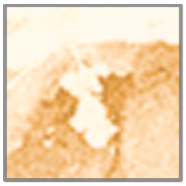

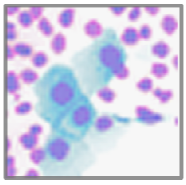
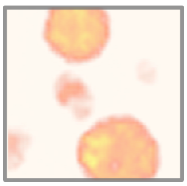
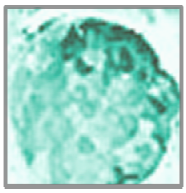


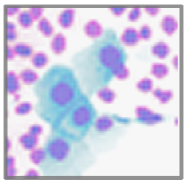
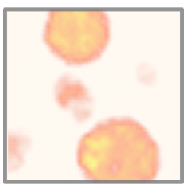
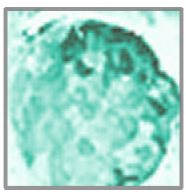

Inaugural
Annual Stem Cell Symposium
At UNSW

Monday August 6 2007
Lecture Theatre & Function Room
Edmund Blacket Building
Prince of Wales Hospital,
Randwick, NSW

INTRODUCTION

Welcome to the first annual *Stem Cell Symposium* at UNSW, an exciting new initiative at the University of New South Wales. Stem cells continue to capture the imagination of both scientists and the lay public alike. Indeed, many view stem cells as the 'medicine of the future'. We are fortunate that Australia enjoys an international profile for our work in stem cell biology and technology, as well as their potential translation to the clinic. Today's Symposium will demonstrate how research linked to UNSW has been integral to this success.

UNSW policy has so far been to let a thousand flowers bloom – this is our first attempt to start to create a garden from these flowers. The objective of our *Stem Cell Symposium* is to promote the exchange of ideas and expertise between stem cell researchers, in particular through cross-disciplinary collaboration between researchers in the Faculties of Medicine, Science, Engineering and Law, and to assist in the development of a University-wide stem cell research strategy. We hope to put UNSW, and through it the state of NSW, at the very forefront of the international stem cell research enterprise.

This first Symposium is therefore intended to showcase the breadth and depth of research talent and expertise in stem cell science across the University and its affiliated institutions. In fact one of the key strengths of this *Stem Cell Symposium* lies in its 'grassroots' origins amongst the wider UNSW research community. This has all occurred at a time of extraordinary public interest and engagement with the topic.

Upon full development, this new initiative will:

- Facilitate world-class 'bench to bedside' stem cell research at UNSW
- Provide scientific advice about stem cells to the community and so enhance the level of public knowledge about stem cells and their potential uses
- Facilitate opportunities for interaction with industry, government, private foundations and benefactors
- Organise regular meetings, invited talks and educational workshops for stem cell researchers

We are very fortunate to have the recently appointed NSW Minister for Science and Medical Research, the Honourable Verity Firth MP, to open our inaugural Symposium. Having recently successfully steered the bill on therapeutic cloning through both the upper and lower Houses of State Parliament, she is well versed on the issues vital to our area.

We also note the involvement of Professor Bob Williamson of the Australian Academy of Science in Canberra. Based at the University of Melbourne, Professor Williamson has been a vocal advocate for stem cell science and its role in the community. We look forward to sharing his vision.

Finally, we are grateful to those institutions and organisations, which have supported the Symposium, as well as our sponsoring industry partners. Without their support, today would not have been possible. Their names are proudly listed on the back cover of this brochure.

We trust you will enjoy the Symposium and use it as a springboard to further advance stem cell research at UNSW and her affiliated institutes.

Professor Bernie Tuch
Convenor *Stem Cell Symposium UNSW*
Director, NSW Stem Cell Network
& Diabetes Transplant Unit
Prince of Wales Hospital

Dr Michael Valenzuela
Convenor *Stem Cell Symposium UNSW*
Clinical Neuroscience Research
Fellow, School of Psychiatry, UNSW
Neuropsychiatric Institute
Prince of Wales Hospital

Dr Juliana Lamoury
Event Manager &
Stem Cell Researcher
Centre for Immunology
St Vincent's Hospital

Inaugural
Annual Stem Cell Symposium
at
UNSW

Program

8.30-9.00 Registration

9.00-9.10 Official welcome by NSW Minister for Science and Medical Research, Hon Verity Firth MP
9.10-9.40 KEYNOTE ADDRESS: Professor Bob Williamson *AO FRS FAA*
"Stem cell research: A precious contract between the community, government, medicine and science"

Morning Session: Neuroscience *Chaired by Professor Perminder Sachdev*

9.40 - 10.00 Dr Michael Valenzuela – "Making Brain from Skin: Propagation of canine dermis-derived neuroprecursors under controlled conditions""
10.00-10.20 Associate Professor Bryce Vissel – "Activin A alters cell fate in the adult hippocampus"
10.20-10.40 Professor Anne Cunningham – "The role of endogenous neural progenitors and pre-existing astrocytes in reactive gliosis in experimental post-traumatic syringomyelia"
10.40-11.00 Dr Jeremy Sullivan – "Cell replacement therapy for hearing loss"

– MORNING TEA –

Lunch Session: Medicine *Chaired by Professor John Shine*

11.20-11.40 Professor Bernie Tuch – "Use of stem cells to develop novel therapies for type 1 diabetes"
11.40-12.00 Associate Professor Ashish Diwan – "Will stem cells cure back pain?"
12.00-12.20 Professor David Ma – "Stem cell research at the BM Transplant Unit of St Vincent's Hospital Sydney & School of Medicine, UNSW"
12.20-12.40 Professor Denis Wakefield – "Corneal transplantation and limbal stem cells"
12.40-13.00 Dr Karen MacKenzie – "Is telomere length a determinant of haematopoietic progenitor cell expansion potential?"
13.00-13.20 Professor Richard Harvey – "Endogenous adult cardiac stem cells and capacity for heart regeneration"

– LUNCH & POSTERS –

Afternoon Session: Science & Society *Chaired by Professor Peter Little*

14.20-14.40 Associate Professor Kuldip Sidhu – "Embryonic stem cells to beta cells or neurons and that is the question"
14.40-15.00 Dr Robert Nordon – "Design and manufacture of microfluidic devices for investigation of stem cell function"
15.00-15.20 Dr Lesley Andrews – "Attitudes to use of surgically removed tissues for research"
15.20-15.40 Ms Irene Nemes – "Therapeutic cloning: the legal minefield"
15.40-15.50 Closing remarks and award of *UNSW Faculty of Medicine* Poster Prize

– DRINKS –

Abstracts - Oral Presentations

KEYNOTE ADDRESS

STEM CELL RESEARCH: A PRECIOUS CONTRACT BETWEEN THE COMMUNITY, GOVERNMENT, MEDICINE AND SCIENCE

Bob Williamson, AO FRS FAA

University of Melbourne

Most of the ethical objections to stem cells science, however honestly held in terms of religious belief, are not sustainable ethically or in terms of community values. It is unethical to appeal to personal philosophy as a justification to block genetic and stem cell medical research that could save lives and prevent handicap for many children and adults in our community. The Human Genome Project has led to a spectacular eruption of scientific data in human molecular genetics and functional genomics (respectively, the structure and function of genes in health and disease). The gene mutations causing most single gene inherited diseases, such as cystic fibrosis and X-linked mental retardation, have been isolated and understood. However, these data have not led to immediate clinical outcomes. The scientific and wider communities are coming to realise that the human genome project's real value is to explain the interaction between genes and environment in terms of susceptibility to complex diseases such as diabetes and obesity, asthma, cigarette addiction and bacterial and viral infection. There is the prospect of using compatible adult or embryonic stem cells as vehicles for gene delivery. I will give examples from our research into cystic fibrosis. While stem cell techniques are still in their infancy, they have the potential to have a real impact both on prevention and therapy for common diseases. However, even the new legal framework in Australia is more restrictive than the frameworks in the U.S.A. or U.K. or Singapore. If Australia is to participate in this research, and obtain the medical, social and technological benefits that will flow from it, we need a political environment and a legal framework that facilitates rather than restricts medical research. We also need scientists at every level who are prepared to go public and defend the ethics of the research that we conduct.

MAKING BRAIN FROM SKIN: PROPAGATION OF CANINE DERMIS-DERIVED NEUROPRECURSORS UNDER CONTROLLED CONDITIONS

Michael Valenzuela*, Kuldip Sidhu, Sophia Dean, Bernie Tuch, Perminder Sachdev

*School of Psychiatry, UNSW and Neuropsychiatric Institute, Prince of Wales Hospital.

The brain's three major cell types originate, like all mammalian skin, from the embryonic ectoderm. There has therefore been increasing interest in the feasibility of deriving neural stem-like cells from both fetal and adult skin. These studies have so far used the neurosphere assay as the propagation method of choice, a technique which readily propagates neural stem-like cells, however is limited by an inherent cellular heterogeneity. Neuronal yield after differentiation has varied from 6-30%. We report here on a system of skin-derived neural precursor (SKiNP) culture which includes the secondary step of adherent monolayer propagation and which leads to substantially higher levels of *in vitro* uniformity and neuronal differentiation.

Method

Small quantities of waste canine skin were obtained from opportunistic veterinary procedures in community dogs. Skin is processed by dissection and enzymatic digestion followed by induction of neural stem-like cells via the neurosphere assay in DMEM media under high mitogenic conditions (bFGF 40ng/ml, EGF 20 ng/ml). Primary neurospheres are then plated in coated flasks, attaching and proliferating with a typical 'bat-wing' morphology. Passaging by 2:1 split occurs every 7 days.

Results

SKiNPs have undergone more than 10 population doublings. PCR analysis confirms conservation of neuroprecursor mRNA including *Nestin*, *CD133* and *NCAM* across passage number. IHC staining for *Nestin* and *NCAM* showed that a majority of cells are positive, indicative of a highly uniform neuroprecursor culture. Differentiation studies confirm that most of these cells develop *in vitro* into a mature neuronal phenotype.

Conclusions

Preliminary results suggest that our two-step fully-defined SKiNP culture system allows for the efficient generation of large numbers of uniform neuroprecursors which can then differentiate to a mature neuronal phenotype. Our choice of canine tissue opens the door to future translational research of autologous cell replacement therapy in dogs with naturalistic neurological conditions.

ACTIVIN A ALTERS CELL FATE IN THE ADULT HIPPOCAMPUS

Andrea Abdipranoto[1], Jin-Sung Park[1], Dr Sally Galbraith[2], James Daniel[1] and Bryce Vissel[1]

[1]Neural Plasticity and Regeneration Research Group, Garvan Institute of Medical Research
[2]Department of Statistics, University of New South Wales

Activin A regulates differentiation of neuroblastoma cells and multipotent neural progenitors *in vitro*. Here we investigate the ability of activin A to regulate cell fate in the adult dentate gyrus (DG), CA3 and CA1 subfields of the injured and intact hippocampus. Mice received an intracerebroventricular (i.c.v) injection of kainic acid (KA, 0.2 μg) or PBS vehicle. Two days after injection mice were then treated by i.c.v infusion of activin A (12.25 ng/ml), follistatin (18.5 ng/ml) or vehicle. Mice were injected with bromodeoxyuridine (BrdU, 300mg/kg/8 hourly/3 days), and sacrificed 6 weeks later. Animals that received an injection of KA displayed significant neuronal loss in the CA3 (55881.946 ± 12230.337 in PBS-injected controls versus 19722.5 ± 3079.264 in KA –injected animals) and CA1 (70574.45 ± 8790.867 in PBS-injected controls versus 40873.876 ± 10317.903). Quantitative real time RT-PCR data demonstrates upregulation of the expression of the β_A subunit of activin following injection of KA with no apparent difference in the expression of the β_B subunit. We investigated the potential of these proliferative cells to express the mature neuronal NeuN. Data indicates that infusion of activin A in KA-injected animals did not results in any significant change in the number of $BrdU^+$ and $NeuN^+$ newly generated neurons in the DG (841.502 ± 267.053), CA3 (610.438 ± 162.381) and CA1 (615.565 ± 130.485) regions of the hippocampus in activin A treated KA-injected animals compared to vehicle controls (DG 1059.068 ± 190.877, CA3 823.611 ± 150.595, CA1 825.776 ± 173.014). However, while activin A did not enhance injury-induced neurogenesis infusion of follistatin to inhibit endogenous activin A resulted in a decrease in the number of newly generated ($BrdU^+/NeuN^+$) neurons in the DG (132.264 ± 30.104), CA3 (197.087 ± 34.683) and CA1 (291.336 ± 43.556) subfields compared to the DG (1059.068 ± 190.877), CA3 (823.611 ± 150.595) and CA1 (825.776 ± 173.014) subfields in vehicle treated KA-injected control animals. We wanted to determine if activin A is capable of stimulating neurogenesis in the intact adult hippocampus. Data indicates that infusion of activin A in PBS-injected animals significantly increased the number of newly generated ($BrdU^+/NeuN^+$) neurons in the DG (377.580 ± 33.455), CA3 (219.835 ± 25.364) and CA1 (204.378 ± 48.829) compared to the DG (191.654 ± 38.779), CA3 (133.241 ± 35.147) and CA1 (61.385 ± 13.821) subfields in vehicle treated PBS-injected animals. Infusion of follistatin did not result in any significant change in the number of newly generated ($BrdU^+/NeuN^+$) neurons in the DG, CA3 and CA1 subfields. Collectively, our results indicate that activin A regulates KA-induced hippocampal neurogenesis and can promote neurogenesis in the intact adult hippocampus.

THE ROLE OF ENDOGENOUS NEURAL PROGENITORS AND PRE-EXISTING ASTROCYTES IN REACTIVE GLIOSIS IN EXPERIMENTAL POST-TRAUMATIC SYRINGOMYELIA

Anne M. Cunningham, Jian Tu, Jinxin Liao, Marcus A. Stoodley

School of Women's and Children's Health and Prince of Wales Medical Research Institute, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia

Post-traumatic syringomyelia (PTS) is a cystic degeneration of the spinal cord that develops in up to 25% of spinal cord-injured patients with many suffering resultant progressive neurological deficit. As after any CNS injury, astrocyte activity and reactive gliosis would be expected among the earliest responses to PTS. These responses may be far from beneficial, as while reactive astrocytes are known to be important for neuroprotection and self-repair, the extracellular matrix proteoglycans they produce inhibit axonal regeneration. In this study, we examined the endogenous progenitor and astrocytic responses to PTS and subsequent glial scar formation. A model of PTS was induced in adult rats by injection of intraparenchymal quisqualic acid and subarachnoid kaolin at C7-T1. Controls included sham-operated and intact animals. Animals received bromodeoxyuridine (BrdU) by either single injection 24 hrs post-syrinx induction or daily injections for 12 days and spinal cords were examined immunohistochemically with antibodies to BrdU and glial fibrillary acidic protein (GFAP) to detect proliferating cells and astrocytes, respectively. We found a positive correlation between cyst size and time after injury up to the 42 day post-injury timepoint. Cell proliferation was significantly increased in the syrinx-induced animals by 7 days post-injury, peaking at 14 days post-injury before declining. By 56 days post-injury more than half of the BrdU-labeled cells expressed GFAP. Reactive astrocytes, the majority of which were not BrdU-labeled, also contributed significantly to the glial scar surrounding cysts. We concluded that this injury induces proliferation of spinal cord neural progenitors, some of which differentiated into mature astrocytes but, in addition, significant hypertrophy and migration of pre-existing astrocytes occurred. Our results provide for the first time insight into the contribution of endogenous neural progenitor cells and pre-existing spinal cord cells to the neuropathology of this condition.

CELL-REPLACEMENT THERAPY FOR HEARING LOSS

Jeremy Sullivan

Garvan Institute of Medical Research, Neuroscience Program

The overall aim of our research is to develop a stem cell therapy to treat hearing loss. The primary cause of acquired hearing loss is the degeneration of the sensory cells in the inner ear that are sensitive to sound. These specialised cells (auditory hair cells) are unable to be repaired or replaced naturally by the body, making hearing loss permanent and irreversible. Unlike auditory hair cells, olfactory sensory neurons continue to be produced throughout life. These new cells are the progeny of stem cells located within the olfactory epithelium. Adult stem cells from the olfactory epithelium have practical and clinically relevant advantages for cell therapies. Our experiments also indicate that these cells possess the ability to differentiate into auditory hair cell-like cells. The long-term goal of our research is to use these adult stem cells to develop autologous cell-replacement therapies for hearing loss.

USE OF STEM CELLS TO DEVELOP NOVEL THERAPIES FOR TYPE 1 DIABETES

Bernard Tuch

Diabetes Transplant Unit, Prince of Wales Hospital

The incidence of type 1 diabetes is continuing to rise with at least 130,000 Australians with this disorder. Whilst administration of insulin subcutaneously is life saving and will lower blood glucose levels, the fine regulation of a ß cell cannot be achieved in this way. The only form of cell therapy for diabetes being practiced clinically at present is with human islets. Whilst beneficial to the few, their availability is quite limited, and their long term benefit uncertain. Stem cells are a potential source of ß cell surrogates, with both embryonic and adult sources being examined. Initial experiments with embryonic stem cells (ESC) tried to differentiate them into ß cells via the ectodermal pathway of development. Few insulin-producing cells were made by this method with a number of features different from those of pancreatic ß cells. More recently attempts to differentiate ESC via an endodermal pathway commenced, the first step being exposure to activin A ± butyric acid. A series of subsequent steps using different reagents in the culture medium has resulted in production of insulin-containing cells, but there are some features of adult pancreatic ß cells missing. They are acute glucose-responsiveness and the absence of insulin-containing secretory granules. Other strategies being examined to produce ß cell surrogates from ESC include genetic manipulation, co-culturing with fetal pancreas and seeding the cells onto scaffolds.

Adult stem cells might also be of benefit in developing a cell therapy for type 1 diabetes. Sources of cells being examined for this purpose include the nose, liver, fetal and adult pancreas, cord blood, bone marrow and peripheral blood.

In summary, the conversion of stem cells into insulin-producing cells is accelerating the progress being made with cell therapies for diabetes, with clinical translation the ultimate goal.

WILL STEM CELLS CURE BACK PAIN?

Ashish Diwan, MBBS, MS, PhD(UNSW)

Chief of Spine Service, Department of Surgery, St George Clinical School

Every year nearly 200,000 Australians become chronic back pain sufferers, that is, they have lumbar region pain for more than 3 months, adding to a large pool of existing sufferers. In the year 2001 the direct health related costs for managing back pain were $1.2billion whilst the indirect cost including loss of national productivity and human capital was $9billion. The intervertebral disc present between bony elements in the spinal column appears to be the main source and originator of back pain in most of these individuals. Current therapies for ongoing debilitating back pain consist of pain management strategies and spinal reconstruction surgery including spinal fusion and disc replacement. There are no preventative strategies to slow down or to reverse degeneration of intervertebral discs.

The intervertebral disc is an oligo-cellular structure that consists of a proteoglycan rich central nucleus pulposus and a collagen-1 rich outer annular ring. The properties of both of these regions provide the characteristic visco-elasticity to a spinal column motion segment.

In collaboration with the Department of Haematology at St Vincent's Hospital over the past three years my team has investigated the potential of Adult Bone Marrow Derived Mesenchymal Stem Cells to be utilised as a novel therapy for patients suffering from chronic back pain. Our efforts have resulted in us demonstrating

1. that rat BM-MSC's can survive and differentiate into disc cells in a rat intervertebral degenerative disc model.
2. that human $CD34^-$ cells survive and differentiate into disc like cells in a rat intervertbral disc.
3. that co-culture of BM–MSC's with discal tissue can lead to a change in the phenotype of the MSC's to disc like cells
4. that the xenografting in #2 (above) does not incite an immune response, raising the possibility of allogenic transplantation in clinical settings
5. that a combination of growth factors from the TGF_ Super Family can help MSC's differentiate into disc like cells.

Based on our work and work of others in the area we have initiated a first-in-human study to evaluate the safety of Autologous Bone Marrow Derived MSC's for chronic back pain subjects who have a single level disc disease.

It is not clear whether pain relief will be obtained by the ability of these cells to produce good quality extra-cellular matrix and create an appropriate mechanical correction of the motion segment, or whether the benefit of the cell therapy will be derived by other mechanisms.

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STEM CELL RESEARCH AT THE ADULT BM TRANSPLANT UNIT OF ST VINCENT'S HOSPITAL SYDNEY & SCHOOL OF MEDICINE, UNSW

David D Ma

Our department has had a successful clinical BMT unit for many years and has been a pioneer in treating leukaemia and other haematological diseases using bone marrow derived stem cells. Earlier research focus was on haemopoietic engraftment and differentiation. In recent years, we have undertaken world-leading clinical trials using bone marrow stem cell transplantation to treat autoimmune diseases. "Bench to clinics" research has been an integral part of our transplant programme. Recently, we have turned our attention to the possibility of differentiation of BM derived stem cells (SC) to cells of non-haemopoietic lineages and its potential applications. This new research of adult BM derived stem cells has led us to investigate further the factors and molecular pathways controlling stem cell differentiation and has opened the possibilities of using BM derived stem cells for treatment of non-haemopoietic diseases including cardiac, neurological and orthopaedic diseases. Our current studies include: Haemopoietic SCT for autoimmune diseases, granulocyte colony stimulating factor mobilised autologous CD133+ progenitor cells for treatment of refractory ischaemic heart disease, potential of human adult stem cells as treatment for Parkinson's disease, and stem cell therapy for repair of the degeneration of intervertebral disc. Some highlights of our current collaborative projects in cardiac, neurological and orthopaedic areas will be discussed.

CORNEAL TRANSPLANTATION AND LIMBAL STEM CELLS

Denis Wakefield

School of Medical Sciences UNSW

Corneal disease is the commonest cause of treatable blindness and corneal transplantation is the commonest and most successful form of transplantation. Despite this the identity and characterisation of limbal stem cells is not well defined. Human corneal epithelial stem cells (HCESC) are responsible for the maintenance and repair of the corneal epithelium and reside at the corneal limbus. There are no reliable markers for HCESC and stem cell identification is a critical step prior to the widespread use of stem cell therapies. In corneal deficiency states, limbal cells may be transplanted; this is thought to provide transient amplifying cells (TAC) that migrate onto the recipient cornea to replace the denuded host epithelium. Such transplantation is not universally successful and there maybe no evidence of the persistence of donor cells in the graft. Improved techniques for HCESC transplantation are needed, particularly as immunosuppressive therapy, with the risks of life-threatening side effects, maybe needed to prevent rejection.

We have recently identified an extensive panel of potential markers for limbal stem cells by comparing the gene expression in foetal corneal epithelium with that of gene expression in primary cultured limbal epithelial cells. We reasoned that the foetal cornea in the early stages of embryology would express genes similar to that of the adult limbal epithelium. Differential gene expression of foetal limbal tissue was compared with the expression of the central foetal cornea. A “focussed” Stem Cell microarray differential expression experiment was also performed on p63 positive primary cultured adult limbal epithelial cells and on the same cells passaged 5 times. Of 266 genes examined, 34 were differentially highly expressed (>2 fold) in the limbus (compared to the central cornea) and primary cultured adult limbal epithelium (compared to cells after 5 passages). RT-PCR and immunohistochemistry (IHC) was performed on several of the preferentially up-regulated genes using foetal and adult corneas and cultured primary limbal epithelial cells. BrdU label-retaining studies in corneal organ cultures verified that CK14, 15, CDH3 and Wnt4 were expressed in slow cycling cells. These and other studies are helping to define the phenotype of human corneal stem cells.

IS TELOMERE LENGTH A DETERMINANT OF HAEMATOPOIETIC PROGENITOR CELL EXPANSION POTENTIAL?

Karen MacKenzie

Stem Cell Biology Program, Children's Cancer Institute Australia

The ability of haematopoietic stem cells (HSCs) to self renew is essential for lifelong production of mature blood cells and is a critical issue in HSC transplantation. However, the expansion potential of HSCs, varies dramatically between individuals. This variability is particularly striking in HSCs derived from umbilical cord blood (CB). Substantial evidence indicates that the length of chromosomal-end structures, called telomeres, is a determinant of the replicative lifespan of certain types of human somatic cells, such as fibroblasts. As in fibroblasts and other somatic cells, telomeres in haematopoietic cells shorten with each cell division. Excessive telomere shortening has been demonstrated in inherited and acquired blood disorders, including aplastic anemia and myelodysplastic syndromes. It has been proposed that replicative exhaustion, due to critical telomere shortening in HSCs contributes to the development of these disorders. However, a direct link between telomere length (TL) and the expansion capacity of HSCs was not previously demonstrated. We have investigated this relationship using CD34+ progenitor cells derived from CB. Our results showed that TL and expression of the telomere maintenance enzyme, telomerase, in CD34+ cells predicts erythroid expansion ($p<0.01$ and $p=0.01$ respectively). These results were corroborated by a correlation between proliferation of erythroid cells and telomere loss ($p=0.01$). In contrast, no correlations were found between TL, telomere loss or telomerase activity and the expansion of monocytic, granulocytic or megakaryocytic cells. There was also no correlation between TL or telomerase activity and clonogenic progenitors, including primitive progenitors derived from long-term culture. Another striking observation was that telomerase was dramatically up-regulation as progenitors underwent erythroid differentiation, but was down regulated during differentiation of other myeloid cell types. Together, these results provide new insight to the regulation of TL and telomerase activity during myeloid cell expansion and demonstrate that TL is an important determinant of CB-derived erythroid cell proliferation.

ENDOGENOUS ADULT CARDIAC STEM CELLS AND CAPACITY FOR HEART REGENERATION

Richard P. Harvey, Joan Li, Vashe Chandrakanthan, Owen Prall, Corey Heffernan, Munira Xaymardan, Ishtiaq M. Ahmed, Kate Kollar and MK Menon

Victor Chang Cardiac Research Institute, 384 Victoria Street, Darlinghurst, NSW 2010, Australia; and Australian Stem Cell Centre.

Ongoing and latent mitogenic potential in the adult mammalian heart is now well established and numerous stem cell-like populations have been described, including those within side-population cells, or defined using cell surface or nuclear markers of the haemopoietic stem cell (c-kit, Sca1) or embryonic cardiac progenitor (Isl1) lineages. However, the relationship between populations defined by these markers is unknown, and few studies have formally quantified cardiac progenitor cell populations relative to stem cell characteristics other than marker expression. We have surveyed various fractions of the non-diseased murine adult cardiomyocyte interstitium for cells with colony-forming ability in a colony-forming unit-fibroblast (CFU-F) assay and for lineage potentiality in vitro. A single fraction defined as Pdgfra+/Sca1+/Pecam1-/Nkx2-5-GFP- was enriched for CFU-F activity. Pdgfra+ cells and CFU-F are found within the myocardial walls, but also in the aortic root and cardiac valves. Analysis using GFP expression from a Pdgfra-GFP knockin allele suggest that CFU-F in the aortic root and coronary arterioles includes cells located within the tunica adventitia, previously described to have smooth muscle potential. CFU-F cells have secondary colony forming ability, and can differentiate with high efficiency in vitro into cardiomyocytes, smooth muscle, adipocytes, chondrocytes and osteocytes, but not haemopoietic cells. Embryonic studies suggest an epicardial origin for Pdgfra+ cells in development. Our study defines one class of endogenous cardiac precursor cell that is clonogenic, self-renewing and multipotent for a subset of mesodermal lineages. These cells may represent perivascular precursor cells with pro-vasculogenic potential during homeostasis and ischaemic repair. Their behaviour in disease and pro-regenerative models, and their ability to form cardiomyocytes in vivo are under investigation.

EMBRYONIC STEM CELLS TO BETA CELLS OR NEURONS AND THAT IS THE QUESTION

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Human embryonic stem cells (hESC) derivatives are emerging as a promising source of tissues in regenerative medicine. The first set of hESC lines was derived in 1998 by Prof James Thomson in Wisconsin, and now more that 414 hESC lines have been produced worldwide and only few are characterized fully and none so far suitable for immediate therapy. Stem cell biology as a field, in a very short period, have shown a tremendous progress particularly in our understanding of the directed differentiation process in hESC to achieve desired lineages of cells derived from the three germ layers e.g. ectoderm, mesoderm, and endoderm. Primarily ectoderm derivation from hESC to neurons seems to be a default pathway and hence a significant progress is made in this direction with some headways made to clinical stage (for Parkinson and spinal cord injury). However, derivation of endoderm lineages from hESC such as beta cells for type 1 diabetes seems to be a difficult path so far. Here I will review are efforts to achieve lineage specifications from hESC to beta cells or neurons as targets for future cell therapy. The conflict between scientific reality and institutional priority in hESC research is a question that may be hampering further progress in this field.

DESIGN AND MANUFACTURE OF MICROFLUIDIC DEVICES FOR INVESTIGATION OF STEM CELL FUNCTION

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Microfluidics is an emerging field that has given rise to a large number of scientific and technological developments over the last few years. Often referred to as 'lab-on-a-chip', microfluidic devices are manufactured from silicone rubber (PDMS) using soft lithography techniques. The technology allows one to design and manufacture flow channels at the micron level, and to specify precise concentration gradients and flow conditions. The technology has recently been applied to the study of stem cell microenvironment including investigation of adhesion, chemotaxis and high-density cell culture.

We have developed a hollow fibre membrane bioreactor for production of therapeutic cell products derived from haematopoietic or mesenchymal stem cells. Cells grow at densities that are 10-100 fold higher than standard tissue culture, because nutrients are continuously dialysed against fresh media. A membrane microfluidic device has also been developed for study and optimization of high-density growth of haematopoietic stem cells at lab-scale. We will present an overview of this technology and our capability for custom manufacture of microfluidic devices for various biological studies.

ATTITUDES TO USE OF SURGICALLY REMOVED TISSUE FOR RESEARCH

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Background
Further development in stem cell research is required for it to progress to clinically useful therapeutic cloning. Sources of oocytes for this research, and ultimately clinical use, are being sought. One option is to extract oocytes from prophylactically removed ovarian tissue from women who are at high genetic risk of ovarian cancer. In order to determine if this is an acceptable option to such women, a survey of attitudes to this type of research was conducted.

Methods
Patients of the Prince of Wales Hospital Hereditary Cancer Clinic at high risk of ovarian cancer, who have been offered prophylactic oophorectomy, were mailed a questionnaire regarding their attitudes to stem cell research, therapeutic cloning, and donation of tissue for such research.

Results
215 eligible women were mailed the survey. 107 returned responses. The remainder were either lost to contact or declined to participate.
Of the 107 respondents, 99% felt that stem cell research should continue with the current or lesser restrictions, and that therapeutic cloning would be or may be an important source of future health care. No participant thought this type of research should be discontinued. 75% would definitely agree to having surgically removed tissue used for research and the other 25% would agree if they knew exactly what the research involved. None said they definitely would not agree to surgically removed tissue being used for research. The same results were found when asked specifically about surgically removed ovaries.
Similar findings applied to tissue in general and ovaries in particular being specifically used for stem cell research (73% and 26%), with one respondent saying they definitely would not agree to such use.
A slightly lower number said they would definitely agree to body tissue or ovaries being used for therapeutic cloning (70%), or would do so if they knew exactly what the research involved (29%). Again, one respondent said they would definitely not agree to use of their tissue for therapeutic cloning.
Qualitative responses indicated that this group of women were strongly in favour of stem cell research with a view to therapeutic cloning. Concerns included commercialisation, adherence to regulations and assurances that the ovaries would not be used for human cloning.

Conclusions
These findings indicate that women undergoing prophylactic oophorectomy may be a willing source of oocytes for stem cell research and therapeutic cloning.
Subsequently, a study to utilise prophylactically removed ovaries to develop techniques to harvest and mature oocytes for stem cell research has been commenced.

THERAPEUTIC CLONING: THE LEGAL MINEFIELD

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In 2002 the Australian Parliament enacted legislation which prohibited both therapeutic and reproductive embryonic cloning. Just four years later, in December 2006, this same legislation was amended, reversing the prohibition on therapeutic cloning, while retaining the ban on reproductive cloning.

The Prime Minister, sensing the political mood, allowed a conscience vote. This contrasted with his decision several months earlier, against introducing any changes to the 2002 Act, despite 54 Recommendations having been made by a Statutory Review Committee.

Opponents of the Bill used the “slippery slope” argument, fearing unintended consequences. Proponents of the Bill were influenced by the hope of finding cures for debilitating diseases, the threat of a scientific brain drain, and the modest increment to what had been decided in 2002. Gender played a vital role, as women voted overwhelmingly in favour of the Bill.

Approval of the legislation had as much to do with the careful drafting of the provisions as with any rational, social, or scientific factor. The legislation is narrow in scope, retains an absolute prohibition on reproductive cloning and contains strict regulations with heavy criminal penalties.

The Act requires a review after 3 years. A number of questions remain. Will Parliament be asked to extend the legislation further? Does stem cell research demand a global, rather than a local approach, by way of an International Covenant? What is the likelihood of scientists pushing the boundaries of the prohibitions? What should be the role of law/morality in this debate? Does the legal status of a cloned embryo need further examination? What if a cloned embryo fails to be destroyed within the 14 day statutory period, either through human error or through intent? Will the embryo have a separate legal standing recognized by law? These are just some of the questions which will need addressing as the law tries to keep up with science.

Abstracts - Poster Presentations

P01

GENERATION *IN VITRO* OF T LINEAGE CELLS FROM HUMAN ADULT HAEMATOPOIETIC STEM CELLS

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Reconstitution of the T lymphocyte compartment of the adaptive immune system occurs through recruitment of $CD34^+$ Haemopoietic Stem Cells (HSCs) to the thymus. Thymic output decreases with age, and it is not known whether the decline is driven by stem cell or organ quality. T cell progenitor subsets of the HSC compartment have been described in mice, but not for human adults, and definition of this cell type would be a foundation for safe and effective stem cell transplants. We phenotyped HSCs from (GCSF-mobilized) adult peripheral blood and examined their growth potential in the OP9/Delta-like1 culture system, which provides an *in vitro* model for thymic development. A population of cells from normal adult donors were [$CD38^+$, Kit^+] and decreased with age (n=21 patients, age range 22-71, $p<0.05$). Based on these results we selected a gating strategy for FACS sorting of CLPs: primary gating by [$CD45^{mid}$, $CD34^+$, $Lineage^-$], and then sorting to either [$CD38^+$ AND Kit^+] or [$CD38^-$ OR Kit^-]. Delta-like1 co-culture supported differentiation of mobilized HSCs (n=10 patients) to $CD7^+$ and then to $CD4^+$ $CD8^+$ double positive cells, as well as $CD4^+$ SP cells. $CD38^-$ -selected cells developed similarly, but via a $CD38^+$ stage. Control cocultures without the T-lineage -promoting Delta signal developed to $CD19^+$ and [$CD4^+$, $CD14^+$] phenotypes, demonstrating the precursor's potential to form other (B-cell and myeloid) lineages. In conclusion, we have identified a candidate population of human adult lymphoid progenitors and have demonstrated that they can differentiate *in vitro* to the T cell lineage.

P02

IN VITRO CHARACTERIZATION OF ADULT MURINE HEART-DERIVED CARDIAC PROGENITOR CELL POPULATION

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The adult mammalian heart possesses multipotent stem cell-like populations in the myocardial interstitium. Most studies, however, have used only one or two surface markers to identify cell populations with stem cell activity. We have formally surveyed cardiac interstitial cells for such activity using Sca-1, PDGFRa, PECAM1 and Nkx2-5-GFP as markers, establishing spatial distribution of over 12 sub-fractions and their potential for self-renewal and mutipotentiality *in vitro*. We showed that only the Sca-1$^+$/PDGFRa$^+$/PECAM1$^-$ fraction had colony-forming units-fibroblast (CFU-F). In a single cell replating assay, these colonies formed secondary colonies at ~15% efficiency. Nkx2-5 is a cardiac homeodomain transcription factor and we have demonstrated expression of an Nkx2-5-GFP knockin allele in all of the interstitial cell fractions studied. However, only the Sca-1$^+$/PDGFRa$^+$/PECAM1$^-$/Nkx2-5-GFP$^-$ and not the Sca-1$^+$/PDGFRa$^+$/PECAM1$^-$/Nkx2-5-GFP$^+$ sub-fraction were capable of forming CFU-F indicating that expression of *Nkx2-5* has functional implications. Freshly processed CFU-F contained cells expressed PDGFRa and vimentin. Colonies derived from Sca-1$^+$/PDGFRa$^+$/PECAM1$^-$ cells could differentiate into cardiomyocytes, endothelial cells, smooth muscle cells, adipocytes and osteocytes although not haematopoietic cells, *in vitro*. Differentiation was confirmed by investigating expression of specific markers: 1) cardiomyocytes expressed Nkx2-5, GATA4, connexin-43 and a-Actinin; 2) smooth muscle cells expressed smooth muscle myosin heavy chain; 3) endothelial cells expressed vWF, PECAM1, alkaline phosphatase, VE-Cadherin and caveolin-1; 4) adipocytes were positive for Oil Red O stain; and 5) osteocytes were positive for alizarin Red S and alkaline phosphatase. Culture of Sca-1$^+$/PDGFRa$^+$/PECAM1$^-$ cells in matrigel lead to formation of vessel networks expressing endothelial markers such as alkaline phosphatase, VE-Cadherin and caveolin-1. Our experiments identify a novel fraction of cardiac interstitial cells enriched for cells that are clonogenic, self-renewing and multipotent for differentiation into a variety of mesodermal lineages (cardiomyocyte, endothelial, smooth muscle, osteocyte and chondrocyte) at high efficiency *in vitro*.

P03

ENCAPSULATION OF HESC AS A 3D MODEL TO STUDY THEIR DIRECTED DIFFERENTIATION

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Human embryonic stem cells (hESCs) have an indefinite replicative capacity and can be directed to differentiate into any specialized cells of the body and thus provide invaluable source of transplantable cells and tissues for replacement therapy. However, immune rejection of the grafts continues to be a major issue. We have previously shown that encapsulating hESC and mouse ESC in alginate hydrogel microcapsules before transplantation prevented formation of teratomas after four weeks and three months respectively both in immunodeficient (SCID) and immunocompetent (BALB/c) mice. Encapsulated ESC showed spontaneous differentiations both under in vivo and in vitro conditions. Nevertheless, the viability of encapsulated hESC in the culture significantly decreased after one week. In this study, we aim at optimising the culture condition in order to maintain viability of encapsulated hESC so as to use this as a feeder-free three dimensional model system to study differentiation. The configurations of alginate capsules and culture conditions were modified to enhance the viability of hESC. hESC were harvested as single cells and encapsulated in alginate capsules (1.1% VS 2.2% alginate) and cultured in the presence and absence of conditioned media (CM) from human fetal fibroblasts (HFF) in 20% knockout SR medium. hESC were examined for morphology, viability, proliferation rate and expression of gene markers at different time points. The viability and proliferation rate were significantly higher in hESC cultured in CM for over 2-3 weeks time period. Interestingly, hESC formed clusters in microcapsules when cultured in the presence of CM but not in 20% knockout serum-replacement media. RT-PCR analysis showed that the recovered hESC clusters from capsules continued to express pluripotent markers, Oct-4 and Nanog over two weeks. Together these data show that hESC can be effectively maintained in a pluripotent state that continues to proliferate inside the alginate microcapsules. These encapsulated hESC can be coaxed to differentiate.

P04

DNA METHYLATION STATUS OF SOME DEFINITIVE ENDODERM-SPECIFIC GENES DURING DIRECTED DIFFERENTIATION OF hESC CLONAL LINES BY ACTIVIN A

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Regenerative cellular therapies using hESC has been extensively studied to potentially create a cure for type I diabetes that affects more than 140,000 Australians to date. hESC having the ability to self proliferate indefinitely *in vitro* provides an unlimited source of cells for developing cellular therapies. The path from definitive endoderm to functional glucose responsive insulin secreting β-cells is complex. The present study focused on the developmental pathway of β-cell derivation, from hESC to pancreatic precursors – endodermal cells. We studied the 'epigenetic' profiles (methylation) of endoderm-specific genes; *Foxa2*, *GATA4* & *Sox17*, using a technique called Combined Bisulfite Restriction Analyses (COBRA). We demonstrated for the first time that these genes were unmethylated at their promoter regions in an undifferentiated state. This was substantiated by data demonstrating their gene expressions by RT PCR. Treatment with activin A for five days induced differentiation of hESC to definitive endoderm, and that also led to a significant ($P < 0.01$) upregulation of gene and protein expressions. Demonstration of some gene expressions in the absence of activin A treatment raised the possibility of the existence of partial methylated status on promoter regions in the above genes. The existence of partial methylated status on these genes was confirmed by inducing global demethylation using 5'aza-2-deoxycytidine. In conclusion, *Foxa2*, *GATA4* & *Sox17* genes responsible for definitive endoderm formation are partially methylated at their promoter regions in an undifferentiated stage of hESC. 5'aza-2-deoxycytidine, a global demethylation agent can promote complete demethylation on these genes leading to their gene and protein expressions. This study forms the basis of understanding the mechanism of definitive endoderm formation in hESC. The latter plays a significant role in achieving sufficient numbers of β cells from hESC as a potential cell therapy for type 1 diabetes.

P05

GLYCOGEN SYNTHASE KINASE-3β INHIBITION PRESERVES HAEMATOPOIETIC STEM CELL ACTIVITY DURING EX VIVO EXPANSION

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Unrelated umbilical cord blood (UCB) has emerged as a viable stem cell option for patients lacking matched family donors, however widespread application is limited by the reduced numbers of Hematopoietic Stem Cells (HSCs) in transplanted UCB units leading to greater graft failure and delayed haematopoeitic recovery. *Ex vivo* expansion of HSCs may overcome inferior haematopoietic recovery by increasing HSC dose. Current ex vivo expansion methods commonly result in the loss of HSC activity. Wnt signaling plays an important role in the regulation of HSCs. Activation of Wnt can be mediated through the inhibition of glycogen synthase kinase-3_ (GSK-3_) that prevents degradation of _-catenin, the main downstream effector of Wnt. Here we show that GSK-3_ inhibition induces _-catenin accumulation in human HSCs and promotes relocation of _-catenin from the cytoplasm to the cell nucleus to induce the transcription of Wnt target genes, c-myc and HoxB4, both promoting stem cell activity. In addition, GSK-3_ inhibition down-regulates cyclin D1 while up-regulating cdk inhibitor $p21^{Waf1}$ resulting in reduced cell cycling. The increased engraftment human HSCs and improved output of the multilineage progeny of the injected cells was seen in the bone marrow of NOD/SCID mice transplanted with HSCs treated with GSK-3_ inhibitor. Thus activated Wnt and reduced cell cycling both induced by GSK-3_ inhibitor act to preserve HSCs during *ex vivo* expansion. In addition, GSK-3_ inhibition up-regulates the expression of CXCR4 that is critical for stem cell homing to the bone marrow, reduces apoptosis and increases the adherence of HSCs to the bone marrow stroma. The latter may account for the increased engraftment of human HSCs in mice that received cells treated with GSK-3_ inhibitor. Collectively our results show that GSK-3_ inhibition acts to sustain stem cell activity during ex vivo expansion.

P06

NEUROPEPTIDE Y (NPY) AND THE Y1 RECEPTOR ARE REQUIRED FOR THE PROLIFERATION OF ADULT MOUSE OLFACTORY PRECURSORS

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The existence of stem cells in the central nervous system suggests the potential of nervous tissues to regenerate in the adult mammal. The regenerative capacity of the olfactory neuroepithelium has been well studied, and although the neuropeptide, NPY, has been implicated as an olfactory stem cell growth factor, little is known about the molecular events that control stem cell activity. The aim of this study is to examine the effects of NPY pathways during olfactory neurogenesis. Primary olfactory neurosphere cultures were prepared from adult wild type, NPY knockout and Y1 receptor knockout mice. Olfactory neurospheres were shown to be three-dimensional aggregates of proliferating cells that formed secondary neurospheres and were multipotent. The numbers of olfactory neurospheres derived from wild type mice increased more compared to NPY and Y1 knockout mice when grown over three weeks ($p<0.05$). Single cell deposition experiments showed that secondary neurospheres were formed from wild type, but not NPY or Y1 knockout neurosphere cultures. Extracellular antibody markers were used to isolate putative populations of stem cells from the olfactory neurosphere cultures. Homogeneous populations of horizontal basal cells ($ICAM^{+ve}$), globose basal cells (GBC^{+ve}) and glandular cells ($CD15^{+ve}$) were isolated from the olfactory neurosphere culture. The number of cells retrieved following flow cytometry was calculated as a percentage of the total number of cells sorted. A higher percentage of $ICAM^{+ve}$, GBC^{+ve} and $CD15^{+ve}$ were retrieved from the Y1 knockout animals compared to WT or NPY knockout animals. Interestingly, the $ICAM^{+ve}$ cells retrieved from Y1 knockout animals did not survive in culture (n= 3) and only the $CD15^{+ve}$ cells derived from wild type mice formed secondary neurospheres (n=6). These results indicate NPY and the NPY Y1 receptor are required for the normal proliferation of adult olfactory precursors. As we move closer to identifying the olfactory stem cell, factors needed for enriching this population of cells for use in cell-based therapies are needed, and NPY is at least one of these factors.

P07

MODELING THE ADHESION OF HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cells (hESCs) have previously been grown on 3-D scaffolds manufactured from poly (lactic co-glycolic acid), (PLGA). Although complex cellular structures were observed, there is a lack of specific information regarding cellular adhesion to the scaffolds and interaction between the cells and the scaffold surface. Assessing adhesion of hESCs in a 3-D environment is technically difficult; therefore we have developed a 2-D model to allow for simple investigation of the adhesion of hESCs to the surface of PLGA. We have tested the adhesion efficiency of single pluripotent hESCs on uncoated PLGA surfaces and PLGA surfaces coated with ECM proteins including; collagen I, collagen IV, laminin, and fibronectin. Our results demonstrated that laminin is the most effective agent in facilitating single pluripotent hESC adhesion to a PLGA surface. In addition, the combination of laminin and PLGA significantly enhances hESC adhesion efficiency. Immuno-inhibition of integrin receptors by antibodies revealed that integrins _3_1 and _6 were mostly responsible for the binding of pluripotent hESCs to laminin/PLGA coated cover slips. The adhesion conditions optimized in the 2-D model were then applied in a 3-D environment resulting in efficient seeding of 3-D PLGA scaffolds. In conclusion a 2-D model can be potentially used for quantitative assessment of cellular adhesion to a range of biomaterial surfaces.

P08

SURVIVAL OF FOUR TYPES OF HUMAN CELL TRANSPLANTS IN ATHYMIC RAT SPINAL CORD

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While the potential of stem cell treatments in the repair of spinal cord injury in humans is widely recognised, there are still many issues to be addressed before these treatments become a reality. In this study we have investigated four human cell types; embryonic neuronal progenitor cells (ESC), undifferentiated bone marrow mesenchymal stem cells (BMSC), multipotent stem cells from the olfactory mucosa (OSC) and mature olfactory ensheathing cells (OEC) to assess their suitability for spinal cord transplantation. Cells were either transfected with green fluorescent protein (GFP) or pre-labelled with CDFA cell tracer prior to injection. 0.5µl of each cell suspension (100,000 cells/µl) was injected 1mm deep and 1mm lateral to the midline of athymic rat spinal cord at vertebral level T10 and T11. Animals were euthanised at 24hrs and 1 week. All cell transplants were clearly visible using fluorescent label or antibodies against human mitochondria at 24hrs. By 1 week, OSC and OEC were present in similar numbers but only a few ESC and BMSC could be detected. Changes to experimental paradigms such as increasing cell number and changing differentiation conditions did not improve their detection after transplantation probably due to poor survival or rejection. While immunocompromised athymic rats (rnu/rnu) were used as hosts to overcome xenograft rejection by T cells, it is possible that natural killer cells have also contributed to graft rejection in these animals.

P09

REPROGRAMMING HUMAN FETAL FIBROBLASTS TO PLURIPOTENT STATE AFTER FUSION WITH hESC

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Mouse and human ESC have been shown to have the ability to reprogram somatic cells after fusion[1-3]. The efficiency of reprogramming is low and the generated hybrid cells are unlikely to be applied to clinical research because of their tetraploid DNA content. In the present study the Envy line with green fluorescent protein[4] and a puromycin resistant human fetal fibroblasts[5] were used for fusion. hESC and tHFF as single cells (5_10^6 of each cell type) were mixed in the presence of polyethylene glycol PEG 3400. 24 hours after treatment, cell culture media was supplemented with puromycin. Selection in the presence of puromycin was carried out after 20 days and the positive clones dissected out under the fluorescence microscope. More than 50 stem cell-like colonies were observed to be both GFP-positive and able to survive in the presence of puromycin for more than 20 days. These colonies have the morphology similar to normal Envy line with growth rate slower than parent line. After 20 days, the colonies were passaged and RNA extracted from the hybrid cells. Primary results have shown that these cells do express pluripotent markers, i.e. Oct4 and Nanog. Thus, the data indicate that the drug resistant GFP-positive colonies are stem cell like and are pluripotent. Currently the efficiency of reprogramming (approximately 50hESC-like colonies/ 7 million cells) in our hands is 6 times higher than that was reported before (Cowan et al., 2005). However, when these fibroblast-derived stem cells were cultured to passage 3, the growth of these stem cell- like colonies was slowed down significantly. The higher efficiency of reprogramming human fetal fibroblasts to pluripotent state after fusion with hESC will play a significant role in facilitating the formation of pluripotent stem cell-like colonies without the use of human embryos and for developing potential patient-specific stem cell lines for therapeutics.

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P10

CHARACTERISATION OF LATE OUTGROWTH ENDOTHELIAL PROGENITOR CELLS FROM HUMAN PERIPHERAL BLOOD

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Endothelial progenitor cells (EPCs) may potentially be used in cellular therapies to treat vascular disorders such as ischaemic heart disease. Currently, EPCs remain poorly understood and there is a need for further characterisation studies.
We have established a method to detect one type of EPCs (late outgrowth) from the peripheral blood. This methodology will become an important tool in our continued research, aimed at defining and understanding heterogeneity within the human adult EPC compartment. Peripheral blood mononuclear cells (MNCs) from normal donors (n=8) were obtained by Ficoll density centrifugation and plated on collagen 1 using a medium to promote endothelial differentiation.
Cultures consistently gave rise to colonies at 2-3 weeks at very low frequency (1 colony per 180 million MNCs plated). Cells displayed typical cobblestone morphology, were highly proliferative and were subcultured while keeping a consistent morphology. FACS analysis demonstrated markers of endothelial (CD34, CD31, vWF) but not of haematopoietic (CD45) lineage. In addition, inducible antigens (CD62E, CD106, CD54) were up-regulated upon TNF stimulation. Cells formed tube-like structures on Matrigel and incorporated acetylated LDL.
Cells were confirmed to be of endothelial lineage thus demonstrating that a population of EPCs exists in the peripheral blood of adults. This *in vitro* system will be used in our next study to detect late outgrowth EPCs from patients with chronic ischaemic heart disease.

P11

COMPARISON OF PHENOTYPIC AND MOLECULAR CHARACTERISTICS OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS AND INTERVERTEBRAL DISC CELLS

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Intervertebral disc (IVD) contains chondrocyte-like cells in the nucleus pulposus (NP) surrounded by fibroblast-like cells in the annulus fibrosus (AF). Bone marrow mesenchymal stem cells (BM MSCs) provide a source to treat IVD degeneration. This project aimed to characterise BM MSCs and IVD NP and AF cells. **Method:** BM MSCs were isolated from donors by density gradient centrifugation and negative selection using antibody complexes. IVD tissues were collected from discarded disc specimens of patients undergoing disc replacement. NP and AF were separated and the cells were obtained using enzyme digestion method, respectively. Both the BM MSCs and IVD cells were cultured in appropriate medium supplemented with FBS and cells characterised by flow cytometry and QT-PCR. **Result:** Morphology of BM MSCs appeared different from both cultured AF and NP cells. BM MSCs had a fibroblastic appearance, whilst cultured AF and NP cells had a more raised and triangular shape. For protein expression analysed by flow cytometry, significant differences were noted in CD105 and CD166 expression between BM MSCs, AF cells and NP cells: for CD105, BM MSCs was 33 ± 7%, NP 10 ± 4%, and AF 15 ± 6%, ($n>6$, $p<0.02$ & 0.09 respectively); and for CD166, BM MSCs 64 ± 7%, NP 8 ± 2%, and AF 9 ± 5% ($n>5$, $p<0.01$ & 0.01 respectively). These three types of cells exhibited similar patterns of expression for 9 other proteins. Gene expressions of Collagen I, Collagen II, Aggrecan and Sox9 were also analysed. Collagen II and Aggrecan were highly expressed in IVD cells and expression of these 2 genes was extremely low or undetectable in BM MSCs. **Conclusion:** We found that BM MSCs and the IVD cells have different morphology. BM MSCs expressed CD105 and CD166 antigens at higher level as compared to AF and NP cells. In terms of gene expression, Collagen II and Aggrecan were higher in IVD cells compared to BM MSCs. These markers may be used in distinguishing between BM MSCs and IVD cells. This observation provides useful information for ongoing studies on the potentials of BM MSC in the treatment of severe IVD degeneration.

P12

MODELLING NEURONAL DIFFERENTIATION IN HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS USING CYTOKINES

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Recently, an increasing number of reports have shown that adult human mesenchymal stem cells (hMSCs) are capable of differentiation into a wide range of cell types, including neural-like cells. The present study aims to: (1) Model neuronal differentiation in hMSCs using a neurotrophic cytokine cocktail; (2) Examine alterations in the gene and protein expression profile post-cytokine exposure; and (3) Demonstrate that the observed morphologic conversion to neuronal-like morphology, results from active and dynamic processes. hMSCs were isolated from bone marrow obtained from haematologically normal donors using density-gradient centrifugation and lineage-committed cell depletion, adherence to cultureware, and expansion in MAPC medium containing 10% FBS. Neuronal differentiation was performed using an adherent culture system with a fibronectin coating, and application of a cytokine cocktail (bFGF/EGF/PDGF) for 3 weeks. Prior to differentiation, neuronal markers Nestin, NR4A2 and TH, and pluripotency marker OCT3/4, could be detected in hMSCs at low levels by real-time PCR. Following 3 weeks of neuronal differentiation, expression was upregulated greater than 40-, 7-, 1.5- and 9-fold respectively, with concomitant downregulation of Collagen I (mesodermal) and CSPG4 (glial) by greater than 1.5- and 2-fold respectively, and induction of MAP2 (neuronal) expression. Neural protein expression was also detected by immunofluorescent staining for Nestin, MAP2 and Beta-III-Tubulin. Live cell microscopic examination of hMSCs after neuronal differentiation revealed cells to actively extend and retract cellular processes, develop refractile cell bodies, and form short transient branches. Furthermore, a degree of dynamic cell-cell interaction was observed. We conclude that the morphological changes observed in hMSCs are a true response to neuronal differentiation stimuli and involves active processes, unlike the cellular shrinkage/toxicity phenomenon observed in response to chemical stimuli; also, neuronal differentiation was successful in driving hMSCs towards an early neuronal fate, as evidenced by altered marker expression.

P13

MULTIPLE SCLEROSIS DRUG IFN-β1B AND CYTOKINES ALTER THE KYNURENINE PATHWAY OF TRYPTOPHAN METABOLISM IN MESENCHYMAL STEM CELLS

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In most tissues, including the brain, the essential amino acid tryptophan (Trp) is degraded primarily by the kynurenine pathway (KP), which generates neuroactive metabolites including the neurotoxin quinolinic acid (QUIN) and kynurenic acid (KYNA), a neuroprotectant. Kynurenine (KYN) metabolites have been implicated in diverse aspects of physiology and pathophysiology, such as Multiple Sclerosis and AIDS dementia complex.

However, the role of Trp metabolism and their functional consequences in stem cell biology such as mesenchymal stem cells (MSCs) has been poorly explored. MSCs are multipotent progenitor cells, which possess unique immunomodulatory properties, and they are suitable for autologous transplantation.

To explore the Trp metabolism in MSCs, we have sought in this study to investigate the expression and regulation of the KP in both mouse and human MSCs. Cultures of MSCs were examined for the expression of the KP enzymes and production of KP metabolites by real-time RT-PCR, immunocytochemistry, HPLC and mass spectrometry. We demonstrate for the first time that mouse and human MSCs constitutively express all KP enzymes at both RNA and protein levels including indoleamine 2,3 dioxygenase (IDO), the first and rate-limiting KP enzyme. Moreover, when cultured in the presence of specific induction factors namely IFN-γ, or current multiple sclerosis therapies such as IFN-β1b, the IDO gene expression together with KYN and KYNA production were altered in MSC cultures.

These results suggest that: a) MSCs possess a complete, functional expression of the KP and b) KP induction in MSCs could lead to neurotoxicity and compromise differentiation and engraftment of endogenous and/or transplanted stem cells. Furthermore, the results also generate the hypothesis that the selective inhibition of the KP in MSCs could minimize neuronal death and optimize stem cell differentiation, which might lead to novel therapeutic strategies.

P14

CHARACTERISATION OF MESENCHYMAL PROGENITOR CELLS IN Y-RECEPTOR TRANSGENIC MICE

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The isolation and characterisation of mesenchymal stem cells has proven to be difficult due to their rarity within bone and the lack of specific markers for their identification. NPY-immunoreactive fibres have been shown within bone marrow and also around bone lining and marrow cells suggesting a role for NPY in the regulation of bone mass.
Adult-onset deletion of Y2 receptors solely from the hypothalamus of mice, as well as germline deletion of Y2 receptors, results in increased bone formation due to elevated osteoblast activity. Data from our lab suggests that this is due to a greater number of mesenchymal progenitor cells in the $Y2^{-/-}$ mice and a down-regulation of Y1 receptor expression on $Y2^{-/-}$ bone cells. *In-situ* hybridisation on femur sections reveals the presence of Y1 but not Y2 receptor mRNA in osteoblasts, suggesting a direct role for the Y1 receptor on bone precursor cells. Moreover, germline $Y1^{-/-}$ mice have a similar increase in bone mass and formation to $Y2^{-/-}$ mice although their phenotype appears to be mediated via non-hypothalamic routes.
We have isolated two highly proliferative mesenchymal progenitor populations which exhibit multipotentiality from bone cells by flow cytometry using Sca-1, a stem cell antigen, negative selection of hematopoietic cells as well as elimination of $CD51^{-}$ erythroid precursor cells. Preliminary data suggest that there is no difference between $Y1^{-/-}$ and wildtype mice in the number of cells present in either mesenchymal progenitor population however the $Y1^{-/-}$ cells are more proliferative *in vitro*. RT-PCR analysis on RNA from wildtype mice shows that, in culture, both cell populations exhibit strong Y1 receptor expression.
These data support a direct role for the Y1 receptor on the ability of bone precursor cells to develop into mature mineral producing osteoblasts which could have powerful therapeutic implications in diseases such as osteoporosis.

P15

DIFFERENTIATION AND MOBILIZATION OF SCA-1+/CD31- CARDIAC SIDE POPULATION CELLS IN A MURINE MYOCARDIAL ISCHEMIC MODEL

Simon Liang, Terence Tan and Beng Chong

Myocardial infarction is the most common cause of heart failure and remains one of the leading causes of morbidity and mortality in humans. Stem cells are important in the maintenance and repair of adult tissues. Hoechst effluxing cells, termed side population (SP) cells are a rare subset of cells found in adult tissues that are highly enriched for stem and progenitor cell activity. Recent studies have suggested that Sca-1+/CD31- cardiac SP cells are capable of differentiation into cardiomyocytes in vitro. However, the response of cardiac SP cells to myocardial injury remains unknown in vivo. In this study, we directly transplanted the Sca-1+/CD31- cardiac SP cells into the myocardial infarction region. After two weeks, the transplanted cells were found to express cardiomyocyte or endothelial cell markers. Importantly, transplanted into non-infarct myocardium after ischemia, these cells were found in injured myocardium. Consistent with their homing property, these cells showed higher expression of CD184, a chemokine receptor, which is responsible for hematopoietic stem cell migration. Our results, therefore, have suggested that the Sca-1+/CD31- cardiac SP cells are able to mobilize into injured myocardium from non-ischemic myocardium and differentiate into cardiomyocytes as well as endothelial cells in the acutely infarcted mouse heart. Understanding and enhancing such processes may hold enormous potential possibilities for therapeutic myocardial regeneration in ischemic heart disease.

P16

BMP-2 ENHANCES TGF-_3 - MEDIATED CHONDROGENESIS OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS TOWARDS NUCLEUS PULPOSUS-LIKE CELLS IN ALGINATE BEAD CULTURE

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Regeneration of the intervertebral disc (IVD) is a new therapeutic horizon for treating back and neck pain. Bone marrow (BM) mesenchymal stem cells (MSCs) are pluripotent stem cells capable of differentiating into various cell types including chondrocytes. However, it remains uncertain whether MSCs can be induced *in vitro* to differentiate into nucleus pulposus (NP) – like cells. In this study, we investigated the capacity of BM MSCs to differentiate into NP-like cells using a growth factor - based induction system. The MSCs were encapsulated in alginate beads and induced for differentiation using serum-free media containing 10ng/ml transforming growth factor-_3 (TGF-_3) or its combination with bone morphogenetic protein-2 (BMP-2) for up to 21 days. Real-time PCR, histo/immunohistochemical staining, Western blot and ^{35}S-incooperation assay were carried out to evaluate NP phenotype development and involved signalling pathways. Results showed that gene expression of NP markers, aggrecan, types II & IX collagens, melanoma inhibitory activity and Sox9 were up-regulated at higher levels by TGF-_3/BMP-2 than TGF-_3 alone. The gene expression study of Smad pathway showed a decreased Smad3 and an increased Smad8 during differentiation. Blocking BMP-2 signaling by noggin completely suppressed BMP-2 enhanced marker gene expression, confirming a critical role of BMP-2 in chondrogenesis of MSCs. Pharmacological inhibition of MEK1/2 of MAPK signaling pathway resulted in an increase in aggrecan and type II collagen mRNA levels. Protein expression also showed stronger NP phenotype by TGF-_3/BMP-2 stimulation. The differentiated MSCs are functional by presenting proteoglycan biosynthesis. In conclusion, the combination of TGF-_3 with BMP-2 in alginate bead culture is superior to the conventional TGF-_ induction method for chondrogenesis of BM MSCs towards NP cell phenotype *in vitro*. This system provides a potential to supply NP-like cells for IVD regeneration and also can be used as an *in vitro* model to investigate the underlying molecular mechanisms of disc cell phenotype induction.

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