Technology Strategy Board
Driving Innovation

MRC Medical Research Council

MRC–TSB iPSC Grand Challenge Workshop

1st August 2012

Thistle Hotel, Birmingham

Workshop Report

The workshop was attended by 31 delegates from across the UK iPSC research landscape (Annex 1), who met to discuss the emerging opportunities and challenges in iPSC research and identify how the UK research community could best work together to progress the field.

Rob Buckle (MRC) and Zahid Latif (Technology Strategy Board) welcomed the delegates and introduced the objectives of the meeting, which were;

- To provide an update on new strategic investments and the resource these will provide;
- To inform MRC and TSB as to any areas where further thinking is required concerning future UK support;
- To provide input to Pfizer re. the proposed IMI iPSC banking call.

In relation to these objectives, a number of issues were highlighted for discussion in considering how future iPSC collections/banks might best be configured:

- Do we need a strategic approach in determining which diseases and patients should be targeted given that creating iPS cell lines is expensive and resource intensive?
- What inclusion criteria should be used to optimize the quality and translational potential of iPSC lines? The utility of any cell line will be affected by issues such as
    - its scientific quality
    - the availability of adequate controls
    - the constraints on use of that cell line as dictated by intellectual property, ethical and consent issues
    - the accompanying patient information available for each line.
- What assays should be used to characterize each line – what core information is needed to ensure cell lines are of use to the wider research community?
- What degree of centralisation should we be seeking – whether in banking, annotation, databases or technical support?
    - to use resources as efficiently as possible
    - to increase collaborative effort and impact
    - to attract inward investment.

Presentations

1. IMI StemBANCC

Zameel Cader (University of Oxford), the Academic Coordinator of the StemBANCC Consortium, provided an overview of the **Stem** cells for **B**iological **A**ssays of **N**ovel drugs and predi**C**tivetoxi**C**ology' programme. The consortium had been put together under the Innovative Medicines Initiative (IMI), a joint undertaking between the European Commission (EC) the pharmaceutical industry association EFPIA seeking to boost pharmaceutical innovation in Europe. StemBANCC and will start in October 2012 running for 5 years, with a committed budget of €26m from the EC, to be matched by contributions from participating pharma

partners. The consortium comprises 11 countries, with 25 academic/non-profit research organisations/SMEs and 11 EFPIA participants.

The main objectives of StemBANCC are to

- provide access to well characterized, genetically diverse iPSC-derived cell types for pharma and academia in Europe, focusing on 500 subjects in 8 disease groups, spanning neurological and psychiatric disorders and diabetes
- provide cell-based assays for predictive toxicology (focus on liver, heart, kidney)
- establish assays for the study of disease biology and compound efficacy & toxicity for drug screening assays
- optimize protocols for the appropriate culture, differentiation, expansion and maintenance of iPSC-derived cell-types
- foster the implementation of a central test facility for the maintenance of the biobank beyond the funded period.

The project will be delivered as 11 different work packages grouped within 6 themes and managed through a project office, steering committee (EFPIA and academic work-package leaders) and scientific advisory board.

Identified challenges to the project include; establishing standardisation for cell type identity, identifying molecular/cellular phenotypes relevant to disease, being able to work with polygenic rather than just monogenic disorders and being able to produce enough quality cells of consistency to use within industrial assays. A number of ethical considerations were still to be addressed including country specific requirements, and it was noted that no pan-European process is in place and currently 90% of the subject recruitment is in the UK. Cell line availability would be addressed by a steering committee which would be convened to evaluate requests beyond the consortia with the view to making the resource as accessible as possible.

This coordinated and collaborative approach should provide the opportunity to enable the establishment of a robust ethical and governance framework for iPSC research and develop a repository of human iPSCs via common processes.

2. WT-MRC Human iPSC Initiative

Fiona Watt (Kings College London) gave an overview of the planned WT-MRC human iPSC platform, to be launched later in 2012[1]. Its goal was to combine iPSC technology with genomics, proteomics and stem cell biology to discover how:

- genetic variation affects cellular function
- genetic lesions result in disease phenotypes.

Addressing these questions and building the platform is intended to create an open access resource for the wider biomedical community.

One of the issues to overcome in the process will be to determine the functional significance of genetic variation between individuals and/or between healthy and diseased tissue. This would be done by creating 'cell observatories' that can examine numerous cell stimuli with high throughput, quantitatively measuring responses at the single cell level and relating specific responses to specific genetic alterations. Analyses could be endpoint kinetic assays, live cell imaging or at the proteomic level to assess phenotype. Genetic analyses will also be carried out using large sample numbers (~1000 donors), to enhance the signal to noise ratio and identify expression QTL, tissue state dependence and indirect cell phenotype interactions.

The various workstreams would be carried out across several different UK centres (WT Sanger Institute, Kings College London (KCL), Dundee, European Bioinformatics Institute) specialising in each facet of the research. KCL will focus on a low throughput, collaborative approaches to

[1] *HIPSCI* - www.hipsci.org

develop new protocols for generating disease relevant differentiated cell types and utilise information from HTS screens for improved iPSC generation, expansion and differentiation.

3. Future IMI iPSC Banking Call

Ruth McKernan (Pfizer Neusentis) introduced the forthcoming IMI proposal to establish an iPSC repository capable of supporting research into disease mechanisms and drug development in pharma. It was highlighted that while iPS cells provide a significant opportunity to validate disease pathways using human data, there was currently a disjointed effort across the US and Europe in response to this, providing only limited access to quality-controlled patient derived iPS cell lines. A specific need for industry going forward would be the provision of differentiated iPSC cells with full phenotypic information at scale and within a defined time line. Accordingly, the forthcoming IMI call hoped to capitalise on progress in the derivation and characterisation of iPSCs and provide an opportunity to consolidate European efforts, avoiding duplication, and maximising their impact. It was considered that the UK was well placed to play a leading role in delivering this goal.

The proposed IMI bank would ideally provide access to quality-controlled stem cell lines with information linking the associated genotypic, phenotypic and associated clinical data via a searchable on-line catalogue. Going forward with such an initiative should provide benefits to multiple stakeholders - from academic researchers, through industry and healthcare physicians - and be self-sustaining as it supported the development of new drugs, therapies and business opportunities.

The IMI process was ongoing and the final call specification would not emerge for some months, but its likely scope was outlined, including the putative timelines, work packages and budget (~€50m over 5 years). While the potential benefits to numerous parties were clear, a number of hurdles would need to be overcome, and these were presented as open questions; e.g. who could deliver such a programme, the nature of the business model to deliver sustainability, the roles of stakeholders and incorporation of current activities, whether a single master bank or multiple banks were required, possible distribution hubs etc. At this point no clear conclusion was evident but it was apparent that a coordinated effort would be needed if a successful UK-based bid was to be developed that could deliver the desired outcomes.

Thematic Discussions

4. Derivation technologies

Discussion on cell line derivation technology was led by Ludovic Vallier (WT/MRC Stem Cell Institute Cambridge & WTSI) and focussed on reprogramming methods where costs and variability were major considerations. Most approaches now pursued non-integrative technologies, with contrasting methods utilising Sendai virus (£100/line, plus a requirement for continual engagement with the supplier company) versus episomal reprogramming vectors which are cost effective (pence/line). mRNA reprogramming may also have utility. Large variability across iPSC derivatives was considered to be a significant problem, although this might largely reflect culture conditions, while the level of analysis needed re. epigenetics / DNA methylation was currently ill-defined. There was some controversy as to the number of cell lines that needed to be evaluated per donor (with views ranging from single to 5-10 isolates) or whether it was the differentiation processes itself which required constraint. It was also highlighted that it may be prudent not to be locked to a single technology as emerging technologies and future scientific advances could easily render it obsolete.

5. Cell phenotyping

Chris Denning (Nottingham) was the lead discussant on cell phenotyping, addressing where and at what level validation is needed, and highlighting the variety of ‘omics’ technologies that

could be applied. It was generally agreed that this was best undertaken upstream at the progenitor cell stage rather than just at the point of terminal differentiation. The potential utilities of current and emerging cell platforms for disease modelling and drug screening were discussed. IPSCs offer the advantage of expandability coupled to the genetic manipulations that can be employed, while direct reprogramming, for example as recently demonstrated from fibroblasts to neural cells, could potentially supplant iPSCs in the future once the technology has matured. The desirability of generating a marker panel (of banked RNA, tissue) from gold standard mature cell types was discussed, as a reference point for validation, although it was noted that the production of mature phenotypes from hESC or reprogrammed cells was not yet possible.

6. Disease collections

Discussion on disease collections and making them useful was led Charles ffrench-Constant (MRC Centre for Regenerative Medicine, Edinburgh). The issue of ensuring sufficient sample size was highlighted, and it was agreed that blood/serum bio-repositories should ideally be established alongside patient / population cohorts for all interested parties to use. In doing so it was critical that these be linked to meticulous records of clinical and lifestyle history, and that consent for blood/fibroblast donation should be non-restrictive. Lastly comparable methods of iPSC generation should be used where possible. Consistency, documentation and banking were key elements in establishing and future-proofing such collections, and it was considered that the community would benefit from the establishment of a guidance document setting out best practice in this area. It was emphasised that iPSC collections should ideally be based on well phenotyped patient /population collections, for which a number of well known epidemiological collections existed in the UK.

7. Quality control and stem cell banking

Glyn Stacey (UK Stem Cell Bank) outlined issues relating to establishing quality control in cell line generation and banking. An International Stem Cell Banking Initiative[2] established through the multinational International Stem Cell Forum had already established consensus on standards and guidance for stem cell line banking and the supplementary requirements for clinical grade stem cells. An ISCBI meeting held in February 2012 had further discussed the requirements for iPSCs, a report from which is attached at Annex 2. Identified issues to be addressed included; the number of clonal isolates required to assure a representative in vitro model, pluripotent potential, the need to report details of the isolation method used (which may influence the iPSC properties), IP constraints and material transfer agreements, and the need for standards/definitions regarding banking and nomenclature. It was suggested that part of any cell bank's remit should include the evaluation of generic features of iPSCs such as canonical markers, expression of reprogramming factors as well as self-renewal capacity and pluirpotency. However, the availability of reliable and simple tests for pluripotency was questioned. Determining the epigenetic profile of the cell lines might also be part of this remit, although what "ground state" the lines should be tested against was unclear, while stable reference cell lines would be needed as controls. It was added that the robustness of cell phenotype during culture needed to be established. Addressing all of these issues by the bank(s) would be costly and time consuming and the approach to be taken would ultimately depend upon the balance of requirements and fit for purpose.

Robert Downey (UK Biobank) provided an overview on high through-put sample handling and the logistics of implementing a large scale or "industrialised" approach, which would be required for any large-scale iPSC banking project. A number of components needed to be considered in providing material for research customers, with successful supply dependent upon the interface between infrastructure, organisational structure and skills, available

[2] *ISCBI - www.stem-cell-forum.net/ISCF/initiatives/international-stem-cell-banking-initiative*

technology and scientific production methods. Experience and capability in this area is available in the UK, with a course in biobanking available to help build further capacity.

8. Donor consent and traceability

Discussion on donor consent and traceability were also led by Glyn Stacey. UK regulations are in place and include a Code of Practice for the use of human stem cell lines[3]. Furthermore, best practices which include a national donor consent form for hESC derivation are in place through the human embryonic stem cell coordinators (hESSCO) group[4] and could be readily adapted for tissue donation to support iPSC derivation. It was noted that donor information underpinning informed consent must include the donor being fully informed of all possibilities of research with derived cell lines, while donations should be anonymised and altruistic, with no donor benefit. It was highlighted that generic consent forms covering all disease areas are available though work being progressed within NIHR Biomedical Research Centres, and could be used to support work in this area.

Summary and Conclusions;

In this emerging area of stem cell research, the UK has world-leading expertise, the skills base and resources in patient/population-based research that can be integrated to help progress the use of iPSCs as a platform for research into disease mechanisms and the development of new therapeutics. There are many issues to be resolved for the potential of this technology to be fully delivered but several strategic initiatives are currently being established in this area with a view to ensuring that the UK can compete at the forefront of this field. Much will be gained by continued communication and collaboration across the iPSC research community, and a major opportunity is available through the forthcoming IMI call to establish a pan-European iPSC repository. It was agreed that it would be beneficial for the UK community to join forces in assembling a unified bid under this initiative, and in so doing seek to add value to other activities already underway in this domain

Annexes

1. **Workshop participants**
2. **iPSC banking – draft report from the International Stem Cell Banking Initiative**

[3] *UK Code of Practice - www.mrc.ac.uk/Utilities/Documentrecord/index.htm?d=MRC003132*
[4] *Franklin et. al., (2008) Regen. Med. 3(1) 105-116*

Annex 1.

MRC–TSB iPSC Grand Challenge Workshop Participants

First Name	Surname	Affiliation
Rubina	Ahmed	NC3Rs
Peter	Andrews	Sheffield
Richard	Archer	TSB
Lyle	Armstrong	IMI StemBANCC / Newcastle
Zam	Cader	IMI StemBANCC / Oxford
Amanda	Carr	UCL
Sally	Cowley	IMI StemBANCC / Oxford
Chris	Denning	Nottingham
Paul	Downey	UK Biobank
James	Ellis	WT Sanger Institute
Charles	ffrench-Constant	MRC Centre Edinburgh
Karen	Kennedy	WT Sanger Institute
Cay	Kielty	Manchester
Tilo	Kunath	MRC Centre Edinburgh
Ruth	McKernan	Neusentis (Pfizer)
Willem	Ouwehand	WT Sanger Institute
Steve	Pollard	UCL
Rachel	Russell	Neusentis (Pfizer)
Austin	Smith	WT Centre Cambridge
Glyn	Stacey	UK Stem Cell Bank
Vasanta	Subramanian	Bath
Christian	Unger	Sheffield
Ludovic	Vallier	MRC Centre Cambridge & WTSI
Veronica	Van Heynigen	MRC Human Genetics Unit Edinburgh
Fiona	Watt	KCL
Lorraine	Young	Nottingham
Robin	Buckle	MRC
Jane	Itzhaki	Wellcome Trust
Zahid	Latif	TSB
Helen	Page	MRC
David	Pan	MRC

Annex 2

INTERNATIONAL STEM CELL Forum

NIBSC | Department for Business Innovation & Skills | life technologies | PEPROTECH

Report on the 'ISCBI Workshop on the delivery of High Quality Induced Pluripotent Stem Cells (iPSC) Resources', held February 21st-22nd 2012, at the National Institute for Biological Standards and Control, Potters Bar, UK

Sponsored by: Department of Business, Innovation and Skills
The International Stem Cell Banking Initiative
PeproTech
Life Technologies

Organised by: NIBSC and the UK Science & Innovation Network

iPSC banking – report from the 'ISCBI Workshop on the delivery of High Quality Induced Pluripotent Stem Cells (iPSC) Resources', held February 21st-22nd 2012, at the National Institute for Biological Standards and Control, Potters Bar, UK

Earlier this year, the International Stem Cell Banking Initiative (ISCBI), a global network of pluripotent stem cell banks that work together to promote and facilitate 'best practice' in stem cell research and the delivery of pluripotent cells for clinical use, held the '**ISCBI Workshop on the delivery of High Quality iPSC Resources'** at the National Institute for Biological Standards and Control, Potters Bar, UK. This network of banks is an initiative funded by the International Stem cell forum (ISCF,) an international funding body comprising 20 members, established to promote and facilitate research and harmonisation through a number of well-targeted global initiatives. The aim of this workshop, organised by the UK Stem Cell Bank , University of Massachusetts and the British Consulate-General Boston, was to bring together experts in a number of key stem cell areas along with invited speakers

The workshop started with a welcome by Glyn Stacey, Director of the UK Stem Cell Bank followed by a Keynote Session consisting of three talks on '**The Landscape of iPSC Derivation and Use'**.

Pete Coffey (University College, London, UK) spoke on **'The Clinical Application of iPSC for Eye Disease'** and described and discussed the work of 'The London Project to Cure Blindness'. This project focuses on age related macular degeneration (AMD) and comprises both the 'wet' and 'dry' types of this disorder. Both types lead to atrophy of the retinal pigment epithelium (RPE). Currently, 'wet' AMD is treated by injections into the back of the eye very 6 to 8 weeks or by the translocation of peripheral RPE into the maculae. There is currently no treatment available for the dry form of the disorder.

Can pluripotent stem cells be used to generate RPE? This has been demonstrated using human embryonic stem cells (hESCs) and indeed this source of RPE might prove of use in the treatment of AMD. Methods of graft delivery are being evaluated and include delivery on an artificial membrane to facilitate attachment of the RPE cells to Bruch's membrane at the back of the eye. Animal studies performed in both rats and pigs have demonstrated that the transplantation of RPE does improve the sight of the animals.

What is the advantage of using pluripotent cell lines to make RPE for use in the clinic? The cost saving treating a patient with this type of therapy is substantial. It costs approximately £15,000 to keep a person blind and £4,000 for treatment with RPE cells. To date patients treated with RPE have recovered some vision for up to 8 years (patient monitoring still in progress there have been no failures to date). There are some 700,000 cases of age related macular regeneration in the UK. Treatment with RPE would save the NHS around £2,000,000. Attention is now being turned to the potential use of iPSC to produce cells for therapy as this would ameliorate the need for immunosuppression required to prevent the rejection of the transplanted cells by the patient. ACT has conducted a clinical trial and has reported that human embryonic stem cell (hESC) derived RPE cells transplanted into patients have resulted in improved vision. These results are currently being debated by the scientific community since the results are open to interpretation.

Peter Andrews (University of Sheffield, UK) discussed **'Culture Adaptation of ES cells'**. Although focussing on hESC, Peter believed that the principles described in his talk could be applied to iPSC. He talked about the selection process for a hES cell within a heterogenous population that pre-disposes the cell fate to that of self-renewal, suggesting that nullipotency is the ultimate end-point for this type of cell. Nullipotency, a recessive trait, can also be reversed. Karyotypic changes are also observed in culture adapted cells. These changes do not appear to be random, the most common are gains of chromosomes 12p, 17q and X and these are the same changes as those seen in embryonal carcinomas. The most recent ISCI studies which looked at 125 ES lines and 11 iPSC lines demonstrated in addition to the gains on chromosomes 12p,17q and X, changes in chromosomes 1 and 20. Changes are observed during extended passaging of cells and these changes are observed in both bulk passaging

(approximately 30%), using enzymes, as well as in manual passaging (approximately 14%). The ISCI study also looked at the SNP profiles of the cell lines and demonstrated that in 20% of cells a minimal amplicon on chromosome 20 was observed which contained 3 genes one of which BCL2L1, is a potential candidate gene for driving culture adaptation. What causes this culture adaptation? There is no difference in attachment or cell cycle time between normal and 'adapted' cells. However, more normal cells apoptose in culture that their abnormal counterparts. There is a trend to add rho kinase (ROCK) inhibitors to enzymatically treated cells in culture to prevent apoptosis, does this make the cells in these culture more susceptible to genetic changes? Peter concluded by suggesting that it is inevitable that culture adaptation will arise. This adaptation may encompass both genetic and epigenetic changes. These in turn, could prove problematic for the future applications of these pluripotent cells. Nevertheless, these adaptations provide an opportunity to study and control stem cell fate and to model cancer progression. Caveat emptor: a normal karyotype does not preclude karyotypic changes in a cell!

Yoshinori Yoshida (Centre for iPSC Cell Research and Application, Kyoto, Japan) talked about the **'Analysis of differentiation capacity from ES and iPS cells'**. He discussed the potential use of pluripotent cells for cellular therapies and asked whether there was indeed significant differentiation potential between different types of pluripotent cells. Cells derived from different sources using various derivation methods may display distinct patterns in their potential to produce lineages representative of the three germ layers.

Yoshinori went on to discuss whether there were differences between pluripotent cells used in cardiomyocyte differentiation. He did indeed demonstrate a huge difference between lines. He tried to improve and standardise the differentiation conditions using a system described by Kattman, S. et al.(Cell Stem Cell (2011), vol **8,** pp228-240) using the Activin A and BMP4 pathways to drive differentiation to cardiomyocytes. He was able to differentiate the majority of lines but he described one karyotypically normal clone of iPSC that would not differentiate into cardiomyocytes and would not produce teratomas.

For the purposes of banking cells and choosing clones, Yoshinori also looked at the propensity of the lines to differentiate down the neural lineage, using this lineage to determine 'good' versus 'bad' clones using criteria such as KLF4 and miR371-3 as described by Kim et al (Cell Stem Cell (2011), vol **8,** pp695-706) but in his hands these markers were not shown to be reliable indicators of neural differentiation.

Are there differences between the expression profiles of hESC and iPSC? Building on the work of Lister, R., et al. (Nature (2011), vol **471,** pp68-73) and Ohi, Y et al.,(Nature Cell Biology (2011) **13**, 541–549)An extensive study of a range of iPSC clones/lines derived from different tissue sources and a number of hESC revealed some differences in the methylation of theTCERG1L, C9orf64 and TRIM4 promoters across lines. However, it appears that there is in fact no major difference in the gene expression patterns between iPSC and hESC cell lines.

Day 2, Session 1 started with a session entitled **'iPSC Derivation Methods'** and featured three talks on reprogramming cells using different technologies. The first talk given by **Pauline Lieu** (Life Technologies, USA) was entitled **'Efficient Method to Generate Integration – free iPSCs from Different Cell Types and Novel Live Alkaline Phosphatase Substrate for PSC (pluripotent stem cell) Identification'.** Pauline discussed the need for efficient technologies to generate integration-free iPSC. She discussed the current methods used to generate these lines and the problem of low reprogramming efficiency. The use of Sendai virus, a virus that does not integrate into the nucleus and is sold in a kit form as 'Cytotune™' by Life Technologies was described. Sendai virus has a higher reprogramming efficiency than messenger RNA and Lentivirus and can be used efficiently to reprogramme peripheral blood mononuclear cells. It takes 3-4 weeks to generate a line using this technology.
Reprogramming using Sendai virus can be achieved in a feeder-free environment. Anti-Sendai virus antibodies can be used to determine that the virus is no longer present in the system (achieved after approximately 5 passages). The emergence of reprogrammed colonies can be tracked using a live Alkaline Phosphatase stain (Life Technologies) which does not damage the cells.
Although this method increases the efficiency of reprogramming, there is a difference observed between the reprogramming efficiences of normal versus diseased fibroblasts

Kerry Mahon (Stemgent, USA) spoke on **'RNA reprogramming for iPSC derivation'**. Kerry described the Stemgent technology using mRNA, first described by Warren et al , (Cell Stem Cell (2010); **7**:618-630). Stemgent have produced a kit containing synthetic mRNA for Oct4, Sox2, KLF4, c-myc and Lin28 that are used in the reprogramming of cells by daily application of mRNA (18 days) combined with BR18R a soluble protein that blocks interferon signalling and allows the cell to survive repeat administration of single stranded mRNA. This method of reprogramming is useful to both academia and to industry as it produces cells that have not seen a virus (integrating or non-integrating) and are therefore does not require screening for integration sites or loss of virus from the cells. The reprogramming is safe, efficient, is the fastest method to produce functional iPSC (less than one month), and is amenable to high throughput and automated methods. Clonal line establishment is typically 85-90% .The iPSC should show a closer fidelity to hESC and could potentially be used in the establishment of a QC standard. The reprogrammed cells can be produced under GMP conditions as the reagents used are Xeno-free Reprogrammed colonies are identified using a live antibody to TRA-1-60 or TRA-1-80. The karyotype of the cells is normal and cells have been shown in vitro and in vivo to produce cells representative of all three germ layers.

As with the Cytotune™kit, the reprogramming efficiency is lower with diseased rather than normal fibroblasts. We have developed a new kit that should make the reprogramming less laborious and more efficient. This kit includes miRNA in addition to mRNA and the

reprogramming is carried out in a feeder-free system. The time required to generate colonies in this system is typically 10-14 days from start of transfection.

Kwang-Soo Kim (McLean Hospital, Harvard Medical School, USA) spoke on **'Protein-based reprogramming for iPSC derivation'**. He started out discussing the problems with cell therapies for Parkinson's disease where technically challenging, ethically contentious foetal cells are used. The use of iPSC in this setting would be an advantage as these could circumvent the difficulties. The production of dopaminergic neurons for therapy could be monitored by the expression of Pitx3 gene which is considered to be the 'gold standard' marker of this cell type. Delivery Chung S. et al (PNAS (2011) vol 108 pp 9703-9708).However, the majority of human iPS cells are generated by retroviral or lentiviral vectors, resulting in multiple chromosomal integration and residual reprogramming genes and the remaining transgenes and chromosomal disruptions could be harmful and may cause genetic, molecular, and cellular dysfunctions such as tumor formation.

Therefore, to use these cells for clinical application it is critical to establish non-genome integrating and/or gene-less reprogramming methods. He suggested the generation of iPSCs by direct protein delivery without any virus or DNA vector as these protein-based iPSCs may represent safe pluripotent cells. How could this be achieved? By Streptolysin O-induced membrane permeability or protein delivery using CPP fusion? The work of Chang was discussed (Chang, M-Y et al. PLOS (2010)5(3): e9838) who generated iPSC from rat neural cells and fibroblasts usng Streptolysin O.

He then went on to describe work using cell penetrating peptide (CPP) or protein transduction domain (PTD): short peptides capable of overcoming the cell membrane barrier. This approach was originally described back in 1988 by Frankel and Pabo (Frankel, A et al.Cell(1988); **55**:1189-1193; and Science (1988);**240**:70-73) where the HIV-TAT protein with a short basic segment at amino acid 48-60 was able to penetrate the cell membrane and activate and activate HIV specific genes. Other naturally occurring peptides with a high percentage of basic amino acids are also able to cross the cell membrane. These are known as CPP (Ziegler, A. et al (2005) Biochemistry; **44**:138-148 and El-Sayed, A et al. (2009) AAPS J.; **11**: 13–22). This type of direct protein transduction technology would enable the delivery proteins without the risks associated with viral systems. Initial experiments using human newborn fibroblasts have revealed that that CPP can successfully reprogramme these cells to iPSC which exhibit similar morphology, cell growth characteristics, cell surface and genomic markers to those seen in hESC (Kim,D. et al (2009) Cell Stem Cell; **4**:472-476). These cells also produce all three germ layers *in vitro* and *in vivo*.

Just how similar or indeed how different are hESC and iPSC? There are a number of publications that reveal that hESC are more efficient at producing certain cell types than iPSC (Hu, B-Y et al (2010) PNAS; **197**:4335-40 and Feng, Q et al (2010) Stem Cells;**28** :704-712). It might be that viral reprogramming is not the best way to reprogramme cells. Rhee (Rhee, Y-H et al (2011) J. Clin.Invest.; 121 : 2326-2335) has demonstrated that human iPSC

generated by different methods can efficiently generate neural Progenitor Cells and dopaminergic neurons that can be used to successfully treat rats. Residual expression of reprogramming factors was observed in differentiated cells reprogrammed using lentiviral delivery systems. Neural precursor cells derived from lentiviral/retroviral-hiPS cells were not highly expandable and exhibited early senescence wheras those from cells reprogrammed using CPP could be expanded without senescence. What causes these problems in the iPSC? Genomic instability, copy number variation, aberrant epigenomic reprogramming?
Reprogramming using protein based methods has been shown to produce cells that better resemble hESC than those generated by viral methods. Unpublised data by Park (2011) using hepatocytes reveals that using protein based delivery of reprogramming factors produces cells with significantly better genome integrity compared to virus-based iPSCs. Therefore, the use of protein based delivery systems provides an ideal source of human iPS cells for drug discovery, disease modeling, and reprogrammed cells that may be suitable for clinical translation. However, low efficiency of iPS generation, the production of pure reprogramming proteins and the low efficiency of protein delivery still need to be addressed for this to be a viable delivery method.

Session 2 entitled **'Differentiating iPSCs and hESCs : Characterisation and Quality Control'** comprised three technical talks the first of these was given by **Paul Sartipy** (Cellectis, Sweden) on **'Cardiomyocytes derived from human pluripotent stem cells' .** Peter outlined the applications for human cardiomyocytes . These included studies on cardiomyocyte function, modesl for normal and abnormal cardiac development, safety pharmacology,target identification and validation, functional genomics, in vitro disease modeling, cell replacement therapy and customized screening assays. He went on to discuss the models available for efficacy and safety testing during pre-clinical drug discovery **.** These models include cell lines, primary cardiomyocytes, engineered cardiac tissue, explanted hearts and cardiac tissue, large and small animal. Cardiomyocytes can be derived from hPSC in a number of different ways (Burridge et al. (2012) Cell Stem Cell;**10** :16-28). However, interline variability in cardiac differentiation can arise due to genetic background, derivation method, culture conditions and passage number, efficacy of reprogramming and cell type of origin, levels of expression of endogenous growth factors and /or receptors, epigenetic status and kinetics of differentiation
It is the optimization of exogenous and endogenous signaling that will ultimately determine the efficiency of cardiac differentiation. There are a plethora of different factors involved in the differentiation of hPSC into cardiomyocytes (Burridge et al. (2012) Cell Stem cell; **10** :16-28). The molecular and functional characteristics of hPSC derived cardiomyocytes are well defined and include the expression of cardiac markers and ion-channels, the display of ventricular-, atrial-, and nodal-like action potentials, functional blocking of ion-channels and respone to

pharmacological stimuli. However, the developmental status of hPSC-derived CMs are in some aspects immature and display a fetal phenotype:

This fetal phenotype may reduce the general applicability of hPSC-derived CMs for in vitro drug testing however it might be beneficial with respect to cell transplantation in regenerative medicine.

Can these cells be used in the study of cardiotoxicity (Sartipy, P and Björquist, P (2011) Stem Cells;**29**:744-748 and Mandenius C., et al (2011) J. Appl. Toxicol.; **31**:191-205) ?

Cardiotoxicity endpoints such biomarkers of life threatening ventricular arrhythmia, predictive biomarkers of drug-induced myocardial injury and contractility studies can indeed be studied using hPSC derived cardiomyocytes. Action potential recordings in hES-CMC demonstrate the presence of cardiac phenotypes (Jonnson, M et al (2010) Stem Cell Res;**4**:189-200).

Cardiomyocytes are selected for in vitro testing on the basis of a number of parameters: Electrophysiological selection (Beat frequency <50bmp, APD90>300ms) and pharmacological selection (response to cisapride). Ion channels in the heart enable the heart to beat in rhythm. Are these channels present in hPSC? Blocking of the inward potassium channel, hERG causes a phenomenon called 'early after depolarizations' this can result in 'torsades de pointes',tachycardia and other arrhythmias. This effect is also seen in hESC derived cardiomyocytes .

Nalos L (2012) B J Pharm; **165**:467-478 made a comparison of the inhibition of the rapid activating delayed rectifier potassium channel (I_{Kr}), using blocking drugs in five models used to screen pro-arrhythmia. This study included hESC derived cardiomyocytes and the selective response obtained from the hES-CMC model was comparable to the *in vivo* animal models included.The hESC derived cardiomyocytes also lend themselves to analysis by micro electrode arrays (MEA) which allows changes in field potential to be measured. These can be used as a surrogate way to measure arrhythmias and Q-T prolongation. In this system signal retrieval is non invasive and interpretation is similar to an ECG. Recently clusters of hESc derived cardiomyocytes have been used to examine field potential duration to hERG channel inhibitors and the results showed a difference between respondive and non-responsive clusters (Yamazaki, K et al. (2012) Toxicol In Vitro; **26:335**-42). This establishes an assessment system with potential to influence the QT interval, using pharmacologically selected clusters. He concluded that although pure populations of cardiomyocytes can be derived from hPSC with relatively high yield there are differences between hESC/hiPSC lines in terms of cardiomyocyte differentiation efficiency. hPSC derived cardiomyocytes display many critical functional properties of human cardiomyocytes, but in some aspects, hPSC cardiomyocytes display a foetal phenotype. hPSC derived cardiomyocytes are useful tools for drug testing. They are especially useful for predicting the effects of I_{Kr} blockers. However, standardization of assays/cells is required and more research is needed to achieve the "adult" cardiomyocyte phenotype and to generate preparations of pure ventricular-, atrial-, and nodal-like CMs

David Hay (University of Edinburgh, UK) spoke on '**The generation of metabolically active and predictive hepatocytes from pluripotent stem cells' .** In his talk David described how hepatic endoderm can be produced from both hESC and iPSC by directed differentiation via definitive endoderm (Terrace J. et al (2007) Stem Cells and Development;**16**:771-778. Hay D. et al. (2008) PNAS ;**105**:12301-6; Hay D. et al (2008) Stem Cells.;**26**:894-902, Terrace J et al (2009) Experimental Cell Res.; **315**:2141-2153 Terrace, J et al (2010) Experimental Cell Res.; **316**: 1637-1647; Sullivan G (2010) Hepatology;**51**:329-35). These cells express genes characteristic of *bona fide* hepatic endoderm but what can these cells be used for? They can be used for a wide range of purposes:novel liver models, metabolic disease models, the assessment of novel medicines and the discovery of novel biomarkers. This system can be automated since the process of differentiation is highly reproducible. The differentiation process be divided into a number of well defined stages and differentiation to hepatocyte-like cells can be achieved in 14 days. However maintaining stable differentiated cells is proving to be problematic using currently available substrates including matrigel™. David described the screening of polymer libraries to identify cell culture substrates capable of increasing the phenotypic stability of both primary and hPSC derived 'hepatocytes' (Hay et al (2011) Stem Cell Res; **6**:92-102). Polymer library microarrays were produced and screened for the attachment of stem cell derived hepatic endoderm and long-term fuction using high content screening. Six polymers produced hits during the screen and these were subjected to detailed investigation. One polymer 134 displayed superior hepatic maturation and function over the other candidates and was selected for extensive characterisation alongside matrigel, a commonly used biological substrate. Unlike on matrigel where differentiated cells displayed phenotypic instability and a reduction of phenobarbital-induced CYP3A and albumin levels with time, polymer 134 displayed a stable morphology, higher levels of inducible CYP3A and better albumin levels than on matrigel at the same late timepoints. Other parameters such as integrin expression were also studied on these cells and revealed differences between adhesion substrates.

These cells were also shown to be a valuable tool in the prediction of drug and metabolite toxicity.

David concluded by describing the work of the company Fibromed, a company working towards the provision of a genotyped and phenotyped biobank of cells that can be used to produce PSC as starting materials for the generation of hepatocytes which can be utilised in discovery technologies to build novel products.

Simone Haupt (Life and Brain, Germany) discussed **'Human pluripotent stem cells-a source for neurological disease modelling'.** Induced pluripotent stem cells can be used to generate a wide range of disease models such as schizophrenia, spinal muscular atrophy, Retts syndrome, Parkinsons etc. However in protocols to differentiate cells into neural cells there is no standardisation and no stable intermediate cell populations. This has a knock-on effect resulting in impaired scalability and banking, poorly defined culture conditions, impure

populations, protracted differentiation, low yield, lengthy and complex differentiation procedures and batch-to-batch variations. Indeed the situation is not helped when the hPSC source material shows differences in the potential to differentiate (Osafune, K. et al. (2008) Nat. Biotech.; **26**:313-315, Hu B-Y et al. (2010) PNAS; **107** :4335- 4340). How can we work towards standardisation? In a recent paper (Bock, C. et al (2011) Cell;**144**:439-52) produced a reference map of iPSC and hESC variation. By assessing the epigenetic and transcriptional similarity of ES and iPS cells the authors predicted the differentiation efficiency of individual cell lines.

The combination of assays (gene expression and DNA methylation) yields a scorecard for quick and comprehensive characterization of pluripotent cell lines. Recently a well characterized set of iPSC cells have been described (Boulting, G. et al. (2011) Nat Biotechnol.;**29**:279-86). This set comprises 16 lines that have been assessed for their pluripotential capacity and ability to terminally differentiate. Therefore, these lines provide a robust resource for the study of the basic biology of PSCs.

A couple of stable PSC derived neuronal intermediates, neural stem cells, have been described and can be used for neuronal modeling: A rosette-type of cell (Koch, P. et al. (2009)PNAS;**106**: 3225-3230) and a primitive neuronal precursor (Li, W. et al (2011) PNAS; **108**:8299-8304). These intermediates enable the cells to be captured in a self-renewing state from where they may be extensively expanded. These cells could then be directed to generate defined neuronal and glial cell types in an efficient manner. The rosette type of neural cells is a homogeneous pure neural stem cell population that can self-renew (> 100 Passages) and this renewal is independent of genetic background (hESC). These rosette type cells are also long-term neural epithelial-like stem cells (Lt-NES) that display a stable tripotent differentiation potential (neuronal, glial and oligodendrocytes), form functional synapses and are amenable to regional patterning.

Lt-NES cells derived from differenct sources exhibit similar characteristics and can be used as a tool to model neurological disease (Falk, A. et al. (2012) PLOS ONE;**7**:e29597). These cells cells reliably differentiate into functional neurons and glia. The Lt-NES cells also respond to morphogenes by altering regional marker expression and neurotransmitter phenotype. Genetic profiling reveals that both hESC and iPSC derived Lt-NES express genes seen in neural progenitors and the neural stem cell niche. In addition, pluripotent cell lines cluster together and are distinct from the lt-NES cell lines. Lt-NES inter-profile clustering is also independent of the cell of origin (hESC or hiPSC). These cells also exhibit a distinct ventral anterior hindbrain identity. Morphologically Lt-NES appear to resemble human foetal neural stem cells. However, Lt-NES and foetal NS are distinct neural stem cell types representing different developmental stages.

Lt-NES-derived neurons can be used for studying human disease such as Alzheimer's disease (AD) (Koch, P. (2012) Am. J. Pathol.;**180**:2404-2416) as Lt-NES cells can be used to address patho-physiological changes in familial monogenic forms of AD and might provide a valubale

platform for the development of pharmaceutical compounds. They have also been used to study Spinocerebellar ataxia type 3 (MDJ) (Koch, P. et al. (2011) Nature; **480**;543-546). Summarising Simone said that hPSC are a good source for neurological disease modeling. LT-NES provide a standardized resource for human neurons that are independent of genetic background (hESC line or hiPSC line). They are capable of self-renewing and therefore scalable. LT-NES exhibit a stable differentiation potential along with regional identity. They are also amenable to genetic modifications. A characteristic of great value is that these cells are cryopreservable (able to survive repeated freeze-thawing) and when thawed, can be successfully differentiated into neurons. Thus, these cells lend themselves to automated plating in a multiwell format and could provide a robust source of cells for pharmacological screening.

Lt-NES are human cells for studying human diseases. They can be used as a as platform to express candidate genes/mutants in a physiological cellular context. When used in reprogramming these cells can facilitate the study of pathogenic processes since hiPSC-derived lt-NES reflect genotype and expression levels found in disease-relevant somatic cells. However, the 'one size fits all' approach is inappropriate for neural disease modelling and repair . Tight temporal control over patterning seen in hPSC cell-derived neural precursor cells and at early stages cells are fully competent to respond to extrinsic developmental cues (Zeng, H. et al 2010 PLOS ONE;**7**:e11853). Run–through protocols can be manipulated accordingly to derive cells that express gene profiles which reflect distinct rostrocaudal and dorsoventral neural identities. Therefore, we need to design bespoke derivation methods tailored to the specific pathology and application. These protocols should be adaptable to industrialized methods (GMP, robustness, scalability, costs) and provide well characterized cell types (publications/applications).

The third session was a session on '**Establishling iPSC lines from donors and preliminary characterisation'**. In this session there were 2 talks. The first, by **Ludovic Vallier** (University of Cambridge, UK) was entitled **'Human Induced Pluripotent Stem Cells: Old challenges and new opportunities.** In his talk Ludovic discussed the human iPSC facility in Cambridge, UK. This facility, established in 2009, promotes and facilitates the use of PSCs by clinicians and academics to study and exploit these cell types in disease modelling and cell therapies. The focus is on cardiac, neuronal, blood and metabolic disorders. Over the last 2 years more than 400 lines have been generated from 70 patients with a 90% success rate. Cells for reprogramming are sourced from skin biopsies. The dermal fibroblasts generated are reprogrammed using the 4 Yamanaka factors delivered using lentiviruses or retroviruses. From biopsy to the characterisation of a hiPSC takes 2 to 3 months. Studies using blood derived endothelial progenitor cells have revealed this cell type as an efficient cell type to reprogramme making it a viable option to for high throughput generation of iPSCs. The EPSCs can be reprogrammed using Sendai virus which does not integrate into the host genome. Genome-wide analysis of iPSCs and the parental cells from which they were derived has been carried out in collaboration with the Sanger centre and this work has demonstrated

that the profiles of the iPSCs are almost identical to the parental lines. Cells were differentiated into neuroectoderm by inhibiting activin and BMP signalling and endodermal differentiation was achieved using a combination of activin, BMP-4 and FGF-2.

However, it was demonstrated that the variability in differentiation capabilities of cell lines derived from different individuals is on a par with the variability seen between lines derived from the same patient. Both iPSC and hESC can be differentiated using the same protocols but there is still a need to standardise these differentiaton methods to ensure reproducibility.

Ludovic concluded his talk by discussing the fact that there are still a number of challenges that need to be addressed in the production of iPSC. These include the optimisation of the reprogramming factors used, the cell type (this influences the quality of the iPSC), derivation conditions, delivery of reprogramming factors, scale -up and characterisation. With respect to characterisation, this will probably move from phenotypic analysis and qPCR to genome wide studies (exome, histone and methylome analysis). Another important consideration in this area is the ethical governace of the human material used to generate the cell lines. Appropriate consent should be in place for the downstream application of these cells. Indeed it might require further regulatory input to assure that all the ethical issues are addressed.

The second talk given by **David Kahler** (New York Stem Cell Foundation, USA) detailed the **'Derivation and Characterisation of iPSC lines in the NYSCF Personalised Medicine Bank'**

This bank is not actively banking at present but is still setting up. The foundation is privately funded and focusses on high risk/low return projects that would not be funded from other sources.

This personalised medicine approach will be achieved via high throughput screening. Using standard protocols where skin biopsies and fibroblasts are the starting materials. The reprogramming method will probably use Sendai virus. The cells will be from healthy and diseased subjects that are over the age of 18 where informed consent has been sought and a medical history taken.

David has a background in flow cytometry and this could be put to great use in selecting early reprogrammed cells by sorting on day 7 with the markers CD13, SSEA4 and Tra-1-60 and replating the cells on inactivated feeders. This would clean up the cultures and enrich for iPSCs.

Once the hiPScs have been generated they will be characterised using a panel of markers and assessed for their ability to differentiate down the three germ layers.

David finished his talk by emphasising that the challenges that the NYSCF personalised medicine bank faces to fulfil its remit are constraints on the time and labour (skilled technicians) for this resource intensive activity. This brought the morning session to a close.

The afternoon session started with a Moderated discussion session on 'Critical points for iPSC banking for clinical use' led by **Glyn Stacey** a number of topics were discussed. From this discussion it was established that early evaluation of disease needs to be addressed: to

establish how many patients to capture the range of genotypes presented in the disease of interest and to determine what is the likely penetration of the genetic trait in an *in vitro* model. What about the choice of tissue? Recent work in Yamanaka's laboratory indicates that a broad range of tissues generate iPSCs equally well, although no data has been presented on their relative epigenetic states.

It should also be borne in mind that the reprogramming method may influence research data. In the deviation of lines, lengthy isolation times may lead to higher levels of genetic change/mutation, but there is a balance to be struck since clones that appear later and survive may be more stable. Viral transduction methods are effective and produce lines of replication capacity of at least 50-100 passages. However, the quality of cells may be influenced by the reprogramming technique, methodological variations and the donor cell type stability during protocol. Low efficiency methods could also be selective for certain genotypes. It was thought that these variables might induce greater differences between individual cell lines than the genotype or defect and this is an area that requires further study.

Intellectual property (IP) is also a serious constraint. The situation is reasonably clear with use of Yamanka factors but there may be constraints on other techniques used in the generation of lines. Plans for banking which do not address the IP issue could collapse at the first stage of signing Material Transfer Agreements (MTAs) for the lines. Possibly a model where central funding secures ownership of lines and IP within project would remove this issue. There was strong interest in establishing commonality in MTAs but Universities and companies would require a very strong draw with clear benefits to them, in order to establish these.

There are many issues associated with the derivation and development of cell lines identified to be of fundamental importance to many areas of research, development and commercial exploitation. Care should be taken with ethics and the specific nature of the consent, ie. the potential to commercialise the cell lines, export cell lines to other countries, use for a wide range of purposes etc.

Another point to consider are how many "clones" should be isolated from each donor to ensure a representative clone can be identified (ideally this number should be between 5 and 10). It might be possible to preserve large numbers by simple preservation methods in a multiwell tray format, as has been achieved for primary hepatocytes.

Early screening of the lines should be performed to give an indication of pluripotency. The generation of small pre-master banks would give adequate material for these early selection procedures.

The characterisation of the cells must address two key features: self-renewal capacity and pluripotency. Self-renewal is different to pluripotent potential and there is a need for more data and possibly new markers to distinguish between these two functions.

There is also a need for a straightforward test for pluripotency that can be used in research laboratories on a regular basis.

Methylation studies giving broad epigenetic profiles could be applied to the the testing of cell banks to reveal differences between clones of the same line. However, a comment was made that very detailed testing could make the supply of cells financially non-viable .However, it should be noted that the costs of genetic testing are coming down significantly.

Banks should ask for information on the iPSC isolation methods, so that details such as lengthy isolation procedures (which are more likely to induce genetic changes) can be registered

Banks should evaluate generic features of iPSCs such as those established by ESTOOLS (see Annex 2A).Fundamental characteristics need to be met for a cell line to be accepted as an iPSC (the group proposed that the cells need to satisfy 10 as a minimum).

Definitions of a clone, cell bank etc needs to be clarified. Minimum standards for banks need to be addressed (see the ISCBI (2009) Stem Cell Rev. and Rep.; **5:**301-314). Standardised nomenclature and reporting criteria (eg Luong et al (2011) Cell Stem Cell;**8**:357-9) are needed for PSC lines. It was noted that the point of sampling at many cell banks was post-thaw from a sample of the bank (not prior to freezing) and the European Medicines Agency representative emphasised the need to demonstrate equivalence of the pre- and post- cryopreservation cultures of the cell lines. Testing for mycoplama and sterility was also recommended to banking centres since this is a critical sterility test and mycoplasma infection can have a devastating effect on the cell lines.

With respect to differentiation broadly speaking iPSC and hESC seem to perform equivalently. There is a need for stable reference cell lines such as those described by Bock (Bock, C (2011) Cell; **144**:439-452). There is also a requirement for good examples of positive and negative control materials for genomic and phenotypic studies as well as standard reference materials for benchmarking studies. These materials would aid in the standardisation of procedures and would facilitate QC. Currently differentiation protocols are long, complex and expensive which is the opposite of what industry needs. There is a requirement to refine these protocols and if possible make them more robust and reproducible with better yields of pure cell populations.

Although hPSC demonstrate useful functional attributes, both iPSC and hESC are still producing only foetal phenotypes. This needs to be addressed to see if we can generate cells with the relevant functional and morphological characteristics of adult cell types.

The meeting ended after this session. It had been an informative meeting that addressed many contemporary and relevant questions. The general feeling was that it had been well worthwhile participating since it had brought together eminent scientists in the field, with industry, funding bodies and regulators and had highlighted the challenges ahead for this exciting dynamic field of research and development where best practice is essential to the delivery of high quality iPSC resources.

Annex 2A

Minimal Criteria for the classification of putative iPS cells for further study (as suggested by ESTOOLS)

- Stable ES cell like morphology and growth pattern
- Expanded in culture as established line for > 10 passages
- Viable frozen stocks
- Human ES cell surface antigen profile: Expression of SSEA3, SSEA4, TRA-1-60/TRA-1-81, L-ALP (TRA-2-54 or TRA-2-49) – quantitated by flow cytometry
- Express key endogenous pluripotency-associated genes: Oct4, Nanog, Sox2, Rex, TDGF, assessed by:
  - qRT.PCR
  - immunostaining/western blot
- Neural differentiation in vitro – immunostaining for TuJ1 and GFAP
- Primary evidence of pluripotency in embryoid body or other in vitro differentiation assays by qRT-PCR for lineage markers
- Transgenes down-regulated
- Diploid karyotype

Note: *These minimal criteria should be met before putative iPS cells are entered into further study, unless a strong case can be made that one or other criterion should not exclude the cells from specific experiments. For example, it should be noted that, although no SSEA3(-) or SSEA4(-) human ES cells have yet been identified, rare polymorphisms in the human population indicate that SSEA3(-) or SSEA4(-) human ES or iPS cells might be encountered.*

Advanced characteristics that should be assessed for putative iPS cells

- Array CGH or SNP analysis of genetic integrity
- DNA fingerprint confirming identity with somatic cell of origin
- Teratoma formation
- Detailed evidence of differentiation in vitro to three germ layers with functional markers
- Copy number of transgenes with evidence of silencing; or evidence of transgene deletion or non-incorporation
- Gene expression profile – quantitative assessment by TLDA
- Methylation status of Oct4 and Nanog promoters
- X chromosome activation/inactivation status for female cells
- Comprehensive transcriptomic analysis by microarray or high throughput cDNA sequencing (for selected lines)

Note*: ESTOOLS can provide central resourcing for some aspects of advance phenotyping when all minimal criteria have been documented provided the cell line is made freely available to other ESTOOLS partners for research use.*