“Islet equivalent” stem cells and diabetes

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Submitted for the examination of MD (Res) at Imperial College London
All this work was carried out in the

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ABSTRACT

Type I diabetes mellitus is a metabolic disorder caused by an autoimmune destruction of the insulin-producing beta cells in the islets of Langerhans. Despite insulin treatment, diabetes results in the development of micro and macro-vascular complications that are associated with a high rate of morbidity and mortality. Curing diabetes would be the best treatment option for these patients. Pancreas transplantation is limited by the shortage of organ donors and by the high risk associated with this surgical procedure. Islets transplantation is a more recent non-invasive technique with a low risk of complications. It is however restricted by the shortage of organs and has variable success rates. Additionally, it is well established that after infusion of islets, a substantial percentage of them are lost immediately post-transplant through the instant blood-mediated inflammatory reaction. A promising option would be the use of stem cells as 'islet equivalents'. These cells have the ability to proliferate and the capacity to differentiate into insulin-secreting cells leading to islet repair and regeneration of new beta cells. The cell source would not be limited and the cells could be transplanted in a non-invasive method.

This work examines the efficacy of two sources of stem cells in restoring euglycaemia in a diabetic animal model. It demonstrated the success of pancreas derived progenitor cells but not haematopoietic stem cells in reversing this diabetic phenotype. This work also examines whether these stem cells expressed tissue factor as a trigger for provoking coagulation leading to significant early cell loss. It demonstrated that both stem cell types are prothrombotic and that this effect can be mitigated by local treatment of these cells with anti-thrombin cytotopic agent. The results were however inconclusive whether this procoagulant effect was via TF-dependent or TF-independent mechanism.
Finally, this work includes the completion of a phase I trial demonstrating the safety and tolerability of the infusion of autologous expanded progeny of an adult CD34+ stem cell subset to patients with type I diabetes mellitus and a successful renal transplant. However, there was no convincing evidence to suggest efficacy of these cells in reducing insulin requirements in these diabetic patients.
ACKNOWLEDGMENTS

I would like to acknowledge my family in particular my mother for being there for me whilst jumping all hurdles in my career but especially whilst writing this thesis. I would like to thank my sister for her patience and support during the whole process. Without them, I would not have been able to achieve what I have done so far.

I would like to thank Tony Dorling for believing in me and for all his ongoing help and support throughout my MD journey even after moving to Kings College. He is my main supervisor and is a great academic and teacher. He guided me patiently through all the animal work and laboratory-based experiments as well as writing up. I would also like to thank Vassilios Papalois who gave me so much encouragement over the last few years and who without, this clinical trial would have not have happened. He is my second supervisor who guided and supported me through the clinical trial. I would like to express my gratitude to Terry Cook who took over my supervision at the last minute and was of great help whilst writing this thesis. I would also like to thank Liz Lightstone for helping me on several occasions when I ran into difficulties during my MD. In addition, it would not have been possible to complete my thesis without Nicky Kumar who is always generous with her time and advice and the encouragement of my friends in the research office especially Marie and Michelle.

I would like to acknowledge Nagy Habib and Paul Shiels and their teams who kindly provided me with the stem cells that composed the basis of my MD, and the team at Imperial College Stem Cell Laboratory and John Goldman Centre for Cellular Therapy, at Hammersmith Hospital where the clinical trial product was manufactured.
Finally, I would like to thank David Taube for being an inspirational mentor over many years and who gave me this opportunity and making it possible for me to undertake this degree.
DECLARATION

Except where acknowledged, I declare that this thesis is entirely my own work and is based upon research carried out in the department of renal and transplant medicine at Imperial College and Imperial College Healthcare NHS trust between December 2007 and June 2011.

I undertook all of the experimental stem cell work in the diabetic animal model. All clotting assays and immunocytochemistry studies were performed by myself. All basic statistical analyses were also performed by me.

Professor Charles Pusey was the Principal investigator of the clinical trial. I joined the team of the trial after the protocol has been established and the initial ethics approval was pending. The clinical trial was given ethical and local approval in 2007 with further amendments approval in 2008. Once part of the team, I was involved in every process related to the study from then onwards. This included applying approvals for minor amendments, consenting, screening and enrolling patients into the trial. Patients were consented by Prof Vassilios Papalois in my presence. Once the patient was recruited into the study, I was responsible for organising the various steps of the protocol by collaborating with the different departments, assessing the patient at follow up visits and completing the Case Report Forms. I also worked closely with the study monitor at every stage to ensure all the necessary steps have been taken for a safe and successful clinical study.
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PUBLICATION


Presentation

Phase I safety and tolerability study following the infusion of the autologous expanded progeny of an adult CD34+ stem cell subset (Insulincytes) to patients with type I diabetes mellitus and a successful renal transplant. R Charif, S Marley, MY Gordon, P Tait, J Apperley, J Davis, C Pusey, N Habib, D Taube And V Papalois. Oral presentation at BTS March 2010.

Poster

Phase I safety and tolerability study following the infusion of the autologous expanded progeny of an adult CD34+ stem cell subset (Insulincytes) to patients with type I diabetes mellitus and a successful renal transplant. R Charif, S Marley, MY Gordon, P Tait, J Apperley, J Davis, C Pusey, N Habib, D Taube And V Papalois. Poster at ATC May 2010.
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<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>ABOc</td>
<td>ABO compatible</td>
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<tr>
<td>ABOi</td>
<td>ABO incompatible</td>
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<tr>
<td>AGE</td>
<td>Advanced glycation end products</td>
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<tr>
<td>Anti-HLA</td>
<td>Anti-human leucocyte antigen</td>
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<td>ASC</td>
<td>Adult stem cell</td>
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<td>AVR</td>
<td>Acute vascular rejection</td>
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<td>BMSC</td>
<td>Bone marrow derived stem cells</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Deceased donor</td>
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<tr>
<td>CITR</td>
<td>The Collaborative Islet Transplant Registry</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CRF</td>
<td>Case Report Forms</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CT</td>
<td>Clotting time</td>
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<tr>
<td>CyA</td>
<td>Cyclosporine</td>
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<tr>
<td>DAH</td>
<td>Diffuse alveolar haemorrhage</td>
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<tr>
<td>DAPI</td>
<td>4,6 diamino-2-phenylindole</td>
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<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
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<tr>
<td>DSA</td>
<td>Donor specific antibody</td>
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<td>EBMT</td>
<td>European Blood and Marrow Transplant Group</td>
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<td>EBV</td>
<td>Epstein Barr virus</td>
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<td>EC</td>
<td>Endothelial cell</td>
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<td>EDIC</td>
<td>Epidemiology of Diabetes Interventions and Complications</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EPC</td>
<td>Endothelial progenitor cells</td>
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<td>ESC</td>
<td>Embryonic stem cell</td>
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<td>ESRD</td>
<td>End stage renal disease</td>
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<td>FBC</td>
<td>full blood count</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FDP</td>
<td>Fibrinogen degradation products</td>
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<td>FFA</td>
<td>Free fatty acids</td>
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<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
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<td>FVIIa-AT</td>
<td>Activated factor VII-Antithrombin</td>
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<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
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<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<td>GAL</td>
<td>Galactose-α1, 3-galactose</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
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<td>Hba1c</td>
<td>Glycosylated haemoglobin</td>
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<td>HBSS</td>
<td>Hanks’ buffered saline solution</td>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency</td>
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<tr>
<td>HMWK</td>
<td>High-molecular weight kininogen</td>
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<td>HP</td>
<td>Human plasma</td>
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<td>HSC</td>
<td>Haematopoietic stem cell</td>
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<td>HSCT</td>
<td>Haematopoietic stem cell transplant</td>
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<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<tr>
<td>hADSC</td>
<td>human adipose-derived mesenchymal stem cell</td>
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<tr>
<td>hTF</td>
<td>Human tissue factor</td>
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<tr>
<td>hTFPI</td>
<td>Human tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>HTLV</td>
<td>Human T-lymphotrophic virus</td>
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<td>IA-2</td>
<td>Insulinoma-associated protein 2</td>
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<td>IBMIR</td>
<td>Instant blood-mediated inflammatory reaction</td>
</tr>
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<td>IBMTR</td>
<td>International Bone Marrow Transplant Registry</td>
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<td>ICC</td>
<td>Immunocytochemistry</td>
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<td>Islet equivalents</td>
</tr>
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<td>IL-2</td>
<td>Interleukin 2</td>
</tr>
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<td>Interleukin 3</td>
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<tr>
<td>INFγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IPF1</td>
<td>Insulin promotor factor 1</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
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<tr>
<td>I/R</td>
<td>Ischaemia reperfusion</td>
</tr>
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<td>ISSCR</td>
<td>International Society of Stem cell Research</td>
</tr>
<tr>
<td>ITR</td>
<td>Islet Transplant Registry</td>
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<tr>
<td>LD</td>
<td>Live donor</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>mADSC</td>
<td>mouse adipose-derived mesenchymal stem cell</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein 1</td>
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<tr>
<td>MHV-3</td>
<td>Murine hepatitis virus type-3</td>
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<tr>
<td>Millimoles/L</td>
<td>mmol/L</td>
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<tr>
<td>MMTT</td>
<td>Mixed meal tolerance test</td>
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<tr>
<td>MNC</td>
<td>Mononuclear cell</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity onset diabetes of the young</td>
</tr>
<tr>
<td>MP</td>
<td>Mouse plasma</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>mTF</td>
<td>Mouse tissue factor</td>
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<td>NCT</td>
<td>Nicotinamide</td>
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<td>NFkB</td>
<td>Nuclear factor-kB</td>
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<td>NGN-3</td>
<td>Neurogenin-3</td>
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<tr>
<td>Omniceyes</td>
<td>Day 7 human adherent CD34+ Omniceyes</td>
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<tr>
<td>PAK</td>
<td>Pancreas after kidney transplant</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBSC</td>
<td>Peripheral blood stem cells</td>
</tr>
<tr>
<td>PDPC</td>
<td>Pancreas derived progenitor cell</td>
</tr>
<tr>
<td>Pdx1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PEC</td>
<td>Porcine endothelial cell</td>
</tr>
<tr>
<td>PRA</td>
<td>Plasma calcification assays</td>
</tr>
<tr>
<td>PTA</td>
<td>Pancreas transplant alone</td>
</tr>
<tr>
<td>pTF</td>
<td>Porcine tissue factor</td>
</tr>
<tr>
<td>rATG</td>
<td>Rabbit anti-thymocyte globulin</td>
</tr>
<tr>
<td>rhAPC</td>
<td>recombinant human activated protein C</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SKPT</td>
<td>Simultaneous kidney and pancreas transplant</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythromatosus</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
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<tr>
<td>TAT</td>
<td>Thrombin-antithrombin complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TF+</td>
<td>Tissue factor positive</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TIDM</td>
<td>Type I diabetes mellitus</td>
</tr>
<tr>
<td>TLN</td>
<td>Thrombalexin</td>
</tr>
<tr>
<td>TMA</td>
<td>Thrombotic microangiopathy</td>
</tr>
<tr>
<td>TTP</td>
<td>Thrombotic thrombocytopenic purpura</td>
</tr>
<tr>
<td>VOD</td>
<td>Veno-occlusive disease</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α-minimal essential medium</td>
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Equal efficacy of intravenous and intra-pancreatic day 7 adherent CD34+ Omnicytes treatment of STZ-induced diabetic mice in stabilising glucose levels (Unpublished data)
Reproduced with permission from A Dorling

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1. INTRODUCTION

Insulin dependent diabetes mellitus, also called Type I diabetes mellitus (TIDM) is a metabolic disorder characterised by hyperglycaemia due to insulin deficiency. It is common and the incidence varies from 8 to 17 per 100,000 population per year in Northern Europe and the U.S. with the highest incidence of 35 per 100,000 in Scandinavia to the lowest incidence of 1 per 100,000 in Japan and China (Kasper 2005).

Type I diabetes causes an estimated 5–10% of all diabetes cases (Daneman 2006) or affects 17 - 34 million people worldwide (WHO 2013). The incidence of type I diabetes has been increasing by about 3% per year (Aanstoot 2007). Much of the data on epidemiology of type I diabetes in young people have been generated by large collaborative efforts that were centered on standardised registry data, such as the DIAMOND Project worldwide and the EURODIAB Study in Europe (Green 2001; Group 2006). The EURODIAB register is an international collaborative effort, which encompasses population-based registers of new cases of TIDM in children aged less than 15 years from 20 centers, distributed across 17 countries in Europe. In a relatively recent Lancet study, Patterson and the EURODIAB Study Group provided updated estimates of trends in incidence of Type I diabetes in individuals younger than 15 years in Europe from 1989 to 2003. 15-year incidence data collected by 20 population-based registries in 17 countries were used to estimate rates of increase in geographical regions in Europe. Model-based rates of increase were then used to predict the number of new cases throughout Europe by 2020. The prediction is that between 2005 and 2020, new cases of type I diabetes in European children younger than 5 years will double and that the prevalence of cases in those younger than 15 years will increase by an alarming 70% (Patterson 2009).
Type I diabetes mellitus is caused by autoimmune destruction of the insulin-producing beta cells in the pancreas. Despite insulin treatment, diabetes often results in metabolic defects leading to the development and progression of micro and macro-vascular complications. Macrovascular disease leads to an increased prevalence of cardiovascular disease, while microvascular damage results in diabetic retinopathy, nephropathy and neuropathy. These late complications are associated with a high rate of morbidity and mortality. It has been shown that these diabetic complications are less common and less severe in patients with well-controlled blood glucose levels (Nathan 2005).

Curing diabetes would be the best treatment option for these patients to restore euglycaemic state. Replacement of the destroyed insulin-producing tissue, usually by whole pancreas transplantation, can cure these metabolic abnormalities but is greatly limited by the shortage of deceased organ donors and by the high risk associated with this surgical procedure. This means that this curative option is only offered to a selective group of patients. Allotransplantation of pancreatic islets is a more recent relatively non-invasive technique with a low risk of complications. Although it is a more attractive option, it is again restricted by the falling number of organ donors and has had a variable success rates in different transplant centres. An ideal and promising treatment option to cure T1DM would be to use ‘islet equivalents’ which are primarily derived from a stem or progenitor cell population shown to have the ability to proliferate extensively and the capacity to either differentiate into insulin-secreting cells in sufficient numbers or promote islet repair by the regeneration of new beta cells. The source of these cells would hence not be limited and they could be transplanted in a similar non-invasive method to pancreatic islets with a low risk of complications.
1.1 Pancreas development and anatomy

1.1.1 Embryological development

The pancreas forms from the embryonic foregut and is therefore of endodermal origin. Pancreatic development begins with the formation of ventral and dorsal anlage or buds. The ventral pancreatic bud becomes the head and uncinate process, and comes from the hepatic diverticulum. The ventral system also gives rise to the hepatobiliary system. At approximately the eighth intrauterine week of life, differential rotation and fusion of the ventral and dorsal pancreatic buds results in the formation of the definitive pancreas. As the duodenum rotates to the right, it carries with it the ventral pancreatic bud and common bile duct. After reaching its final destination, the ventral pancreatic bud fuses with the much larger dorsal pancreatic bud. At this point of fusion, the main ducts of the ventral and dorsal pancreatic buds fuse, forming the duct of Wirsung, the main pancreatic duct (Carlsson 2004; Kozu 1995). Figure 1 illustrates the stages of embryological development of the pancreas.

Differentiation of cells of the pancreas proceeds through two different pathways, corresponding to the dual endocrine and exocrine functions of the pancreas. In progenitor cells of the exocrine pancreas, important molecules that induce differentiation include follistatin, fibroblast growth factors, and activation of the Notch receptor system. Development of the exocrine acini takes place through three successive different stages. These include the predifferentiated, protodifferentiated, and differentiated stages, which correspond to undetectable, low, and high levels of digestive enzyme activity, respectively.

Progenitor cells of the endocrine pancreas arise from cells of the protodifferentiated stage of the exocrine pancreas. Under tight control of several pancreas-specific transcription factors, these cells differentiate to form two lines of committed endocrine precursor cells. Among these factors are pancreatic and duodenal
homeobox 1 (Pdx1), neurogenin-3 (NGN-3) and Insulin gene enhancer protein ISL-1.

The first line of committed cells, under the direction of PAX-6 forms alpha-(α) and gamma-(γ) cells, which produce glucagon and pancreatic polypeptides respectively. The second line, influenced by PAX-4, produces beta-(β) and delta-(δ) cells, which secrete insulin and somatostatin respectively. Insulin and glucagon can be detected in the human fetal circulation by the fourth or fifth month of fetal development (Carlson 2004).

1.1.2 Anatomy and physiology

The pancreas is a composite gland of both exocrine and endocrine components. Pancreatic beta cells are found in the islets of Langerhans, which are of various sizes and contain a few hundred to a few thousand endocrine cells. Islets are anatomically
and functionally separate from pancreatic exocrine tissue. Exocrine cells secrete pancreatic enzymes and fluid directly into ducts that drain into the duodenum. Normal subjects have about one million islets that in total weigh 1 to 2 grams and constitute only 1 to 2% of the mass or parenchyma of the pancreas (Bray 1994).

Islets vary in size from 50 to 300μm in diameter. They are composed of several types of cells. At least 70% are beta cells, which are localized in the core of the islet and secrete insulin. These beta cells are surrounded by alpha cells that secrete glucagon, smaller numbers of delta cells that secrete somatostatin and pp cells or gamma cells that secrete pancreatic polypeptide as shown in figure 2. All of the cells communicate with each other through extracellular spaces and through gap junctions. This arrangement allows cellular products secreted from one cell type to influence the function of downstream cells. As an example, insulin secreted from beta cells suppresses glucagon secreted from alpha cells (Constanzo 2011).

A neurovascular bundle containing arterioles and sympathetic and parasympathetic nerves enters each islet through the central core of beta cells. The arterioles branch to form capillaries that pass between the cells to the periphery of the islet and then enter the portal venous circulation.

After birth, the maintenance of beta cell mass is the result of a dynamic process consisting of neogenesis, proliferation and apoptosis (Bonner-Weiss 2002). This is a physiological process whose objective is to guarantee the preservation of glucose homeostasis in the presence of different challenges such as obesity and other states of insulin resistance (Fernandes 1997; Swenne 1992). One study by Bonner-Weir and colleagues demonstrated that there was 27% recovery of pancreas weight and 42% of the endocrine pancreas 8 weeks after near-total pancreatectomy in young rats (Bonner-Weir 1983). The same group estimated the beta cell turnover in adult rat islets to be as high as 3% per day (Finegood 1995). In normal or even in diabetic
animal models, chronic high-dose glucose infusion protocols showed increased beta cell mass, beta cell function, neogenesis and indices of beta cell replication (Bonner-Weir 1989; Sako 1990; Topp 2004).

Figure 2: Schematic representation of the anatomic relationship in an islet of Langerhans. The insulin-secreting beta cells (in blue) are in the centre closest to the blood supply and are surrounded by the glucagon-producing alpha cells (in orange). On the outside are the delta cells (in yellow), which make somatostatin, and the PP cells (in green), which make pancreatic polypeptide. Adapted with permission from: Robertson RP. In: UpToDate, Basow DS (Ed), UpToDate, Waltham, MA, 2013. Copyright © 2013 UpToDate, Inc.

1.2 Type I diabetes mellitus

TIDM is characterised by the permanent destruction of insulin-secreting β-cells within the pancreatic islets of Langerhans (Atkinson 1994). This process occurs in genetically susceptible subjects, is probably triggered by one or more environmental factors, and usually progresses over many months or years during which the subject is asymptomatic and euglycemic. This long latent period is a reflection of the large
number of functioning beta cells that must be lost before hyperglycemia occurs (McCulloch 1991).

Symptomatic patients typically have lost more than 70% of their beta-cell population, resulting in essentially no insulin production and an inability to regulate plasma glucose levels properly. The beta-cell destruction seen in type I diabetes is most often caused by an autoimmune mechanism. The immune system in such patients loses the immunologic tolerance against beta cell antigens, resulting in an immune response against the islets. This process involves both CD8+ and CD4+ cells and results in insulitis, an inflammatory process, which causes the destruction of pancreatic beta cells (Gepts 1965; Notkins 2001).

There are a number of auto-antigens within the pancreatic beta cells that may play important roles in the initiation or progression of autoimmune islet injury including glutamic acid decarboxylase (GAD), insulin, insulinoma-associated protein 2 (IA-2), and zinc transporter ZnT8 (Boitard 1992).

In the latter stage of the natural history of the disease, there is little insulin secretion as manifested by low or undetectable serum C-peptide levels. C-peptide is co-secreted with insulin by the beta cells as a by-product of the enzymatic cleavage of pro-insulin to insulin. Thus measurement of C-peptide provides a validated means of quantifying endogenous insulin secretion being closely related to the amount of functioning beta cell mass (Palmer 2004). The natural history of diabetes is illustrated in figure 3.
Although the natural history of diabetes has been described in the literature, there are no published data on the actual rates of transient or complete remission in patients with new diagnosis of type I diabetes. There are some studies that investigated the residual insulin secretion at the time of diagnosis for type I diabetes. It has been widely accepted that insulin secretion is profoundly impaired at the time of diagnosis of type I diabetes. However, recent studies suggest that on the whole, insulin production may be more substantial at diagnosis than had been previously appreciated and that residual insulin production may persist in a subgroup of patients with autoimmune diabetes (Sherry 2005). Steel et al. studied insulin secretory responses to a mixed-meal tolerance test (MMTT) patients with new-onset type I diabetes. They found the average response to be 52% of the response of normal control subjects but with maximal stimulation, the impairment in response was more profound at 30% of normal response. In same study, they also found that insulin secretion in response to MMTT declined gradually to a level at 2 years that was 28 ±
8.4% of the response at diagnosis (Steele 2004). By 24 months, 47% of patients had C-peptide levels that were below the lower limit of detection.

1.3 Vascular complications in type I diabetes mellitus

Diabetes results in metabolic defects leading to both microvascular and macrovascular disease. These complications are associated with significant morbidity and mortality as has been shown in many studies (Barzilay 1992; Krolewski 1988). Microvascular complications include diabetic retinopathy, nephropathy and neuropathy while macrovascular disease leads to coronary artery disease, peripheral vascular disease and cerebrovascular complications. Mechanisms for vascular disease in diabetes include the pathologic effects of advanced glycation end product accumulation, impaired vasodilatory response attributable to nitric oxide inhibition, smooth muscle cell dysfunction, overproduction of endothelial growth factors, chronic inflammation, hemodynamic dysregulation, hypercoagulability, and enhanced platelet aggregation (Vinik 2002). Hyperglycaemia induces the glycation of mitochondrial proteins resulting in the generation of superoxide anions. The latter are key in the pathogenesis of microvascular diabetic complications via activation of the polyol pathway, the hexosamine pathway, formation of advanced glycation end products (AGE), activation of protein kinase C and nuclear factor F-kB (NFκB) in endothelial cells. Some of these processes may persist even after correction of hyperglycaemia, providing a molecular explanation for the concept of ‘glycaemic memory’ (Vella 2010). In the pathogenesis of macrovascular disease, free fatty acids (FFA) may have similar effects on mitochondrial oxidative stress pathways, particularly in states of insulin resistance (Vella 2010).

Common risk factors for vascular disease in patients with diabetes include hyperglycaemia, dyslipidaemia, hypertension, smoking, and obesity. The association
of hyperglycaemia with vascular complications was confirmed in the prospective Diabetes Control and Complications Trial (DCCT). This trial demonstrated that intensive insulin therapy, aiming for lower glucose levels, resulted in lower rate of microvascular complications (DCCT 1995; DCCT 2006). At best, tight diabetic control by intensive insulin therapy reduces the risk of microvascular complications by 35-90% when compared to conventional therapy (DCCT 2002). In addition, the observational Epidemiology of Diabetes Interventions and Complications (EDIC) follow-up study to the DCCT, reported extended benefit of risk reduction of retinopathy and nephropathy over the next 10 years in the intensive insulin therapy compared to conventional therapy, despite the absence of a difference in glycaemic control between the 2 groups during the post-DCCT trial period. The same EDIC study also demonstrated that tight glycaemic control in type I diabetes decreased fatal and nonfatal cardiovascular events (Nathan 2005).

1.4 Current therapies for type I diabetes mellitus

1.4.1 Insulin

Exogenous insulin replacement has been the primary therapeutic option for controlling plasma glucose levels. However, even modern insulin regimens consisting of short-, medium- and long-acting, genetically synthesised insulin fail to match the sensitivity of nutrient-induced, endogenous insulin production, potentially resulting in undesirable patient outcomes. While intensive management of diet and insulin therapy is desirable as it has been shown to reduce complications, patients with type I diabetes are susceptible to hypoglycaemia, which remains a limiting and potentially life-threatening side effect. This risk of hypoglycaemia is increased with intensive insulin therapy and is particularly problematic in patients with brittle type-I diabetic disease and in those who suffer hypoglycaemic unawareness, a
manifestation of autonomic neuropathy (DCCT 1995; DCCT 2006). These complications and side effects make insulin administration a suboptimal treatment option for this group of patients.

1.4.2 Beta cell - based treatment

The ideal treatment for type I diabetic patients is restoration of euglycaemia by replenishing the pancreatic islets through either repair or regeneration or both. In view of evidence for regenerative potential of the pancreas (section 1.1.2), some researchers attempted the use of effective and safe immunoregulatory strategies in an attempt to block the autoimmune process causing diabetes and ultimately preserve beta-cell mass by facilitating endogenous mechanisms of beta cell regeneration. Such immunomodulatory approach should be done shortly after or even before the clinical presentation of type I diabetes since larger beta cells mass would be preserved. Studies with chronic use of immunosuppressive agents such as steroids, azathioprine, or combination of both or cyclosporine demonstrated a slower decline or even some improvement in serum C-peptide levels in patients with new diagnosis of type I diabetes (Assan 1990; Cook 1989; Elliott 1981). Other studies used immunomodulatory therapies such as humanized anti-CD3 or rabbit polyclonal anti-T cell globulin. However, this strategy was not effective as only minority of patients experienced short-term insulin independence despite increased C-peptide levels. Treated patients did show better beta cell function and lower insulin requirement up to 1 year post therapy when compared to placebo group but this effect was not long lasting (Keymeulen 2005; Saudek 2004).

For long term type I diabetes, immunointervention regimens alone would not be successful as most of the beta cell mass would have been destroyed already (section 1.2) by the time patients are diagnosed. For these patients, the strategy
should combine immunointervention to prevent ongoing autoimmunity with beta cell replacement. Because type I diabetes is caused by loss of function of a single cell type, cell-based treatment focusing on replacing lost beta (β)-cell populations is possible. These therapies include whole pancreas organ transplantation, islet allograft transplantation and islet “equivalent” cell therapy based on stem cells. These potential strategies are summarised in figure 4.

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**Figure 4: Potential strategies for treatment of recent onset and long term type I diabetes adapted from (Couri 2008a)**

1.5 Pancreas transplantation

Whole pancreas transplantation remains the current gold standard of cell-based therapies for type I diabetes mellitus. It can offer a sustained normoglycaemic state by restoring glucose-regulated endogenous insulin secretion in these patients. This in turn improves the quality of life, reduces the long-term risk of vascular damage and eliminates the risk of hypoglycaemia (Vardanyan 2010). This surgery was first
performed in Minnesota in 1966, and by 2008 over 30,000 pancreas transplants had been performed worldwide; 22,000 in the US and 8,000 elsewhere (Gruessner 2008; Kelly 1967). The majority of these transplants (60-70%) were simultaneous kidney pancreas transplants (SKPT), less frequently pancreas after kidney (PAK) in patients with end stage renal failure due to diabetic nephropathy. Fewer than 4% of cases were pancreas combined with heart, liver, or intestine. Pancreas transplant alone (PTA) represented only 7% of the 2008 total pancreas transplants but this has increased to 25% in 2012 (United Network for Organ Sharing 2012).

1.5.1 Pancreas transplant outcomes

The mortality, morbidity and results of pancreas transplantation have improved over the years but vary with surgical experience, patient and donor selection. Patient survival rates range from 95-98% at one year, 90-92% at 3 years and 85-88% at 5 years. (Arbor Research Collaborative for Health; Gruessner 2011; United Network for Organ Sharing 2012).

Graft survival is defined as sustained exogenous insulin independence evidenced by normal fasting glucose concentrations and normal glycosylated haemoglobin (Hba1c) values. Long-term graft function remains highest in patients receiving an SKPT followed by PAK and PTA. Graft survival varied between 86-92% at 1 year and 60-76% at 5 years in SKPT recipients. This compares to graft survival rates of 78-88% at 1 year and 45-55% at 3 years for pancreas transplants alone (Arbor Research Collaborative for Health; Gruessner 2011; United Network for Organ Sharing 2012).
1.5.2 Benefits of pancreas transplantation

Successful pancreas transplantation restores euglycaemia almost immediately post transplantation. Recipients experience normal fasting and post-prandial glucose levels and Hba1c returns to normal levels. It results in independence from exogenous insulin as a consequence of restoring pancreatic islet function. The impact of successful pancreas transplantation on the secondary complications of diabetes have been mainly studied and reported in SKPT recipients. Morbidity and mortality have decreased markedly during the past several decades after pancreas transplantation. Recipients who become insulin independent report better quality of life despite the need for immunosuppression (Becker 2001; Landgraf 1996). The best patient survival was observed in recipients of SKPT with prolonged pancreas graft function (Reddy 2003; Smets 1999; Weiss 2009). Figure 5 shows patient survival in diabetic patients receiving SKPT transplant compared to those receiving a kidney transplant alone. Patient survival of those who were transplanted with a SKPT has also been reported to be superior to patient survival observed with waitlisted patients who remain on dialysis (Witczak 2007). In addition, stabilization or improvements in microvascular complications, such as diabetic retinopathy, nephropathy and neuropathy, have been reported after successful pancreas transplantation (Fioretto 1998; Navarro 1997). Positive impact on macrovascular disease progression after SKPT has also been demonstrated (Jukema 2002; Sutherland 2001). Hypoglycaemia and hypoglycaemic unawareness are rare post pancreas transplant. Pancreas transplantation results in improvement of glucose counter-regulation after hypoglycaemia so that symptom recognition of hypoglycaemia is restored and occurs at higher blood glucose concentrations.
1.5.3 Limitations to pancreas transplantation

Although pancreas transplantation has a high success rate, it is a major surgical procedure with associated mortality rates between 1 and 3%. The technical failure rate in the United States is around 8%. Common complications encountered early after pancreas transplantation includes increased cardiac morbidity as well as hospitalisation and systemic infection. Beyond the first year, complications are usually related to long-term immununosuppression (White 2009). Accordingly, it is currently recommended only for patients with type I diabetes who do not respond to conventional, insulin-based treatment and who are deemed fit to undergo this surgical risk. An unavoidable and major restriction of whole-pancreas transplantation is the limited availability of organ donors in general and of donor pancreata specifically that are suitable for transplantation.
Donor selection in pancreas transplantation significantly impacts on outcome (Sutherland 2001). An ideal donor would be defined as a donation after brain death, between 10 and 40 years of age, with a BMI less than 28kg/m$^2$ and cause of death other than cerebrovascular disease (Fridell 2010). Hence there is reluctance to accept higher-risk organs resulting in higher non-recovery or discard rates compared to other abdominal donor organs (Gruessner 2011; Muthusamy 2012).

The combination of donor and recipient selection, organ shortage and the high risk of complications associated with pancreas transplantation makes this treatment option suitable only for a minority of diabetic patients.

### 1.6 Islet transplantation

Given the complications of whole pancreas transplantation, newer research efforts have explored transplanting only pancreatic islet cells. Islet transplantation has the potential to restore homeostatic insulin secretion and hence normoglycaemia in patients with type I diabetes. Importantly, it is less invasive than whole pancreas transplant. The process of islet transplantation is summarised in figure 6. A mixture of highly purified specialized enzymes (Collagenase) is used to isolate islets from the pancreas of a deceased donor. Collagenase solution is injected into the pancreatic duct that runs through the head, body and tail of the pancreas. Delivered this way, the enzyme solution causes distension of the pancreas, which is subsequently cut into small chunks and transferred into so-called Ricordi’s chamber, where digestion takes place until the islets are liberated and removed from the solution. Isolated islets are then separated from the exocrine tissue and debris in a process called purification. A radiologist usually performs the transplant where the islets are infused slowly through an intravenous catheter into the portal vein of the liver of the recipient. Blood flow within the vein carries the islets into the liver tributaries where they lodge.
within the sinusoids and establish vascular connections. Possible risks of the procedure include bleeding and thrombosis.

Because the islets are fragile, the process of islet preparation and purification is difficult and results in significant proportion of islets damaged or lost. Typically a patient receives at least 10,000 islet "equivalents" per kilogram of body weight, extracted usually from two donor pancreata. Patients often require two or three islet infusions to achieve insulin independence. Some transplants have used fewer islet equivalents taken from a single donated pancreas.

Figure 6: The process of clinical islet transplantation for the treatment of diabetes mellitus (Naftanel 2004)

1.6.1 Islet transplant outcomes

Although it is theoretically advantageous procedure, it has not yielded the tangible success seen with whole-pancreas transplants. Insulin independence was first achieved with this method in 1989, but it required significant immunosuppression to maintain graft survival (Scharp 1990). Over the following decade, developments of
more effective and less toxic immunosuppressive regimens as well as improved harvesting techniques have significantly increased the success rate of islet transplantation. In 2000, the novel Edmonton protocol, a steroid-free immunosuppressive regimen, enabled seven patients to remain insulin-independent for an average of one-year post procedure (Shapiro 2000). Figure 7 shows that in 2000 islet graft survival of the Edmonton protocol became comparable to pancreas transplant graft survival of the mid 1980's. However these results have been difficult to reproduce and 9-year islet graft survival rates even among the original Edmonton patients remain below 10% (Robertson 2004a). Initially successful islet transplant had an uncertain long-term islet survival, with the majority of patients requiring multiple islet infusions to achieve normoglycaemia.

The Collaborative Islet Transplant Registry (CITR) data, collected across international clinical islet programmes showed that between 1999 and 2009, 571 allogeneic islet transplant recipients received 1,072 infusions from 1,187 donors. The United States contributed 66% of these transplants and the remaining 34% were preformed in Europe and Australia. 31% of transplant recipients received a single islet infusion, 47% received two infusions, 20% received three and 2% received 4-6 infusions (CITR 2012). CITR data also reported that 60% of patients, including patients with multiple infusions, achieve insulin independence with the first year, but by the third year, the percentage that remain euglycaemic is closer to 35%. As described in section 1.6, a significant proportion of the islet equivalent will be lost in the purification process in addition to further islet mass loss early post transplantation into the liver. Up to 50% of islets can be lost during the isolation process and hence the frequent need for 2-3 pancreata to prepare sufficient islets for a single recipient (Leitao 2008). The mechanism of the additional early graft loss following transplantation is by a process termed the instant blood-mediated inflammatory reaction (IBMIR). This process is explained in detail later in section 1.10.6.
Figure 7: Comparison of rates of progress of allogeneic islet transplantation versus pancreas transplantation as therapy for type I diabetes. The first allogeneic islet transplant to be reported as successful appeared in 1980, 20 years earlier than the Edmonton series was reported. The first pancreas transplant to be reported as successful appeared in 1966, 18 years earlier than the 1984–1987 series was reported. At 15 months post-transplant, the Edmonton series of allogeneic islet transplants for the years 2000–2005 compared favourably with the success rate of pancreas transplants for the years 1984–1987 (Robertson 2010)

1.6.2 Benefits of islet transplantation

Allotransplantation of pancreatic islets is a relatively recent non-invasive technique, which has had variable success rates in different transplant centres. It is less efficient in reversing diabetes but is associated with a lower risk of immediate post-procedure complications and lower morbidity rate when compared to whole pancreas transplantation. Although long-term insulin independence is rarely achieved in the vast majority of patients, there are important benefits by partial function. The occurrence of severe hypoglycaemic events decreases and hypoglycaemic
unawareness abates. Improvements in HbA1c levels are seen and patients exhibit persistent C-peptide function (Bromberg 2006; Ryan 2005).

There are published data that report stabilization of diabetic retinopathy and neuropathy following islet transplantation (Thompson 2008; Warnock 2008). Fiorina et al. demonstrated improvement in diabetic microvascular and macrovascular complications as well as improved patient and graft survival rates in patients with islet after kidney transplant when compared with recipients of kidney transplant alone (Fiorina 2003; Fiorina 2005).

1.6.3 Limitations to islet transplantation

Although islet transplantation is relatively non-invasive, at least 50% of islet recipients experience at least one adverse event, which are either related to immunosuppression or complications associated with the infusion procedure (CITR 2012). These adverse events remain less serious that the complications following pancreas transplantation. Islet transplantation is more restricted than pancreas transplantation by the limited number of deceased donors particularly given that very often at least two pancreata are required to isolate an adequate number of islets per recipient. This is due to the complex and difficult processes required to isolate and purify the islet transplant as well as early loss post transplantation in the hepatic portal vein. Another potential consequence of islet transplantation is a high rate of sensitization. Olack et al. and others showed that sensitization occurred in early diabetic patients who had been treated with multiple islet preparations from multiple donors, leading to the development of anti-human leucocyte antigen (anti-HLA) antibodies (Mohanakumar 2006; Olack 1997; Rickels 2006). This antibody formation and in turn sensitization may preclude the ability to undergo future transplantation.
whether it is islet, pancreas or kidney due to decreased probability of finding a compatible graft.

The body of literature that supports the benefits of islet transplantation or secondary complications is small as the number of studies is limited. Khan and Harlan concluded that there is no enough evidence to advocate the use of allogeneic islet transplantation over conventional diabetes therapy. The current results cannot outweigh the risks or consequences of islet transplantation (Khan 2009).

In summary, islet transplantation still faces major challenges; especially those related to cell loss during the process of islet isolation and the losses related to the graft site, apoptosis, rejection, autoimmunity and immunosuppression. The main strategies to optimize islet transplantation should aim at improving the processes of isolation, purification and implantation of the islets as well as avoiding alloimmune injury. This will lead to better islet outcomes, reduction in the need for multiple infusions and decreasing allosensitisation risks but there is a lot to be done before reaching that goal.

1.7 Islet xenotransplantation and its limitations

As a solution for organ shortage and the availability of suitable donor pancreata, xenotransplantation has been investigated as a new source of islets for transplantation. Xenotransplantation refers to the transplantation of tissues or organs from one species to another. Since the early 1990’s, there have been few published cases of clinical islet xenotransplantation (Elliott 2011; Groth 1994; Valdes-Gonzalez 2005). More recently over the last decade, significant progress has been made, with pig islets providing sustained normoglycaemia in a small number of non-human primates in which diabetes has been induced (Cardona 2006; Cardona...
These results were encouraging and indicated that pig islet xenotransplantation may offer clinically viable treatment option for diabetes in the long-term. However, it also raised many novel medical, logistic, safety and ethical issues. Disease transmission and permanent alteration to the genetic code of the animals used have also been causes for concern.

Successful clinical application of islet xenotransplantation currently is inhibited by a number of immunological and non-immunological barriers. To date no xenotransplantation trials have been entirely successful due to the events arising from the response of the recipient’s immune system. This response, which is generally more extreme than in allotransplantation, ultimately results in the loss and rejection of the xenograft after transplantation. Following transplantation into the portal vein, similar to early islet allotransplant loss, islet xenotransplants face immediate destruction by the non-specific instant blood-mediated inflammatory reaction. In addition and depending on the source of the islets, preformed xenoreactive natural antibodies may contribute to IBMIR which is then followed by a largely T-cell mediated acute rejection. This immune process leads to further loss of the surviving islets (Van der Windt 2012). The mechanism of these processes is described in section 1.10.4.

There are also several technical and logistical obstacles for xenotransplantation. These challenges include the difficulty in isolation and preparation of pig islets which are significantly influenced by the donor age. Deriving islets from pigs of different ages has different advantages and disadvantages. For example, islets from neonatal pigs are smaller in size compared to adult pigs and hence they harder to isolate and purify. On the other hand, neonatal islets have high resistance to hypoxia and maintain a proliferative capacity after transplantation. This may result in a functional islet mass after transplanting a smaller number of islets (Emamaullee 2006; Korbut 1996). The optimum age of donor pigs is the subject of ongoing investigation.
There have been several strategies used to attempt to overcome some of these hurdles for islet xenotransplantation. These include genetic engineering of pigs to avoid the effects of the IBMIR, more targeted and efficacious immunosuppressive regimes, investigating alternative anatomical sites for islet xenotransplantation and immunomodulation by co-transplantation of mesenchymal stem cells of either donor or recipient origin (Van der Windt 2012).

Finally, safety of clinical islet xenotransplantation remains a concern mainly the potential for the transfer of pig microorganisms and viruses to the islet recipient such as porcine cytomegalovirus. There have been guidelines issued by the US regulatory authorities that address this problem (Emamaullee 2006).

In view of these many hurdles, there is still some way to go before islet xenotransplantation becomes an achievable therapeutic option for type I diabetes.

1.8 Stem cells for beta-cell substitution

The number of islet and pancreas transplants, as currently practiced, has an extremely limited impact on the type I diabetes burden. Ultimately, alternate sources of transplantable cells are needed because the current donor pool of 2000 pancreata annually is minor compared to the need (United Network for Organ Sharing 2012). In addition, both transplants are associated with the development of serious complications. Stem cell therapy can offer the greatest potential of producing an unlimited source of cells and if adequately differentiated, they can become fully mature and functional beta cells. If autologous, stem cells could minimize the need for immunosuppression. However, targeted immunosuppression may be required to prevent the autoimmune destruction of the new beta cells (McCall 2009).
Stem cells have the unique function of asymmetric division (Wagers 2004). They have 3 general properties; first, they are capable of long-term self-renewal; second, they are undifferentiated; and third, they can give rise to specialised cell types. These important characteristics distinguish them from other cell types. Stem cells possess the ability of producing undifferentiated daughter cells or generating progenitor tissue-specific or organ-specific specialised cells when given appropriate physiological signals or experimental conditions. A stem cell is pluripotent when it has the ability to differentiate into any of the three germ layers (endoderm, mesoderm and ectoderm) and to give rise to any fetal or adult cell type. This compares to a multipotent stem cell that can give rise to cells of multiple but limited cell types (Alison 2009). Stem cell can be classified as either embryonic stem cells, which are pluripotent or adult stem cells, which are multipotent.

For stem cell therapy to work in type I diabetes, scientists need to recreate ex-vivo in cell culture what happens in the embryo. The stem cell must first be induced to make definitive endoderm, then differentiate into pancreatic endoderm or PDX1-expressing and finally reach a stage in which it secretes insulin in response to high glucose concentrations. The molecular details of this differentiation pathway are still not completely identified. Detailed understanding of the sequence of events, the signaling pathways and quantitative pattern of gene expression during differentiation is essential for designing and producing fully functional insulin-producing cells as shown in figure 8 (Mayhew 2010).
To achieve the promise of novel stem cell-based therapies, scientists must be able to easily and reproducibly manipulate the cells so that they possess the necessary characteristics for successful differentiation, transplantation and engraftment. To be useful for transplant purposes, stem cell must be reproducibly made to:

- Proliferate extensively and generate sufficient quantities of tissue, which is often a technical hurdle.
- Produce progeny that can differentiate into the desired cell type(s)
- Survive in the recipient after transplant
- Integrate into the surrounding tissue after transplant and improve the function of the damaged tissue.
- Function appropriately for long duration
- Avoid harming the recipient in any way
- Avoid the problem of immune rejection

### 1.8.1 Embryonic stem cells

Embryonic stem cells (ESC) are undifferentiated cells derived from the inner cell mass of a blastocyst, usually harvested 4-5 days after fertilization. First isolated in humans in 1998, these cells express high levels of telomerase activity and are capable of differentiating into all three embryonic germ layers, ectoderm, mesoderm and endoderm. Pluripotency is the main characteristic that distinguishes ESC from adult stem cells. Because of their pluripotency and potentially unlimited capacity for self-renewal, ESC therapies have been proposed for regenerative medicine and tissue replacement. They can produce limitless numbers of themselves for continued research or clinical use. However, isolating the embryoblast or inner cell mass results in the destruction of the fertilized human embryo, which raises ethical issues. The other major concern with the possible transplantation of ESC into patients as treatment is their ability to form tumours including teratomas (Hussain 2004).

Murine and human embryonic stem cells can be differentiated into insulin-producing cells by manipulating culture conditions. In vitro differentiation of ESC can generate embryoid bodies, which, after selection for nestin-expressing ESC, were stimulated to differentiate towards a beta-cell-like phenotype (Lumesky 2001; Segev 2004). With
manipulation of culture conditions and the use of transcription factors associated with beta-cell lineage resulted in higher yield of cells with properties of functional beta cells (Blyszczuk 2003; Kahan 2003). Transplantation of ESC-derived insulin-producing cells reversed diabetes in rodents, indicating that these cells do synthesise and release insulin (Blyszczuk 2003; Hori 2002). ESC lines are not assumed to be identical and problems in control of differentiation and teratoma formation remain issues to be overcome. It has been shown that animals develop tumours when they are transplanted with ESC-derived insulin-producing cells (Fujikawa 2005).

Ethical and safety concerns of ESC remain extra hurdles for their use in practice. These issues need to be addressed and resolved before these cells can become readily available option for use as clinical therapies.

1.8.2 Adult or tissue Stem cells

Adult stem cells (ASC) are multipotent undifferentiated cells found among differentiated cells in a tissue or an organ. The primary role of adult stem cells is to maintain and repair the tissue in which they are found. Adult stem cells typically generate the cell types of the tissue in which they reside. However, many experiments over the years have shown that stem cells from one tissue may exhibit the ability to give rise to specialised cell types of other tissue, a phenomenon known as plasticity (Lakshmipathy 2005). ASCs have been identified in many organs and tissues including the brain, bone marrow, skin, liver, heart and the pancreas. They exist in very small number and are thought to reside in a specific area of each tissue where they remain quiescent for many years until they are activated by tissue injury (Walker 2009).
If these adult stem cells can be grown in cell culture and manipulated to generate specific cell types then they can be used to treat injury or disease such as developing insulin-producing cells as therapy for type-I diabetes. A potential advantage of using ASCs is that they can be autologous. In that case, patient’s own adult stem cells could be expanded in culture and then reintroduced into the patient. This would avoid the need for immunosuppression to prevent rejection, which may be needed in allogeneic ASC or ESC. This has not yet been determined in human studies.

Different sources of adult stem cells have been proposed for the production of beta-cells. There have been studies that reported the generation of insulin-producing cells from induced pluripotent stem cells, haematopoietic stem cells, stem cells derived from the pancreas and non-pancreatic organ-bound stem cells.

### 1.9 Sources of adult stem cells for beta cells

#### 1.9.1 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSC) are pluripotent stem cells that are artificially derived from adult somatic cells that have been reprogrammed into ESC-like state. Somatic cells are modified by inducing the expression of certain stem cell genes and proteins (Yu 2007). Two studies reported the successful generation of mature insulin-producing cells from murine and human iPSC (Jeon 2012; Zhang 2009). iPSC would be an ideal source for cell replacement therapy because it has been shown that they can be derived from patients including those with diabetes (Maehr 2009). These cells can be used to generate autologous beta cells for transplantation avoiding the controversial use of ESC and the need for long-term immunosuppression. Despite these advantages, reprogramming of adult somatic cells to obtain iPSCs can pose significant risks that could limit their use in humans. If
viruses are used to genomically alter the cells, the expression of oncogenes may potentially be triggered and hence their propensity to form tumours (Yu 2007). However, with rapid advances in iPSC technology, many subsequent studies have already reported iPSC generation using non-integrating methods of gene delivery (Mayhew 2010). At present, tumourigenicity of iPSC cell remains a serious concern making them unsuitable candidates for cell-based therapy until future studies prove their safety.

1.9.2 Bone marrow derived stem cells

Bone marrow derived stem cells (BMSC) have been studied for many years and are therefore the best-characterised type of stem cells. They are multipotent, capable of self-renewal and well known as a source of stem cells for blood cells. They have also been shown to have the ability under control of local factors in certain microenvironments to differentiate into other cell types after migration to the site of damage in several tissues (Herzog 2003; Korbling 2002).

Three distinct populations of stem cells are thought to reside in the bone marrow: the haematopoietic stem cells (HSC), the mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC). Haematopoietic stem cells express CD34 and CD133 on their cell surface and give rise to all lineages of blood cell differentiation while mesenchymal stem cells give rise to the stromal cells of the bone marrow, including the ostogenic, chondrogenic and adipogenic lineages (Gangaram-Panday 2007).

HSCs are rare cells, with a frequency of 1 in $10^4$ to 1 in $10^5$ amongst bone marrow cells. Human HSCs can be isolated by selection based on expression of the cell surface sialomucin CD34 (Nielson 2008). Stem cells in hematopoietic tissue have
been used for haematological reconstitution for many years. MSCs have a significant immunosuppressant property and are able to differentiate into several tissues. EPCs are capable of angiogenesis and are widely used in regenerative therapy. All three types of stem cells have been used in cell-therapy and are candidates for the treatment of diabetes (Couri 2008b).

Many experiments over the last decade demonstrated the plasticity of BMSCs in vitro and their role in pancreatic endocrine regeneration post injury in vivo. Early work by Ianus et al. suggested that grafted bone marrow stem cells could differentiate into functional pancreatic beta cells in diabetic mouse model. BMSC tagged with green fluorescent protein (GFP) were injected into irradiated mice. After 6 weeks, GFP positive cells were found within the pancreatic islets of the recipient mouse accounting to only 1-3%. These cells were shown to express insulin and other transcription factors associated with beta cells. They also responded to glucose stimulation by secreting insulin. So the researchers concluded that these cells have effectively differentiated into pancreatic beta cells although there were low levels of engraftment. However, all subsequent in vivo studies have failed to replicate these results but did demonstrate that BMSC are able to stimulate beta-cell regeneration in damaged pancreatic tissue by a variety of mechanisms (Ianus 2003).

In the same year, another group injected irradiated mice with GFP positive BMSC followed by streptozotocin to induce pancreatic islet damage. At the end of the study, these cells were found in the pancreatic islets but they did not produce any insulin, glucagon or Pdx-1. However, there was an increase of insulin-secreting bromodeoxyuridine (BrdU) positive cells within islets. BrdU is an analogue of thymidine and is commonly used in the detection of proliferating cells in living tissues. Additionally, treated mice had near-normal glucose levels 5 weeks post streptozotocin injection. The authors concluded that while BMSC did not seem to
differentiate into pancreatic beta cells, they still have the potential for being host cells in beta-cell replacement therapy (Choi 2003).

Hess et al. repeated a similar experiment but reversing the steps by injecting irradiated mice with streptozotocin causing pancreatic injury first followed by GFP positive BMSC transplantation. Similar to Choi et al., they found that GFP positive BMSCs preferentially engraft to the damaged pancreatic tissue and most of them did not secret insulin. However, they did demonstrate that a small proportion of these cells in the islets did produce insulin showing that BMSC can differentiate into insulin-producing phenotype. However, they noted that this small population was too few in number to explain the reversal of hyperglycaemia and the increased levels of systemic insulin. This effect was observed prior to the detection of GFP positive insulin-producing cells and did not occur in control animals. These findings led to the conclusion that the reduction in hyperglycaemia was attributable to a rapid rise in endogenous insulin stimulated by BMSC transplantation rather than differentiation of the BMSC into beta cells (Hess 2003).

The ability of BMSC to transdifferentiate into insulin-producing cells by culture in high glucose conditions and exposure to beta-cell stimulating factors was also demonstrated by Tang et al. These differentiated cells expressed multiple pancreatic genes including insulin, GLUT2 and Pdx-1 and were sensitive to glucose stimulation by secreting insulin in response. Moreover, they resulted in normoglycaemia 1-week post transplantation into streptozotocin induced diabetic mice confirming their effect in vivo (Tang 2004).

Hasegawa et al. confirmed previous data that acute pancreatic injury was necessary to get improvements in hyperglycaemia and islet regeneration. They also found that pancreata with regenerated islets were surrounded by BMSC-derived haemopoietic cells. Most of the BrdU-positive cells were found within close vicinity of these
hematopoietic cells, suggesting that they were involved in the proliferation and differentiation of pancreatic progenitor cells. Finally, they also concluded that mobilization of BMSC-derived endothelial and hematopoietic progenitor cells was critical for beta-cell regeneration. Endothelial progenitor cells are important in neovascularisation, which is an important process of tissue regeneration (Hasegawa 2006).

The relationship between BMSC-derived endothelial progenitor cells and beta-cell regeneration, in response to pancreatic injury was examined in another study. Mathews et al. found an increase in BMSC-derived and endogenous endothelial cells along with an increase in the total number of blood vessels in the animal with time after streptozotocin injection, irradiation and GFP positive BMSC transplantation. These researchers concluded that these endothelial progenitor cells were recruited to the pancreas in response to beta-cell injury (Mathews 2004).

MSC have also been shown to home to and promote repair of pancreatic beta cells in rodents (Lee 2006). The question of possible contribution of immunosuppressive qualities of MSCs to beta cell regeneration was addressed in a more recent study. Hyperglycaemia in streptozotocin-induced irradiated mice was reversed by concomitantly administration of bone marrow cells and syngeneic or allogeneic MSCs. Neither bone marrow cell nor MSC transplantation was effective alone. Successful treatment of diabetic animals was not due to the reconstitution of the damaged islet cells from the transplant, since no donor-derived beta cells were found in the recovered animals, indicating a graft-initiated endogenous repair process. In addition, MSCs were shown to have significantly suppressed beta cell specific T-cell proliferation in the pancreas protecting newly formed beta cells from destruction by T-lymphocytes (Urban 2008).
In summary, this evidence suggests that BMSCs can be manipulated to differentiate into beta cells in vitro but they do not differentiate into insulin-producing cells in vivo. Despite this, published data have established that all 3 populations of BMSCs (HSC, MSC and EPC) are able to stimulate beta-cell regeneration and support pancreatic growth in vivo by different mechanisms making the bone marrow an attractive option for cell-therapy of type I diabetes.

1.9.3 Pancreas derived stem cells

The most logical place to search for a means to regenerating new beta cells would be in the pancreas itself. It is known that beta-cell mass increases markedly during development and that it can fluctuate with physiological changes such as obesity and pregnancy (Bonner-Weir 2000). The exact origins and nature of cells required in vivo to repair pancreatic damage remains a complex and controversial problem. It has been shown that significant regeneration of pancreatic tissue, including beta-cell mass, occurs in adult rats after a 90% pancreatectomy (Bonner-Weir 1993). Dor et al. used genetic tracing studies to demonstrate that existing beta cells are the primary source of new beta cells in vivo (Dor 2004). However, many other studies have found evidence of the involvement of pluripotent stem cells. Various different candidate populations of pancreatic resident progenitor stem cell have been described in the literature including pancreatic-duct epithelial cells, islet derived progenitor cells, and exocrine tissue, all of which appear capable, to a variable degree, of differentiating into insulin-producing cells in vitro.

Wang et al. used pancreatic duct ligation to instigate a regenerative response in rats and discovered that there was considerable islet cell hyperplasia composed mostly of beta-like cells. A beta-like cell is similar to an actual beta cell but did not definitively express all markers at the exact same levels of normal beta cells. Using
bromodeoxyuridine labelling, the investigators demonstrated that this hyperplasia could not entirely be accounted for by proliferation of existing beta cells alone. The study suggested that these new beta cells were derived from progenitor stem cells. He proposed that exocrine duct cells differentiated and proliferated to form new beta cells (Wang 1995). Subsequently, further studies described the role of ductal cells in islet regeneration. Bonner-Weir et al. also demonstrated that pancreatic ductal epithelial cells could dedifferentiate into progenitor cells capable of proliferating and forming new beta cells and acini (Bonner-Weir 2004). One study by Gao et al. addressed the factors that affect transdifferentiation of pancreatic ductal cells and they established that these endocrine cells could be derived from cytokeratin 19 positive ductal cells and that this differentiation could be inhibited by serum-contained factors, although these specific growth factors were not identified (Gao 2003).

The second potential group of stem cells is islet-derived stem cells. The evidence for this group of stem cells came from studies that showed a population of insulin-containing cells reappearing in the islets after total destruction of the beta cell mass with streptozotocin (Guz 2001). Nestin-positive cells have been proposed to represent a population of islet-derived stem cells (Zulewski 2001). However, later evidence suggests that although nestin is a functional protein expressed in beta-cell precursors, it might also be present in other different populations in the pancreas and hence is not a specific marker for endocrine stem cells but important functional protein in a variety of stem cell sources (Wang 2005). Xu et al. reported that beta cell replacement post pancreatic injury in a mouse model involved Ngn3 positive endocrine progenitor cells in the ductal lining. These cells were found to be pluripotent and were capable of differentiating into all four pancreatic endocrine cell subtypes (Xu 2008).
One interesting study described the generation of beta-like cells by reprogramming differentiated exocrine pancreas cells in vivo using a combination of transcription factors. These beta-like cells did not aggregate in vivo to form islet-like clusters but were similar to beta cells in morphology and function. They were able to mitigate hyperglycaemia by secreting insulin (Zhou 2008).

Based on currently published data, it is generally accepted that the pancreas in the adult has a small population of cells that are capable of continuous self-renewal and can differentiate into cells of the pancreas. These cells would hold several advantages over other sources of stem cells. They combine the ability for prolong proliferation with an already partial differentiation towards endocrine phenotype. This might facilitate a more readily and less complicated differentiation strategy towards islet cells than that of other sources.

1.9.4 Hepatic and other non-pancreatic stem cells

Extra-pancreatic organ-bound stem cells can differentiate into islet cells. Hepatocytes, human adipose tissue-derived mesenchymal stem cells and splenocytes have been shown to have this ability (Gangaram-Panday 2007). As the liver and the pancreas are both endodermally-derived, it is thought that the liver would be a potential source of pancreatic stem cells. It is easily accessible and has substantial regenerative ability making it ideal tissue for autologous grafting and trans-differentiation cells.

Rodent-liver stem cells and human fetal-liver cells have been differentiated in vitro into insulin-secreting cells by culture methods and/or introduction of beta cell-specific genes. When transplanted, these cells reverse diabetes in rodents (Zalzman 2003; Zalzman 2005). Cells within liver that can differentiate into insulin-secreting cells after
introduction of beta-cell-specific genes have also been seen in vivo after adenoviral gene-delivery into rodents that have been rescued from diabetes for long periods (Ber 2003; Yang 2006). Table 1 summarises the advantages and disadvantages associated with different types of beta cell replacement including stem cells.
<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Autologous source</th>
<th>Insulin produced in vitro</th>
<th>Insulin produced in vivo</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islet cell transplant</td>
<td>Less invasive procedure</td>
<td>Limited donors Risk of sensitisation [need for several infusions] Variable results [short-term insulin independence] Long-term immunosuppression</td>
<td>No</td>
<td>Not applicable</td>
<td>Yes</td>
<td>Improved isolation and delivery methods</td>
</tr>
<tr>
<td>Embryonic stem cells [ESC]</td>
<td>Unlimited supply Pluripotent</td>
<td>Ethical controversy Risk of teratoma No long term studies</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Induced pluripotent stem cells [iPSC]</td>
<td>Unlimited supply without ethical concerns of ESC</td>
<td>Viral and genomic integration with the risk of tumour formation and infections</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>New derivation methods avoiding integration</td>
</tr>
<tr>
<td>Bone marrow stem cells [BMSC]</td>
<td>Easy accessibility Established harvest and delivery protocols Immunoprotective properties Stimulate beta cell regeneration in damaged pancreas</td>
<td>Difficulty in achieving sufficient cell number in vitro for in vivo effect</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Multiple mechanisms for increasing insulin production in damaged pancreas</td>
</tr>
<tr>
<td>Pancreatic stem cells</td>
<td>Already partially differentiated towards beta cells</td>
<td>Difficulty in isolating cells and transdifferentiation factors</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Unclear on origin, role and location</td>
</tr>
<tr>
<td>Hepatic cells</td>
<td>Easy accessibility Regenerative tissue Endodermal origin</td>
<td>Difficulty in achieving sufficient cell number in vitro for in vivo effect</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Transdifferentiation into beta-like cells</td>
</tr>
</tbody>
</table>

*Table 1: Summary of different types of beta cell replacement, advantages and disadvantages Adapted from (Godfrey 2012)*
1.10 Clinical trials in stem cell therapy for type I diabetes

Stem cells in hematopoietic tissue have been used for haematological reconstitution for many years. These bone marrow cells are already routinely collected for current medical procedures such as various autoimmune disorders and haematological malignancy treatments. They have the advantage of being easily accessible. Harvesting BMSC in the form of aspirate bone marrow or mobilised stem cells isolated by apheresis has been standard clinical practice for decades. Thus, the bone marrow could potentially serve as an autologous source for cells and in turn minimise immunosuppression and rejection problems. These factors make the bone marrow an attractive source for potential type I diabetes stem cell therapy.

There have been several phases I/II clinical studies of BMSC therapy in type I diabetes over the last 20 years. One of the earliest studies by Elliott et al. was published without complete outcome data in the Lancet in 1981 (Elliott 1981). The researchers obtained a bone marrow biopsy from the diabetic patient and infused BMSC including both MSC and HSC directly into the patient’s pancreas by arterial catheterization. This trial included 52 patients that had either type I or type II diabetes.

The largest prospective phase I/II study with the longest follow-up comes from the Bone Marrow Transplantation Unit in Ribeirao, Brazil. These researchers based their cell-therapy for type I diabetes on the concept of “immunologic resetting” to stop the islets from being destroyed by the autoimmune response early within 6 weeks of disease presentation. Their 3-stage protocol aims to use an immunomodulatory strategy during the initial phase of the disease when the patient still has an important beta-cell reserve in an attempt to preserve and protect it. It starts with mobilisation of HSC from the bone marrow with low dose cyclophosphamide and granulocyte colony
stimulating factor (G-CSF). These HSC are collected and frozen. This is followed by a conditioning regimen, which includes high dose cyclophosphamide and rabbit anti-thymocyte globulin (rATG), to destroy most of the patient’s lymphocyte clones, which include both autoreactive and non-autoreactive. Finally, the previously collected HSC are re-infused into the patient to regenerate or recover an immune system that will no attack the pancreatic beta cells (Couri 2009a; Voltarelli 2007). There was no control group in this study.

Up to December 2008, 23 patients have been enrolled in the study. During a mean follow up of 29.8 months, 20 patients became insulin independent for some time. 12/23 (52.3%) patients remained free from insulin for a mean of 31 months, 8/23 (37.8%) patients were transiently insulin-free for a mean of 17.7 months post HSC infusion then relapsed. 3 patients were maintained on daily insulin doses. Patients, who experienced an insulin-free period, had improvement in their glycoslated haemoglobin HbA1c, increased mean peak C-peptide levels for prolonged time, up to 4 years after treatment. The severe adverse effects included bilateral pneumonia in 2 patients, 2 cases of autoimmune disease (Graves, hypothyroidism), and a case of transient hypogonadism as well as 9 cases of post-transplant hypospermia. None of the patients developed primary infection or reactivation of cytomegalovirus Epstein-Barr virus. There was no mortality (Couri 2009b).

As there was no control arm in the study by Couri et al, the best comparable would be the control untreated patients in several previous immunomodulatory trials for type I diabetes. One example of such trial is the Canadian-European Cyclosporine trial where it was reported that in the control untreated group only 27 and 10% of patients were in a non–insulin-requiring remission at 3 and 12 months, respectively, after diagnosis (Group 1988; Martin 1992). Similarly, a randomized trial by the IMDIAB Study group compared nicotinamide (NCT) and nicotinamide plus cyclosporine (NCT+CyA) in recent onset insulin-dependent diabetes. This trial
reported remission of 4/27(14.8%), 3/27(11.1%), 0/28 in NCT group, NCT+CyA
group and placebo group respectively at one year after diagnosis (Pozzilli 1994)
Another trial published by Harrison et al. in 1985 demonstrated an increased
remission rate in newly diagnosed type I diabetic subjects treated with azathioprine.
In this study, there was a controlled arm with diabetic untreated patients of similar
demographics to those that were treated. They reported that at 12 months 7/8
(87.5%) patients in the treated group were in remission compared to 1/11 (0.09%) in
the untreated group. Remission was not sustained in the treated group after 1-2
years after treatment (Harrison 1985). If the results of the untreated control group
can be used as the rate of remission during the natural course of type I diabetes then
it can extrapolated that study by Couri et al. does demonstrate some benefit to these
patients.

Based on the Brazilian autologous nonmyeloablative HSC treatment protocol, few
further studies in type I diabetes have occurred worldwide. The first study included
16 patients in China. 5 out of 6 patients, that had type I diabetes for less than 3
months, became insulin independent and one patient had 50% insulin dose
reduction. All other 11 patients that were diagnosed for more than 3 months before
treatment remained on insulin (Couri 2009b). Secondly, the use of autologous
umbilical cord stem cells in children with type I diabetes resulted in no significant
differences in daily insulin doses and in a decline in C-peptide levels after 1 year of
follow-up. This study was conducted at the University of Florida (Voltarelli 2009).
Similar to Brazil, there is a research group in cell-therapy in Argentina that has
undertaken human studies looking at the effect of endovascular implantation of
autologous adult mononuclear CD34+ CD38- cells into patients with type I and type II
diabetes. They reported their outcome data for both studies in an abstract form only
at the 4th International Society of Stem Cell Research (ISSCR) annual meeting in
2006. They stated that there was an increase in C-peptide levels and a reduction in
the daily insulin requirement in 23 type I diabetic patients enrolled in the study. None of the patients became insulin-independent but based on higher C-peptide levels; they concluded that these cells improved pancreatic function in these patients (Feranndez Vina 2006).

Finally, a collaborative multi-centre randomised research project between Chicago and the Brazilian group in Sao-Paulo was planned to start in 2010. No published report of this study is available so far (Couri 2009b).

1.11 Coagulation and beta-cell therapy

1.11.1 The Coagulation cascade

Coagulation is the transformation of flowing blood into a stable gel, and the transformation is the result of complex enzymatic mechanisms that are initiated when blood comes in contact with extravascular surfaces. This process is accomplished when soluble fibrinogen molecules undergo enzymatic cleavage and assemble into insoluble fibrin fibers. Figure 9 shows the classical model of the coagulation cascade involving a regulated series of zymogen activation reaction. At each stage a precursor protein or zymogen is converted to an active protease by cleavage of one or more peptide bonds in the precursor molecule. Other components involved are a non-enzymatic protein co-factor, calcium ions and an organizing surface provided by a phospholipid emulsion in vitro or by platelets in vivo. The final protease generated is thrombin that converts the soluble protein fibrinogen into an insoluble fibrin matrix. This is strengthened further by covalent cross-linking catalyzed by factor XIIIa to form a clot (Adams 2009).
Figure 9: The coagulation cascade showing the intrinsic, extrinsic and the common pathways involved (bigtomato.org/drugs/bigcoagcascade.php)
There are two separate initiation mechanisms for the enzymatic coagulation cascade, both leading to the same end-point, which is fibrin network formation. The two initiation pathways are termed the intrinsic or contact activation pathway and the extrinsic pathway. The terms are derived from the fact that all functional components of the intrinsic pathway are intravascular, whereas triggering component of the extrinsic pathway, tissue factor (TF), is not found in blood, but in extravascular tissue. Although there are major differences in the activation mechanism and the subsequent enzymatic steps, both the intrinsic and extrinsic pathways converge into the common pathway, ultimately leading to fibrin formation (Adams 2009; Ruseva 2011).

TF, also known as thromboplastin, platelet tissue factor, factor III, thromboplastin, or CD142 is a protein present in subendothelial tissue and leukocytes necessary for the initiation of thrombin formation from the zymogen prothrombin. It is a non-enzymatic glycoprotein constitutively expressed on the surface of cells that are not normally in contact with plasma such as macrophages and fibroblasts. Exposure of plasma to these cells initiates the coagulation to repair a damaged blood vessel. Endothelial cells also express TF when stimulated by inflammatory stimuli and cytokines such as endotoxin, tumour necrosis factor or interleukin-6 and probably involved in thrombus formation under pathologic conditions (Ruseva 2011).

The contemporary accepted cell-based model of in vivo coagulation describes a stepwise and overlapping pattern of activation highlighting three distinct phases of the cascade. A simplified version of the 3-phase coagulation cascade is shown in figure 10. The initiation phase of coagulation, by TF, which binds small amounts of circulating factor VIIa, activates factor X to factor Xa and this can then convert trace amounts of prothrombin to thrombin (i.e the extrinsic system). It also activates factor IX and thus linking the intrinsic and extrinsic pathway. The minor amount of thrombin
produced through the extrinsic factor tenase complex pathway is insufficient for complete fibrin formation but is enough to activate platelets through the protease activated receptors (PAR) as well as converting factors V and VIII in the intrinsic pathway to their active forms. This feedback activation of intrinsic system factors by these small amounts of thrombin constitutes the initial amplification phase where there is conversion from extrinsic to intrinsic thrombin generation. This is followed by the propagation phase, which involves the conventional activated tenase (VIIIa:IXa) and prothrombinase (Va:Xa) complexes, and leads to the generation of large amounts of thrombin capable of cleaving fibrinogen. This phase takes place on negatively charged phospholipid surfaces, which in vivo are provided by platelet membranes. The platelet is probably the only cell type on which the propagation phase can occur effectively. When enough thrombin is generated at a sufficient speed to result in a critical mass of fibrin, these soluble fibrin molecules will polymerize into strands and result eventually in an insoluble fibrin matrix (Adams 2009; Ruseva 2011).
Figure 10: Simplified modern clotting cascade pathways. Coagulation is initiated by extrinsic tenase, which forms when factor VIIa binds to tissue factor. Extrinsic tenase activates factors IX and X. In the presence of calcium, factor IXa binds to negatively charged phospholipid surfaces where it interacts with factor VIIIa to form intrinsic tenase, a complex that efficiently activates factor X. Factor Xa binds to factor Va on negatively charged phospholipid surfaces to form prothrombinase, the complex that activates prothrombin to thrombin. Thrombin then converts fibrinogen to fibrin. Activated platelets or monocytes provide negatively charged phospholipid surfaces on which these clotting reactions occur (Bates 2005).

1.11.2 Regulation of coagulation and anticoagulants

Regulation of the pathway occurs at each level, with each phase associated with inhibitory factors, either by enzymatic inhibition or modulation of cofactor activity. The main inhibitory regulatory proteins are tissue factor pathway inhibitor (TFPI), antithrombin, protein C and heparin cofactor II. The serine protease TFPI is the main TF regulator. It neutralizes activated factor Xa and inhibits the TF and activated
factor VIIa complex. It is predominantly produced by endothelium, bound to heparin sulphate as well as other cells including platelets. It is also found in a circulating form bound to low-density lipoprotein (LDL). The administration of heparin or low molecular weight heparin (LMWH) results in the endothelial form of TFPI being released into the circulation leading to systemic anticoagulation. Antithrombin is a serine protease inhibitor exerting its actions preferentially on free enzymes and to a lesser extent on bound serine proteases. The action of antithrombin is greatly enhanced by the administration of heparin or heparin-like molecules expressed on endothelium. Thrombin-binding to constitutively expressed endothelial thrombomodulin activates protein C, which with protein S as a cofactor inactivates factors Va and VIIIa. Heparin cofactor II expressed on liver cells inactivates thrombin in the presence of heparin or heparin-like molecules (Adams 2009).

Heparin is the first anticoagulant drug to be identified. It has high affinity for antithrombin and inactivates serine proteases including factors II, VII, IX and X. At higher doses, heparin cofactor II is activated which results in thrombin inactivation (Kovacs 2003). Hirudin is a polypeptide that was first isolated from the medicinal leech. It binds thrombin at the active site and fibrin binding site forming an irreversible hirudin:thrombin complex (Chang 1989). Both heparin and hirudin cause non-selective systemic anticoagulation.

1.11.3 Coagulation in vitro

The pathways of the coagulation cascade model are reflected in the laboratory analysis of clotting in vitro. When blood is placed in a glass test tube, it clots fairly quickly. Calcium ions are required for this process. Addition of Ethylenediaminetetraacetic acid (EDTA) or citrate prevents clotting by binding calcium. Clotting can be initiated in vitro at a later time by adding back an excess of
calcium ions. Recalcified plasma will clot in 2-4 minutes. Coagulation can be initiated via the “intrinsic” pathway in vitro when factor XII, prekallikrein and high-molecular weight kininogen (HMWK) bind to kaolin, glass, or another artificial surface. Once bound, reciprocal activation of XII and prekallikrein occurs as shown in figure 7. Factor XIIa triggers clotting via the sequential activation of factors XI, IX, X and prothrombin. Hence the clotting time after recalcification can be shortened by the addition of emulsion of negatively charged phospholipids with or without by preincubation of the plasma with kaolin (insoluble aluminium silicate). This would be a measure of the intrinsic coagulation pathway. The clotting time is also shortened by the addition of thromboplastin, which contains tissue factor, and hence this would reflect and would be a measure of the extrinsic coagulation pathway (Adams 2009; Tollefsen 2013).

1.11.4 Coagulation and inflammation

Homeostasis is a defense mechanism to prevent haemorrhage and disseminated thrombosis. Activated by vessel wall injury, it consists of intertwined activation of platelets and the coagulation cascade, tightly controlled by natural anticoagulants and the fibrinolytic system (Levi 2004). Inflammation aims to restore the integrity of damaged tissues, most frequently because of injury or infectious pathogens. The coagulation system and the innate inflammatory response share a common ancestry and are coupled via common activation pathways and feedback regulation systems (Delvaeye 2009). The two-way relationship between both has persisted throughout evolution: coagulation triggers inflammation and inflammation triggers the activation of the coagulation system. Extensive interaction between inflammation and coagulation involves cell receptor-mediated signaling, cellular interactions and the production of cell-derived microvesicles by endothelial cells, leucocytes and platelets.
in addition to enzymes and soluble or cell-expressed regulators that work on both the coagulation and inflammation pathways.

Platelets, tissue factor and thrombin in inflammation are a good illustration of this two-way relationship. After adhering to the injured vessel wall, activated platelets release cytokines, growth factors, and numerous proinflammatory mediators. Inflammatory cytokines induce TF expression in leucocytes and in endothelial cells. The formation of a complex between TF and the coagulation FVIIa or Xa is instrumental in initiating coagulation on negatively charged cell membranes, whereas membrane-bound TF is also capable of signal transduction directly mediating inflammatory reaction (Schouten 2008). Blocking TF activity completely abrogates inflammation-induced coagulation activation in models of experimental endotoxaemia or bacteremia (Levi 1994). A series of feedback and amplification steps lead to the generation of large amount of thrombin, which results in the generation of fibrin polymers and fibrinogen deposition. In addition, the major coagulation enzyme thrombin has many functions beyond haemostasis. It is a key regulator of cell activation by protease activated receptors present on platelets but also on endothelial cells, thus triggering inflammatory pathways (Shrivastava 2007).

Failure of the complex balance between pro- and anticoagulation, or between pro- and anti-inflammatory reactions because of genetic or acquired disturbances may result in disease. Inflammation shifts endothelial cells towards a more prothrombotic state, downregulating anticoagulant defence mechanism and leading to expression and synthesis of thrombogenic molecules such as von Willbrand factor and TF. Thrombotic vascular occlusion can occur in several multisystem disorders, may complicate local procedures such as angioplasty and vascular surgery, and features prominently in the pathology of hyperacute rejection of allografts or xenografts, where it leads to irreversible infarction of the transplanted organ. In this setting, the procoagulant state within the graft develops from the simultaneous effects of
complement activation, dependent on the deposition of anti-graft endothelial cell (EC) antibody, subendothelial tissue factor exposure, loss of proteoglycan linked anticoagulant molecules such as antithrombin III and TFPI from endothelial cell surfaces, downregulation of thrombomodulin, which is internalized after EC activation and widespread platelet activation (Platt 1990). All of which leads to thrombosis and consequently infarction.

1.11.5 Coagulation and Islet transplantation

It is established that islet loss experienced during the isolation, implantation and engraftment period is high. An estimated 50% of the final preparation infused into the patient is lost during engraftment period (Leitao 2008). The large number of islets needed to establish insulin-independence in a diabetic receiving islet transplant is thought to be due to the early loss of a significant proportion of these injected islet cells through both non-immune and immune processes.

The association between coagulation and inflammation plays a major role in the field of islet transplantation. It is well established that after infusion of islets into the portal vein, a substantial percentage of them are damaged in the immediate post-transplant period through an inflammatory response termed the “instant blood-mediated inflammatory reaction” (Bennet 2000a; Bennet 2000b). IBMIR manifest by activation and consumption of platelets, activation of neutrophils and monocytes, and activation of the coagulation and complement systems (Bennet 1999; Titus 2003). IBMIR is potentially triggered by molecules expressed on the cell surface of islets such as tissue factor and collagen residues that are normally not in direct contact with the blood (Moberg 2002). Islet transplant also results in activation of both humoral and cellular responses and the innate immune system may be involved in IBMIR and in
turn contribute to the further loss of a significant fraction of transplanted islets (Bottino 1998; Chandra 2007; Nagata 1990).

Moberg et al. provided evidence for the production of TF by the islet cells as a trigger of the detrimental thrombotic reactions observed in clinical islet transplantation. Coagulation activation and subsequent release of insulin were found consistently after clinical islet transplantation in 4 patients even in the absence of signs of intraportal thrombosis. The endocrine cells were found to synthesise and secrete active TF demonstrated by higher expression of TF in the islet preparations and higher activity of TF when assessed in vitro. Additionally, they successfully abrogated the clotting reaction triggered by the islets in vitro by blocking the active site of TF with specific antibodies or site inactivated factor VIIa making this agent a candidate drug for inhibition of TF in vivo (Moberg 2002).

The same group in a follow up experiment confirmed that islets intended for clinical transplantation produced TF in both membrane-bound form and the alternative spliced form and that factor VIIa inhibitor blocked both forms of TF in vitro. They also demonstrated correlation between initial Thrombin-antithrombin complex (TAT) and FVIIa-antithrombin complex (FVIIa-AT) levels, which increase with the activity of IBMIR, and C-peptide levels 7 days post islet infusion. Patients with initially strong IBMIR showed no increase in insulin synthesis highlighting the important role of TF in this process (Johansson 2005). However, these researchers reported considerable variation in the amount of TF expression in islets from different donors, and in turn suggested that TF expression may be induced. They went on later to suggest that TF expression is probably only one feature in an array of pro-inflammatory events, including the expression of Monocyte chemotactic protein 1 (MCP-1) and IL-8 that are triggered in the islets by the chain of events prior to transplantation (Korsgren 2005).
If early graft loss after intra-portal islet transplant is to be reduced, interventions should be directed against various components of IBMIR or, ideally, against all components by a single agent. The preventive strategies for IBMIR that have been investigated so far include systemic treatment of recipient with heparin but this harbours the risk of systemic complications, especially that of bleeding.

Other strategies included pre-conditioning of the isolated islets before transplant with anticoagulation or anti-inflammation agents and the genetic alteration of the islets. The prior administration of soluble complement receptor-1 reduces this rapid islet destruction both in vitro and in vivo (Bennet 2000a). Rood et al. showed that pre-transplantation complement depletion or anticoagulation with dextran sulphate reduces porcine islet xenograft loss significantly but neither alone sufficient to prevent IBMIR (Rood 2007). Similarly, Titus et al. demonstrated that inhibition of coagulation or inhibition of platelet aggregation by the incubation of islets in allogeneic blood with heparin or Reopro (monoclonal anti-GpIIbIIIa) respectively did not make a substantial improvement in the destruction of the islets in terms of histology or proinsulin release (Titus 2003). Johansson et al. reported successful a dose-dependent inhibition of IBMIR by low molecular weight dextran sulphate in vitro and in vivo in a nonhuman primate model suggesting it use as part of a tentative protocol in clinical islet transplantation (Johansson 2006). On the other hand, the Swedish group encapsulated the islet surface with continuous heparin coating rendering it blood biocompatible in vitro and in a vivo mouse model. They concluded that this novel pre-treatment procedure efficiently inhibited the IBMIR without increasing the risk of bleeding and without inducing acute or chronic toxicity in the islets and hence should be considered for clinical use (Cabric 2007). More recently, pre-conditioning human islets with recombinant human activated protein C (rhAPC) alone or Tirofiban alone or in combination was examined by Akima et al. They found that Tirofiban monotherapy was ineffective, whereas rhAPC monotherapy prevented
IBMIR in a dose-dependent manner preserving islet integrity in vitro. The combination of pre-treatment with both agents worked synergistically to preserve islets suggesting that co-inhibition of the platelet and coagulation pathways is required for the optimal anti IBMIR effect. (Akima 2009).

As mentioned earlier, having identified TF as a trigger for IBMIR post islet transplantation, several groups investigated the effects of blocking TF by various agents as a potential target therapy for elimination of IBMIR (Moberg 2002). Moberg et al. used low molecular mass factor VIIa inhibitor and demonstrated its efficacy in vitro (Johansson 2005; Moberg 2002). The same group investigated the effect of L-arginine, cyclospine A, enalapril and nicotinamide, which all have been previously shown to affect TF and MCP-1 expression in monocytes and endothelial cells, on human islet cells in vitro. Their results led to the conclusion that adding nicotinamide to the culture medium can reduce TF and MCP-1 expression in human islets and in turn can diminish the IBMIR in clinical islet transplantation without the systemic effect of antithrombotic drugs (Moberg 2003). Another group reported the protection of islets from IBMIR by pre-treatment of islets and the recipient with a humanised monoclonal anti-TF antibody (CNTO859) in a nonhuman primate marginal mass model. Although intervention with the antibody did not lead to insulin independence, compared to their matched controls, treated animals had decreased post transplant markers of coagulation, higher fasting C-peptide levels and prolonged graft function. They concluded that this study should be used as a foundation on which to build on a more effective peritransplant strategy to minimize islet loss and maximize islet function (Berman 2007).

In current practice, in most transplant centres, islets are transplanted in heparinised medium to prevent coagulation at the time of infusion followed by systemic coagulation with either intravenous heparin or subcutaneous low molecular weight
heparin for 5-7 days post transplant. None of the trials report adverse effects such as bleeding (Goss 2003; Hering 2005; Shapiro 2000).

1.11.6 Coagulation and islet xenotransplantation

Successful clinical application of islet xenotransplantation is currently not achievable due to a number of immunological and non-immunological barriers. The xenograft islets transplanted into the portal vein are lost very early post transplantation due to IBMIR similar to that observed in islet allotransplantation where TF plays an important trigger to this process. Incompatibilities between the human and pig coagulation-anticoagulation systems makes IBMIR even more problematic in xenotransplantation (Ji 2011; Johansson 2005). Inhibition of TF expression or thrombin formation prevented islet damage in vitro but in vivo anticoagulation does not fully prevent IBMIR suggesting another component to this response. (Ji 2011; Moberg 2002; Ozmen 2002)

Dissimilar to islet allotransplant, in addition to tissue factor triggering inflammation and coagulation, the human complement system is also activated due to preformed xenoreactive natural antibodies, known as XNAs. This results in endothelial damage, inflammation, thrombosis and necrosis of the transplant (Ji 2011; Johansson 2005; Van der Windt 2012). The vascular endothelium of pigs express galactose-α1, 3-galactose (GAL) oligosaccharide against which humans have natural anti-GAL antibodies (Good 1992). The binding of antibodies to GAL antigen after islet xenograft infusion results in almost immediate complement activation leading to the destruction of the graft. Adult pig islets have lower expression of GAL compared to neonatal pig islets but still undergo IBMIR when transplanted (Rayat 2003). In addition neonatal islets from pigs that do not express GAL are less susceptible but does not obliterate the IBMIR (Thompson 2011). This suggested than antibody
binding to other non-GAL antigens may be an initiating factor in complement activation (Van der Windt 2012). Figure 11 shows the mechanisms involved in IBMIR post islet xenotransplantation.

Figure 11: Schematic overview of the instant blood-mediated inflammatory reaction. Tissue factor expression and antibody binding to non-Gal antigens, as well as to Gal on neonatal pig islets, activate coagulation and complement cascades, leading to clotting, direct cellular membrane damage through the membrane attack complex (MAC), and recruitment of macrophages and monocytes through the chemoattractants C3a and C5a (Van der Windt 2012)

1.11.7 Coagulation and stem cell therapy

Although there is ample precedence in islet allo- and xenotransplantation literature demonstrating the important role of anticoagulation, there is very little published at present to support the idea of intravenous, intraportal or intrarterial delivery of stem cell transplants in humans is associated with increased risk of thrombosis. Most of the stem cell transplant data available comes from the field of HSC transplant in haematological settings, which are different to the use of stem cell-based therapy in diseases such as diabetes. All the evidence published for potential causes of
coagulopathy comes from HSC transplant (HSCT) for haematological diseases where there is association with the abnormal bone marrow and the extent of pre-conditioning regime. In the absence of haematological disease, diabetic patients or patient with degenerative disorders responsive to stem cell therapy should in theory have a relatively normal bone marrow and in most of these disorders, pre-conditioning immunosuppressive regime would not be required prior to the stem cell transplant. However, there have been no studies that investigated the state of the bone marrow in these patients prior to stem cell infusion and in turn no evidence to support this assumption.

There is evidence however that suggests three typical haemostatic complications in HSCT, namely veno-occlusive disease (VOD), thrombotic microangiopathy (TMA) and diffuse alveolar haemorrhage (DAH) may be manifestations of thrombosis in the microcirculation. These thrombotic complications are observed following allogeneic or autologous HSCT at slightly different rates and are a major cause of morbidity and mortality. Proliferation of bone marrow endothelial progenitor cells, which is a source of endothelial cell repair is attenuated under the effect of HSCT conditioning regimen, rendering the vascular bed vulnerable (Nadir 2012). These haemostatic impairments are found to be related to administration of chemotherapy, and growth factors, use of intravenous catheters, and graft-versus-host reaction (Nadir 2004; Piguet 1989).

Hepatic VOD develops in 1-22% of patients after HSCT with onset typically within the first month post stem cell infusion. It ranges in severity from mild, reversible disease to severe form associated with multi-organ failure and death (Richardson 1999). Several endothelial injury markers are upregulated in patients with VOD, including P- and E- selectins, TFPI, soluble TF and endothelial PAI-1 (Nurnberger 1998). The role of prophylactic heparin anticoagulation, alone or in combination with other agents, in VOD has been assessed in only one randomised controlled study, which reported beneficial effect of low-dose heparin infusion (Attal 1992). There is no approved
therapy for established hepatic VOD. Current strategies focus on supportive management and anticoagulant or fibrinolytic drugs (Nadir 2012). Defibrotide, a single stranded polydeoxyribonucleotide that exerts anti-thrombotic activity by binding to vascular endothelium, without significant effect on systemic coagulation, has shown encouraging results in VOD management (Chopra 2000).

The incidence of TMA following stem cell transplantation is between 0.1% and 15%. It is significantly lower after autologous HSCT compared to allogeneic transplant (Iacopino 1999). Elevated von Willebrand factor (VWF) antigen levels found in patients with TMA are more likely the results of diffuse endothelial injury and not due to severe VWF-cleaving protease deficiency (Elliott 2003). The optimal management of transplantation-related TMA has not been established. The suspected offending drug is usually discontinued and plasma exchange has been used as a part of treatment because of clinical similarity to idiopathic thrombotic thrombocytopenic purpura (TTP) but with limited efficacy. Other therapies attempted include defibrotide and immunoglobulin G with variable success (Nadir 2012).

Although endothelial cell injury is a common feature of the pathogenesis of HSCT-associated thrombotic complications and the intravascular thrombosis observed with pancreas and islet transplantation, it is unclear if non-HSCT or HSCT for non-haematological diseases share that phenomenon.

1.12 Site of beta-cell delivery

At present, islet or cell-based transplantation, whether allogeneic, autologous or xenogeneic most commonly involves the intravenous delivery of the islets or cells into the portal vein of the liver. The liver was established as the site of choice for islet transplantation in clinical practice following the publication of the first few cases of
successful islet infusion into the liver in 1980s and subsequently in 1990 (Najaran 1980; Scharp 1990; Sutherland 2001). However, it has become increasingly recognised that the liver does not provide the ideal microenvironment for islets due to immunological, anatomical and physiological factors that contribute to the loss of islet mass early after infusion. In both islet allo and xenotransplantation, this method and site of delivery results in a large number of islets lost early post transplant thorough the non-specific IBMIR and rejection as described in section 1.10.5 and 1.10.6 respectively. IBMIR results in fibrin capsule formation surrounding the islets and leads to infiltration of leucocytes mainly polymorphonuclear cells. After activation, these cells secrete reactive oxygen species that cause rapid and direct damage to the islets that are extremely susceptible to oxidative stress. Intraliver infusion is also associated with thrombosis and hepatic tissue ischaemia caused by islet entrapment in to liver sinusoids that leads to endothelial cell activation and functional impairment (Cantarelli 2011).

Revascularization of transplanted islets is essential for proper glucose homeostasis. In view of intra-islet endothelial cells loss during the isolation process, islets are more susceptible to post-transplant stresses and damage as the revascularisation of the intraliver islets is not immediate and often takes several days. Complete circulation is only re-established within 10-14 days post infusion (Carlsson 2002). Additionally, islets placed into hepatic-portal circulation are exposed to higher levels of immunosuppressive drugs that are thought to be largely toxic for islet function (Shapiro 2005). Intra-liver islets are not exposed to arterial concentrations of glucose. Intra-portalally transplanted islets are exposed to intermittent hyperglycaemia of a greater magnitude than seen in normal physiology, an effect that can be toxic to the islets, or, at the very least, challenges the beta cells that have not encountered this environment in the native pancreas (Gray 1989; Juang 1994; Makhlouf 2003;
Yumiba 1992). However, there is little evidence to suggest that this exposure composes a major hurdle for islet survival post transplantation.

In view of the negative consequences of the intravenous islet graft delivery into the liver, there have been attempts to address these barriers by investigating alternative transplantation sites to improve graft survival and engraftment. An ideal islet transplant site would have multiple desirable properties that provide the conditions favouring optimum islet survival. The transplant site should result in minimal early inflammatory reactions and protect islets from cellular immune responses. It should be a well-vascularised microenvironment with good access to nutrients, suitable oxygen tension at physiological pH and free from toxic metabolites and oxygen-free radicals. Additionally, it should be easily accessible technically via minimally invasive approach.

Table 2 reports the various alternative islet transplant sites that have tested in experimental models. Most of these studies have been investigating alternative sites in small animal models. Only a few of these have been transferred to large animal models and finally in clinical practice in humans. The table reveals the advantages and disadvantages for each of the alternative transplant sites in terms of the desired immunological, anatomical, physiological and surgical properties. Adapted from (Cantarelli 2011; Merani 2008). For example extravascular implantation sites such as muscle and kidney capsule provide the benefit of IBMIR avoidance but they do not mimic physiological insulin release. On the other hand, while the pancreas provides the most suitable physiological site for islet transplantation, it poses a challenge in terms of technical accessibility and high risk of acute complications post transplantation. The thymus, testis, anterior eye chamber and brain may confer protection in allotransplantation. They are thought to be immunoprivileged sites and may offer complete or partial protection from allorejection or xenorejection without the need for concurrent immunosuppression. There are specific barriers to each of
these immunoprivileged sites in the clinical context. The thymic site is the most developed with large animal investigations (Cantarelli 2011; Merani 2008).

Although many transplantation sites have been proposed, few have found their way into the clinical setting. Additionally, there have been several studies evaluating the immunological benefit of prevascularised subcutaneous chamber devices and the technology of encapsulation for the transplantation of xenogeneic porcine islets (Pilleggi 2006; Siebers 1997; Sykes 2007). Finally, there are currently few ongoing clinical phase I/II studies testing the safety, feasibility and efficacy of alternative sites for autologous and allogeneic islet transplantation and their results are awaited. While the ideal site for islet implantation in humans is yet to be identified, the liver remains the site for islet transplantation in clinical practice at present.
Table 2: Evaluation of immunological, anatomical, physiological and surgical properties of alternative experimental islet transplant sites. Adapted from (Cantarelli 2011; Merani 2008)

<table>
<thead>
<tr>
<th>Transplant site</th>
<th>Minimal IBMIR</th>
<th>Immune-protection</th>
<th>Well vascularised</th>
<th>Physiological insulin release</th>
<th>Easy access</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>-</td>
<td>_</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pancreas</td>
<td>-</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Muscle</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>++</td>
</tr>
<tr>
<td>Omentum</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>++</td>
<td>_</td>
<td>++</td>
<td>_</td>
<td>++</td>
</tr>
<tr>
<td>Gastric submucosa</td>
<td>?</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Genitourinary tract</td>
<td>?</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>Kidney capsule</td>
<td>++</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>Anterior eye chamber</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>Testis</td>
<td>?</td>
<td>+</td>
<td>?</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>Thymus</td>
<td>?</td>
<td>+</td>
<td>?</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

In HSCT, the cells are infused peripherally and the thrombotic complications are described in section 1.10.7 and do not occur immediately post administration which suggests no direct association to the site or method of infusion. There is no evidence to suggest that a similar response to IBMIR occurs in this setting. In the few stem cell trials or studies published as treatment for diabetes, none report the exact infusion
method or site so it is unclear whether the cells are administered peripherally or intra-portally. There were no reported adverse events related to thrombosis at any site. It is hence unknown what is the ideal site for stem cell transplant in patients with diabetes.

1.13 Potential strategies for anticoagulation therapy

At present, there is no feasible and safe alternative site for islet transplantation to intra-liver delivery. Whilst this remains the only option in clinical practical, there have been attempts to address the main cause of early islet graft loss post intra-portal vein placement. Several strategies has been investigated which aim to minimize the IBMIR thereby enhancing islet engraftment and survival.

The treatment options described so far for the prothrombotic state found after intravenous intra-portal islet transplantation have been either systemic anticoagulation or pre-conditioning of the islet graft pre-transplantation with a variety of therapeutic agents. Systemic anticoagulation has been documented as being of preventive benefit. However, systemic treatment can be complicated by bleeding, involving sites remote from the primary pathology, which at times may be life threatening. Pre-conditioning regimes have mainly been applied to islet transplantation in experimental models and only heparin in reality is used in the clinical setting. An alternative approach is to either the use genetic strategy to target expression of anticoagulant molecules in xenotransplantation or more promising the use of antithrombotic cytotoxic constructs directed to the graft cells and can act locally to prevent coagulation without the undesirable systemic complications.
1.13.1 Genetic constructs or engineering

This work was directed towards the potential use of a genetic strategy for xenotransplantation, where transgenic pigs might be useful as a source of islets. In xenotransplantation, this option might involve pre-transplant perfusion of xenografts with genetic material, or genetic alteration of the islets.

Riesbeck et al. at Imperial College engineered two genetic constructs encoding novel membrane-tethered anticoagulant fusion proteins based on soluble human tissue factor pathway inhibitor (hTFPI) and the leech anticoagulant hirudin. Hirudin is the most potent natural inhibitor of thrombin. Unlike antithrombin III, hirudin binds to and inhibits only the activity of thrombin, with a specific activity on fibrinogen. Both sets of constructs have been expressed in rodent cells and have been shown to bind appropriate coagulation factors (Riesbeck 1997; Riesbeck 1998). When expressed constitutively on the surface of porcine endothelial cells (PEC), anchored by the transmembrane/cytoplasmic tail of human CD4, hTFPI bound to and inhibited factors Xa, VIIa, and porcine tissue factor (pTF), and hirudin retained potent thrombin-binding activity. Hirudin expression significantly modified the procoagulant phenotype of PEC in human plasma, leading to prolongation of clotting time (Riesbeck 1998).

Chen et al. demonstrated that expression of hTFPI-CD4 on PEC effectively prevented pTF-dependent clotting, whereas expression of hirudin-CD4 construct inhibited both pTF-dependent and -independent fibrin generation. When both constructs were co-expressed in the same cell, the potent procoagulant properties of in vitro cultured, IL-1a-activated PEC were almost completely abolished this experimental system. The scientists concluded that these results support the hypothesis that the hTFPI-CD4 and hirudin-DC4 constructs may be useful agents for clinical applications as a safer and more effective way to inhibit thrombosis. Genetic manipulation will allow for targeted expression for local anticoagulant in situations in
which thrombotic complications are anticipated such as islet xenotransplantation avoiding the undesirable effects of systemic anticoagulation (Chen 1999a; Chen 1999b).

Chen et al. further developed the work with the anticoagulant fusion proteins by applying it to heart transplantation in rodent model to demonstrate their effect in an in vivo setting. Because intravascular thrombosis and systemic coagulopathy are prominent features of acute humoral xenograft rejection, they hypothesized that expression of anticoagulants on xenogeneic vascular endothelium might inhibit the process. Hearts from novel transgenic mice strains, expressing hTFPI construct and hirudin respectively, were transplanted into rats. In contrast to control non-transgenic mouse hearts, which were all rejected within 3 days, 100% of the organs from both strains of transgenic mice were completely resistant to humoral rejection and survived for more than 100 days when T-cell-mediated rejection was inhibited by administration of cyclosporine A. The anticoagulant constructs achieved effective inhibition of thrombosis in transplanted cardiac xenografts and offered complete protection against humoral rejection. These results demonstrated the critical role of coagulation in the pathophysiology of acute humoral rejection and the potential for inhibiting rejection by targeting the expression of anticoagulants to graft endothelial cells (Chen 2004).

In summary, the genetic strategy of the expression of anticoagulant fusion protein constructs to target localised thrombosis has been successfully demonstrated in vitro and in vivo. The animal work by Chan et al. covered a spectrum of mouse models including humoral rejection in xenogeneic transplantation. These results are encouraging where it’s envisaged that transgenic pigs might be useful for islet xenotransplantation. However, in practice, the use of this strategy is limited because xenografts remains in the experimental pre-clinical phase and because the gene
therapy approaches have suffered from poor delivery of the genetic material and from side effects related to viral delivery systems.

1.13.2 Anti-thrombotic cytotopic agents

As a clinical strategy for xenografts, gene therapy is limited in its utility and a more practical and promising approach is the use of cytotopic agents developed in the MRC Centre for Transplantation at Kings College in London. Cytotopic agents are engineered protein and peptide agents made to function at the Cell Surface. Cytotopic proteins made by Richard Smith of the MRC Centre have been modified to behave like intrinsic membrane proteins while retaining pharmaceutically acceptable physical properties. The primary modification is based on a natural mechanism used by proteins such as Src and Ras, the myristoyl-electrostatic switch. The anticoagulant cytotopic agent is a soluble compound comprising an antithrombotic agent and a membrane-binding element, where the antithrombotic agent has a weight of less than about 5,000 daltons. It can bind to the cell membrane of cells, tissues and organs to prevent or reduce the formation of blood clots. ‘Thrombalexin’ (TLN) is one such compound, based on hirulog peptide, the active moiety in the leech anticoagulant hirudin, which binds thrombin irreversibly. TLN contains a synthetic structurally minimized and chemically reactive form of a myristoyl-electrostatic switch. In laboratory testing, TLN has been shown to retain potent antithrombin activity when bound to cell membranes and can inhibit fibrin clot formation in a recalcified plasma assay. In these assays, cell-bound hirulog inhibits only clotting that is initiated by the cells, in contrast to when soluble hirulog is added, when all clotting is inhibited, including that induced by the glass tubes, a situation that is analogous to the systemic anticoagulant effect seen in vivo when soluble therapeutics are used. The compound is relatively small in size which means that it is generally less
immunogenic than large agents, especially if it originates from an exogenous source. Another advantage is that it is easy to manufacture and importantly can be manufactured synthetically. Finally, since the compound can bind to cell membranes, it can be administered locally so that the compound has an effect at a specific location rather than having a systemic effect. The compound can be used in the short-term manipulation of organs in transplantation. It can also be used in cell therapies in which it is desirable to confer resistance to coagulation. TLN is currently undergoing pre-clinical development in a £1.3M project funded by a Wellcome Trust Translation Award, with the aim of generating pharmaceutical grade reagent for a Phase I Clinical Trial by 2015 (Dorling 2013; Smith 2001; Smith 2012).

In translational terms, one potential clinical use of such cytotopic constructs has been in prostate cancer therapy. The membrane localizing anti-coagulant hirudin cytotopic analog PTL004 were shown to have comparable anti-migratory and anti-proliferative properties to hirudin, and liposomes bearing this compound are even more effective at inhibition of proliferation of PC3 prostate cancer cells. These results demonstrate the potential of a cytotopic approach using tailed anticoagulants in localized prostate cancer therapy (Galustian 2011).

Another compound (Mirococept), based on applying similar modifications to human complement receptor type 1 is at an advanced stage of development. Mirococept has potent biological activity (including against C5) in multiple pre-clinical models and has undergone Phase 1 and pilot Phase II clinical studies in human renal transplant patients. A large Phase II study in kidney transplantation is funded by the MRC and will start recruiting in 2013 (Dorling 2013; Smith 2001).
1.14 Summary and hypothesis

At present, organ shortage remains a major limiting factor for pancreas transplantation, which is the gold standard therapy for type I diabetes. In addition to limited organ supply for donation, islet transplantation has several hurdles before it becomes a treatment of choice for this disease. Although limited supply is not an issue in xenotransplantation, it has a different set of barriers that need to be resolved before it can be used in clinical practice. Insulin secreting stem cells provide a solution to organ shortage and so may be a novel and promising treatment for type I diabetes. However, based on the evidence available for the IBMIR following intravenous transplantation of islet allo and xeno-transplantation into the liver, it can be postulated that intravenous administration of ‘islet equivalent’ stem cells will provoke a similar immediate inflammatory response and thrombosis triggered by tissue factor. This will lead to significant early loss of the cells infused.

The current study investigates the following hypothesis and attempts to find a therapy to reverse it.

I) Stem or progenitor cells capable of insulin secretion ‘islet equivalent’ are capable of successfully reversing diabetic phenotype in a mouse model and in a phase I human trial.

II) The insulin secreting cells express tissue factor on their cell surface. Following transplantation, this TF expression can initiate the coagulation cascade and cause thrombosis leading to early cell loss following treating diabetic mice with these cells.

III) Treatment of ‘islet equivalent’ cells with anti-thrombotic cytotoxic agent, based on hirulog peptide, prior to transplantation reverses this coagulation cascade partially or completely in vitro. This potentially can be used in vivo as a mechanism to improve
stem cell survival in the diabetic mouse model and reduces the number of cells required to achieve normoglycaemia.
2. MATERIALS AND METHODS

2.1 “islet equivalent” stem cells sources

2.1.1 Pancreatic-Derived Pathfinder cells

The Shiels’ laboratory, part of the Division of Cancer Sciences and Molecular Pathology, in Glasgow have isolated and characterised a novel population of cells from adult rat pancreatic ducts. These cells were found to exhibit the potential to differentiate into both pancreatic and hepatic cells types. They termed this cell population pancreas derived progenitor cells (PDPCs) and published their findings in 2009. The exact methods for the isolation of PDPCs and the rest of the in vitro characterisation work are described in the paper by Stevenson et al. (Stevensen, 2009).

Two subpopulations of PDPC were selected in vitro culture and characterised. These were CD90 or Thy1.1 positive (CD90+) and CD90 negative (CD90-) cells. CD90, also known in rodents as rat and mouse Thy1 and Thy1.1 respectively, is a cell-surface protein which is expressed on a variety of cell types including thymocytes, fetal liver cells, umbilical cord and mesenchymal stem cells in humans, mice and rats. CD90 is now considered a surrogate marker for various kinds of stem cells such as HSC, usually in combination with other markers. The function of CD90 has not been fully elucidated or understood. It has speculated roles in cell-cell and cell-matrix interactions. There is evidence suggesting that it is involved in cell recognition, adhesion and signal transduction (Gerhengorn 2004, Hasegawa 2007, Lee 2006, Munoz 2005, Zhou 2008). Several studies proposed that the expression of CD90 in the adult liver rat oval stem cell population facilitated them to recognize and adhere to stromal tissue as potentially repair cells after injury (Colman, 2004,Hasegawa, 2007,Thorel, 2010,Yagi, 2010).
Both PDPC subpopulations expressed pancreatic and duodenal homeobox-1 (Pdx-1). Pdx-1, also known as insulin promotor factor 1 (IPF1) in humans, is a transcription factor necessary for pancreatic development and beta-cell maturation. The protein encoded by this gene is an activator of several genes, including insulin, somatostatin, glucokinase, islet amyloid polypeptide, and glucose transporter type 2. The encoded nuclear protein is involved in the early development of the pancreas and plays a major role in glucose-dependent regulation of insulin gene expression. Defects in this gene are a cause of pancreatic agenesis, which can lead to early-onset insulin-dependent diabetes mellitus, as well as maturity onset diabetes of the young (MODY). The detection of Pdx-1 transcriptional expression indicated the potential of these PDPC subpopulations to become insulin-producing cells (Stevensen, 2009).

These two subpopulations were distinct when characterised. CD90+ sub-population could be induced to differentiate into hepatic cell types expressing albumin and storing glycogen typical of mature hepatocytes. These differentiated cells showed both morphological and gene expression consistent with hepatic lineage. The same population could be induced into pancreatic lineage forming a 3D islet-like structures. On the other hand, CD90- subpopulation in pancreatic differentiation medium could also be induced into pancreatic lineage but they did not have the same morphology observed with CD90+ cells (Stevensen, 2009).

In pancreatic nicotinamide-containing differentiation media, PDPC subpopulations exhibited different morphology. The fibroblast-like CD90+ developed into islet-like clusters by 28 days which eventually detached from the parent cell layer while the small epithelial-like CD90- cells remained unchanged in morphology at day 28. Reverse transcription polymerase chain reaction (RT-PCR) analysis of undifferentiated cells demonstrated positive pdx-1 expression but no expression of insulin or glucagon in both CD 90+ and CD90- populations. However, differentiated
CD90+ cell clusters were positive for the transcriptional markers of all these three markers compared to only Pdx-1 and insulin in differentiated CD90- cells (Stevensen, 2009).

The isolation of these novel PDPCs into two morphologically distinct subpopulations, which in vitro, can both potentially differentiate into insulin-secreting cells led to the desire to examine in vivo effects of these sorted cells. In addition, the same group in Glasgow was able to isolate and identify similar PDPCs from human pancreatic biopsies. In this study, I investigated the in vivo effect of both rat and human PDPCs isolated in Glasgow on a diabetic mouse model and examined their coagulation properties.

2.1.2 Haematopoietic stem cells “Omnicytes”

Habib’s group at Imperial College isolated a subpopulation of CD34+ cells as a separate putative stem cell source. These cells have small lymphocyte-like morphology, of approximately 7 to 15µm in diameter, adhere to tissue culture plastic and in culture produce a population of cells exhibiting diverse morphologies and expressing genes corresponding to multiple tissue types. This CD34+ subpopulation of cells with these distinct properties was termed “Omnicytes”. CD34+ cells were harvest by leukapheresis of G-CSF mobilised peripheral blood progenitor cells from donors. Typical yield from this process was 5-10x10^10 most of which are mononuclear cells and around 1% are CD34+ (5-8x10^6). These CD34+ cells are separated and cultured. The adherent fraction usually constitute about 1% of the total CD34+ population. So a typical process would provide 5-10x10^6 putative stem progenitor cells. These cells are then cultured in vitro where 3-4 log expansion in number is achievable within 1-2 weeks. This would provide 5-10x10^9 for clinical
applications. The cell expansion was highly reliable and reproducible in their experiments (Gordon, 2006).

The researchers demonstrated that before and after culture, the adherent CD34+ cells and their progeny express an array of gene products as revealed by reverse transcription-polymerase chain reaction analysis, flow cytometry and gel electrophoresis consistent with various tissue lineages. RT-PCR results indicated that adherent CD34+ cells differentiate into cells expressing markers associated with liver, pancreas, cardiovascular, muscle and nerve cell tissue as well as haematopoiesis. They express Pdx-1, NGN-3 and insulin consistent with pancreatic differentiation. It is worth noting that these adherent CD34+ cells express CD38, CD33 and HLA-DR. The expression of HLA antigens potentially can make these cells susceptible to rejection if used as allogeneic rather than autologous transplant. Gordon et al. described the exact methods for the isolation and culture of Omnicytes as well as the rest of the in vitro work in the paper published in 2006 (Gordon, 2006).

In this study, Omnicytes were the source of stem cells for in vitro and in vivo work. I investigated their vivo effect in a diabetic mouse model and examined their coagulation properties. Autologous infusion of insulin-secreting Omnicyte progeny was the source of stem cells in the phase I clinical trial described later 3.5.

2.2 In vivo effects of stem cells on streptozotocin-induced diabetes

The pancreatic beta cells are terminally differentiated and can be ablated by the toxin streptozotocin (STZ). Cell-based therapy that might enable or cause a terminally non-functional tissue to reinitiate repair process and reform a functional organ would be very attractive.
The in vivo effect of these PDP cells and Omicytes in a diabetic mouse model was investigated at Imperial College under supervision of Professor Anthony Dorling. The experiments I performed were based on the hypothesis that these cells could contribute to islet formation in vivo as mediators of pancreatic tissue regeneration and in turn could potentially be a direct cellular therapy for diabetes.

2.2.1 Isolation and maintenance of PDPCs and Omicytes

As described in Stevenson et al. paper, the isolation of rat and human PDPCs was performed in Glasgow. Pancreatic ductal tissue was isolated from 12-month old Albino Swiss (Glasgow) rats, and from undiseased female human pancreatic biopsy. In both instances, tissue was microdissected and minced, prior to seeding in CMRL medium (Invitrogen, Paisley, UK). The PDPCs form a confluent monolayer after approximately 5 weeks in culture. These were then harvested and washed in phosphate-buffered saline (PBS). PDPC were maintained in culture in 20ml of CMRL 1066 medium supplemented with 10% fetal bovine serum (FBS; Sigma, Poole, UK), 2mM glutamate, 1.25mg/mL amphotericin B, and 100U/ml Penicillin, 100 mcg/ml streptomycin (all Invitrogen, Paisley, UK) in T75 flasks with 0.2μm filter caps (Corning, UK) at 37°C in a 5% CO2 atmosphere (Stevensen, 2009).

Gordon et al. described the method of Omicyte isolation in their paper and performed in their lab at Imperial College (Gordon 2006). All blood samples containing the CD34+ cells were diluted at 1:4 in Hanks’ buffered saline solution (HBSS; Gibco, Paisley, UK) before the mononuclear cells (MNCs) were separated by centrifugation over a Lymphoprep (Axis-Shift, Kimbolton, Cambridgeshire, UK) density gradient at 1,800 rpm for 30 minutes (Heraeus, Hanau, Germany). The MNC fraction was collected and washed first in HBSS, then with MACS (magnetic cell sorting) buffer (PBS supplemented with 0.5% bovine serum albumin (FBS) and 5mM EDTA).
CD34+ cells were isolated from MNCs, using the CD34+ positive cell selection kit (MiniMacs; Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated CD34+ cells were plated on 35-mm² Petri dishes in α-minimal essential medium (α-MEM) supplemented with 15% fetal bovine serum and incubated for 2 hours at 37°C and 5% CO₂. After 2 hours, the non-adherent cell fraction was removed by washing the plates three times. Adherent CD34+ cells were cultured in α-MEM supplemented with 30% FBS and cytokines (20 ng/ml stem cell factor (SCF), 1ng/ml GM-SCF, 5 ng/ml IL-3, and 100 ng/ml G-CSF) at 37°C in 5% CO2 in air (Gordon, 2006).

Both these cell populations PDPCs and Omnicytes once isolated and cultured, were delivered to our laboratory in Imperial College to be used in the work performed in this study.

2.2.2 Mouse strain and streptozocin treatment

I performed these experiments at Imperial College. All animal procedures were conducted under the terms of the project licence issued by the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986.

5 groups of female C57Bl/6 mice (4 animals in each group for the PDPCs experiments and 5 animals in each group for the Omnicytes experiments) were made diabetic by intraperitoneal injection of streptozocin (Sigma, Aldrich) at a dose of 250mg/kg on day 0 of the experiment. Streptozotocin, a glucosamine-nitrosourea, is a DNA alkylating agent that enters cells exclusively via the GLUT2 glucose transport protein. It is a toxic glucose analogue that preferentially accumulates in pancreatic beta cells via the GLUT2 glucose transporter. High doses of STZ selectively destruct the insulin - producing beta cells and induce rapid and permanent hyperglycaemia in mice. This usually takes 3-4 days in mice and 7 days in rats. In contrast, the injection
of multiple low doses of STZ generates Hydrogen peroxide and induces the expression of GAD autoantigens. This causes the development of delayed and progressive hyperglycaemia which may take up to 20-30 days. Chemical induction of diabetes with STZ is the most widely used animal mouse model of diabetes mellitus. Genetic and surgical models are rarely used because they require highly technical skills and the percentage of animals sacrificed during the procedures are usually higher compared to chemical induction models (Van Belle 2009). The high dose STZ diabetic animal model was chosen in all the experiments of this study as it is well established, relatively well understood, and it is the most widely used model (Colman, 2004, Mansford, 1968, Van Belle, 2009). It is technically simple and results in rapid and irreversible hyperglycaemia of the mice which is what was required in the study to accurately assess any effect of insulin-secreting “islet-equivalent” on glucose levels and beta cell damage.

STZ was prepared in 20mM citrate buffer (pH 4.5) and used immediately for intraperitoneal delivery. This high dose was used to ensure the destruction and obliteration of beta cells, to the extent that the untreated control mice would not survive long enough to regenerate sufficient pancreatic tissue to alleviate the effects of STZ treatment. In this protocol, diabetes is defined as serial non-fasting glucose levels, 3 days post-STZ administration of ≥20 mM/L. Severe progressive hyperglycaemia under the Home Office licensed and proved protocol is defined as progressive rise in serum glucose to ≥ 30mM/L or clinical signs of hyperglycaemia. Hence, animals with glucose ≥ 30mM/L or a body weight loss of ≥20% were sacrificed.

2.2.3 stem cell infusion

1.5x10^6 cells were injected into the tail vein of the animals on day 3 after STZ administration. The cells administered were one of the following types unsorted male
rat cells, CD90+ male rat cells, CD90- male rat cells, female human PDPCs or day 7 human adherent CD34+ Omicocytes. In the PDPCs experiments, this infusion was repeated on day 10 after STZ administration. All animals receiving Omicocytes had a single intravenous infusion of cells but in the initial experiment performed by Dr Daxin Chen, a scientist in our laboratory at Imperial College, one of treated animal groups were injected with the same number of day 7 human adherent CD34+ Omicocytes directly into the pancreas of the animal instead of intravenously into the tail vein. Control animals were injected with equal volumes of conditioned culture medium into the tail vein at the same time points. Blood glucose was monitored every 3 days for the first 4 weeks then weekly until end of the follow up period. Animals were weighted weekly throughout the experiment duration. Under the Home Office approved protocol, animals were followed up for a maximum of 100 days.

In the PDPCs experiments, the use of xenogeneic model in which male albino swiss rat or human PDP cells were injected into female C57Bl/6 mice facilitated the tracking of the transplanted cells and in turn allowed us to address whether the PDP cells differentiated in vivo to repair damage, stimulated the host tissue to repair damage, or both.

### 2.2.4 Blood glucose measurement

In all experiments, two glucose values were determined from a blood sample obtained from the tail vein of the animal, and measured using the Ascensia® Autodisc™ Blood Glucose Test Sensors (Bayer Healthcare, Berks, UK) following the manufacturer’s recommendations. The mean of the two measurements was recorded. Blood glucose was monitored every three days for the first four weeks then weekly until end of the follow-up period. Animals were weighed weekly throughout the experiment duration.
2.2.5 Immunosuppression

The expression of HLA antigens by Omnicytes made them potentially immunogenic and possibly susceptible to rejection after transplantation. Hence, Cyclosporine (NEORAL® Oral Solution, Novartis), which is a potent immunosuppressive drug, was administrated to all animals involved in the Omnicytes studies whether they were treated with cells or were control animals. Cyclosporine (CyA) binds with the cytosolic protein cyclophilin of lymphocytes especially T-cells. This complex of cyclosporine and cyclophilin inhibits calcineurin, which is responsible for activating the transcription of interleukin 2 (IL-2) and in turn interfering with the activity and proliferation of T-cells. CyA was diluted in normal saline to 15mg/ml and was injected at a dose of 20mg/kg intraperitoneally into all animals daily for 7 days starting on day of cellular therapy or control infusion.

On the other hand, CyA was not used in any of the experiments using rat or human PDPCs, as these cells were not believed to be immunogenic. Earlier preliminary work in our laboratory, by Dr Daxin Chen, demonstrated that these PDPCs are not immunogenic. Administration of cyclosporine at a dose of 20mg/kg as an immunosuppressant had no effect on the efficacy of PDPCs in reducing blood glucose levels in this animal model of diabetes (Data unpublished). Hence, since PDPC are thought to be immunologically null, no immunosuppression was given to the animals in any of these experiments.

2.2.6 Tissue harvest and further analysis

At the end of the experiment follow-up time of 90 days, animals were scarified. Solid organs including pancreata from PDPC transplanted and control mice were removed and snap-frozen in liquid nitrogen, then embedded in Tissuetek OCT compound (RA
Lamb). These were sent to Glasgow for further tissue analysis. Shiels laboratory in Glasgow performed immunohistochemistry and fluorescence in situ hybridisation experiments. The materials and methods for these examinations were described in our paper (Stevensen, 2011).

Similarly, at end of the experiment follow-up time of 35 days, Omnicyte-treated or control animals were sacrificed. Pancreata from CD34+ cell transplant and control mice were removed and snap-frozen in liquid nitrogen, then embedded in Tissuetek OCT compound (RA Lamb). These were delivered to Prof Habib’s group at Imperial College for further tissue analysis (Data analysis not completed).

2.3 In vitro assessment of coagulation properties of stem cells

Similar to islets, we postulated that following intravenous infusion, stem cells capable of insulin secretion i.e. ‘islet equivalent’ (PDPCs or Omnicytes) will provoke an inflammatory response and thrombosis, which will lead to significant loss of cell. The second hypothesis tested in this study is that these stem cells expressed tissue factor on their cell surface that can initiate the coagulation cascade. It has already been demonstrated that TF plays an important role in the mechanism of thrombosis in islet allo and xenotransplantation (Chapter 1.10.5 and 1.10.6). It follows that treatment of TF positive cells prior to transplantation into the diabetic mice with anti-thrombotic proteins, such as hirudin, may reduce the degree of early cell destruction observed due to thrombosis. This can be used as a way to improve the survival of these cells post transplantation and optimise their performance.

To investigate these hypotheses, coagulation or plasma recalcification assays were performed to establish if the cells caused in vitro clotting and attempts were made to identify the potential mechanism by examining tissue factor expression structurally
and functionally as one possible pathway. Pre-conditioning of these insulin-secreting cells with anti-thrombotic cytotoxic agents was preformed to assess if this treatment reverses this procoagulant effect and results in reduced thrombosis and cell loss.

2.3.1 Antibodies

The primary and secondary antibodies used in plasma recalcification assays (PRA) and, immunocytochemistry (ICC), together with appropriate isotype controls, are listed in table 3.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Fluorochrome</th>
<th>Concentration mg/ml</th>
<th>Investigations</th>
<th>Company</th>
</tr>
</thead>
<tbody>
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<td>Rabbit anti-mouse tissue factor IgG</td>
<td>-</td>
<td>1</td>
<td>PRA and ICC</td>
<td>America diagnostica inc.</td>
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<td>10</td>
<td>PRA and ICC</td>
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<td>0.4</td>
<td>PRA</td>
<td>Affinity Biologicals</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>-</td>
<td>0.4</td>
<td>PRA</td>
<td>Santa Cruz biotechnology</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG</td>
<td>FITC</td>
<td>0.4</td>
<td>ICC</td>
<td>Santa Cruz biotechnology</td>
</tr>
<tr>
<td>Donkey anti-sheep IgG</td>
<td>FITC</td>
<td>0.4</td>
<td>ICC</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

Table 3: Primary, secondary and isotype control antibodies used in PRA and ICC

2.3.2 Hirulog-like cytotoxic peptide construct

The anti-thrombotic cytotoxic agent was developed by Richard Smith in the MRC Centre for Transplantation at Kings College. It was kindly donated to our group to perform these experiments and assess its effect and efficacy in the setting of stem cell use in diabetes. The agent used in my experiments is registered as HLL peptide (PTL006; with patents/US2012232014) with a molecular weight of 5272 Daltons. It is a derivative of hirulog prepared by solid phase synthesis disulphide linked to a polyethylene glycol conjugate of phosphatidyl ethanolamine (PEGTAIL-1). It acts as an irreversible thrombin inhibitor with membrane-localising properties. It was prepared as a colourless solution at 50μM concentration and stored at -20°C. In laboratory testing, the peptide was shown to retain potent antithrombin activity when bound to cell membranes and can inhibit fibrin clot formation in a re-calcified plasma
assay. In these assays, cell-bound hirulog-like peptide inhibits only clotting that is initiated by the cells, in contrast to when the soluble peptide is added, when all clotting is inhibited, including that induced by the glass tube (section 1.12.1).

2.3.3 Plasma recalcification assay

1.5 x10^5 cells were suspended in 50μL tris-buffered saline and mixed with 100μL of plasma composed of 90% fresh human plasma and 10% mouse plasma (ICR mouse plasma in sodium citrate; Harlan UK Ltd) and 100% fresh human plasma for rat PDPC and human Omnicytes respectively in a glass tube (Corning, Corning, NY). 100μL of 25 mM Calcium chloride CaCl₂ in tris-buffered saline was added, and the tube was incubated at 37°C in a water bath; the time for fibrin clot to form was determined in triplicate, during which the tubes were continuously agitated by tilting. The mean and standard error of the triplicate clotting time was calculated for each of the re-calcified plasma assay. P-values were calculated using Student’s t-test. Rodent TF binds poorly to human factor VII. To measure rat or mouse TF activity, a mixture of 10% rat plasma and 90% human plasma is used in this assay. The 10% rat plasma provides sufficient rodent factor VII (Morrissey Lab, University of Illinois at Urbana-Champaign, Urbana, IL 61801 http://tf7.org/mlabprotocols.htm).

To test for TF functional expression on the surface of the cells and the effect of the cytotopic agent, in some of the clotting assays, cells were treated with one of these options; 2.5μl of rabbit anti-mouse Tissue factor (100μl/ml), sheep anti-human TF antibody (1mg/ml), 2.5μl of isotype control for 30 min at 4°C or 250 μl of 50mM anti-thrombotic cytotopic agent (described in section 2.3.2) for 10 min at 4°C before determining clotting times. This last option allows the antibody to bind to the cell surface or the cytotopic agent to tether into the cell surface respectively. To test the effect of soluble rather than localised anti-thrombotic cytotopic peptide, 167μl of
50μM of this agent was added to the PBS solution in which the cells were suspended prior to immediately determining the clotting time. This did not allow time for the peptide to tether into the cell surface. Finally, in some assays, TF-dependent thrombin generation requires the participation of FVIIa, and therefore, FVII-deficient plasma (Diagnostic Reagents) was used in a separate assay to determine whether clotting was TF-dependent.

The Plasma recalcification assay used in this study is a technically simple and cheap test. It is a well-established assay, widely used in experimental work and does not require any complex equipment. However, the clotting time does rely on the human eye to detect the formation of a fibrin clot. This factor has the potential to introduce a higher degree of inaccuracy compared to other complex methods. I attempted to minimise this error by taking several steps. There was only one operator for all the assays as I performed all the experiments alone. Additionally, each specific test was performed in triplicate and the mean of all 3 clotting times was calculated and finally the plasma recalcification assay was repeated at least twice on each type of cells assessed.

2.4 Immunocytochemical staining

200μl of fresh cells at a concentration of 2.5x10⁶ cells/ml were suspended in each well of a 96 well tray. The trays were centrifuged at 1800 min⁻¹ for 2 minutes at 4°C. Medium was removed by flick/blotting and the pellet resuspended in 200μl of 1% bovine serum albumin (BSA). 50μl of these cells were pipetted, mixed with BSA and suspended into a well. Another 50μl of 1% BSA were added to make up 100μl in each well. 1-2μl of the primary antibody or control isotype was added to each well then the tray was placed on ice for 30 minutes. PBS was used to wash the cells twice then the tray was centrifuged again as above for 2 minutes. The medium was
removed and 100μl of 1% BSA was added to the remaining pellet in each well. 1-2 μl of the secondary antibody was added and the tray was left on ice for 30 minutes. The cells were washed again twice with PBS as previously described. Cells in each well were diluted with 60μl of PBS. 5μl of this diluted solution was dropped onto a slide and allowed to dry completely. Once the cells were dry, they were fixed with 30μl of cold 4% paraformaldehyde for 15-20 minutes at room temperature. For immune staining, the nuclei were visualized with a mounting medium with DAPI (4,6 diamino-2-phenylindole; Vecta Shield) was added and then slides were viewed under a fluorescence deconvolution microscope (Zeiss Axiovert S100-TV, Zeiss, Germany).

2.5  In vivo assessment of the coagulation properties of stem cells

2.5.1  Mouse strain and stem cell infusion

3 groups of female C57Bl/6 mice (3 animals in each group) were made diabetic by intraperitoneal injection of streptozocin as described earlier in section 2.2.2. On day 3 post injection post STZ, 1.5x10^6 day 7 human adherent CD34+ Omnicytes were injected into the tail vein of the first group. The second group of animals received day 7 human adherent CD34+ Omnicytes that had been treated with the hirulog-like peptide (section 2.3.2) by incubation for 10 minutes at 4° prior to infusion. The third group was a control group where the animals were injected with equal volumes of conditioned culture medium. 30 minutes after intravenous cell infusion, all animals were sacrificed. Immediate collection of mouse blood was performed via intra-cardiac aspiration. This blood was used to obtain plasma and evaluate platelet consumption as indirect evidence for coagulation.
2.5.2 Evaluation of platelet count

Platelet-rich mouse plasma was obtained from blood by centrifugation at 80g for 10 min at 4°C, followed by dilution at 1:20 with 1% ammonium oxalate and 2.5 mM Gly-Pro-Arg-Pro peptide (Sigma, St Louis, MO). Samples were placed in a counting chamber in a moist petri-dish, and the platelets in 1mm² were counted (N). The number of platelets per liter of blood equaled 2Nx10⁹. Mice usually have 3-4 times number of platelets when compared to humans.

2.6 Stem cell product for phase I clinical trial

The isolation and culture of the stem cell product used in the clinical trial entitled “Phase I Safety and tolerability study following the infusion of autologous Expanded progeny of an adult CD34+ stem cell subset (Insulincytes) to patients with type I diabetes mellitus and a successful renal transplant” was performed under GMP conditions at the Imperial College Stem Cell Laboratory and John Goldman Centre for Cellular Therapy, Hammersmith Hospital.

Manufacture of the expanded adherent CD34+ stem cells concentrate took place in three stages; bone marrow mobilisation and leukapheresis, followed by immunoselection and adherence selection of the CD34+ stem cells, and finally involves the expansion and differentiation of the immunoselected adherent CD34+ stem cells in cell factories with the addition of cell growth media supplemented with cell growth cytokines.
2.6.1 Registration and ethics for phase I clinical trial

The trial was registered with ClinicalTrials.gov with identifier NCT00788827 and registered with EudraCT 2006-002328-40. Imperial College, London (Protocol Code HHSC/005) was the trial's sponsor. Medicines and Healthcare Products Regulatory Agency (MHRA) and Local Research Ethical Committee approval was formally granted prior to starting trial. Informed consent was obtained for every patient screened for the trial.

2.6.2 Mobilisation of bone marrow

We know from previous work at Imperial College that “Omnicytes” are haematopoietic stem cells that are small and nucleated and bear the heavily glycosylated mucin-like surface antigen CD34. So they are early non-differentiated haematopoietic cells that are extractable from the peripheral blood circulation of individual donors as described in section 2.1.2.

Granulocyte colony-stimulating factor is commonly used at a dose of 10 mg/kg/day subcutaneously to mobilize peripheral blood stem cells (PBSC) from healthy donors. Mobilisation of cells expressing CD34 antigen peaks in the peripheral blood between days 4 and 5 of G-CSF dosing. Thus, leukapheresis is typically commenced on day 4 or 5 of G-CSF treatment and is repeated until a target number of CD34+ cells are collected (usually ≥4×10⁶/kg). Compared with the graft composition of bone marrow harvests, G-CSF mobilized products contain three to fourfold higher CD34+ doses (Cashen, 2007).

Based on these haematological protocols, patients enrolled in the study received a daily subcutaneous injection of G-CSF filgrastim (NEUPOGEN® is the Amgen Inc.)
at 10µg/kg of for five days to mobilise bone marrow progenitor cells and increase numbers of peripherally circulating CD34+ stem cells.

### 2.6.3 Leukapheresis

After completion of bone marrow mobilisation, the study participant undergoes leukapheresis in the haematology day unit to obtain autologous lymphocytes and monocytes containing the CD34+ stem cells fraction needed for the study. Apheresis is a laboratory procedure for separating out one particular constituent of blood and returning the remainder to the circulation. Leukapheresis is apheresis of white blood cells. It is a standard procedure for the isolation of stem cells from the peripheral circulation via a central or femoral line. It has been routinely used in patients with haematological malignancies requiring HSCT.

At the end of the first leukapheresis, the total CD34+ cell count isolated was measured. If this was found to be below that necessary to obtain sufficient cells for expansion and differentiation (less than $1 \times 10^7 /10\text{mL}$), the patient will undergo a second leukapheresis. In this case, G-CSF treatment was continued for further 24 hours prior to the second leukapheresis on the following day. Following the collection, the labelled leukapheresis product was then transported to the GMP manufacturing site for further processing.

### 2.6.4 Immunoselection and expansion of adherent CD34+ cells

Immuno-magnetic purification of the CD34+ stem cell subset population was carried out in the stem cell laboratory. The collected lymphocytes and monocytes was first washed in PBS/EDTA/HAS followed by the addition of a set volume of specific cell
type reagent composed of CD34+ antigen coated magnetic beads and following mixing, the bag is aseptically connected to the CliniMACS machine. An automated process applies the cells to the separation column, performs a series of washes depending on the programme selected and finally elutes the purified CD34+ cells. The key components of the CliniMACS instrument are an integrated computer, the magnetic separation unit, a peristaltic pump and various pinch valves. The integrated computer controls all components of the instrument and directs the system to perform procedures in a standard sequence. The magnetic separation unit includes a movable permanent magnet and a holder for the selection column. The peristaltic pump controls the flow rate through the tubing set and 11 pinch valves ensure controlled flow of buffer and cell suspension. Once connected, the system works within a closed environment, protecting the product at all stages.

Adherence selection is achieved by incubating the CD34+ cells in tissue grade plastic “cell factories” followed by three washes with more than 100ml saline solution to remove the non-adherent CD34+ cells. Following removal of non-adherent cells, the cells are resuspended in Alpha medium supplemented with 30% human AB serum containing specific concentrations of cell growth cytokines Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), interleukin-3 (IL-3), SCF, and G-CSF and human male blood group AB serum. The cells are then incubated at 37°C in 5% CO2 in air for seven days.

### 2.6.5 Differentiation into “Insulincytes”

At the end of the 7-day expansion, the progeny of the immunoselected adherent CD34+ stem cell were differentiated into insulin and C-peptide expressing cells by the sequential addition of the various reagents and growth factors. Glucose was added on day 7 (2.5mM final concentration) followed by fibroblast growth factor on
day 8 (10ng/mL). Dimethyl Sulfoxide (DMSO, 1% final concentration), butylated hydroxyanisole [100μM final concentration] and exendin-4 (10ng/mL) were all added on day 9. On day 10, glucose (11.1mM final concentration), sodium pyruvate (1mM final concentration), fibroblast growth factor (20ng/mL), epidermal growth factor (20ng/mL) and exendin-4 (10ng/mL) were also added. Finally, the process ends with the addition of glucose (2.5mM final concentration), nicotinamide (10mM final concentration), hepatocyte growth factor (100pg/mL), exendin-4 (10ng/mL) and activin A (2nM final concentration) on day 14. On day 21, the expanded and differentiated cells termed ‘Insulincytes’ were aseptically aspirated from the ‘cell factory’ and following a final wash in saline and samples are taken for sterility, total nucleated cell count and viability. The aspirated cell culture was volume reduced and then packaged for release and use in the study. At this stage, the Insulincytes are sampled for total nucleated cell count and viability, sterility and freedom from mycoplasma contamination.

Figure 12 is a flow chart summarising the manufacturing process from collection of autologous leukapheresis product to harvesting of expanded and differentiated CD34+ stem cell progeny (Insulincytes).
### Figure 12: Summary of the manufacturing process of expanded and differentiated CD34+ stem cell progeny ‘Insulincytes’

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukapheresis product</td>
<td></td>
</tr>
<tr>
<td>Wash and volume adjustment</td>
<td>Mouse anti-human micro-beads added</td>
</tr>
<tr>
<td>Immunoselection of CD34+ cells using the CliniMacs apparatus</td>
<td></td>
</tr>
<tr>
<td>Selection of Omnicytes (by adhesion to plastic)</td>
<td>Expansion of Omnicytes in a cell factory plus growth factors</td>
</tr>
<tr>
<td>Differentiation into Insulincytes</td>
<td></td>
</tr>
<tr>
<td>Volume adjustment and packaging</td>
<td>Quality Control testing and release for autologous infusion</td>
</tr>
</tbody>
</table>

#### 2.6.6 Insulincyte infusion

On day 21, the autologous expanded progeny of adult CD34+ stem cell subset ‘Insulincytes’ were injected into either the body or tail of the pancreas of the participant. This procedure was performed by selective catherisation of the splenic artery under fluorescent guidance by an experienced radiologist. Prophylactic anticoagulation therapy was administered for five days post stem cell infusion to avoid cell thrombosis in the form of 20mg subcutaneous enoxaparin (Clexane prefilled syringes, Sanofi). Figure 13 reveals intra-arterial digital subtraction angiography images of the stem cell product infusion into the pancreas of one of the study patients.
Figure 13: Intra-arterial digital subtraction angiography (IADSA) images of the stem cell product infusion into the pancreas of one of the study patients
2.7 Statistical analysis

Significant differences were tested by Student's t-test between the groups. P value of <0.05 was considered significant. All data were presented as the mean ± standard error (SE) or standard deviation (SD).
3. RESULTS

3.1 Hypothesis I

Stem or progenitor cells capable of insulin secretion ‘islet equivalent’ are able to reverse diabetic phenotype in a mouse model.

3.1.1 In vivo examination of whether rat and human PDPC treatment normalises blood glucose and reverses the diabetic phenotype in a mouse model

To investigate whether PDPCs reversed diabetes, 20 animals were made diabetic by the administration of STZ as described in 2.2.1. STZ injection resulted in progressive hyperglycaemia with blood glucose levels above 20 mmol/L by day 3 in all animals, which met the definition of diabetes in the protocol of the study described in 2.2.1. There were 5 groups and each group was composed of 4 animals. The 4 treated animal groups were administered one of four cell treatment options intravenously 3 days post STZ as described in 2.2.2. These cell treatment options were blinded to me at the time of the experiment and were marked as cells A, B, C, D. Retrospectively, these corresponded to the cell treatment options of mixed population of unsorted rat PDPCs (CD90+ and CD90- cells), CD90+ rat PDPCs, CD90- rat PDPCs, and mixed population of unsorted human PDPCs (CD90+ and CD90- cells) respectively. The subpopulations of rat PDPCs based on CD90 expression were studied here to determine if they have the same in vivo effect despite their distinct morphological differences, gene expression and differentiation capabilities (Stevensen 2009). A second infusion of the same cell therapy for each group was administered to the animals on day 10 after STZ injection. Mice in the
control group were injected with conditioned culture medium into the tail vein at the same time points as the treated animal groups.

All treated animals had gradual reduction in blood glucose after receiving intravenous cellular therapy while blood glucose levels continued to rise in animals of the control group. Prior to reaching 12 days after STZ induction, these control animals had to be sacrificed as glucose measurements reached above 30mmol/L and they became profoundly diabetic. On day 9 post STZ injection, all treated animals had significantly lower blood glucose than the control group. The results of the experiment are shown in figures 14 and 15. Student’s t-test p-value was <0.005 for all treated groups compared to the control group as shown in table 4. Cell-treated animals became euglycaemic with time. They remained diabetes-free until the end of the experiment at 90 days when all the animals were scarified in keeping with the home-office licensing conditions.

Both unsorted rat and human PDPC treated groups demonstrated reduction of blood glucose to eventually normal levels similar to pre-STZ treatment. However, this effect may have been less pronounced in the earlier stage of the experiment in the mixed rat PDPC compared to unsorted human PDPC, CD90+ and CD90- PDPC treated animals as shown in figure 15. By day 39, treated animals all had similar measurements and maintained this effect until the end of the experiment. The explanation for this is unclear but maybe related to the small number of animals or the differences in cellular sub-fractions or batch variation.
Figure 14: Both unsorted human and rat PDPCs are equally efficacious in successfully reversing hyperglycaemia in STZ induced diabetic animals. Mean blood glucose levels in millimoles/L (mmol/L) ± SD are shown versus time in days following STZ treatment (day 0) and post PDPCs infusion (day 3 and day 10).

Figure 15: Equivalent efficacy of CD90+ and CD90- rat PDPCs in reversing the diabetic phenotype of STZ-treated mice. Mean blood glucose levels in mmol/L ± SD are shown versus time in days following STZ administration (day 0) and post PDPCs treatment (day 3 and day 10).
<table>
<thead>
<tr>
<th>Control</th>
<th>36.6 ± 5.0</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted rat PDPCs</td>
<td>22.6 ± 3.5</td>
<td>0.00187</td>
</tr>
<tr>
<td>CD90+ rat PDPCs</td>
<td>16.3 ± 3.2</td>
<td>0.00023</td>
</tr>
<tr>
<td>CD90- rat PDPCs</td>
<td>15.7 ± 2.9</td>
<td>0.00018</td>
</tr>
<tr>
<td>Unsorted human PDPCs</td>
<td>18.5 ± 1.5</td>
<td>0.00022</td>
</tr>
</tbody>
</table>

Table 4: Mean glucose level for each animal group in mmol/L ± SD on day 9 and Student’s t-test results for blood glucose measurements of each of the treated groups compared to the control group.

A second important observation and consistent with the normalisation of blood glucose was that all PDPC-treated animals recovered from an initial loss of body mass and then reached a body weight consistent with their age. The appropriate animal weight was maintained until the end of the experiment as shown in figure 16. This contrasted with the ongoing body weight loss throughout the experiment in the control group with their profound hyperglycaemia until they were sacrificed.

Figure 16: Gain of body weight following the infusion of PDPCs into STZ-treated diabetic mice. Mean body weight in grams ± SD are shown versus time in days following STZ administration (day 0) and post treatment with PDPCs (day 3 and day 10).
In summary, xenotransplanted rat and human pancreas-derived pathfinder cells delivered intravenously completely reversed the diabetic phenotype of STZ-treated mice. All treated animals had normalised glucose measurements irrespective of which cellular therapy option they received. So unsorted rat, unsorted human, CD90+ and CD90- all resulted in the same outcome of normoglycaemia and reversal of weight loss pattern. These data indicate significant insulin secretion has been achieved in all treated animals and in turn would be consistent with ongoing pancreatic tissue regeneration.

3.1.2  *In vivo* examination of whether day 7 human adherent CD34+ Omnicytes treatment normalises blood glucose and reverses the diabetic phenotype in a mouse model

Preliminary work by Dr Daxin Chen was performed in our laboratory prior to starting this study. He used the same method described in 2.2.3 to induce diabetes in 3 groups of animals with STZ. 3 days after STZ administration, one animal group was injected with day 7 human adherent CD34+ Omnicytes (Omnicytes) directly into the pancreas of each animal, one group was injected with a similar number of these cells intravenously into the tail vein of the animal and the third group acted as a control group. All animals received cyclosporine injections as described in 2.2.5. Glucose measurements in all groups were monitored for 33 days.

As demonstrated in figure 17 below, the cellular therapy whether administered into the pancreas or intravenously resulted in stabilisation of the glucose levels while measured glucose levels continued to increase in the control animals. They became progressively and increasingly hyperglycaemic until the end of the experiment (Data
not published but copied with permission from Anthony Dorling and Daxin Chen). At the end of the experiment follow-up time of 35 days, Omnicyte-treated and control animals were sacrificed.

Figure 17: Equal efficacy of intravenous and intra-pancreatic day-7 adherent CD34+ Omnicytes treatment of STZ induced diabetic animals in successfully stabilising glucose measurements compared to control animals. Mean blood glucose levels in mmol/L are shown versus time in days following STZ treatment (day 0) and post cell infusion (day 3). (Unpublished data; permission from A Dorling).

Although the diabetic phenotype was not completely reversed in the treated animals (i.e. normoglycaemia was not induced), the treatment did halt the progression to overt hyperglycaemia. In addition, there was no significant difference on the effect of these cells on the animals between the two cell-delivery modalities. Hence, further experiments were carried out using the simpler approach of intravenous administration of the cellular therapy into the tail of the animals.

To confirm whether treatment with day 7 human adherent CD34+ Omnicytes has the ability to reverse diabetes and to assess whether the earlier findings of Dr Chen can
be replicated, 10 animals were made diabetic by the administration of STZ that resulted in progressive hyperglycaemia with blood glucose levels above 20 mmol/L by day 3 in all mice. On day 3 post STZ, 5 animals were infused with Omnicytes as described in 2.2.2 and 5 animals were infused with equal volumes of conditioned culture medium at the same time and acted as the control group. Both treated and control animals had increasing glucose levels with time at a similar rate. All animals had to be sacrificed between 9 and 12 days post STZ injection due to significant hyperglycaemia and/or weight loss. The results of this experiment are shown in figure 18. Both groups of animals had significant body mass loss by day 6 as demonstrated in figure 19. The observed mean weight of animals in both groups appears to have increased between day 6 and the end of the experiment on day 12. This can be explained by the sacrifice of animals that had ≥20% body weight loss in keeping with the home-office licensing conditions during that period leaving only animals with higher body mass.

**Figure 18:** Day-7 human CD34+ Omnicyte treatment of STZ induced diabetic animals did not reverse hyperglycaemia. Mean blood glucose levels in mmol/L ± SD are shown versus time in days following STZ treatment (day 0) and post cell infusion (day 3)
Although I repeated this experiment several times, I was unable to show that Omnicyte treatment resulted in normalising glucose levels in this diabetic animal mode. Additionally, I was unable to reproduce similar results to those demonstrated by Dr Chan where therapy with these cells led to glucose level stabilisation and partial mitigation of STZ diabetic effect. A possible explanation to this lack of reproducibility maybe related to the variability of the cells and the batches.

In summary, although day 7 human adherent CD34+ Omnicytes under certain culture conditions *in vitro* are able to differentiate into insulin-secreting cells (Gordon 2006), the intravenous administration of these cells did not reverse hyperglycaemia in STZ-induced diabetic animal model. At best, this cellular therapy stabilised glucose levels *in vivo* on one occasion. This effect was not consistent or easily reproducible *in vivo*.
3.1.3 Conclusion of in vivo efficacy of ‘islet equivalents’

The results of in vivo work using PDPCs as cellular therapy demonstrated the complete reversal of the diabetic phenotype of STZ-treated mice. The outcome was similar and sustained for CD90 sorted and unsorted rat PDPCs as well as human PDPCs. Data from this experiment indicate significant insulin secretion has been achieved in all treated animals and would be consistent with pancreatic tissue regeneration by all progenitor cell types.

The Glasgow group took this work further forward in order to identify the mechanism by which PDPCs reversed diabetes in this animal model. Organs and tissues from my experimental mice were sent to Glasgow as described in 2.2.6. Members of Paul Shiel’s group in Glasgow performed further analysis on this tissue. They reported 3 salient findings. Firstly, Immunohistochemical examination was supportive of the hypothesis that observed normoglycaemia in PDPC-treated animals was the result of sufficient insulin production caused by pancreatic beta cells regeneration in these animals. They demonstrated that therapy with rat or human PDPCs was associated with regeneration of beta cell islets that expressed insulin, glucagon and C-peptide in a pattern similar to normal mice at 90 days post therapy (Stevenson 2011). Furthermore, using fluorescence in situ hybridization (FISH), the same group showed that the majority of the new islet tissue was mouse in origin. Only 0.05-0.018% of the signal detected was rat or human in origin. Xeno- signals from human or rat PDPCs were found to be scattered throughout the pancreatic sections indicating that these cells were not forming islet-like structures in vivo in comparison to the in vitro findings (Stevenson 2009). However, they remained unclear if these xeno-signals came from surviving original infused cells or their progeny. Finally, RT-PCR analysis of pancreatic tissue in treated animals detected insulin gene (Ins II) transcripts that
were both rat and mouse in origin. The majority of the transcripts were mouse-derived. The number of rat PDPCs isolated in the pancreatic tissue is sufficient to explain the small proportion of observed rat Ins II mRNA. Significantly, Ins I gene transcript that is normally only produced during embryonic development in the mouse, was also detected in treated animals by RT-PCR studies (Stevenson 2011).

The conclusion of this work was that the presence of small number of rat and human PDPCs in the tissue of treated animals as well as rat gene Ins II transcript indicated that these progenitor cells do home towards damaged tissue and differentiate into insulin-secreting cells without forming islet-like clusters themselves. However, their small number can only have minor contribution to the total insulin made by the regenerative pancreas and so could not account for the normoglycaemic recovery observed in these animals. The presence of Ins I gene transcript in regenerated pancreas was indicative of endogenous repair processes taking place and involved developmental reprogramming within the pancreas. (Stevenson 2011).

Based on these findings, the Glasgow group went on to propose that these adult progenitor cells work by stimulating the process of repair in the damaged pancreas of the animal probably by activating other host cell types to form new islets. However, the exact mechanism of this activation and the nature of host cells involved remain unclear. This suggestions would also explain similar *in vivo* efficacy of both CD90+ and CD90- rat PDPCs despite their differences in differentiation capability and morphology *in vitro*. As it was not possible to determine if the injected PDPCs had undergone division or not, it is hence unclear whether this regenerative effect was due to a subpopulation progeny common to both CD90+ and CD90- cells or applies to all parent PDPCs. This proposal is consistent with other published data demonstrating initiation of pancreatic endogenous tissue regeneration *in vivo* by bone - marrow derived mouse stem cells (Hasegawa, 2007, Hess, 2003, Lee 2006). These host cells are incapable of independent regeneration under these
experimental conditions and require the presence of PDPC to be stimulated directly or indirectly by paracrine effect for repair. Possible candidates for cell types include functionally analogous murine PDPC, islet-derived adult stem cells, Embryonic Stem-like cells surviving from embryonic development, activated NGN-3+ve facultative progenitors and other endocrine or exocrine cells of the pancreas (Zhou 2008, Stevenson 2011).

The in vivo work using the second source of stem cells, Omnicytes, had a different outcome in this diabetic mouse model. The intravenous administration of these cells did not reverse hyperglycaemia but did stabilise glucose levels in one experiment, an effect that was not replicated. Pancreata from Omnicyte-transplanted and control mice were removed respectively and were delivered to the Habib’s group at Imperial College for further tissue analysis. However, this investigation remains incomplete and therefore it is not possible to comment on the engraftment location of the infused cells in treated animals or on the presence or absence of any differences in the pancreatic tissue and in turn islet architecture between treated and control animals. It is also difficult to speculate on the mechanism by which Omnicytes led to glucose stabilisation in the treated mice in one of the experiments.

Several potential explanations can be proposed for the inconsistent effect of Omnicytes in this diabetic animal model. Firstly, these cells came from consented individuals who underwent bone marrow mobilisation and apheresis either as normal volunteers or in most cases due to underlying different haematological disorders or malignancies. So although all of the cells were isolated and cultured in a similar manner in vitro, the cell batches were very heterogeneous in origin. This may have resulted in having a slightly different end product in each of the batches used in these experiments.
Secondly, Gordon et al. did report that the yield of *in vitro* differentiation of day-7 Omnicyte into insulin-secreting cells was very variable and at best was 40% of total cells (unpublished data). In my experiments, day-7 human adherent CD34+ Omnicytes rather than insulin-secreting differentiated progeny were used to treat this diabetic mouse model. So it can proposed that *in vivo* these cells failed to differentiate into insulin-secreting cells or that the number of differentiated insulin-secreting cells was insufficient to have an effect directly or indirectly on the damaged pancreatic tissue of the animals in most of the experiments. The apparent dissimilarity between *in vitro* and *in vivo* behaviour of these cells could be due to the incorrect conditions of the engraftment niche for differentiation into insulin-producing cells.

Thirdly, similar to islet allo and xenotransplantation, it is possible that the intravenous delivery of these cells resulted in a significant early cell loss either due to thrombosis, as suggested in the hypotheses of this study, or due to acute rejection. It is known from earlier characterisation studies that Omnicytes do express HLA antigens (Gordon 2006) making them immunogenic and hence susceptible to alloimmune injury. Cyclosporine was used as an immunosuppressive therapy and was administered regularly in the experiments but may have not protected these cells sufficiently to avoid loss caused by rejection. All these 3 potential explanations are plausible and maybe all contributing to the observed variable outcome of these experiments.
3.2 Hypotheses II and III

II) The insulin secreting cells express tissue factor on their cell surface. Following transplantation, this TF expression can initiate the coagulation cascade and cause thrombosis leading to early cell loss following treatment of diabetic mice with these cells.

III) Treatment of ‘islet equivalent’ cells with anti-thrombotic cytotoxic agent, based on hirulog peptide, prior to transplantation reverses this coagulation cascade partially or completely in vitro. This potentially can be used in vivo as a mechanism to improve stem cell survival in the diabetic mouse model and reduces the number of cells required to achieve normoglycaemia.

3.2.1 Assessment of coagulation and tissue factor expression

In order to test the second hypothesis, I assessed the physical and functional expression of TF on the cell surface of “islet equivalents” using plasma recalcification assays and immunocytochemistry studies respectively.

To test the third hypothesis, PTL006 was used as the cytotoxic agent in this study. It is a hirulog analogue peptide and targets thrombin by binding irreversibly to it. Thrombin is a logical and a good target because of its multiple roles in coagulation. It converts fibrinogen to fibrin, the final step in the coagulation cascade; it amplifies its own generation by feedback activation of FV and FVIII, key co-factors for the prothrombinase and intrinsic tenase complex respectively; and it coordinates platelet activation and aggregation with coagulation. The inhibition of thrombin not only blocks fibrin formation but also attenuates thrombin generation and platelet activation.
as described in section 1.10. Hence inhibiting thrombin would result in reversing thrombosis whether it is due to TF-dependent or TF-independent mechanism. Another advantage to using this agent in this setting is that PTL006 has already been shown to retain potent anti-thrombin activity when bound to cell membranes and can inhibit fibrin clot formation in recalcified plasma assay similar to the assay used in these experiments (Dorling 2013; Smith 2012).

3.2.2 Assessment of contact activation clotting time for human and rodent plasma

It is worth mentioning that in assessing coagulation in rodent stem cells and to measure rodent TF activity with plasma recalcification assays, a mixture of 10% mouse plasma and 90% human plasma was used in the study. We know that rodent TF binds poorly to human factor VII so the 10% mouse plasma provides sufficient rodent factor VII (Morrissey Lab). In addition, in the absence of any cells, using 100% mouse plasma in this recalcification assay to measure the spontaneous contact activation clotting time results in less than half of the clotting time observed with 100% human plasma. This makes it difficult to detect any small significant changes in the time measurements especially in assays that result in shortening of the clotting time.

In a titration assay that I performed, the mean spontaneous contact activation clotting time for 100% mouse plasma (MP) was 62.7 seconds compared to 153.1 seconds for 100% human plasma (HP) as shown in figure 20. Mixing 10% mouse plasma and 90% human plasma resulted in a clotting time of 112.3 seconds. These results are shown in figure 20. This mix provides enough rodent FVII for TF binding and a clotting time that is long enough to detect any significant changes with the additions of cells or agents. Hence this was the composition of the plasma used in all the
following plasma recalcifications assays applied to rodent cells.

Figure 20: Titration assay to assess the contact activation clotting time of human vs. mouse plasma

3.2.3 Tissue factor positive melanoma rat cell line as control cells for plasma recalcification assay

I used TF positive (TF+) melanoma rat cells as control cells to confirm that both the triplicate plasma recalcification assay and the anti mouse/rat TF antibody are working appropriately and successfully. The plasma recalcification method used to assess clotting time (CT) in this experiment has been described in section 2.7 and the results are demonstrated in table 5 and figure 21. The data show significant reduction in CT in the presence of cells that express TF on their cell surface (p≤0.05). Pre-treatment of these cells with an inhibitory rabbit anti-mouse TF (mTF) antibody (Ab) partially corrected that effect when compared to treatment with an isotype control antibody but did not reach statistical significant (p=0.08). However,
the CT significantly increased with pre-conditioning these TF+ rat cells with either higher concentration of mTF antibody at 167μg/ml or the cytotoxic agent PTL006 that binds to thrombin irreversibly (p≤0.05). Finally, the addition of cells into a solution containing PTL006, which having lost the tethering tail is essentially soluble hirulog, resulted in complete reversibility of the procoagulant effect of these cells. The CT was even greater than the spontaneous contact activation CT found for plasma in glass tube (p≤0.05). This perhaps reflects the undesirable systemic over anticoagulation observed when non-localised anti-thrombotic agents are applied.

<table>
<thead>
<tr>
<th></th>
<th>Mean clotting time (seconds ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cells</td>
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</tr>
<tr>
<td>Cells alone</td>
<td>50.0 ± 0.6</td>
</tr>
<tr>
<td>Cells + rabbit IgG (isotype control)</td>
<td>55.0 ± 3.1</td>
</tr>
<tr>
<td>Cells + rabbit anti mouse TF (16.7μg/L)</td>
<td>63.1 ± 5.5</td>
</tr>
<tr>
<td>Cells + rabbit anti mouse TF (167μg/L)</td>
<td>75.0 ± 2.6</td>
</tr>
<tr>
<td>Cells + PTL006 on cell surface</td>
<td>69.0 ± 3.4</td>
</tr>
<tr>
<td>Cells + PTL006 in solution</td>
<td>190.6 ± 7.3</td>
</tr>
</tbody>
</table>

Table 5: Clotting time in seconds ± standard error (SE) for in vitro plasma recalcification assays with no cells, with TF+ melanoma rat cells alone, with the addition of a isotype control antibody, rabbit anti mouse TF antibody or PTL006 on the cell surface or in solution.
Figure 21: Plasma recalcification assay revealing *in vitro* prothrombotic effect of TF+ melanoma rat cells and the significant reversal of this property by pre-conditioning the cells with high concentration rabbit anti mTF, PTL006 tethered to the cell surface or in solution. The CT is on the y-axis is in seconds ± SE

In summary, this experiment with TF+ control cells confirmed that the plasma recalcification assay is working appropriately and that a proportion of the clot formation within this assay was TF dependent. Although I did not confirm that the maximum concentration of anti-TF Ab used was fully saturating the TF on the cell surface of the melanoma cells, 167μl/L was the concentration of the same anti mTF Ab used for examining the coagulant properties of rat PDPCs. This experiment also demonstrated the anticoagulant effect of the cytotoxic peptide PTL006.

It is important to mention at this stage that there were no TF+ human cells available to be used as control cells for confirmation that anti human TF antibody are working appropriately and successfully.
3.2.4 *In vitro* examination of the coagulation properties of PDPCs and whether this property can be reversed with anti-thrombin cytotopic peptide.

Having established that transplanted rat and human PDPCs successfully reverse the diabetic phenotype of STZ-treated animals, the next step was to assess if these cells lead to thrombosis *in vitro* as a potential mechanism for *in vivo* early cell loss post infusion.

To examine if rat PDPCs resulted in coagulation, triplicate plasma recalcification assays were preformed as described in 2.7. The results of these experiments are shown in table 6 and figure 22. The data shows that *in vitro* CT was reduced significantly from 127 seconds to almost 40 seconds in the presence of PDPCs (p≤0.05). The CT for pre-conditioned cells with 167μl/L rabbit anti-mouse TF antibody was not significantly different to that of cells treated with isotype control antibody (p=0.24). However, the CT did significantly rise in the presence of pre-conditioned PDPCs with PTL006 or in the presence of these cells in solution with PTL006. It increased from 39.7 seconds to 57.3 and 174 seconds respectively (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>Mean clotting time (seconds ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td>Cells + rabbit anti mouse TF</td>
<td>39.7 ± 2.0</td>
</tr>
<tr>
<td>Cells + PTL006 on cell surface</td>
<td>57.3 ± 1.5</td>
</tr>
<tr>
<td>Cells + PTL006 in solution</td>
<td>174 ± 4.1</td>
</tr>
</tbody>
</table>

*Table 6: Clotting time in seconds ± SE for in vitro plasma recalcification assays of PDPCs alone, with the addition of an isotype control antibody, rabbit anti mouse TF antibody, PTL006 tethered to the cell surface or cells with PTL006 in solution respectively*
These results indicate that the PDPCs have a prothrombotic property that was not altered by inhibiting tissue factor with the highest concentration of rabbit anti-mouse TF antibody. Despite this apparent lack of TF involvement, the prothrombotic effect of these cells was partially mitigated by the tethered form of cytotoxic peptide PTL006 and was reversed completely when this agent was added into the solution in which PDPCs were suspended. Similar to the findings of TF+ control cells experiment described in 3.2.3, CT for PDPCs in the presence of the soluble form of PTL006 exceeded that of the intrinsic contact activation CT of plasma alone. These results were reproducible on two other sets of rat PDPCs. Due to limited stem cell number, I did not have access to sufficient human PDPCs that would allow
examination of these cells to elucidate whether they exhibit the same effect as rat PDPCs on coagulation or not.

In summary, these findings are consistent with the hypothesis that PDPCs are procoagulant in vitro and they suggest that the prothrombotic phenotype is possibly TF-independent. There are several possible explanations to the observations found in this experiment and will be discussed in 3.2.7.

3.2.5 *In vitro* examination of the coagulation properties of day 7 human adherent CD34+ Omnicytes and whether this property can be reversed with anti-thrombin cytotopic peptide.

To examine if Omnicytes resulted in thrombosis, triplicate plasma recalcification assays were preformed as described in 2.7. To assess reproducibility of the results, I repeated the same experiment with several different batches of day-7 human CD34+ Omnicytes on the same day and on separate days. Two important findings were consistent and reproducible in all these experiments. Firstly, similar to rat PDPCs, the data confirmed that day-7 adherent CD34+ Omnicytes were prothrombotic *in vitro*. They have a procoagulant property demonstrated by shortening of the CT when these cells are added to human plasma. Secondly, thrombin inhibition by the application of PTL006 locally on the cell surface or systemically in the solution resulted in the reversibility of coagulation caused by these cells. In all the experiments, pre-conditioning Omnicytes with anti-thrombin cytotopic PTL006 did significantly increase the CT back to spontaneous contact activation CT when compared to untreated cells. On the other hand, the addition of PTL006 into the solution in which the cells were suspended led to CT elevation beyond the observed baseline CT with plasma alone.
The next question of whether coagulation caused by Omnicytes was TF-dependent or TF-independent was more difficult to answer due to inconsistent results. There was variability in whether TF is functionally expressed on these cells or not. Only 50% of the cell batches demonstrated significant partial reversibility of the coagulant effect by pre-treatment of the cells with sheep anti-human TF (hTF) antibody. Binding of the inhibitory sheep anti-hTF antibody to TF led to significantly longer CT compared to cells treated with isotype control antibody (p≤0.05). The results of one of these experiments are shown in table 8 and figure 21. These findings are suggestive of TF playing a pivotal role in thrombosis caused by these cells since inhibiting it partially mitigated the procoagulant effect. In contrast, the other 50% of the cell batches revealed no significant difference in the CT whether the cells were pre-conditioned with sheep anti-hTF antibody or isotype control antibody (p≥0.05). The results of one of these experiments are shown in table 7 and figure 23. This would indicate that inhibiting TF did not interfere with coagulation caused by these cells and in turn suggests that clot formation within the assay was TF-independent.

<table>
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<tr>
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<td>Cells + sheep IgG (isotype control)</td>
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<td>Cells + sheep anti human TF</td>
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</tr>
<tr>
<td>Cells + PTL006 on cell surface</td>
<td>182.3 ± 12.0</td>
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<tr>
<td>Cells + PTL006 in solution</td>
<td>293.3 ± 29.4</td>
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</table>

Table 7: Clotting time in seconds ± SE for in vitro plasma recalcification assays of Omnicytes alone, Omnicytes with the addition of a isotype control antibody or sheep anti-human TF antibody, pre-conditioning the cells with PTL006 or the addition of PTL006 to the solution in which Omnicytes were suspended respectively
Figure 23: Clotting time in seconds ± SE of in vitro plasma recalcification assays for CD34+ Omnicytes. It reveals the procoagulant effects of these cells and reversibility of this effect by pre-treatment of the cells with sheep anti-hTF Ab, tethered PTL006 or in the presence of PTL006 in solution respectively.

<table>
<thead>
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<th>Condition</th>
<th>Mean clotting time (seconds ± SE)</th>
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</thead>
<tbody>
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<td>109.7 ± 3.5</td>
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<td>Cells + sheep IgG (isotype control)</td>
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<tr>
<td>Cells + sheep anti human TF</td>
<td>128.7 ± 7.4</td>
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<tr>
<td>No cells + FVII deficient plasma</td>
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<td>Cells + FVII deficient plasma</td>
<td>142.3 ± 1.5</td>
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<tr>
<td>Cells + PTL006 on cell surface</td>
<td>172.0 ± 3.0</td>
</tr>
</tbody>
</table>

Table 8: Clotting time in seconds ± SE for in vitro plasma recalcification assays with no cells and in the presence of Omnicytes in normal plasma and in Factor VII (FVII) deficient plasma respectively. It also demonstrated the CT for pre-treated Omnicytes with a control antibody, sheep anti human TF (hTF) antibody or PTL006.
Figure 24: Plasma recalcification assays revealing greater reduction of the CT in the presence of Omnicytes in normal plasma compared to Factor VII (FVII) deficient plasma. CT was unchanged with cells pre-treated with sheep anti human TF (hTF) antibody. The procoagulant effect was reversed when cells were pre-treated with PTL006.

However, it is important to highlight that on this occasion the plasma recalcification assay was performed on Omnicytes using normal plasma and factor VII deficient plasma respectively. As described earlier, the initiation phase of coagulation by the extrinsic system involves binding of TF to a small amount of circulating factor FVIIa (section 1.10.1). Using FVII deficient plasma would be expected to inhibit TF-dependent coagulation from taking place and was used as another test for examining TF activity. As demonstrated in table 8 and figure 24, the addition of cells to normal plasma reduced the CT from 183 seconds to 109 seconds, which is a 40% reduction. Pre-treatment with anti-hTF antibody did not increase the CT. On repeating the
assay using FVII-deficient plasma, the addition of the cells did cause a statistically significant reduction in CT but the percentage change was very small. The CT decreased from 162 seconds to 142 seconds, which is only a 12% reduction. In this experiment, although inhibiting the TF pathway did not prevent the cells from causing significant clotting, it did result in a much smaller percentage reduction in the CT. This suggests that the absence of FVII did impair the clotting induced by the cells i.e. that TF is contributing little to the clotting here which is not consistent with the result of the inhibitory anti-TF antibody test. One possible explanation could be that the inhibitory anti-hTF antibody was either non-functional or used in too small concentration for saturation of TF expressed on surface of these Omnicytes in this experiment. However, they do suggest that for the 50% of omnicytes that presented this pattern in the clotting assays, TF contributed little to the prothrombotic tendency.

In summary, these findings are consistent with the hypothesis that Omnicytes are prothrombotic in vitro and that this effect is reversed with the application of PTL006. It is however unclear if this prothrombotic phenotype is TF-dependent, TF-independent or both. This has several potential explanations which will be discussed in 3.2.7 and certainly needs further investigations.

3.2.6 Do “islet-equivalents” express tissue factor?

Immunocytochemistry is a very well established method for detecting the presence of proteins or antigens on the cell surface. The key in this assessment is the primary antibody used to detect the protein, which is TF in this study. Hence, rabbit anti-mouse TF and sheep anti-human TF antibody were selected for the examination of rat PDPCs and human Omnicytes respectively.
TF+ melanoma rat cells, from the same cell line used in 3.2.3, were tested as positive control cells for immunocytochemistry analysis. Rabbit anti-mouse TF primary antibody and goat anti-rabbit antibody IgG - FITC fluorescein conjugated secondary antibody were used in immunocytochemistry study as described in section 2.5. Once the cells were dry and fixed, mounting medium with DAPI was added and then the slides were viewed under a fluorescence deconvolution microscope. Figure 25 reveals the result of the analysis and demonstrates the absence of the expected FITC green fluorescein since these rat cells are known to express TF on their cell surface. I repeated this experiment on another occasion with the same cell line but obtained a similar negative outcome.

Figure 25: Immunocytochemistry of TF+ melanoma rat cells. There is no evidence of the expected green fluorescein conjugated to the secondary antibody since these rat cells were known to express TF.
These unexpected results could be explained by a problem in the cells themselves or in the experimental technique either due to human error in applying the method or due to non-functionality/low concentration of the antibody used. The same rat cells were positive for TF in the functional assays described in 3.2.3 indicating that they were not the source of the negative outcome. Additionally, the primary rabbit anti-mouse TF antibody used here successfully inhibited TF appropriately in the functional assays in 3.2.3 and 3.2.5 and so this would go against it being the limiting step for the expected positive outcome. It is hence more likely that human error in the experimental technique have led to this negative result. Due to time limitation, I did not explore this likely occurrence. Ideally, I should have performed further analysis to identify the limiting step in the experimental method and in turn correct it to get the appropriate expected outcome prior to testing other cells.

Despite unsuccessful outcome with the positive control rat cells and due to time constraint, I proceeded to perform further immunocytochemistry analysis to examine if TF expression on cell surface of rat CD90+ PDPCs and day-7 human adherent CD34+ Omnicytes is the possible trigger for initiating coagulation caused by these cells. In immunocytochemistry experiment of rat PDPCs, the same primary and secondary antibodies as in the study of rat TF+ melanoma cells described earlier were used. For immunohistochemistry assessment of Omnicytes, sheep anti-human TF primary antibody and donkey anti-sheep IgG - FITC fluorescein conjugated secondary antibody were used and applied as described in section 2.5.

The results of these experiments demonstrated no apparent evidence for physical expression TF on the cell surface of neither rat PDPCs nor Omnicytes. Figures 26, 27 and 28 show the lack of the green fluorochrome that would be expected to be observed in the presence of TF on the surface of these cells. The same negative
results were obtained on repeating these immunocytochemistry studies on a different set of both rat PDPCs and Omnicytes at a different time point. In view of limited stem cell source, these analysis were not performed on rat CD90- PDPCs and human PDPCs.

Figure 26: Immunocytochemistry analysis of rat PDPCs cells. There is no evidence of the green fluorescein conjugated secondary antibody possibly suggesting possible absence of TF on the cell surface these cells
Figure 27: Higher power of immunocytochemistry analysis of rat PDPCs cells

Figure 28: Immunocytochemistry of day-7 human CD34+ Omniceyes. There is no evidence of the green fluorescein conjugated secondary antibody suggesting possible absence of TF on the cell surface of these cells
It is not possible to make any conclusions from these immunocytochemistry analyses since the same experiment on positive control cells did not give the expected affirmative outcome. Therefore, the absence of green fluorescein in this setting does not necessarily confirm the lack of TF expression on the cell surface of PDPCs and Omnicytes respectively.

3.2.7 Conclusion of in vitro examination of “islet equivalents”

Findings of in vitro assessment of PDPCs did support the hypothesis that these cells are prothrombotic and that this property can be reversed partially or completely by tethered and soluble form of PTL006 respectively. The data also suggested that the prothrombotic phenotype is possibly TF-independent.

However, it is difficult to be certain and conclude that PDPCs do not express TF and that their procoagulant phenotype is TF-independent before investigating this further. One possible explanation for the results obtained is that insufficient TF inhibition occurred to demonstrate a significant effect on coagulation and in turn prolong CT. The highest concentration of rabbit anti-mTF antibody used in the plasma recalcification assays may have not saturated TF expressed on the surface of these PDPCs. Hence, prior to making any conclusion, it is essential to investigate this possibility by titrating increasing concentrations of anti-mTF antibody in the plasma recalcification assays and reassessing the functional expression of TF on PDPCs on each occasion. Additionally and in view of immunohistochemistry technique failure, other experimental methods, such as flow cytometry and RT-PCR, should also be applied to these cells to investigate whether TF is expressed on their surface or not. If these assessments confirm negative expression for TF, only then it can be
concluded that PDPCs do not express TF and can be speculated that they cause coagulation \textit{in vitro} via a TF-independent mechanism. This second explanation is also possible but less likely.

Although in most cases, coagulation \textit{in vivo} is triggered by TF, there is published literature reporting the identification of an inducible prothrombinase that acts in a TF-independent pathway, termed Fgl2/fibroleukin. It has been shown to contribute to immunologically mediated thrombosis in experimental and human viral hepatitis and in acute vascular xenograft and allograft rejection (Ghanekar 2004; Marsden 2003). Levy et al. demonstrated that the pathways by which vascular thromboses are elicited in viral hepatitis are mechanistically distinct from the classical pathways of coagulation induced by trauma or bacterial lipopolysaccharide (LPS). Activated endothelial cells and macrophages express distinct cell-surface procoagulants important for both the initiation and localisation of fibrin deposition in viral-induced liver disease. Fgl2/fibroleukin was initially identified as one of these procoagulants induced by murine hepatitis virus type-3 (MHV-3) infection (Parr 1995). The same group went on to successfully generate an fgl2/fibroleukin-deficient mouse. They did not demonstrate any baseline alterations in standard haematological and coagulation profiles or bleeding times between mice infected with the MHV-3 whether they were fgl2/fibroleukin-deficient or not. They did however show that fibrin deposition and liver necrosis were markedly reduced and that survival was increased in fgl2/fibroleukin-deficient animals. There was failure of induction of fgl2/fibroleukin mRNA expression and lack of procoagulant activity in peritoneal macrophages isolated from these deficient mice. In contrast, the response to LPS, which induces TF/FVII-dependent procoagulant activity in peritoneal macrophages, was unaffected in fgl2/fibroleukin deficient mice compared to gfl2/fibroleukin positive mice (Marsden 2003). In the same paper, they addressed the relevance of fgl2/fibroleukin in human viral hepatitis. They demonstrated the expression of Fgl2 mRNA in endothelial cells
and macrophages of liver from hepatitis patients. They also showed that expression of fg2/fibroleukin mRNA and protein varies markedly in patients with marked chronic hepatitis B versus those with minimal chronic viral hepatitis B. Expression of f2gl/fibrleukin is highly associated with fibrin expression.

In 2001, Levy et al. reported the isolation and functional expression of a distinct human prothrombinase termed Fgl2/fibroleukin. Human Fgl2-encoded protein is a serine protease that bypasses the TF/FVII extrinsic pathway and directly cleaves prothrombin to thrombin. Stimuli known to induce expression of the fgl2/fibroleukin mRNA and protein such as interferon gamma (INFγ) treatment, MHV-3 viral infection and xenotransplantation are distinct from those that elicit TF/FVII-dependent fibrin generation such as LPS. In contrast to FXa, the Fgl2/fibroleukin prothrombinase activity is not inhibited by anti-thrombin III and is not dependent on FVII. The function of constitutively expressed Fgl-2 remains to be conclusively determined and is the subject of ongoing studies (Levy 2000; Yuwaraj 2001).

Grant et al. examined the role of fgl2 in acute vascular rejection (AVR) in xenografts. They reported that porcine kidney xenografts undergoing AVR showed induction of Fgl2 on graft vascular endothelial cells (Ghanekar 2004). They also demonstrated that deletion of Fgl-2 in the donor ameliorates AVR in a mouse-to-rat cardiac xenotransplantation model. Grafts from deficient mice had reduced fibrin deposition but developed cellular rejection. There was up-regulation of Fgl2- mRNA expression while TF mRNA levels declined in association with progression of AVR in donor grafts from wild type mice (Mendicino 2005). They explained that the diverging pattern of total graft mRNA TF and Fgl-2 expression during xenograft rejection suggests that these genes respond to different regulatory stimuli, consistent with in vitro studies that have shown that induction of TF expression on porcine EC by human serum is predominantly mediated by antibody and complement, whereas Fgl-2 expression is not induced by media containing complement or isolectin B4 (a
substitute for anti-alpha-GAL-specific antibody) (Ghanekar 2004). They speculated that Fgl-2 may contribute to the previously described TF-independent coagulation activity of porcine endothelial cells, complete neutralization of which required the addition of hirudin, a thrombin inhibitor (Chen D 1999a).

The same group also examined the role of this prothrombinase in allorejection. In a heterotopic cardiac transplant model, treatment of wild-type mice with neutralizing anti-Fgl2 antibody ameliorated histological evidence for allorejection and intravascular fibrin deposits and resulted in increased graft survival. They examined kidney biopsies from patients who had undergone renal transplantation. Human Fgl-2 mRNA transcripts and proteins expression was in association with fibrin deposition location and correlated with the presence of rejection (Ning 2005).

This prothrombinase Fgl2/fibroleukin could be a potential candidate for the trigger of the clotting effect of rat PDPCs in vitro via a TF-independent pathway by directly cleaving prothrombin and generating thrombin. This would also explain the discordant reversal of this effect by the hirulog-like cytotopic PTL006 as it binds thrombin irreversibly. However, Fgl-2 has been reported in the literature so far to be an inducible prothrombinase and its role in the absence of an inducible stimulus such as viral infection, rejection, or INFγ is not determined. In the in vitro studies, PDPCs were not stimulated with one of the known stimuli for Fgl-2/fibroleukin prior to the plasma recalcification assay but they have been in culture in the presence of added growth factors for a number of weeks. This could have been a trigger for the prothrombinase induction though unlikely.

One can speculate that PDPCs may constitutively express Fgl-2 at low levels sufficient to result in coagulation in vitro but whether this effect is sustained and significant in vivo remains to be determined. This question can be answered firstly by investigating whether fg2l/fibroleukin mRNA and protein are expressed in PDPCs or
not and secondly by performing clotting studies of the animals in the immediate period after intravenous infusion of PDPCs to determine the level and in turn significance of coagulation activity as a result of this cellular therapy. These tests could include the extent of platelet consumption and the levels of fibrinogen and fibrinogen degradation products (FDP) which are indirect measure of thrombin generation. FDP are components of the blood produced by clot degeneration by the action of plasmin on deposited fibrin. The most notable subtype of FDP is D-dimer (section 1.10). If this effect does take place in vivo then PDPCs can potentially be lost through early thrombosis following transplantation into diabetic animals. Treatment of these ‘islet equivalent’ cells with anti-coagulation proteins, such as PTL006, prior to transplantation could reduce the degree of early cell destruction due to thrombosis.

It has been shown that infused PDPCs do survive and reverse diabetic phenotype in STZ treated diabetic mice (section 3.1.2). This indirectly suggests that even if this thrombotic event takes place after transplantation of these cells, it does not lead to complete destruction of all the cells. Human PDPCs come from pancreatic tissue of biopsies or unsuitable transplant organs making the source of the cells limited to a certain extent. In turn, pre-treatment of the cells with anti-thrombin cytotoxic agent such as PTL006 prior to infusion can be used as a way to optimise the number of cells required for reversing diabetes.

Similar to PDPCs, findings of in vitro assessment of Omnicytes are consistent with the hypothesis that these cells are also prothrombotic and that this effect can be reversed with the application of PTL006. It is however unclear if this prothrombotic phenotype is TF-independent, TF-independent or perhaps involving both mechanisms. The other question left unanswered is whether the prothrombotic phenotype and lack of efficacy at reversing diabetes in mice are connected.
The variability in the in vivo results with different batches of Omnicytes reported in 3.1.2 parallels the variability observed in in vitro functional assays and so one possible explanation is the heterogeneity of phenotype between batches rather than inconsistencies with experimental technique. The inter-variability of different cell batches may have been influenced by the origin of the cells, which was diverse, and by the accurate reproducibility of the same end product (3.1.3). We know that Omnicytes have varying capacity to differentiate into insulin-secreting cells in culture, therefore it can be speculated that this cell inter-variability may have led to a varying extent of TF expression on the cell surface and hence the mixed results obtained.

However, there are several findings of some of these experiments that introduces some doubt about the antibody used and suggest that the variable capability and efficacy of the anti-hTF antibody in inhibiting TF could be a more likely plausible explanation for the inconsistent results. One disadvantage and dissimilar to the anti-mTF antibody, the human antibody was not tested on TF positive human control cell line prior to using it to assess Omnicytes in the plasma recalcification assays. This is due to the lack of such a cell line but the control experiment would have been useful in testing the anti-hTF antibody and reassuring if the results were as expected positive. Doubt about the antibody was confirmed using FVII deficient plasma in the clotting assay. It demonstrated that the absence of FVII did impair the clotting induced by the cells suggesting that TF might be involved, although only to a small extent.

On the other hand, when used to assess Omnicytes, the anti-hTF antibody did not always result in a negative outcome. In fact, in 50% of the assays, the results were significantly positive and consistent with the antibody inhibiting TF. This points to functional anti-TF antibody but perhaps on occasions too small antibody concentration used to saturate the TF on the surface of Omnicytes which may well have differing degree of TF expression. Further experiments are needed with higher
concentrations of anti-hTF to determine if this is sufficient to unify and then reproduce the results and in turn support the conclusion that Omnicytes cause clotting via a TF-dependent pathway.

In summary, for now it can be concluded that day-7 human adherent CD34+ Omnicytes have a universal procoagulant property but the universal functional expression of TF in these cells is not confirmed. There is however some evidence to suggest that TF-dependent mechanisms may be involved in thrombosis caused by these cells. Finally, this universal prothrombotic effect of Omnicytes was successfully mitigated by pre-treatment of the cells with anti-thrombin cytotopic agent. This leads to the potential use of this agent as a localised anti-thrombotic therapy to improve cell survival and performance post infusion.

3.2.8 In vivo assessment of the coagulation properties of day 7 human adherent CD34+ Omnicytes

Functional assays described earlier in 3.2.5 confirmed that Omnicytes are prothrombotic in vitro and that this effect can be reversed with the cytotopic agent PTL006. The next step was to assess if these cells cause coagulation in vivo and whether pre-treatment with this agent protects against this process. This was addressed by assessment of indirect evidence for coagulation in animals that have been infused with untreated and pre-treated Omnicytes respectively.

3 groups of mice were made diabetic by STZ administration and on day 3 post STZ, the first group of 3 animals were infused with untreated Omnicytes and the second group received Omnicytes that had been pre-treated with the PTL006 as described in sections 2.2.2 and 2.2.3. The third group was a control group where the animals were injected with equal volumes of conditioned culture medium. Half an hour after
intravenous cell infusion, all animals were sacrificed. Immediate collection of mouse blood was performed via intra-cardiac aspiration. This blood was used to obtain plasma and evaluate platelet consumption as indirect evidence for coagulation following control or cellular therapy. Mean platelet count was calculated as described in section 2.5.2.

The results are shown in table 9 and figure 29. Cellular infusion resulted in 43% reduction in mean platelet count from $1.04 \times 10^6$ to $0.59 \times 10^6$ indicating platelet consumption and in turn clotting but it did not reach statistical significant ($p=0.07$). On the other hand, infusion with Omnicytes pre-treated with anti-thrombin agent PTL006 protected against this effect as demonstrated by preserved mean platelet count in this group of animals. This trend is very suggestive that the cellular treatment is a trigger of a coagulation leading to probable fibrin clot formation, thrombosis and resulting in a thrombocytopenia as platelets are sequestered in the clot. The results also suggest that this effect is preventable by the presence of PTL006 tethered to the surface of the cells infused. *In vivo*, this means that the agent will have a localised anti-thrombotic effect without the unwanted and undesirable side effects of systemic anticoagulation. Further experiments are needed to confirm the reproducibility of these results and to determine if this prothrombotic effect is significant and consistent with different batches of Omnicytes.

<table>
<thead>
<tr>
<th></th>
<th>Mean platelet count $\times 10^6$/μL ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control animals</td>
<td>$1.04 \pm 0.29$</td>
</tr>
<tr>
<td>Animals injected with CD34+ Omnicytes</td>
<td>$0.59 \pm 0.14$</td>
</tr>
<tr>
<td>Animals injected with pre-treated CD34+ Omnicytes with PTL006</td>
<td>$1.40 \pm 0.15$</td>
</tr>
</tbody>
</table>

*Table 9: Mean platelet count $\times 10^6$/μL ± SE in control animals, animals treated with day-7 human adherent CD34+ Omnicytes and the same cells pre-treated with anti-thrombin PTL006*
Figure 29: Mean platelet count \( \times 10^6/\mu L \pm SD \) in control animals, animals treated with day-7 human adherent CD34+ Omnicytes and the same cells pre-treated with anti-thrombin hirulug-like PTL006 respectively. It shows platelet count reduction with cell infusion while this count is preserved when pre-treated cells were infused.

I was unable to repeat this experiment to confirm my initial findings due to limited supply of Omnicytes. Additionally, due to restricted source of rat and human PDPCs, I did not get the opportunity to perform these experiments using PDPCs to assess in vivo procoagulant properties of these cells. Other assays that could also be used to detect coagulation activity are indirect measurements of in vivo thrombin generation in the plasma collected after stem cell infusion such as the levels of fibrin degradation products including d-dimers and the levels of activation peptides such as prothrombin fragment.
3.3 Phase I Safety and tolerability study following the infusion of autologous expanded progeny of an adult CD34+ stem cell subset (Insulincytes) to patients with type I diabetes Mellitus and a successful renal transplant

Professor Charles Pusey was the Principal investigator of the trial. There were 5 co-investigators: Prof Vassilios Papalois, Prof David Taube, Prof Jane Apperley, Dr Paul Tait and Prof Nagy Habib. The clinical trial monitor was Niekol Rombaut. I joined the team of the trial after the protocol has been established and the initial ethics approval was pending. Once part of the team, I was involved in every process related to the study from then onwards. This included several amendments of the protocol, applying for MHRA and ethics approvals for these amendments, consenting, screening and enrolling patients into the trial. Once a patient was recruited into the study, I was responsible for organising the various steps of the protocol by collaborating with the different departments (The haematology department, Imperial College Stem Cell Laboratory and John Goldman Centre for cellular therapy stem cell lab and the radiology department), assessing the patient at follow up visits and completing the Case Report Forms (CRF). I also worked closely with the study monitor at every stage to ensure all the necessary steps have been taken for a safe and successful clinical study.

Details for the formal registration of the trial, MHRA approval and ethics approval were described earlier in section 2.6.1.

3.3.1 Rationale to the study

As reported earlier in section 2.1.2, the Habib’s group at Imperial College isolated a subpopulation of CD34+ cells as a separate putative stem cell source that they
termed "Omnicytes". These isolated CD34+ Omnicytes are able to differentiate into multiple tissue types ex vivo including hepatocytes (Gordon 2006). In defined conditions in culture, up to 40% of the cells produce insulin and these Insulincytes have the ability to stabilise blood glucose levels in STZ-induced mice (section 3.1.2).

The same group at Imperial College had already performed a phase I trial of stem cell administration to patients with liver insufficiency. Undifferentiated progeny of adherent CD34+ stem cell subset were injected into the portal vein or hepatic artery of five patients with liver disease. Each patient received up to 2x 10^9 of these cells. The procedure was well tolerated with no adverse events. There was no evidence of clinical benefit but moderate improvements in biochemical parameters were seen. 2 patients had lower bilirubin levels, 1 patient had increased albumin level and 1 patient had improved liver enzymes. None of the patients in the study experienced worsening of their liver function (Gordon 2006).

Encouraged by the results of the in vitro and the limited in vivo Omnicyte experiments as well as the liver phase I trial, this study was set up to assess the safety and tolerability of the infusion of autologous expanded progeny of an adult CD34+ stem cell subset (Insulincytes) to patients with type I diabetes mellitus and a successful renal transplant. These cells had several advantages; they were haematopoietic in origin so easy to mobilize from bone marrow by G-CSF and to harvest peripherally by leukapheresis as explained in sections 2.6.2 and 2.6.3 respectively. As they were adult stem cells, the ethics of embryonic stem cell therapy and the increased risk of malignant transformation were avoided. Finally, they were autologous in origin and hence escaped the need for enhanced immunosuppression. Patients included in the trial were diabetic patients who had received a successful kidney transplant and hence already on long-term immunosuppression. This had the additional benefit of minimising the chances of damage to the newly infused insulin-
secreting cells by recurrence of the original destructive autoimmune process that resulted in developing diabetes in these patients.

3.3.2 Objectives

The primary end point of the study was to assess the safety and tolerability of expanded adult CD34+ stem cell infusion into the pancreas of a diabetic renal transplant recipient.

The secondary end point was to assess the impact of this cellular therapy on the endocrine pancreatic function as measured by serial biochemical analysis and insulin requirements.

3.3.3 Patient population

The study was to include a total of maximum 10 patients who were type I diabetics with a functioning kidney transplant. These patients were to be under the care of Imperial College Kidney and Transplant Centre and were followed up at either St. Mary's or Hammersmith transplant clinics. The study population was subsequently amended, with ethics approval, to include type II diabetic renal transplant recipients who were on insulin, had significant diabetic complications and were active on the pancreas transplant waiting list. Participation in the study did not preclude patients who were on the pancreas transplant waiting list from receiving a transplant and did not affect their position on the waiting list.
3.3.4 Consent and screening criteria

Patients from the diabetic and transplant clinics who complied with the full inclusion criteria, as shown in table 10, were invited to enter the study and were asked to give their written consent. With informed consent, these patients were then screened for suitability for trial enrolment. The screening process for these patients included full medical history and examination, a series of blood tests and a Magnetic Resonance Imaging (MRI) scan of the pancreas assessing volume and excluding any abnormality. The screening tests are summarised in table 11. The screening visit results were recorded in the CRF for each individual patient.

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
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<tbody>
<tr>
<td>Age 16 to 65 years of age</td>
<td>Age &lt;16 or &gt;65 years old.</td>
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<tr>
<td>Type 1 or Type 2 diabetic with successful kidney transplant</td>
<td>Chronic pancreatitis and poor exocrine pancreatic function</td>
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<tr>
<td>Stable kidney graft function</td>
<td>Recent recurrent GI bleeding or spontaneous bacterial peritonitis</td>
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<tr>
<td>No previous rejection episodes</td>
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<tr>
<td>No steroids as part of immunosuppression</td>
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<tr>
<td>Follow up of &gt; 1 year post kidney transplant</td>
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<tr>
<td>WHO performance scores of less than 2</td>
<td>Pregnant or lactating women</td>
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<tr>
<td>Life expectancy of at least 3 months</td>
<td>HIV or life-threatening infection</td>
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<td>Ability to give informed consent</td>
<td>Inability to give informed consent</td>
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<td></td>
<td>Hypersensitivity to G-CSF</td>
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<td></td>
<td>Patients who have been included in any other clinical trial within the previous month.</td>
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</table>

Table 10: Inclusion and exclusion criteria for recruitment into the study
<table>
<thead>
<tr>
<th>Screening tests</th>
<th>Medical history</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical examination</strong></td>
<td>Weight, Height, WHO performance</td>
</tr>
<tr>
<td></td>
<td>All systems – Cardiovascular, respiratory, Gastrointestinal, neurological, genitourinary as well examination of the eyes and skin</td>
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<tr>
<td><strong>Blood tests</strong></td>
<td>Haematology</td>
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<td></td>
<td>FBC, Clotting screen</td>
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<td></td>
<td>Clinical chemistry</td>
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<td></td>
<td>Full renal profile, liver function test, amylase, LDH, CRP</td>
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<td></td>
<td>Pancreatic endocrine function</td>
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<td></td>
<td>Fasting glucose, HbA1c, C-peptide</td>
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<td></td>
<td>Virology and microbiology</td>
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<td></td>
<td>HBV, HCV, HIV-1/2, HTLV-1/2, HSV, CMV, EBV, syphilis and toxoplasmosis</td>
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<td></td>
<td>Tissue typing</td>
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<tr>
<td></td>
<td>Donor specific antibodies (DSA)</td>
</tr>
<tr>
<td></td>
<td>Anti- ABOi titres*</td>
</tr>
<tr>
<td><strong>Radiology</strong></td>
<td>MRI Pancreas</td>
</tr>
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</table>

* If the patient received an ABO - incompatible (ABOi) kidney transplant

Table 11: screening tests for suitable patients who fulfilled the study inclusion criteria. Haematology included full blood count (FBC) and clinical biochemistry included Lactate dehydrogenase (LDH), C-reactive protein (CRP), Virology Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human immunodeficiency virus (HIV), Human T-lymphotrophic virus (HTLV), Herpes Simplex Virus (HSV), Cytomegalovirus (CMV) and Epstein Barr virus (EBV)

### 3.3.5 Protocol summary

Patients who had successful screening were entered into the study and underwent the trial protocol. This started with bone marrow progenitor cell mobilisation into the peripheral blood with daily subcutaneous G-CSF injection for 5 days to harvest CD34+ cells by leukapheresis on day 5. If the CD34+ product yield was low, the patient had a second leukapheresis on day 6 after another day of G-CSF as described in section 2.6.2 and 2.6.3 respectively. The second stage of the manufacture of the final stem cells product ‘Insulincyte’ entailed immunoselection and adherence selection of the CD34+ stem cells. This was performed under GMP.
conditions at the Imperial College Stem Cell Laboratory and John Goldman Centre for cellular therapy at Hammersmith Hospital. The final stage of product manufacture involved the expansion and differentiation of the immunoselected adherent CD34+ stem cells in cell factories with the addition of cell growth media supplemented with cell growth cytokines. The manufacturing method of the final cell product has been explained in sections 2.6.4 and 2.6.5. The process from harvest of the CD34+ cells until having the end stem cell product ‘Insulincyte’ ready for treatment use required 21 days. On day 26 after the start of bone marrow mobilisation, autologous expanded progeny of an adult CD34+ stem cell subset were infused into the pancreas of the patient via selective catheterisation of the splenic artery under fluorescent guidance by an experienced radiologist as described in section 2.6.6. In order to avoid thromboembolic complications, the patient received subcutaneous injections of low molecular weight heparin (pre-filled syringes of 20mg Enoxaparin sodium, Clexane®, Sanofi) for a total of 5 days post stem cell infusion. The patient was followed up for total of 90 days from the start of the protocol. A schematic summary of the protocol is shown in figure 30.

Figure 30: The timeline summary of the study protocol
During the process of bone marrow mobilisation, the patient was monitored on regular basis by hospital visits on days 0, 1, 3 and 4. During these visits, data were collected on symptoms, adverse events and toxicity. The outcome of all visits was documented in individual CRF of each trial patient. Additionally, during the first 4 weeks of the study, and in keeping with the trial protocol, the patient was admitted twice. The first inpatient stay was on day 4 to allow for leukapheresis and CD34+ cell isolation on day 5 which maybe repeated on day 6. The second inpatient stay was on day 25 to allow for stem cell product infusion on day 26. In both occasions, the patient did not stay longer than 2 nights unless an adverse event or a complication had developed.

3.3.6 Assessment of adverse events and endocrine pancreatic function

After the stem cell infusion and during the study follow up period, patients attended the hospital for assessment at 6, 8 and 12 weeks after the start of bone marrow mobilisation and G-CSF administration (day 0). During these visits, data were collected on symptoms, adverse events and toxicity. The patient underwent physical examination which included weight and performance score. In addition, blood tests were also taken which included full blood count (FBC), clinical chemistry (full renal profile, liver function test, amylase, CRP), pancreatic endocrine function screen (random blood glucose, glycosylated haemoglobin (Hba1c) and C-peptide). DSA samples were collected at screening, prior to the stem cell infusion and week 12 at the end of the study. Similar to DSA, anti-ABO titres were also collected at the same time points in ABO incompatible renal transplant recipients. Table 12 summarises the protocol of the trial including the screening and follow up examination and tests performed at each visit.
<table>
<thead>
<tr>
<th></th>
<th>Baseline screen</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 26</th>
<th>Day 27</th>
<th>Day 28</th>
<th>Wk 6</th>
<th>Wk 8</th>
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<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Weight</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>X</td>
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<td>WHO performance score</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
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<td>Clinical chemistry</td>
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<td></td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>Fasting or random glucose</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
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<td>X</td>
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<tr>
<td>Glycosylated Hb</td>
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<td></td>
<td>X</td>
<td>X</td>
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<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>Amylase</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Insulin dose</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Virology screen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G-CSF</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td>Leukaphe resis</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>X</td>
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<td>MRI pancreas</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adverse event</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

Table 12: Summary of all the screening and follow up examination and tests performed according to the protocol throughout the study for each patient
3.3.7 Potential benefits and risks

It was clearly explained to the patients that the primary aim of the study was to assess safety and tolerability of this cellular treatment and that the secondary aim was to assess for efficacy. Potential benefits of this treatment could include reducing the incidence of hypoglycaemic episodes, reducing the mean daily insulin requirements and improving diabetic control as would be illustrated with better Hba1c.

Several potential risks were also explained to the patient. Firstly, published data has revealed that G-CSF-based PBSC mobilisation is generally safe, well tolerated and almost all donors complete the mobilisation and collection procedures. However, retrospective and prospective studies have identified transient, but not insignificant, morbidities experienced by G-CSF mobilised donors. In a retrospective analysis of 41300 donors registered with the International Bone Marrow Transplant Registry (IBMTR) or European Blood and Marrow Transplant Group (EBMT), the rate of serious complications from G-CSF mobilization and PBSC collection was 1.1%. Many of these complications were associated with central venous catheters, which were inserted in 20% of donors (Cashen 2007). The most common symptoms were bone pain, headache, fatigue, and nausea. More serious side effects, although very rare, nonetheless can occur included transient splenomegaly, very rarely splenic rupture and potential toxicity related to procoagulant effects of G-CSF in high-risk individuals. In patients with underlying autoimmune disease, such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and multiple sclerosis, the use of G-CSF to mobilise cells before planned autologous stem cell transplantation, has been associated with flares of the disease in these patients (Cashen 2007). In view of this last potential side effect and having kidney transplant recipients as trial participants,
one of the concerns in this study was possible graft dysfunction related to an alloimmune response triggered by G-CSF. However, there is sufficient evidence in the literature in solid organ transplant including kidney transplantation that this risk is minimal and the use of G-CSF in this population group was safe (Hurst 2011).

Secondly, the potential small risk of possible malignant transformation of the stem cells. However, the stem cell product ‘insulincyes’ are adult differentiated stem cells and so have finite ability to proliferate making this transformation risk minimal. Lastly, the patients were informed of several possible serious but unlikely complications associated with the stem cell infusion procedure. These were arterial or venous complication including thrombosis during catheterisation or after cell infusion into the pancreas and the risk of pancreatitis. Low molecular weight heparin was administered for 5 days post infusion to reduce this thromboembolic risk. Infections might be introduced but were unlikely and if any infections did occur, appropriate antibiotic administration would be a very effective treatment.

### 3.3.8 Patient Demographics

Patients that met the inclusion criteria and had no exclusion criteria were approached. A total of 7 patients were consented and screened and all were successfully enrolled in the trial. Table 13 shows patient demographics. MRI of the pancreas in all of these patients revealed a similar result of an atrophic small native pancreas with no significant abnormality.
<table>
<thead>
<tr>
<th>Gender</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

Mean age at start of study (years) | 54.6 ± 4.2 |

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Caucasian</th>
<th>Indoasian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DM</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

Mean body mass index (BMI) at the start of study | 29.6 ± 5.8 |

Mean insulin requirement pre-treatment (iu/day) | 61.7 ± 26.4 |

<table>
<thead>
<tr>
<th>Type of kidney transplant</th>
<th>Live donor ABOc</th>
<th>Live donor ABOi</th>
<th>Deceased donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of kidney transplant</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Mean Creatinine (μmol/L) | 127.4 ± 12.9 |

Table 13: Demographics of the 7 patients enrolled in the study. ABO compatible (ABOc) and ABO incompatible (ABOi)

3.3.9 Stem cell infusion

Only 5 out of 7 patient had the stem cell product infusion. The bone marrow of patient study numbers 3 and 6 did not mobilise enough in response to G-CSF resulting in insufficient CD34+ cells to complete cell selection and expansion. These two patients had low CD34+ cell harvest yield of less than 0.01% of total cell number and hence did not complete the study. Table 14 shows the number of expanded progeny of adult CD34+ stem cell subset infused into patients who had successful bone marrow mobilisation with acceptable CD34+ cell yield.
<table>
<thead>
<tr>
<th>Patient study number</th>
<th>Peak total White cell count post G-CSF x10^3 (WBC/μL)</th>
<th>Day of peak WBC post G-CSF</th>
<th>Number of leukapheresis</th>
<th>Expanded cell number received x10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35.7</td>
<td>3</td>
<td>2</td>
<td>153.8</td>
</tr>
<tr>
<td>2</td>
<td>54.3</td>
<td>5</td>
<td>1</td>
<td>87.2</td>
</tr>
<tr>
<td>3</td>
<td>34.1</td>
<td>5</td>
<td>1</td>
<td>Study discontinued</td>
</tr>
<tr>
<td>4</td>
<td>32.3</td>
<td>4</td>
<td>1</td>
<td>70.0</td>
</tr>
<tr>
<td>5</td>
<td>54.1</td>
<td>5</td>
<td>1</td>
<td>96.7</td>
</tr>
<tr>
<td>6</td>
<td>37.3</td>
<td>3</td>
<td>2</td>
<td>Study discontinued</td>
</tr>
<tr>
<td>7</td>
<td>49.3</td>
<td>5</td>
<td>2</td>
<td>84.6</td>
</tr>
</tbody>
</table>

Table 14: The peak total white cell count after bone marrow mobilisation with G-CSF, the number of leukapheresis required for CD34+ harvest and the total number of expanded cells infused into the patients

3.3.10 Outcome of the phase I study

The primary end point of the study was achieved. Treatment protocol was tolerated well without any complications or major side effects related to the treatment. There were no adverse events in the short or medium-term follow up for all the patients until the end of the study. The only reported side effects were those of bone pain, fatigue, and nausea related to G-CSF administration which are common and very well recognized symptoms associated with G-CSF in the literature. One patient had a small haematoma at the site of the femoral vein catheter insertion as access for leukapheresis. This was managed conservatively with analgesia alone and resolved rapidly on follow up.

PBSC mobilisation with G-CSF and the application of this cellular therapy were both safe and did not result in graft dysfunction or in any rejection episodes. Donor specific antibodies remained negative pre and post treatment. There was no change in anti-ABO titres pre and post procedure in 2 recipients who had received an ABOi kidney transplant.
In addition, there were no complications or adverse events associated with the stem cell infusion during or after the procedure. There was no intra-arterial or venous thrombosis and no episodes of pancreatitis. Table 15 demonstrates mean creatinine and mean amylase pre and post stem cell infusion (p≥0.05). In Imperial college Healthcare NHS trust biochemistry laboratory, the reference range for serum creatinine is 55-110 μmol/L and for serum amylase is 0-90 IU/L.

<table>
<thead>
<tr>
<th>Patient study number</th>
<th>Mean creatinine (μmol/L) pre infusion</th>
<th>Mean amylase (units/L) pre infusion</th>
<th>Mean creatinine (μmol/L) post infusion</th>
<th>Mean amylase (units/L) post infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>112.7</td>
<td>33.6</td>
<td>109.7</td>
<td>40.0</td>
</tr>
<tr>
<td>2</td>
<td>122.8</td>
<td>106.3</td>
<td>124.0</td>
<td>140.2</td>
</tr>
<tr>
<td>3</td>
<td>116.1</td>
<td>46.0</td>
<td>n/a*</td>
<td>n/a*</td>
</tr>
<tr>
<td>4</td>
<td>149.1</td>
<td>50.5</td>
<td>143.0</td>
<td>70.6</td>
</tr>
<tr>
<td>5</td>
<td>125.6</td>
<td>44.0</td>
<td>115.2</td>
<td>56.5</td>
</tr>
<tr>
<td>6</td>
<td>144.5</td>
<td>69.0</td>
<td>n/a*</td>
<td>n/a*</td>
</tr>
<tr>
<td>7</td>
<td>130.8</td>
<td>60.5</td>
<td>101.3</td>
<td>70.3</td>
</tr>
</tbody>
</table>

* Study discontinued in patient 3 and patient 6

Table 15: Mean creatinine in μmol/L and mean amylase units/L for the study patients before and after receiving expanded progeny of CD34+ stem cell infusion

There were no convincing data to demonstrate the efficacy of this cellular therapy on diabetes in these patients and so the secondary end point was not achieved. In 4 out of 5 patients that completed the study, there was no significant change in their daily mean insulin requirement following the stem cell infusion and until the end of the study (p≥0.05). Hba1c and C-peptide were not significantly altered pre and post treatment (p≥0.05). These results are shown in table 16. In one type 2 diabetic patient, there was significant reduction in the mean daily insulin requirement post
stem cell treatment with sustained good glycaemic control as shown in table 16 (p<0.05). There was a transient fall in this patient’s Hba1c which started prior to the stem cell infusion as shown in figure 31. It is worth noting that this patient had 2 kg weight loss during the 12-week study period. Since this patient is a type II diabetic with normal C-peptide, it can be speculated that the decrease in insulin dose may be related to the combination of the small weight loss along with the improved healthier lifestyle, which was reported by the patient throughout the trial. After the end of the study, routine blood tests performed in transplant clinic as part of the patient's usual follow up visit assessment showed that this affect was not sustained. The patient gradually increased the insulin dose over the following weeks after the study due to suboptimal glycaemic control with increasing Hba1c. These results were not observed with the second type II diabetic included in the study.

<table>
<thead>
<tr>
<th>Patient study number</th>
<th>Hba1c (%) pre infusion</th>
<th>C-peptide (pmol/L) pre infusion</th>
<th>Mean insulin dose (iu/day) pre infusion</th>
<th>Hba1c post infusion</th>
<th>C-peptide post infusion</th>
<th>Mean insulin dose (iu/day) post infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.3</td>
<td>&lt;33</td>
<td>31.5 ± 4.5</td>
<td>7.7</td>
<td>&lt;33</td>
<td>32.2 ± 4.3</td>
</tr>
<tr>
<td>2</td>
<td>5.4</td>
<td>2041</td>
<td>98.5 ± 7.5</td>
<td>5.6</td>
<td>2043</td>
<td>70.0 ± 7.6**</td>
</tr>
<tr>
<td>3</td>
<td>7.7</td>
<td>&lt;33</td>
<td>52.0 ± 3.7</td>
<td>n/a*</td>
<td>n/a*</td>
<td>n/a*</td>
</tr>
<tr>
<td>4</td>
<td>8.5</td>
<td>186.5</td>
<td>71.5 ± 4.3</td>
<td>8.8</td>
<td>201</td>
<td>73.5 ± 3.5</td>
</tr>
<tr>
<td>5</td>
<td>7.9</td>
<td>&lt;33</td>
<td>30.5 ± 2.5</td>
<td>8.0</td>
<td>&lt;33</td>
<td>32.1± 1.7</td>
</tr>
<tr>
<td>6</td>
<td>10.0</td>
<td>&lt;33</td>
<td>48 ± 5.2</td>
<td>n/a*</td>
<td>n/a*</td>
<td>n/a*</td>
</tr>
<tr>
<td>7</td>
<td>5.9</td>
<td>491.5</td>
<td>65.0 ± 2.6</td>
<td>6.1</td>
<td>127.3</td>
<td>62.5 ± 3.7</td>
</tr>
</tbody>
</table>

* Study discontinued in patient 3 and patient 6. ** p<0.05 for t-test insulin dose pre and post-infusion.

Table 16: Glycaemic control and insulin requirements for the study patients before and after receiving expanded progeny of CD34+ stem cell infusion
Figure 31: Glycaemic control in type II diabetic renal transplant recipient (patient 2) before and after receiving expanded progeny of CD34+ stem cell infusion

3.3.11 Conclusion

The findings of this phase I trial confirm that bone marrow mobilisation with G-CSF to manufacture an autologous haematopoietic adult stem cell product which can be delivered via intravenous catheterisation and infusion into the pancreas of diabetic renal transplant recipient is a safe and well-tolerated process. It can be used as a method for delivering a potential cellular therapy. However, the trial was not successful in demonstrating efficacy of this stem cell product in improving diabetic control by reducing insulin requirements in the study patients. This maybe explained by several potential reasons affecting the various stages of the process. These include the number and variability of the cell source and in turn the quality of the end stem cell product, the method of delivery of this product resulting in possible early loss of the cells after infusion and finally probable inappropriate niche in the pancreas for cell engraftment, regeneration and repair.
In this trial, 2 patients were unable to complete the study due to low CD34+ cell yield despite 6 days of bone marrow mobilisation with G-CSF. As shown in table 15, this did not correlate with the peak white cell count reached in these patients which was not dissimilar to other patients that achieved an acceptable CD34+ cell yield. In the haematology literature, although many factors including the patient’s age, diagnosis and amount of prior therapy have been shown to predict the likelihood of collecting a sufficient number of autologous repopulating cells, factors affecting the capacity of normal donors to mobilise CD34+ cells following G-CSF are less well established. Reports have indicated in some cases wide inter-individual variation among normal subjects given G-CSF for PBSC mobilisation. In an IBMTR analysis, 60% of donors required more than one leukapheresis procedure to collect the target number of CD34+ cells, and 15% required three or more. It has been shown that donor age, steady-state CD34 level, and both the total dose and schedule of G-CSF may impact CD34+ cell mobilisation in normal donors (Cashen 2007).

The study patients did not have haematological disorders but were recipients of a kidney transplant and on long-term immunosuppression which may have affected their bone marrow and hence their response to G-CSF. So the lower yield of CD34+ cells in a couple of the patients can be explained by a similar observation of variable response to G-CSF in normal donors but this effect may have been exacerbated by maintenance immunosuppression. There is no published data that specifically addresses this hypothesis specifically with CD34+ stem cells but a recent study by Reinders et al examined bone marrow-derived MSC from patients with end-stage renal disease (ESRD) and found them to be phenotypically and functionally similar to healthy controls. They concluded that these cells would be suitable for autologous clinical applications in renal transplant recipients (Reinders 2013). However, MSCs are different from haematopoietic stem cells as described earlier in 1.9.2. They are CD34- and are usually isolated from bone marrow aspirate without mobilisation and
then expanded in culture (Reinders 2013). Nonetheless, the study does demonstrate that one type of bone marrow-derived stem cells of patients with ESRD is similar to the general population and this makes it more likely to extend to the other types of stem cells within the bone marrow.

In their published paper, Gordon et al stated that 1-2 weeks of expansion and culture of CD34+ Omnicytes would provide $5-10 \times 10^9$ progeny cells for clinical applications (Gordon, 2006). Interestingly, $2 \times 10^9$ ($3 \times 10^7$cells/kg body weight) undifferentiated progeny of adherent CD34+ stem cell subset constituted the cellular therapy in phase I trial in five patients with liver disease led by the same group (Gordon, 2006). However, these undifferentiated cells were not used in this study. The cells remained in culture for 21 days in total to allow differentiation into Insulincytes prior to infusion. It is known that expansion of these cells in culture peaks between 7-10 days and that they have a finite ability to proliferate (personal communication with Gordon et al). Hence leaving cells in culture for longer period of time to allow differentiation is likely to result in a smaller number of cells at the end of the process. In this study, this prolonged period resulted in a mean of only $9.8 \times 10^7$ cells ($1.2 \times 10^6$cells/kg body weight) available for infusion on day 26 of the study. In the largest published HSC trial to treat diabetes, the study design was very different to the protocol of this study but they used approximately $3 \times 10^6$cells/kg body weight as cellular therapy (Couri 2009a; Voltarelli 2007). This is similar to the cell number used in our study but significantly less than the number of HSC used in the liver study by Gordon et al.

The study design for this trial probably assumed that the effect of these cells is likely to be direct by cell engraftment into the damaged pancreas, secreting insulin and acting like an islet transplant. If this hypothesis were true, then it would be difficult to estimate the total number of Insulincytes required to confer the desired therapeutic effect in these diabetic patients. We know from the islet transplant literature that insulin independence might be achieved with approximately 8-10,000 islet
equivalents (IE)/kg body weight which is what is currently required in islet transplantation. A mass that usually demands two or more deceased donors (Kaufman, 2002). In addition, historical data in the Islet Transplant Registry (ITR) and published reports indicate that a minimum of 6,000 IE/kg need to be transplanted to increase the likelihood of a functional islet graft. However, there does not appear to be any conclusive data correlating the number of transplanted islet equivalents with those that actually engraft post-transplant (www.fda.gov/ohrms/dockets/ac/00/backgrd/3604b1b.doc). Stem cells used in cellular therapy for diabetes do not necessary form islet-like morphology and are in many cases transplanted prior to differentiation so it would be very difficult to estimate the number of stem cells required to give the same effect as 6,000 IE/Kg in islet transplantation.

The quality of the stem cell end product as well as the quantity is an important factor that impacts on the outcome. We know from in vitro studies that Omnicytes are not homogenous despite universal expression of CD34 and adherence to plastic in culture. Although cell expansion was highly reliable and reproducible in in vitro experiments, only up to 40% of these cells differentiated into Insulincytes (Gordon, 2006). This variable ability of these cells to differentiate into insulin-secreting cells in culture would in turn result in a variable end product after 21 days in the study. In vivo, only one experiment showed efficacy of day 7 Omnicytes in stabilising glucose levels in an animal diabetic model (section 3.1.3).

In the trial, the composition of the expanded progeny of CD34+ stem cell product that was infused into the patient was not formally examined. On each occasion, the end product was sampled for total nucleated cell count and viability, sterility and freedom from mycoplasma contamination as quality control testing but not formally tested to confirm the proportion of Insulincytes in each batch. This would have been very informative and could have enabled a more accurate calculation of the true number of insulin-secreting cells infused per kg body weight into each patient. Assuming best
yield of 40% differentiation into insulin-secreting cells, this equates to a maximum mean of $4.8 \times 10^5$ cells/kg body weight of Insulincytes administered to each patient in the study. This cell number is 10 times less than the average number of stem cells used as therapy in the stem cell literature. Hence, it can be proposed that actual number of Insulincytes infused and delivered to the pancreas in the study patients could have been insufficient to show any significant therapeutic effect and that the cell number required for such as effect is much larger. Delivering a larger number of autologous Insulincytes can test this proposal. This is however as described limited by several factors including the original number of CD34+ isolated, the differentiation capacity of the parent cell and the cultures and growth factors used in the expansion and differentiation process to obtain optimal results.

The third potential hurdle that may have resulted in the lack of efficacy of the stem cell product in this phase I study could be due to early cell loss as a result of thrombosis as described in the islet allo and xeno-transplant literature (sections 1.11.6 and 1.11.7). This potential problem is part of my overall study hypothesis and was partly assessed in vitro (section 3.2.5) and in vivo (section 3.2.8) during the study. I demonstrated in these experiments the procoagulant property of day-7 CD34+ Omnicytes that are parent cells to the exact cells infused in the trial. However, if this property can be extrapolated to the progeny of Omnicytes, then Insulincytes can be assumed to be prothrombotic triggering coagulation and leading to cell destruction after delivery into the pancreas. Systemic prophylactic coagulation with LMWH was administered after the stem cell infusion but the timing of this may be too late to prevent the immediate cell loss from taking place. More experiments are needed to confirm that Insulincytes are prothrombotic in vitro and in vivo by demonstrating evidence of coagulation and examining the underlying mechanism for it in order to establish a better preventative strategy.
Finally, the niche where the Insulincytes were delivered i.e. pancreas may have been an unsuitable environment lacking the appropriate conditions and signals needed for cell engraftment, differentiation and repair. As described in section 1.9.2, many studies demonstrated that bone marrow derived stem cells are able to stimulate beta-cell regeneration in damaged pancreatic tissue by a variety of mechanisms (Ianus 2003). Choi et al showed that BMSC did not differentiate into pancreatic beta cells but they still have the potential for being host cells in beta-cell replacement therapy (Choi 2003) and Hess et al concluded from their studies that hyperglycaemia reduction in treated animals was attributable to a rapid rise in endogenous insulin stimulated by BMSC transplantation rather than differentiation of the BMSC into beta cells (Hess 2003). This is also consistent with the findings when PDPCs used as cellular therapy in the STZ-induced diabetic mice (Stevenson 2011).

It is worth highlighting that in all of the animal studies, the injury to the pancreatic tissue was acute and probably reflected early changes in the diabetic disease model. If Insulincytes, which are haematopoietic in origin, have a similar effect to other BMSC reported in the literature then it can be proposed that they are likely to stimulate beta-cell repair and regeneration by another host cell via an indirect paracrine fashion rather than directly as assumed in the study design. This means that the damaged pancreas needs to have viable tissue including probably residual islets but more importantly other host cells in order to have the capacity for regeneration and repair. Patients selected for this study were mainly type I diabetics who have had a long-standing diabetes and in turn extremely likely to have lost all their beta-cell mass in their early years of disease progression. All study patients, apart from one type II diabetic, had low or undetectable levels of C-peptide indicating lack of insulin production and therefore complete or almost complete destruction of beta cell mass. In addition, all MRI scans of the pancreas performed as part of the screening process for each study patient demonstrated atrophic pancreas, which is
again consistent with mostly scarred rather than viable pancreatic tissue. This reasoning probably explains the study design and timing of cellular therapy in the Brazilian stem cell study. The study had a 3-stage protocol aimed at the use of an immunomodulatory and regenerative strategy during the initial phase of the disease when the patient still has an important beta-cell reserve in an attempt to preserve and protect it (Couri 2009a; Voltarelli 2007). Patients that were enrolled in the study were within 6-week period of their diabetic disease presentation which is in contrast to the study patients in this trial.

In summary, there are several possible explanations for the lack of efficacy of the cellular therapy in this phase I trial. Further experimental work is necessary to try and solve the main challenge to the success of the process.
4. DISCUSSION AND FUTURE WORK

As highlighted in the introduction, type I diabetes mellitus is a metabolic disorder characterised by hyperglycaemia due to insulin deficiency. It is common and the incidence of TIDM has been increasing by about 3% per year (Aanstoot 2007). It is caused by autoimmune destruction of the insulin-producing beta cells in the pancreas. Despite insulin treatment, diabetes often results in metabolic defects leading to the development and progression of micro and macrovascular complications. Macrovascular disease leads to an increased prevalence of cardiovascular disease, while microvascular damage results in diabetic retinopathy, nephropathy and neuropathy. These late complications are associated with a high burden of morbidity and high rate of mortality. It has been shown that these diabetic complications are less common and less severe in patients with well-controlled blood glucose levels (Nathan 2005).

Restoring euglycaemia by replacement of the destroyed insulin-producing tissue would be the gold standard treatment for these patients. As discussed in chapter 1, this can be achieved with pancreas transplantation but this is greatly limited by the shortage of deceased organ donors and by the high risk associated with this surgical procedure. Therefore this curative option is not available to all patients. Islets transplantation is a more recent treatment option performed by a relatively non-invasive technique with a low risk of immediate complications. Although it is a more attractive option, it is restricted by a variable success rates and by the limited number of organ donors particularly as 2-3 pancreata are required per recipient to achieve insulin independence. More importantly, a substantial percentage of islets are lost immediately post infusion into the portal vein through an inflammatory process termed the instant blood-mediated inflammatory reaction. It is well established that TF play a vital role in this process resulting in thrombosis and destruction of a significant proportion of the islet transplants. Xenotransplantation faces a similar
challenge to islet allotransplantation in addition to the specific immunological barrier associated with preformed xenoreactive natural antibodies. Both these processes contribute to the loss of the xenograft. There are also several technical and logistical obstacles for xenotransplantation.

A promising and emerging treatment option for type I diabetes would be the use of stem cells as ‘islet equivalents’. Stem cells of various origins have been shown to have the ability to proliferate extensively and are able to differentiate into insulin-secreting cells that can either function directly or act indirectly leading to islet repair and regeneration of new beta cells. Most of the evidence points to an indirect paracrine effect on existing beta cells rather than the direct differentiating of these stem cells into adequate numbers of insulin-secreting cells that can result in euglycaemia (section 1.9). This cellular therapy would mean unlimited source of cells that can be transplanted in a non-invasive method with low risk of complications. However, what is not known from the literature is whether stem cells result in triggering coagulation after intravenous infusion in a similar fashion to islet transplants resulting in stem cell thrombosis and destruction.

This work has examined the efficacy of two sources of stem cells in restoring euglycaemia in an STZ-induced diabetic animal model. I demonstrated the success of specific population of pancreas derived progenitor cells in reversing this diabetic phenotype. The Shiel’s group in Glasgow performed subsequent analysis on the tissue harvested from the diabetic animals treated with this cellular therapy. They found evidence supporting the proposal that in this animal model, these PDPCs correct hyperglycaemia indirectly by triggering repair of existing islets and regeneration of new islets from the recipient’s own tissue rather than directly differentiating into sufficient number of insulin-secreting cells themselves (Stevenson 2011).
More recently, the Shiel’s group in Glasgow has published a study that demonstrated that the presence of the same population of PDPCs from adult rat led to functional repair of renal damage in ischaemia reperfusion (I/R) injury model in mice. PDPC-treated animals had superior renal function at day 14 after I/R injury, in comparison to saline-treated controls. Similar to the study in diabetic mice, FISH analysis demonstrated that the majority of repaired kidney tissue was mouse not rat in origin. Rat PDPCs were only detected at a frequency of 0.02%. The group concluded that PDPCs appear to again act indirectly in a paracrine way, stimulating host repair and regeneration rather than differentiating into renal tissue directly (McGlynn 2013). It is not published whether these researchers replicated the same results with human PDPCs. It would be exciting if this application could be extended to a different model of kidney injury or injury model of a different organ. Successful outcome would advocate the use of PDPCs as a cellular therapy to repair and regenerate damaged tissue irrespective of the tissue type. So far, this has been proven \textit{in vivo} for the pancreas and the kidney. My and these experimental results would encourage testing these cells as therapy for diabetes and acute kidney injury in a phase I clinical trial respectively.

In this work, I also demonstrated the lack of efficacy of day-7 human adherent CD34+ Omnicytes in reversing diabetes in the same mouse model. On one occasion, this cellular therapy resulted in stabilisation of blood glucose but this effect was not reproducible. Several explanations have been proposed to explain these findings including the possibility of early cell loss through thrombosis as observed after islet transplantation.

As emphasized in the introduction, TF is thought to play a major role in initiating the IBMIR leading to significant early cell loss to thrombosis following allo and xeno islet...
transplantation. I believe this work is the first to test the hypothesis of early cell destruction by a similar thrombotic process after cellular infusion of 2 different populations of stem cells: PDPCs and CD34+ Omnicytes. It examined the procoagulant properties of both stem cell types and attempted to establish whether these stem cells express tissue factor as a trigger for provoking coagulation by immunocytochemistry and functional plasma recalcification assays.

The study demonstrated that both stem cell types are prothrombotic in vitro and that this effect can be mitigated by pre-treatment of these cells with the anti-thrombin cytotopic agent ‘PTL006’. One of the advantages of this agent is its local anticoagulant effect, shown by the limited rise in clotting time to equal or lower than the baseline contact activation clotting time when the cells were pre-conditioned with PTL006. This is compared to soluble hirulog that resulted in clotting time significantly exceeding the baseline clotting time. This would probably translate to local versus undesirable systemic effect in an in vivo setting and should be explored in future work.

The in vivo work performed using Omnicytes is very suggestive that the infusion of this cellular treatment is a trigger for coagulation leading to probable fibrin clot formation and resulting in a thrombocytopenia as platelets are sequestered in the clot. The results also suggest that this effect is preventable by the presence of PTL006 tethered to the surface of the cells infused into these animals as demonstrated by the preserved platelet count at the end of the experiment. The outcome of this work points to a possible connection between the prothrombotic phenotype of Omnicytes and their lack of efficacy at reversing diabetes in mice. However, the work is incomplete and further investigations are necessary to firstly replicate the findings of this in vivo experiment using Omnicytes, secondly test the same hypothesis using PDPCs in vivo and finally extend the evidence for coagulation activity with other assays such as indirect measurements of thrombin generation in
the plasma collected after stem cell infusion. If these findings are found to be reproducible then the next step should be to re-examine the efficacy of untreated stem cells compared to PTL006-treated stem cells in reversing diabetic phenotype in STZ-induced animal model. Repeating the same work using different cell number infused would help to establish the minimal number of cells required for the desirable outcome. Based on the in vitro work, it would be expected to reveal that pre-conditioning of the cells with anti-thrombin cytotoxic agent improves the performance of the cells and reduces the necessary cell number to achieve normoglycaemia.

This work is however inconclusive whether the procoagulant phenotype of PDPCs and Omnicytes is due to TF-dependent or TF-independent process. Although the data from plasma recalcification assays using PDPCs suggest that the prothrombotic effect is possibly TF-independent, it is not possible to make this conclusion before further experiments are carried out. Functional assays should be performed with higher concentration of anti-mTF antibody to ensure saturation and in turn complete inhibition of TF. The procoagulant property may be suppressed in an antibody concentration-dependent manner, which would support the involvement of TF. This has not been excluded in this study. Furthermore, TF expression on these cells should be examined again with improved successful immunocytochemistry as well as other experimental methods such as flow cytometry and RT-PCR. These assessments will help propose a more definitive mechanism for this prothrombotic property. If TF was found consistently not to play a role, then a possible explanation in that case could be the involvement of an inducible prothrombinase that acts in a TF-independent pathway, termed Fgl2/fibroleukin. It has been shown to contribute to immunologically mediated thrombosis in experimental and human viral hepatitis and in acute vascular xenograft as well as allograft rejection (Ghanekar 2004; Marsden 2003).
The data resulting from plasma recalcification assays applied to Omnicytes did confirm generalised procoagulant property of these cells but it did not confirm universal functional expression of TF. However, there was some evidence to suggest that TF-dependent trigger may be involved in thrombosis caused by these cells. Some of the negative outcomes raised doubt about the anti-hTF antibody efficacy and concentration used especially in the absence of control TF+ human cells. For the above findings to become conclusive and similar to PDPCs, functional assays should be performed using Omnicytes with higher concentration of anti-hTF antibody to ensure saturation and in turn sufficient inhibition of TF as well as examining TF expression on these cells with various experimental techniques.

Interesting, since the end of this study, recent evidence has been published investigating thromboembolism following mesenchymal stem cells transplantation and demonstrating TF as a trigger for procoagulation (Tatsumi 2013). The paper had several salient findings. It showed that within 24 hours of the intravenous administration of mouse adipose-derived MSCs (mADSCs), animal survival rate increased from 15% to 100% by reducing the number of cells infused from $1.5 \times 10^5$ to $1.5 \times 10^4$ and was similar to infusing control PBS. Histological assessment of the mice demonstrated no evidence of systemic intravascular hypercoagulation but did reveal multiple fibrin clots formed in the right ventricle and the pulmonary arteries with mADSCs at the core of these fibrin clots. The researchers concluded that administered mADSCs triggered thrombus formation around the cells and initiated pulmonary embolism immediately after the administration (Tatsumi 2013).

Tatsumi et al. investigated the procoagulant property of mADSC with clotting assays, and rotation thromboelastometry. They found that clotting times of plasma in the presence of mADSCs were shortened in a cell-number dependent manner and also depended on the length of incubation period as a single cell suspension. The clot formation time of whole blood was also shortened and was dependent on the
incubation period. In addition, the researchers showed that CT was similar using normal mouse plasma and FIX deficient plasma but significantly prolonged using FVII-deficient plasma suggesting that TF played a critical role in mADSCs-mediated coagulation. This suggestion was further strengthened by the demonstration that preconditioning cells with several concentrations of anti-TF monoclonal antibody resulted in shortening of the CT in an antibody-dependent manner. They also showed that TF was strongly expressed on the cell surface of mADSCs using immunostaining, flow cytometry and by RT-PCR. Hence, their second conclusion was that mADSCs have a significant procoagulant property against plasma as well as whole blood, that this property was enhanced during cell suspension period and that the resultant clot formation was mediated by TF expressed on the cellular surface which accelerates the extrinsic blood coagulation pathway. The paper revealed similar findings in human MSC including adipose-derived (hADSC), bone marrow-derived and periodontal ligament-derived MSCs (Tatsumi 2013).

The same group demonstrated the use of two strategies to minimise TF-triggered intravascular coagulation. Firstly, pre-treatment of hADSCs with recombinant human thrombomodulin which resulted in the suppression of coagulation in anticoagulant agent concentration-dependent manner and secondly, avoiding single cell suspension by in vitro use of uncultured hADSC which demonstrated almost no coagulant activity and minimal expression of TF on the cell surface. In vivo transplantation of $1.5 \times 10^5$ uncultured cells did not cause animal death and did not show any histological thrombotic changes in any organs. The paper’s final conclusion stated that TF has a critical role in promoting MSC-mediated coagulation in animal experiments and that its expression is likely to lead to thromboembolism. The authors proposed that extra interventions are required to weaken this procoagulant effect (Tatsumi 2013). The findings by Tatsumi et al. complements this study and supports the hypothesis that stem cells including bone marrow-derived stem cells
whether haematopoietic or mesenchymal have a universal procoagulant phenotype which results in thrombosis probably via a TF-dependent trigger leading to cell loss. This property clearly becomes very important when taking stem cell therapy forward to clinical practice. Considerations should be given to the stem cell number administered and to strategies for thromboembolic events avoidance in order to improve safety, tolerability and efficacy of these cellular therapies.

Additionally, this work included the completion of a phase I clinical trial demonstrating the safety and tolerability of the infusion of autologous expanded progeny of an adult CD34+ stem cell subset to patients with type I diabetes mellitus and a successful renal transplant. The rationale of the study led to several unique points in the design of the trial which allowed assessment of different aspects of safety of this cellular therapy compared to previous trials. The cellular product here consisted of an expanded and differentiated progeny of CD34+ cell population as opposed to mobilised undifferentiated haematopoietic stem cells that are usually isolated and stored to be used at a later time point in other studies. The study population composed of long-standing diabetic patients with a functioning renal transplant contrasting the newly diagnosed diabetic patients with no previous history of end stage renal disease or of a kidney transplant enrolled in other studies. Finally, patients did not receive preconditioning treatment before administering the cellular therapy, which is usually the case in protocols of previous studies.

This trial demonstrated the safety and tolerability of the use of G-CSF and bone marrow mobilisation in renal transplant recipients without initiating an alloimmune response or cause any harm to the kidney graft. It also revealed the safety and tolerability in using differentiated progeny of haematopoietic stem cells delivered as cellular therapy by selective catheterisation approach. There were no clinically observed thromboembolic events. The administration of LMWH after infusion of the
cell product may have contributed to this result but it is difficult to make any conclusion as the study was not designed to specifically address this possible hurdle.

Despite achieving its primary end point, there was no convincing evidence in the phase I study to suggest efficacy of this cellular treatment in reducing insulin requirements of these patients. Several possible explanations have been proposed including the quantity and consistency of the cells delivered, early cell loss due to thrombosis which was not detectable clinically, unsuitability of the niche for cell engraftment and the lack of viable tissue for islet repair and regeneration.

Since the efficacy of human PDPCs has been demonstrated in vivo, this cellular therapy would be a good candidate and a better alternative to be assessed in another phase I clinical trial for safety and efficacy. Using Omnicytes again as potential cellular therapy would certainly need further investigations to identify the limiting step and to unravel the reason(s) for lack of efficacy in the diabetic animal model and resolving these challenges prior to clinical practice. In addition, further in vivo work would help to determine the number of stem cells optimal for infusion to achieve efficacy safely and to support the clinical application of localised anti-coagulant treatment such as an anti-thrombin cytotoxic agent to prevent thrombosis and avoid potential harmful thromboembolic events. A final point, the published evidence favours an indirect effect of stem cell therapy for repair and regeneration of new islets and therefore the presence of viable pancreatic cells may be crucial. This could be in the form of patients with new diagnosis of TIDM or patients with type II diabetes where complete beta cell destruction has not taken place and where residual insulin/C-peptide is still being secreted. These patients may be a more suitable study population for inclusion in future trial.
In summary, this work provided increased insight to the widespread procoagulant phenotype of two different sources of stem cells that vary in origin and characteristics. It also provided evidence for potential localised treatment in the form of a cytotopic agent to minimise this effect \textit{in vitro} and in limited \textit{in vivo} experiments. TF was found to play a pivotal role in stem cell-mediated coagulation although the evidence is not conclusive and further work is necessary. This prothrombotic property will be very important when designing clinical trials of stem cell therapy. Finally, I demonstrated the safety and tolerability of the infusion of autologous expanded progeny of an adult CD34+ stem cell in a clinical phase I trial. There were several limitations to this study and future work is required which can build upon my work presented in this thesis to take this potential therapy forward.


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