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MEETING REPORT

Transgenesis and genome analysis, Nantes, France, June 6th 2011

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On June 6 2011 was held in Nantes, France, the international meeting "Transgenesis and genome analysis" (http://www.ifr26.nantes.inserm.fr/ITERT/TRM/). This meeting is the third one of its kind to be organized by the Transgenic Rats Nantes facility (http://www.ifr26.nantes.inserm.fr/ITERT/transgenese-rat/). The meeting was supported by academic institutions, such as the University of Nantes, INSERM UMR 643, IFR26, CNRS and Biogenouest, as well as private companies, such as Sigma-Aldrich, Stem Cells, Inc and Charles River. Importantly, the meeting received support and co-sponsorship from the International Society for Transgenic Technology. Around 80 participants, from France but also from different European countries, attended the meeting and registration prices were kept as low as possible to facilitate the participation of students.

The meeting aimed to provide an update on recent technical developments in the generation of transgenic animals and in genome analysis. It was intended for Master, PhD and medical students with a background in molecular biology and genetics as an introduction to future work in these rapidly developing areas of research.

Zoltan Ivics (Max Delbrück Center for Molecular Medicine, Berlin, Germany) described the use of transposons, which are discrete pieces of DNA with the ability to change their positions within the genome via a "cut and paste" mechanism called transposition (Mátés et al. 2009; Ivics and Izsvák 2010). Thus, transposons can be viewed as natural DNA transfer vehicles that, similar to integrating viruses, are capable of efficient genomic insertion. Under laboratory conditions, transposons are used as bi-component gene vector systems, in which virtually any DNA sequence of interest (be it a fluorescent marker, an shRNA expression cassette, a mutagenic gene trap or a therapeutic gene construct) can be mobilized by trans-supplementing the transposase in a regulated and highly efficient manner. This methodological paradigm opened up a number of avenues

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for genome manipulations in vertebrates (Ivics et al. 2009) including the generation of transgenic cells in tissue culture, the production of germline-transgenic animals for basic and applied research, forward genetic screens for functional gene annotation in model species and therapy of genetic disorders in humans. Sleeping Beauty (SB) was the first transposon ever shown capable of gene transfer in vertebrate cells, and use of a recently developed hyperactive variant of the SB transposase (SB100X) demonstrated that it is possible to establish a transposon-based, non-viral vector system that is capable of stable gene transfer coupled with long-term gene expression at an efficiency comparable to that of viral strategies. One obvious immediate application of transposon-based technologies is germline transgenesis in laboratory animals. Transgenic animal facilities worldwide could immediately adapt their standard operating procedure to the use of transposon-based plasmid vectors for highly efficient and reliable production of laboratory stocks. The SB100X hyperactive transposon system yields unprecedented stable gene transfer efficiencies following non-viral gene delivery into therapeutically relevant primary cell types, including stem cells (VandenDriessche et al. 2009), and thus may facilitate the clinical implementation of ex vivo and in vivo gene therapies. Indeed, the first clinical application of the SB system will help to validate both safety and efficacy of this approach. Further, it is now becoming amenable to create highly complex libraries of gene knockouts and to thereby establish new models of human disease for therapeutic and pharmaceutical intervention in animal models (Izsvák et al. 2010). Finally, recent advances in iPS reprogramming should facilitate the identification of genetic determinants involved in physio-/pathological pathways in cells derived from patients with specific genetic diseases. Thus, transposon-based technologies have enormous potential to develop powerful genomic tools with the vision of creating a bridge between physiology and genetics (Grabundzija et al. 2011).

Philippe Duchateau (Cellectis, Romainville, France) gave an overview of meganucleases for genome engineering (Galetto et al. 2009). Rare cutting endonucleases have emerged as powerful tools for precise genome engineering. Very different types of proteins have been used as designable scaffolds to generate artificial endonucleases cleaving chosen sequences, including Zinc Finger Nucleases (ZFNs), natural meganucleases from the I-SceI family (MNs), and more recently, Transcription Activator-Like Nucleases (TALENs). Natural meganucleases, also called Homing Endonucleases (Traver et al. 2009), are the most specific endonucleases in nature, and thus, should provide ideal scaffolds for the creation of new genome engineering tools. We have conducted a genome-wide study to characterize the potential of the meganuclease platform. Our results demonstrate that (i) efficacy of MNs induced genome editing is locus-dependent (Muñoz et al. 2011), with epigenetic modifications heavily impacting the process (ii) cleavage by MNs can induce targeted mutagenesis (TM) or homologous gene targeting (HGT) with the HGT/TM ratio being very stable throughout the genome (Grizot et al. 2011; Paredes and Maggert 2009). Moreover, several MNs were tested for their ability to induce targeted modifications in primary cells, including stem cells. Depending on the method of vectorization, frequencies could reach several percent.

Ignacio Anegon (Director of INSERM UMR 643 and of the Transgenic Rats Nantes facility) gave an update about the generation of genetically engineered animals using zinc-finger nucleases (ZFNs). The generation of genetically modified animals or plants with gene-targeted deletions or modifications is a powerful tool to analyze gene function, study disease and produce organisms of economical interest. Until recently, the generation of animals with gene targeted manipulations has been accomplished by homologous recombination (HR) in embryonic stem (ES) cells or cloning through nuclear transfer and has been limited to a few species. Recently, a new technology based on the use of gene-targeted ZFNs was developed and used for the generation of organisms with gene-targeted deletions and/or modifications when combined with HR (Rémy et al. 2010). The presentation covered the use of ZFNs for the genetic engineering of animals, with particular emphasis on their recent work involving the generation of gene-specific knockout rats (Geurts et al. 2009, 2010: Menoret et al. 2010) and ongoing work on ZFN-promoted HR in rat zygotes. Microinjection of plasmid or mRNA for ZFNs into rat zygotes allowed targeted, rapid, complete, permanent and heritable disruption of several endogenous loci. The application of ZFNs to generate gene-targeted knockouts in species where

ES cells or cloning techniques are not available is an important new development to answer fundamental biological questions and develop models of economical interest such as for the production of humanized antibodies. Further refinements of ZFN technology in combination with HR may allow knock-ins in early embryos even in species where ES cells or cloning techniques are available. The new Transcription Activator-Like Nucleases (TALENs) have been also used to generate KO rats at very high rates of efficiency by microinjection of mRNA or plasmid DNA encoding for TALENs (Tesson et al. 2011).

Axel Schambach (Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany) who described the recombinant retroviral vectors (Baum et al. 2006) based upon alpha-, gammaretroviruses and lentiviruses represent well characterized tools for the correction of inherited disorders by stable gene transfer. Especially their feature to stably transduce a variety of cells offers a broad spectrum of potential applications in basic biology and regenerative medicine. In the first part of the talk, he explained how to convert an infectious agent into a vehicle for therapeutics (Schambach and Baum 2008) or stem cell modification (Schambach et al. 2010) and list the advantages and disadvantages of specific retroviral families, e.g. why lentiviral vectors are capable of infecting non-dividing tissues. For translating retroviral and lentiviral vectors into the clinical arena, the risk for severe adverse events related to insertional mutagenesis still needs to be overcome and should be taken into account a priori when designing vectors for gene therapy or stem cell modification. So called self-inactivating (SIN) vectors, which lack enhancer/promoter sequences in the U3 region of the long-terminal repeats, showed a more beneficial safety profile and allowed the inclusion of promoter elements of choice (e.g. tissue or lineage specific promoters). The talk focused on how a retroviral vector needs to be constructed to make it a safe delivery tool. Points to consider when setting up the vector architecture and the different modules which can be incorporated into a SIN vector to reach a therapeutic window were explained with emphasis on novel developments and concepts on vector design (Warlich et al. 2011).

Tuan H. Nguyen (INSERM UMR 948, Nantes, France) described lentiviral vectors production for transgenesis and as new tools for episome and protein delivery**.** Lentiviral vectors have become essential research tools owing to their ability to efficiently transduce non dividing and dividing cells, to shuttle and integrate small as well as large genetic element and maintain stable long-term transgene expression. One of the most interesting developments in the applications of lentiviral vectors is their use for generating transgenic animals as an alternative method to standard DNA pronuclear injection (Singer and Verma 2008). The advantage of lentiviral vector-mediated transgenesis is that it is simple, rapid, versatile and highly efficient. This technology has for instance generated transgenic animals from various species, including transgenic nonhuman primates. The Rat Transgenic Nantes facility has established a platform of genetic engineering and lentiviral vector-mediated rat transgenesis (http://www.ifr26.nantes.inserm.fr/ITERT/), which provides an all-round services including lentivector construction, high quality and titre production of lentiviral vectors, the generation of transgenic rat using pervitelline injection of oocytes, and the genotyping of animals (Rémy et al. 2010a, b). He presented an overview of the state of the art technology for lentiviral vector production and purification. Examples of lentiviral transgenesis were given by the generation of transgenic rats. In the last part, he presented recent developments in the field of lentivectorology showing that lentiviral vectors can be a platform for efficient delivery of circular episomes or of proteins (Sarkis et al. 2008). These new generations of lentiviral vectors will be helpful for applications requiring a transient transgene expression, for instance gene editing in embryos with meganucleases, zinc-finger nuclease, Cre, etc. for generation of transgenic animals.

Laurent Tesson (Transgenic Rats Nantes facility, INSERM UMR 643, Nantes, France) reviewed the technical aspect for the analysis of transgene integration: insertion site identification and copy number quantification. Genetically engineering models have been created to study diseases or gene function. DNA pronuclear injection or retroviral delivery systems were used to make transgenic animals with success. For both techniques, transgene integration is a random event within the genome, often with insertion of variable copy number in a single site for DNA pronuclear injection or with insertion of a single copy at multiple sites for lentiviral vector-mediated transgenesis. Insertion site identification can have practical

benefits: it allows following segregation of multiple transgene integration sites in the progeny, it enables the development of efficient genotyping assays (zygosity assay), and it facilitates the characterization of positional effects on phenotype. PCR-based methods have been developed to find a determined sequence upstream or downstream from the known transgene sequence into the genome. These methods are referred as genome or chromosome walking. Four major methods are used: random PCR, inverse PCR, panhandle PCR and ligation-mediated PCR (LM-PCR). Many improvments (Tonooka and Fujishima 2009; Bryda and Bauer 2010) were done since publications and LM-PCR seems to be the standard method but not the universal one. Depending on the founder line, adoption of different methods could be advisable. All these methods have both advantages and disadvantages but can be performed in any lab with minimal molecular biology expertise. Copy number quantification and zygosity could be performed by real-time PCR with accuracy and speed. The use of the comparative cycle threshold method (2-ΔΔCt) achieves detection of two-fold differences (Tesson et al. 2010). This technique could be helpful in the establishment of breeding programs for transgenic colonies and in experiments in which gene dosage effects could have a functional impact.

Michael Bader (Max-Delbrück-Center for Molecular Medicine (MDC), Berlin-Buch, Germany) presented data on the transgenic rodent models for hypertension research**.** The research on pathophysiological mechanisms involved in the etiology of cardiovascular diseases, including hypertension and diabetes, requires the use of whole animal models. Besides genetically hypertensive rat strains, such as the spontaneously hypertensive rats (SHR), rodents with targeted alterations in their genome have proven to be valuable models to study these diseases and develop novel therapeutic strategies. Transgenic rats overexpressing mouse renin and transgenic mice overexpressing rat angiotensinogen became hypertensive supporting the important role of the renin-angiotensin system (RAS) in cardiovascular diseases (Mullins et al. 1990, Kimura et al. 1992). Local overexpression of angiotensin-converting enzyme 2 (ACE2) in the vascular wall of SHR rats significantly ameliorated their hypertension and verified the important role of the ACE2/angiotensin(1–7)/Mas system as counterregulator of the classical RAS (Rentzsch et al. 2008). Using additional transgenic and KO animal models for this system, furthermore, its relevance for metabolic control was revealed (Santos et al. 2010). Based on these findings they tested an orally active angiotensin(1–7) compound in a novel transgenic rat model for diabetes type II (Kotnik et al. 2009) and showed its antidiabetic activity. Thus, generating genetically modified rodents, they could establish novel disease models and define the ACE2/angiotensin(1–7)/Mas system as a novel potential drug target for hypertension, diabetes and the metabolic syndrome.

Gilles Blancho (INSERM UMR 643-Institut de Transplantation—Urologie—Néphrologie CHU Nantes–France) gave an overview on the use of cloned and transgenic animals in xenotransplantation. Xenotransplantation is transplantation between individuals from different species. It is in fact an old procedure initiated in patients in the early twenteeth century, by pioneer surgeons that at least allowed showing the technical feasibility of a vascularized transplantation. Its interest regained in the 90th, because of a constant and worldwide organ shortage (in case of success, xenotransplantation could offer an unlimited supply) and because of the development of the new techniques such as transgenesis and gene therapy in large animals (Le Bas-Bernardet et al. 2008). In fact, the pig has been identified as the potential donor of organs and not primates because of important ethical issues and risks of virus transmission. Whereas pigs offer advantages, such as size and physiology compatibilities with humans, rapidity and easiness of reproduction, less ethical issues, they bring nevertheless drawbacks due to immunological discordance and also a viral risk. The immunological discordance is mainly due to the existence in humans of preformed antibodies (Ab) directed against an antigen (Ag) that is a disaccharide galactose α1-3 galactose (Gal) expressed on the majority of mammalian cells. Once anti Gal Ab bind their specific target, they activate endothelial cells and complement leading to a rejection so fast that it is called hyperacute rejection (HAR). Transgenic pigs expressing human complement regulatory molecules have been generated and have brought an advantage by being protected from HAR, but nevertheless are secondary rejected. Lately, Gal KO animals have been generated and are currently under investigation. The most promising

trend of xenotransplantation is currently concerning pancreatic islets and neurons that are obviously less rejected.

Abdelhadi Saoudi (INSERM UMR1043, CNRS UMR5282 & University Toulouse III, Toulouse, France) described a strategy to identify in rodents susceptibility genes of immune diseases in humans. Immune diseases, such as autoimmunity and allergy, represent major health problems. Compelling evidence shows that these diseases are influenced by environmental and genetic factors. Although extensive efforts have been put into linkage and association studies of large human patient cohorts, little is known about the non-MHC genes that modulate immune diseases. The identification of such genes in humans is hampered by the genetic heterogeneity of human populations and studies on animal models can overcome these difficulties. In this regards, LEW and BN rats, that show an inverse Th1/Th2 polarization and differ in their susceptibility to immune diseases, represent a valuable model (Bernard et al. 2010). Th1-mediated autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), can be induced in LEW, but not in BN rats. BN rats are susceptible to Th2-mediated allergic disorders to which LEW rats are resistant (Mas et al. 2004). They identified in (LEW x BN) F2 intercrosses one locus on chromosome (c) 9 named *Iresp3* that exerts a major control on heavy metals induced Th2-mediated disorders. This locus co-localize with *Eae4* that controls the susceptibility to EAE. Reciprocal BN/LEW c9 congenic lines have refine the localization of *Iresp* 3 to 117 kb. Fine mapping of this region using LEW and BN interval-specific congenic rat lines, and association studies of single-nucleotide polymorphisms with inflammatory phenotypes, led to localize the gene of influence to *Vav1*, which codes for a signal-transducing protein in hematopoietic cells. Vav1 from BN rats bears a tryptophan in position 63 ($Vav1^{63W}$) instead of an arginin in the LEW rat ($Vav1^{63R}$). This polymorphism is responsible for Vav1 constitutive activation, revealed by its tyrosine hyperphosphorylation and increased guanine nucleotide exchange factor activity. Moreover, this polymorphism induces a marked reduction in Vav1 protein levels, associated with a reduction of calcium mobilization after TCR engagement. Thus, these data indicate that the LEW $Vav1^{63R}$ variant predisposes to Th1-meditaed EAE and protects from Th2-mediated $HgCl_2$-induced IgE response, while the BN Vav1-63 W variant shows opposite effects. On these bases, studies conducted on seven human cohorts (12,735 individuals) demonstrated an association of rs2546133-rs2617822 haplotypes in the first VAV1 intron with multiple sclerosis (CA: odds ratio, 1.18; CG: odds ratio, 0.86; TG: odds ratio, 0.90). The risk CA haplotype also predisposed for higher VAV1 messenger RNA expression. VAV1 expression was increased in individuals with multiple sclerosis and correlated with tumor necrosis factor and interferon-γ expression in peripheral blood and cerebrospinal fluid cells (Jagodic et al. 2009). Together, these data show that animal models through comparative genomics can point to disease genes that genome wide association studies failed to hit.

Kathryn Blair (Wellcome Trust Center for Stem cell Research, Pembroke College, Cambridge University, England) described rat embryonic stem cells: understanding the biology of a new tool for genetic modeling. Embryonic stem (ES) cells were first derived from pre-implantation mouse embryos in 1981 (Evans and Kaufman 1981; Martin 1981) and are defined by their capacity to self-renew (Ying et al. 2008) and to contribute to all adult tissues, including the germ line, upon reintroduction to the embryo. It took 27 years to derive verified, germline competent, embryonic stem cells from another species: the rat. When the Erk-pathway and Gsk3β are inhibited by small molecules in the presence of feeders, rat ES cells can be derived and expanded while maintaining pluripotency marker expression and chimera contribution capacity comparable to mouse ES cells. The advent of rat ES cells presents new opportunities for genetic studies in this valuable model organism (Hirabayashi et al. 2010, Kawamata and Ochiya 2010; Blair et al. 2011). However, rat ES cells have so far proved less karyotypically stable and less robust in long-term culture than mouse ES cells (Buehr et al. 2008 and Li et al. 2008). Preliminary evidence also suggests that rat ES cells may be in a more permissive state than mouse ES cells, which allows transit to extra-embryonic lineages. Understanding the biological mechanisms that underlie differences in mouse and rat ES cell behavior will hopefully lead to the development of defined, optimized conditions for their self-renewal and enhance their usefulness as a tool for genetic studies. The development of optimized conditions for the culture

of rat ES cells may in turn help us to capture the pluripotent state in species which have been recalcitrant to ES cell derivation.

Ludovic Vallier (Anne McLaren Laboratory For Regenerative Medicine, Department of Surgery Department, Cambridge University) gave an overview in genome editing in human pluripotent stem cells. Human pluripotent stem cells can be generated from embryos at the blastocyst stage (human embryonic stem cells or hESCs) or from reprogrammed somatic cells (induced pluripotent stem cells or hIPSCs) (Rashid et al. 2010a). These cells combine the property to grow indefinitely in vitro and the capacity to differentiate into a broad number of cell types. Thus, human pluripotent cells represent a unique opportunity for regenerative medicine since they could enable to production of infinite quantity of cell types with a clinical interest such as liver and pancreatic cells. Importantly, hIPSCs could allow the production of patient specific cell types which are fully immuno-compatible with the original donor thereby avoiding the use of immune suppressive treatment during cell based therapy. However, the use of hIPSCs in the context of genetically inherited human disease will require correction of disease-causing mutations in a manner that is compatible with clinical applications. The methods currently available, such as homologous recombination, lack the necessary efficiency and also leave residual sequences in the targeted genome. His group has established a solid experience with hIPSCs by deriving more than 300 lines from 50 patients suffering from diverse diseases (Vallier et al. 2009 and Rashid et al. 2010b). In addition, his group has developed an approach to edit the mammalian genome of hIPSCs using s combination of ZFNs and *piggyBac* technology in hIPSCs. Using this approach, he successfully achieves bi-allelic correction of a point mutation (Glu342Lys) in the *SERPINA1* gene that is responsible for a_1-antitrypsin deficiency (A1ATD) in hIPSCs. Genetic correction of hIPSCs restored the structure and function of a_1-antitrypsin in subsequently derived liver cells in vitro. These results provide a proof of principle for the potential of combining hIPSCs with genetic correction to serve as a platform for autologous cell-based therapies for genetic disease.

In conclusion, the 3rd transgenesis meeting of the Transgenic Rats Nantes facility was rich with new information and with ample opportunity for discussions and exchanges. The overall very positive balance of this meeting by the quality of scientific presentations and the number of participants are incentives to renew the experience in 2 years.

The local organizing committee hopes to welcome many participants in 2013 to the 4th transgenesis meeting organizing by the Transgenic Rats Nantes facility.

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