-cell-only syndrome. Second, he described a germ cell nuclear protein, BTBD18, that occupies pachytene piRNA-producing loci. Ablation of the gene in mice disrupts piRNA biogenesis, prevents spermiogenesis, and results in male sterility. Transcriptome profiling, chromatin accessibility, and RNA polymerase II occupancy demonstrate that BTBD18 facilitates expression of pachytene piRNA precursors by promoting transcription elongation. He then focused on monospermic fertilization which is essential for the onset of development. One sperm is required, but two are embryonic lethal. A major arbiter of this constricted window of opportunity is the zona pellucida that surrounds ovulated eggs and pre-implantation embryos. Using gene-edited mice, he documented that the N-terminus of ZP2 is necessary and sufficient for sperm binding to the zona matrix independent of glycans. Immediately following fertilization, high concentrations of zinc released from cortical granules inhibit forward motility of sperm. This transient block to zona penetration provides a temporal window for ovastacin, a metalloendopeptidase exocytosed from cortical granule, to complete cleavage of ZP2 which precludes additional sperm binding and ensures monospermic fertilization.

### **Aging of spermatogonial stem cells by Jnk-mediated glycolysis activation: long live the spermatogonial stem cells!**

by Takashi Shinohara (Kyoto University)

Dr. Shinohara graduated from Kyoto University and obtained his M.D. in 1993. He started research under Dr. Tasuku Honjo, cloning human PD-1 in his first paper. He jokingly pointed out that joining Dr. Ralph Brinster's lab as a postdoctoral fellow may have cost him a share in a Nobel prize, but he still thinks that SSCs are far more important than PD-1. While in the Brinster lab, he mostly worked on spermatogonial culture and identification of spermatogonia antigens. He returned to Kyoto University and became a full Professor in 2004. Because spermatogonial stem cells (SSCs) are immortal by serial transplantation, SSC aging in intact testes is considered to be caused by a deteriorated microenvironment. Dr. Shinohara described a cell-intrinsic mode of SSC aging by glycolysis activation. Using SSCs cultured for 60 months, he found that aged SSCs proliferated more actively than young SSCs and showed enhanced glycolytic activity. Moreover, they remained euploid and exhibited

stable androgenetic imprinting patterns with robust SSC activity despite having shortened telomeres. The increased proliferation of aged SSCs was caused by Wnt7b expression, which likely occurred due to decreased polycomb complex-2 activity. Aberrant Wnt7b expression activated c-jun N-terminal kinase (JNK), which downregulated mitochondria numbers by suppressing Ppargc1a, thereby resulting in decreased reactive oxygen species (ROS) and enhanced glycolysis. Analyses of a Klotho-deficient aging mouse model and 2-year-old aged Brown Norway rats confirmed JNK hyperactivation and increased glycolysis. Therefore, not only microenvironment but also intrinsic activation of JNK-mediated glycolysis contributes to SSC aging.

### **Fertility preservation for pre-pubertal boys after chemotherapy-induced infertility**

by Sandra Ryeom (University of Pennsylvania)

Dr. Ryeom is an Associate Professor in the Dept. of Cancer Biology at the Perelman School of Medicine at the University of Pennsylvania. She is the Chair of the Cancer Biology Graduate Group and the Co-leader of the Tumor Biology Program at the Abramson Cancer Center. Her lab is focused on understanding the role of endothelial cells in benign and malignant disease and their contribution to tumor growth and metastatic progression. Gonadal damage is a common and unfortunate consequence of the treatments used to cure pediatric malignancies in boys. Studies have shown that maintaining fertility is a critical aspect of quality of life for these survivors of childhood cancer. Because prepubertal boys cannot produce semen for cryopreservation, fertility preservation options for these patients do not currently exist. Dr. Ryeom described her work that demonstrated that testicular endothelial cells (TECs) are a key population in the SSC niche for SSC self-renewal *in vitro*. Co-culturing human SSCs with human TECs permitted significant expansion and long-term survival of SSCs. Endothelial cells from different organs are transcriptionally distinct, and Dr. Ryeom showed that TECs can be stimulated with FGF-2 to secrete GDNF. She also showed a protective effect of TECs on germ cells after busulfan treatment in mice. For SSCs to be utilized for fertility preservation, they can either be transplanted back into infertile donors or differentiated *in vitro* to generate mature spermatozoa for assisted reproductive approaches. Dr. Ryeom shared preliminary work on culturing human testis cells and TECs as a starting point for *in vitro* differentiation.

## **Session 2 - Emerging and translational research**

Chaired by Marisa Bartolomei

### ***In vitro* gametogenesis in humans**

by Kotaro Sasaki (University of Pennsylvania)

Dr. Sasaki is an Assistant Professor at the University of Pennsylvania School of Veterinary Medicine. Prior to joining the University of Pennsylvania, he completed postdoc training in the Mitinori Saitou Lab at Kyoto University, Japan. His major research interest involves understanding the molecular mechanisms of human germ cell and gonadal development using single cell genomics and their *in vitro* reconstitution using pluripotent stem cells.

The germ cell lineage is the most fundamental component of the life cycle of multicellular organisms, ensuring propagation of genetic information across generations. However, the mechanism underlying germ cell specification in primates, including humans is

largely unknown. Using cynomolgus monkeys as a model organism for early post-implantation development of primate embryos, Dr. Sasaki's group is working to identify the origin and the developmental roadmap of primordial germ cells in primates. In combination with an in-vivo dataset, he described work to reconstitute human germ cell fate *in vitro* from pluripotent stem cells. These studies provide the basis for the mechanistic dissection of the human germ cell specification pathway as well as for reconstitution of human gametogenesis *in vitro*.

**The power of one: novel perspectives of single-cell technologies in gametogenesis**

by Sue Hammoud (University of Michigan)

Dr. Hammoud is an Assistant Professor at the University of Michigan in the Department of Human Genetics. Dr. Hammoud's lab is investigating the cellular and genetic factors required to make a healthy and developmentally competent gamete. Dr. Hammoud showcased the power of single cell RNA-sequencing to uncover the cellular diversity, molecular subtypes and regulatory programs underlying populations of testicular somatic cell types that are necessary for proper germ cell development and maintenance and hence male fertility. She described single cell RNA-sequencing on 35,000 cells from the adult testis, of which ~5,000 single cells were from the somatic compartment to achieve an unbiased analysis of somatic population heterogeneity in the mouse testis. Targeted clustering and marker gene analysis confirmed the presence of all major cell types, and also identified a previously unexpected adult interstitial cell population that expresses Tcf21. The Tcf21 population surrounds the seminiferous tubules. Lineage tracing experiments demonstrate that the embryonic Tcf21 somatic cell population is a cell of origin for multiple somatic cells in both the male and female gonad. *In vitro* these cells can be induced to differentiate into fetal Leydig-like and smooth muscle cells. The regenerative activity of this population is currently be tested.

**Assisted reproductive technologies and adverse perinatal outcomes: the what, why & HOW?**

by Monica Mainigi (University of Pennsylvania)

Dr. Mainigi is the William Shippen, Jr. Assistant Professor of Human Reproduction, in the Division of Reproductive Endocrinology and Infertility at the University of Pennsylvania. Her laboratory focuses on examining the effects of ART on the oocyte, embryo and resulting offspring in both the human and in a mouse model. Greater than 200,000 children in the United States are born yearly with the aid of assisted reproductive technologies (ART). Recent epidemiological studies have suggested that these treatments are associated with an increased risk of adverse perinatal outcomes, including fetal growth restriction, low birth weight, preterm labor, preeclampsia and some rare genetic and epigenetic diseases. Given that many ART treatments, including *in vitro* fertilization (IVF), utilize multiple clinical and laboratory interventions to generate a cohort of embryos capable of implantation and development, it is critical to examine each intervention individually in order to assess its relationship, if any, to the described adverse perinatal outcomes. She described studies in a mouse model of superovulation that showed changes in fetal and placental growth following embryo transfer into a superovulated environment. She also showed an increased umbilical artery resistance and changes in vascular density in these placentas. She conducts parallel studies in human

and finds differences in natural killer cell numbers and function in the endometrium following superovulation. Future studies will utilize co-culture experiments using microfluidics platforms to elucidate underlying mechanisms. These studies are expected to allow us to optimize clinical practices to minimize complications and maximize the chance of a healthy pregnancy and offspring.

**Stem cell therapies for male infertility**

by Kyle Orwig (University of Pittsburgh)

Dr. Orwig is a Professor of Obstetrics, Gynecology and Reproductive Sciences and Director of the Fertility Preservation Program at the University of Pittsburgh School of Medicine. Research in the Orwig laboratory focuses on stem cells, germ lineage development, fertility and infertility, interests he developed during his time in Ralph Brinster's group between 1999 and 2003. Chemotherapy and radiation treatments for cancer or other conditions can cause permanent infertility. Adult patients have the options to cryopreserve eggs, sperm or embryos before treatment that can be used to achieve pregnancies using established reproductive technologies. Those options are not available to prepubertal children who are not yet producing mature eggs or sperm. This is an important human health concern because most children will survive their cancers with their entire reproductive lives still in front of them. Clinics in the U.S. and abroad are cryopreserving ovarian tissues for girls and testicular tissue for boys in anticipation that those tissues can be used in the future to produce mature eggs and sperm. Therefore, the medical and research communities are obligated to responsibly develop next generation reproductive technologies that will enable survivors to use their cryopreserved tissues. Dr. Orwig described his translational research in a non-human primate model progress developing spermatogonial stem cell transplantation and testicular tissue grafting. He demonstrated that live offspring can be generated from sperm produced in autografts and xenografts of cryopreserved primate testis tissue and provided evidence for the safety of germ cell transplantation to restore spermatogenesis in the monkey model stating that these technologies may be ready for translation to the human clinic.

**Keynote Lecture**

**Mammalian germ cells are determined after PGC colonization of the genital ridge**

by David Page (Whitehead Institute, MIT)

Dr. Page is the Director and President of the Whitehead Institute for Biomedical Research, Professor of Biology at MIT, and an Investigator of the Howard Hughes Medical Institute. He is a Member of the National Academy of Sciences, the National Academy of Medicine, and the American Academy of Arts and Sciences. Dr. Page's laboratory explores fundamental differences between males and females in health and disease, both within and beyond the reproductive tract. Dr. Page prefaced his talk by reviewing the incidence of teratoma formation in 129 mice and the history of embryonic carcinoma cells. He then provided evidence that the potential of mammalian primordial germ cells is restricted after they colonize the nascent gonads. In mammals, primordial germ cells (PGCs) are induced in the embryonic epiblast, well before they migrate to the nascent gonads. In fish, frogs, and birds, the germ line segregates even earlier, through the action of maternally inherited germ plasm. Evidence suggests that, across the vertebrata, migrating PGCs

retain a broad developmental potential, regardless of whether those PGCs were induced or maternally segregated. In mammals, this potential is indicated by expression of a network of pluripotency factors, and by the ability to give rise to teratomas and pluripotent cell lines. How the germ line loses this developmental potential remains unknown. Genome-wide analyses of embryonic human and mouse germ lines revealed a conserved transcriptional program, initiated in PGCs after gonadal colonization that demarcates definitive germ cells from soma. Through genetic studies in mice and pigs, he demonstrated that one such gonad-induced factor, the RNA-binding protein DAZL, is necessary *in vivo* for the loss of developmental potential; DAZL's absence prolongs expression of a Nanog pluripotency reporter, facilitates derivation of pluripotent cell lines, and causes spontaneous gonadal teratomas. Based on these observations in humans, mice and pigs, he proposed that germ cells are determined after gonadal colonization in mammals. He suggested that germ cell determination was induced late in embryogenesis – after organogenesis has begun – in the common ancestor of all vertebrates, as in modern mammals, where this critical transition is induced by the somatic cells of the gonad. Failure of this process of germ cell determination likely accounts for the origin of testis cancer in humans.

## Summary

This symposium marking the 25<sup>th</sup> anniversary of SSC transplantation showcased remarkable progress in our understanding of SSC biology, germ lineage specification, testicular microenvironment, translational approaches to harness the power of SSCs for fertility preservation in humans, and genetic modification of animals. It is tempting to speculate what the next 25 years will bring: Germ cell transplantation and associated technologies may find clinical application to restore fertility in survivors of childhood cancer, may inform strategies to achieve human spermatogenesis *in vitro*, may elucidate germ lineage differentiation from pluripotent cells *in vivo* and *in vitro*, and may serve as a platform for germline modification and dissemination of genetic material in domestic animals. Of course, it is the very nature of scientific discovery that the next breakthroughs can hardly be anticipated. Similarly, nobody would have predicted 25 years ago how far we have come after the advent of SSC transplantation.

*Ina Dobrinski<sup>1</sup> and Kyle Orwig<sup>2</sup>*

<sup>1</sup>Dept. of Comparative Biology & Experimental Medicine, University of Calgary, Calgary, AB, Canada;  <https://orcid.org/0000-0002-0695-2606> and <sup>2</sup>Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA;  <https://orcid.org/0000-0002-7952-8419>

**Further Related Reading, published previously in the *Int. J. Dev. Biol.***

**Symposium in honor of Ralph L. Brinster celebrating 50 years of scientific breakthroughs**

Richard R. Behringer and Takashi Shinohara  
*Int. J. Dev. Biol.* (2013) 57: 333-339  
<https://doi.org/10.1387/ijdb.120248rb>

**A history of mammalian embryological research**

H Alexandre  
*Int. J. Dev. Biol.* (2001) 45: 457-467  
<http://www.intjdevbiol.com/web/paper/11417885>

**The use of transgenic animals in biotechnology**

K M Ebert  
*Int. J. Dev. Biol.* (1998) 42: 1003-108  
<http://www.intjdevbiol.com/web/paper/9853831>

**Thirteen years of manipulating the mouse genome: a personal history**

A Bradley, B Zheng and P Liu  
*Int. J. Dev. Biol.* (1998) 42: 943-950  
<http://www.intjdevbiol.com/web/paper/9853825>

**Mouse chimaeras revisited: recollections and reflections**

A K Tarkowski  
*Int. J. Dev. Biol.* (1998) 42: 903-908  
<http://www.intjdevbiol.com/web/paper/9853820>

**Germ cells and germ cell transplantation**

A McLaren  
*Int. J. Dev. Biol.* (1998) 42: 855-860  
<http://www.intjdevbiol.com/web/paper/9853815>

**Transplantation of testis germinal cells into mouse seminiferous tubules.**

T Ogawa, J M Aréchaga, M R Avarbock and R L Brinster  
*Int. J. Dev. Biol.* (1997) 41: 111-122

