

Induced pluripotent stem cells: production and utility in regenerative medicine

The BioPark Hertfordshire, Welwyn Garden City, AL7 3AX: 7th October 2010

"The production of iPS cells from dermal fibroblasts has generated intense interest in the utility of such cells for research purposes and clinical applications. iPS cell production currently requires the use of transcription factor gene delivery to reprogramme cells into iPS cells. Hence, both gene delivery technology and iPS cell characterization and subsequent cell differentiation are critical aspects of iPS cell biology. This meeting will address both issues" Meeting Chairs: *Professor Peter Andrews*, University of Sheffield, UK and *Dr Chris Denning*, University of Nottingham, UK

This event has CPD accreditation

9:00 – 9:30 **Registration**

9:30 – 9:40 **Introduction by the Chairs:** *Dr Chris Denning*, University of Nottingham, UK and *Professor Peter Andrews* University of Sheffield, UK

Chair: *Professor Peter Andrews* University of Sheffield, UK

9:40– 10:10 **Induction of pluripotency in adult stem cells**
Dr Holm Zaehres, Max Planck Institute, Germany

10:10 – 10:40 **High throughput iPSC derivation using LEAP**
Dr Scott Cribbes, European Application Support Manager for CynTellect Inc

10:40– 11:10 **Development of human ES and iPS cell models for Huntington's Disease**
Nicholas Allen, Cardiff University, UK
Huntington's disease (HD) is a devastating neurodegenerative disorder. Despite the fact that the HD gene, with its mutant CAG expansion, was cloned in 1993 and numerous animal models have provided extensive understanding of the disease, relatively little progress has been made in translating findings to develop HD therapeutics. The recent development of ES and iPS technologies now allows human cell models to be developed that will hopefully provide novel and more efficacious platforms for disease analysis and drug discovery, that will complement existing animal studies. We are developing cell models for HD based on the directed differentiation of human HD ES and iPS cell lines to forebrain neurons. Available HD human ES cell lines carry mutant HD alleles with 40-45 expanded CAG repeats, whilst iPS cell lines allow more extensive allelic series of HD alleles to be studied, for example up to 180 CAG repeats. The goal is to establish HD disease models, through biomarker analysis and in vitro phenotypic screens, and to develop robust assays for HD dysfunction suitable for future drug screens.

11:10-11:15 **Speakers Photo**

11:15 – 11:35 **Morning Break**

11:35 – 12:05 **Human ESCs into specific endodermal cell types**
Dr Ludvic Vallier, Laboratory for Regenerative medicine, University of Cambridge, UK
Generation of hepatocytes from human embryonic stem cells (hESCs) could represent an advantageous source of cells for cell therapy approaches as alternative to liver transplantation. However, the generation of hepatocytes from hESCs remains a challenge especially using conditions compatible with clinical applications. Here, we report a novel method to differentiate hESCs and hiPSCs into hepatic cells using defined culture system, which recapitulate essential stages of liver development. Importantly, the cells generated under these conditions exhibited hepatic functions in vitro and in vivo. Therefore, this approach represents toward the generation of hESCs derived hepatic cells for cell based therapy.

12:05– 12:35 **Stem cell states and the single cell**
Professor Peter Andrews, University of Sheffield, UK
After prolonged culture, ES cells are subject to the selection of genetic variants. Accumulating evidence suggests that the 'stem cell compartment' in both ES and other stem cells may be composed of distinct substates. One aspect of culture adaption is that it alters the population dynamics of ES cultures, particularly affecting the behavior of substates within the stem cell compartment. Understanding the nature of these substates may provide insights into the mechanisms that control self renewal, commitment to differentiation and lineage selection of ES and, ultimately iPS cells. Inevitably these same mechanisms may also play a role in cancer progression

12:35 – 1:05

Derivation and utility of cardiomyocytes from human pluripotent stem cells

Dr Chris Denning, University of Nottingham, UK

We have demonstrated that functional cardiomyocytes can be derived from human embryonic stem cells, potentially offering a novel cell source for drug screening, disease modelling and cell replacement. However, before these goals can be realised, several issues must be tackled. We have sought to standardise feeder-free culture methods that function in 14 hESC lines derived in 5 different countries, impacting on the ability to improve downstream technologies. Thus, we have demonstrated industrial scale automation of hESC culture to meet demands of commerce. Standardised culture also provides a platform from which differentiation to the cardiac lineage can be improved and directed. Moreover, high efficiency genetic modification has been demonstrated in 11 hESC lines, potentially providing new routes to RNAi library screening for genome analysis. We have also generated transgenic hESC lines that express puromycin N-acetyltransferase from the cardiac specific MYH6 promoter, allowing enrichment of cardiomyocytes to close to 100% purity by incubation with the antibiotic puromycin. This set of technologies is now being applied to proof of principle studies in drug screening and engineering in vitro disease models produced either by genetic modification or by exploitation of induced pluripotency (iPS) technology.

1:05– 2:00

Lunch and poster viewing

Chair: *Dr Chris Denning, University of Nottingham, UK*

2:00 – 2:30

Novel human iPSC lines for Spinal Muscular Atrophy

Dr Rafael J. Yáñez-Muñoz, Royal Holloway-University of London, UK

Spinal muscular atrophy is a neurodegenerative disease in which the death of spinal motor neurons leads to progressive paralysis, with severity ranging from difficulty in walking to death in early childhood. Human motor neurons are not available from patients, but can be generated from iPSCs, which allows in vitro studies that would not be possible otherwise. We have generated novel iPSC lines from several members of a consanguineous family in which members display a variety of disease severities despite being identical at the known loci relevant to the disease. We are currently characterising these cell lines.

2:30 – 3:00

Lentiviral vector-induced genotoxic reprogramming in the absence of ectopic transcription-factor expression.

Dr Nicole Kane, University of Glasgow, Scotland

Retroviral vectors (based on gammaretroviral or lentiviral (LV) backbones) have widely been employed as a gene transfer system for somatic cell reprogramming to induced pluripotent stem (iPS) cells due to their high gene transfer efficiency, and enhanced rate of reprogramming when compared with non-integrating viral vectors or non-viral systems. However, mutagenic effects of retroviral vectors are well documented in both laboratory and clinical gene therapy studies, principally as a result of dysregulated host gene expression in the proximity of specific integration sites. Ergo, it is possible that vector genotoxicity can contribute to reprogramming of somatic cells, either alone or in combination with expression of relevant TFs. We report induction of a pluripotent-like state from normal human fibroblasts in the complete absence of ectopic expression of pluripotency/reprogramming factors but considerable transcriptional changes and insertional mutagenesis-associated detrimental changes in genomic structure and integrity induced by LV-mediated GFP gene transfer. This study demonstrates that vector induced effects may also play a substantial role in iPSC derivation and that a more complete understanding of molecular mechanisms underlying iPS reprogramming is required. This is an important consideration when analysing data from iPS cells obtained from integrating systems even in circumstances where the majority of the proviral construct is excised, and has implications for interpretation of studies using iPS cells.

3:00 – 3:30

Reprogramming: from Technology to Biology

Dr Keisuke Kaji, Edinburgh University, Scotland

We have developed a non-viral reprogramming system with multiprotein expression vector and Piggybac transposon/transposase in 2009. In addition to improving the strategy, currently we are using the system to understand the mechanism of the reprogramming process, how the cells go back to the pluripotent state by expression of Oct4, Sox2, Klf4 and c-Myc.

3:30 – 3:50

Afternoon Tea/Coffee

3:50– 4:20

Extrinsic Factors in Human Embryonic Stem Cell and induced Pluripotent Cell Maintenance

Professor Sue Kimber, University of Manchester, UK

Our understanding of how to maintain human Embryonic Stem cells in culture with and without feeder cells is growing. hESC are poised to proliferate, initiate differentiation or die depending on extrinsic signals regulating their behaviour. In this talk I will outline our findings on how different exogenous factors from the medium and substrate environment impinge on the cells to influence their fate.

4:20 – 4:50

Application of induced pluripotent stem cells in modelling human immunodeficiency disorders

Dr Sayandip Mukherjee, UCL , London

Induced pluripotent stem (iPS) cells generated from patient samples can potentially provide a platform for dissecting the molecular mechanisms of inherited disorders, design of drug screening protocols, and also for testing the efficacy and safety profiles of gene replacement therapies. We have focussed on chronic granulomatous disorder (CGD) which is a rare inherited neutrophil disorder and affects four in a million. iPS cells generated from skin biopsies of CGD patients will be employed for studying the efficacy of lentiviral vectors delivering a codon optimized gp91phox transgene, and as a proof of principal study to establish their application in bone marrow reconstitution experiments in murine models of CGD.

4:50 - 5:00

Chairmans's summing up

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About the Chairs

Professor Peter Andrews received his D.Phil from the University of Oxford in 1975. Following postdoc positions at the Institut Pasteur in Paris and Sloan-Kettering Institute in New York, he was a research scientist at the Wistar Institute in Philadelphia. He is currently the Arthur Jackson Professor of Biomedical Science in the University of Sheffield, where he is also co-director of the Centre for Stem Cell Biology. His research has focused both on embryonal carcinoma cells, the malignant counterparts of embryonic stem (ES) cells, as well as on the biology of human ES cells themselves.

Dr Chris Denning- PhD in Cancer Gene Therapy at Beatson Institute for Cancer Research, University of Glasgow, 1997; Postdoctoral Research Fellow - gene targeting in mouse ES cells, Institute for Stem Cell Research, University of Edinburgh, 1997-1998; Postdoctoral Research Fellow - gene targeting in somatic cells; first targeted gene disruption in animals other than mouse, Roslin Institute 1998-2001; Principal Investigator, University of Nottingham, 2001-2003; Medical Research Council Fellow in Stem cell biology, University of Nottingham, 2003-2006; Lecturer in stem cell biology, University of Nottingham, 2006-2008; Associate Professor & Reader in stem cell biology, University of Nottingham 2008

About the speakers

Professor Hans Schöler is a Director of the Max Planck Institute for Molecular Biomedicine in Münster (Germany), Professor of the Medical Faculty of the Westfälische Wilhelms-Universität Münster, and Adjunct Professor of Biochemistry at the University of Pennsylvania, School of Veterinary Medicine, Department of Animal Biology in Philadelphia (USA). Professor Schöler majored in Biology in Heidelberg (Germany), receiving his diploma in 1982. In 1985 he was awarded the Ph.D. degree "summa cum laude" at the Center for Molecular Biology in Heidelberg under supervision of Professor Dr. Peter Gruss. From 1986 until 1988 Professor Schöler was head of a research group at Boehringer Mannheim (now Roche) in Tutzing, and from 1988 until 1991 he worked as a staff scientist at the Max Planck Institute for Biophysical Chemistry in Göttingen. In 1991 he commenced as head of a research group at the European Molecular Biology Laboratory (EMBL) in Heidelberg. In 1999, Professor Schöler moved to the University of Pennsylvania (USA), where he served as Professor for Reproduction Physiology at the „School of Veterinary Medicine“ and Director of the „Center of Animal Transgenesis and Germ Cell Research“. Professor Schöler returned to Germany in 2004, where he since is director of the Department for Cell and Developmental Biology at the Max Planck Institute for Molecular Biomedicine in Münster. The main research interests of Professor Schöler are molecular biology of cells of the germline (pluripotent cells and germ cells), transcriptional regulation of genes in the mammalian germline, molecular development of reprogramming the genome of somatic cells after nuclear transfer into oocytes or fusion with pluripotent cells. Since 2005, Professor Schöler is head of the Managing Board of the Stem Cell Network North Rhine-Westphalia, and he was appointed Member in the central ethics committee for stem cell research ("Zentrale Ethik-Kommission für Stammzellenforschung").

Dr Nicole M Kane is a Postdoctoral Research Associate at the British Heart Foundation Glasgow Cardiovascular Research Centre at the University of Glasgow. Her research is focused on the genetic manipulation of human embryonic and induced pluripotent stem cells to further delineate pluripotency and differentiation commitments, in particular to a cardiovascular lineage.

Dr Sue Kimber obtained her BA (Natural Sciences) and PhD from University of Cambridge before undertaking a postdoctoral position with Prof Azim Surani. She has worked on early mouse and human development in Cambridge, London, Copenhagen and most recently Manchester over the last 20+ years. In 2006 she set up the North West Embryonic Stem Cell Centre in Manchester where she and her colleagues are using hESC as human developmental models and generating new hESC lines and their differentiated derivatives suitable for clinical therapy.

Dr Keisuke Kaji, obtained his PhD in 2003 at the Tokyo Institute of Technology. In the same year he joined Dr. Brian Hendrich's group in the Institute for Stem Cell Research (ISCR) at the University of Edinburgh, as a postdoc. he studied the role of Mbd3 and found that the epigenetic molecule was important for lineage commitment in ES cells and development of pluripotent cells in peri-implantation mouse embryos. In 2008, He started my own group in the ISCR and developed a non-viral reprogramming strategy. Currently his group is working to improve the technology and reveal the mechanism of the reprogramming.

Rafael Yáñez is a Senior Lecturer with Royal Holloway-University of London, UK. Dr Yáñez held Lecturer appointments with King's College London and University College London, and received his PhD and BSc in Biochemistry and Molecular Biology from the Autonomous University of Madrid, Spain. He has researched on molecular virology of DNA viruses, therapeutic gene repair and lentiviral vector development. Rafael Yáñez led the team that published the first *in vivo* demonstration of high transduction efficiency by integration-deficient lentiviral vectors. He is currently developing these lentivectors for further applications of research and clinical relevance, with particular interest in neurodegenerative diseases.

Dr Ludovic Vallier is a member of the Department of Surgery and junior principal investigator in the newly opened Anne McLaren laboratory for regenerative medicine (LRM, Cambridge). The Vallier laboratory study mechanisms controlling differentiation of pluripotent cells pancreas and liver. These studies use human Embryonic Stem Cells and human induced pluripotent stem cells as an *in vitro* model of development in combination with functional analyses. Overall, the objective of the Vallier laboratory objective is not only knowing how to control differentiation of human ESCs into specific endodermal cell types (including pancreas and liver progenitors), but also to generate fully functional cell type for clinical applications. hIPSCs and liver metabolic diseases.

REPROGRAMMING OF MEFs BY NON-VIRAL TRANSFECTION OF mRNA ENCODING 4 REPROGRAMMING FACTORS

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Introduction: The groundbreaking work of Yamanaka's group (2006) proved the possibility to reprogram Mouse Embryonic Fibroblasts (MEFs) into stem cell-like cells (induced pluripotent stem cells or iPS) by viral introduction of 4 types of plasmid DNA, each encoding a defined factor (Oct4, Sox2, Klf4, cMyc). Since then, laboratories all over the world tried to improve the efficiency or safety, among others by using other (e.g. Nanog, Lin28) and/or less factors, by using other transfection methods (e.g. non-integrating vectors, protein-based reprogramming). In the past decade, it became clear that mRNA, however always believed to be too unstable, can indeed transfect cells efficiently. Because mRNA does not integrate into the host genome, it is a perfect candidate to safely introduce the factors into MEFs.

Results: Our group has elaborate experience in mRNA transfection by non-viral means. Because mRNA induced protein expression is in situ transient, it is imperative that expression of the 4 factors in MEFs lasts long enough to induce reprogramming. Therefore, we introduced mRNA encoding luciferase (by complexation with liposomes) in two cell lines (HeLa and MSCs) and ensured that luciferase levels were detectable up to 9 days post transfection. Then, we subjected MEFs to either a single or double mRNA transfection with 4 different mRNAs, each encoding a factor (Oct4, Sox2, Klf4, cMyc). Flow cytometry following immunostaining proved successful transfection of all introduced factors. Preliminary results, based on morphology and immunohistochemistry, indicate that iPS colonies have indeed been formed.

Conclusion: mRNA not only has the potential to introduce the 4 above-mentioned factors in MEFs and thereby induce their reprogramming, it also has several advantages, such as no risk of insertional mutagenesis and lower immunogenicity (no CpG motifs). Preliminary results are very promising with regard to iPS colony formation, however confirmation by means of teratoma formation is necessary.

References: Takahashi K, Yamanaka S. *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* (2006) Cell

Rejman J, Tavernier G, Bavarsad N, Demeester J, De Smedt SC. *mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers.* (2010) J Control Release

DERIVATION OF INDUCED PLURIPOTENT STEM CELLS (iPS) CELLS FROM A PATIENT WITH ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY (ARVC)Azra Fatima¹, Sven Dittmann², Shao Kaifeng¹, Guoxing Xu¹, Martin Lehmann¹, Matthias Linke³, Ulrich Zechner³, Hans-Christian Hennies^{4,6}, Ines Stork², Stephan Rosenkranz^{5,6}, Martin Farr², Hendrik Milting², Jürgen Hescheler^{1,6}, and Tomo Šarić^{1,6}

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Abstract : Human iPS cells are very similar to human embryonic stem (ES) cells but do not require an embryo for their derivation. They can be derived from patients with complex genetic defects to create *in vitro* disease models and thus represent an opportunity to study disease pathophysiology, develop new drugs and test methods for delaying disease progression or reversing its phenotype. We have generated iPS cells from a patient suffering from arrhythmogenic right ventricular cardiomyopathy (ARVC) carrying a novel spontaneous heterozygous autosomal dominant mutation in the gene desmin (N116S). The mutation affects filament formation leading to protein aggregates in ventricular myocardium *in vivo*. Lentiviral overexpression of combination of four transcription factors Oct4, Sox2, cMyc and Klf4 from a single vector (gift of Gustavo Mostoslavsky, Boston University, USA) was used to induce pluripotency in the patient-derived dermal fibroblasts. These iPS cells show a human ES cell-like colony morphology, express pluripotency markers at the protein (alkaline phosphatase, Tra-1-81, Tra-1-60, OCT4, NANOG, and SSEA4) and transcript level (OCT4, SOX2, NANOG, REX1), and exhibit the methylation pattern in promoter regions of OCT4 and NANOG genes, which is undistinguishable from that of conventional ES cells. In addition, these iPS cells carry the same genotype and disease-specific mutation as parental somatic cells, form teratomas in immunodeficient animals and differentiate to spontaneously beating cardiomyocytes *in vitro*. Further analyses are being carried out to assess the functional properties of ARVC-specific cardiomyocytes so as to determine whether they recapitulate the patient's disease phenotype *in vitro*. Thus, the ARVC-specific iPS cells generated in this study may serve as a replenishable source of cardiomyocytes for disease modelling and drug discovery.

HETEROGENEITY OF HUMAN INDUCED PLURIPOTENT STEM CELL CULTURES AND THEIR DIFFERENTIATION CAPABILITIES

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Enforced expression of a combination of transcription factors could reprogram somatic cells into induced pluripotent stem cells (iPSCs). However, it has been reported that iPSCs cultures are highly heterogeneous containing colonies morphologically resemble embryonic stem cells (ESCs) yet different in terms of protein expression and differentiation capabilities. In this study, we have used MSUH001-iPSCs line, derived by transfecting 4 factors (Oct4, Sox2, Lin28 and Nanog), to characterize the heterogeneity nature of the cell line. Although the colonies were round and tightly packed with single cells, immunocytochemistry against a typical pluripotent marker Oct4 revealed that iPSCs cultures contained three types of sub-populations; 1) Oct4-negative 2) Oct4-partially positive and 3) Oct4-positive populations. Further immunocytochemical analysis revealed that the cultures were also heterogeneous in terms of SSEA3 and Tra-1-60 expression. To determine the differentiation capacity of MUSH001-iPSCs, we compared retinal differentiation capability of iPSCs with that of Shef3-hESCs. Flow cytometry revealed that iPSCs are superior at differentiating into retinal progenitor cells (RPCs) as almost 30% cells co-expressed RPC markers Pax6 and Chx10 compared to less than 2% Pax6/Chx10 co-expressing cells differentiated from hESCs. When partially reprogrammed colonies were induced to differentiate into RPCs, they failed to express the markers. Through our data, we emphasize that rigorous monitoring and selection of fully reprogrammed iPSCs are prerequisite prior to differentiation.