STEM CELL-BASED REGENERATIVE PHARMACOLOGY
FOR THE TREATMENT OF DIABETES MELLITUS

BY

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<td>AA</td>
<td>Activin A</td>
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<tr>
<td>ADSC</td>
<td>Adipose Derived Stem Cells</td>
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<tr>
<td>AFS</td>
<td>Amniotic Fluid-Derived Stem Cell</td>
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<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<td>ANOVA</td>
<td>Analysis of Variation</td>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>bHLH</td>
<td>Basic Helix-Loop-Helix</td>
</tr>
<tr>
<td>BM-MSC</td>
<td>Bone Marrow Derived Mesenchymal Stem Cells</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DE</td>
<td>Definitive Endoderm</td>
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<tr>
<td>dL</td>
<td>Deciliter</td>
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide Triphosphate</td>
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<tr>
<td>EB</td>
<td>Embryoid Body</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
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<td>ES</td>
<td>Embryonic Stem Cell</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<tr>
<td>FAM</td>
<td>6-Carboxyfluorescein</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>FOXA2/HNF3B</td>
<td>Forkhead Box Protein A2 / Hepatocyte Nuclear Factor 3 Beta</td>
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<tr>
<td>GAD65</td>
<td>Glutamic Acid Decarboxylase 65</td>
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<tr>
<td>GCK</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Glucose Transporter 2</td>
</tr>
<tr>
<td>GTE</td>
<td>Gut Tube Endoderm</td>
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<tr>
<td>GvHD</td>
<td>Graft Versus Host Disease</td>
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<tr>
<td>H-2Kk</td>
<td>Mouse Major Histocompatibility Complex Class I</td>
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<td>HBSS</td>
<td>Hank’s Buffer Salt Solution</td>
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<tr>
<td>HEK293T</td>
<td>Human Embryonic Kidney 293T Cell</td>
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<td>HH</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>HNF4A</td>
<td>Hepatocyte Nuclear Factor 4 Alpha</td>
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<td>HO-1</td>
<td>Heme Oxygenase – 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
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<tr>
<td>IA-2</td>
<td>Islet Tyrosine Phosphatase Il-2</td>
</tr>
<tr>
<td>IDO</td>
<td>Indolamine 2,3-Dioxygenase</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon – Gamma</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IL-1α</td>
<td>Interleukin – 1 Alpha</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin – 1 beta</td>
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<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>iPS</td>
<td>Induced Pluripotent Stem Cell</td>
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<tr>
<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
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<tr>
<td>KLF4</td>
<td>Krueppel-Like Factor 4</td>
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<tr>
<td>L-PK</td>
<td>L-Pyruvate Kinase</td>
</tr>
<tr>
<td>MAFA</td>
<td>Musculaponeurotic Fibrosarcoma Oncogene Homolog A</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic Activated Cell Sorting</td>
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<tr>
<td>MG</td>
<td>Milligram</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>ML</td>
<td>Milliliter</td>
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<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity Onset Diabetes of the Young</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stromal Cell</td>
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<tr>
<td>NEAA</td>
<td>Nonessential Amino Acid</td>
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<tr>
<td>NEUROD1</td>
<td>Neurogenic differentiation 1</td>
</tr>
<tr>
<td>NG</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NGN3</td>
<td>Neurogenin 3</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Nonobese Diabetic / Severely Compromised Immunodeficient</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NRSF</td>
<td>Neuronal Restrictive Silencing Factor</td>
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<tr>
<td>OCT4</td>
<td>Octamer-Binding Transcription Factor 4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>OGDH</td>
<td>Mitochondrial 2-Oxoglutarate Dehydrogenase</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD-MSC</td>
<td>Placenta Derived – Mesenchymal Stromal Cells</td>
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<tr>
<td>PDX1</td>
<td>Pancreatic and Duodenal Homeobox 1</td>
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<tr>
<td>PE</td>
<td>Pancreatic Endoderm</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
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<tr>
<td>PP</td>
<td>Pancreatic Progenitor</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/Sterptomycin</td>
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<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
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<tr>
<td>QPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RA</td>
<td>Retinoic Acid</td>
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<tr>
<td>RISC</td>
<td>RNA-Induced Silencing Complex</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RNAi</td>
<td>RNA Interference</td>
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<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
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<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>shRNAmir</td>
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<td>siRNA</td>
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<td>Sex Determining Region Y Box 17</td>
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<td>TAMRA</td>
<td>6-Carboxy-Tetramethylrhodamine</td>
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<td>T1DM</td>
<td>Type 1 Diabetes Mellitus</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
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<td>TGF-β</td>
<td>Transforming Growth Factor - beta</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor – alpha</td>
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<td>μg</td>
<td>Microgram</td>
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<tr>
<td>μl</td>
<td>Microliter</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Cell Growth Factor</td>
</tr>
<tr>
<td>WNT3A</td>
<td>Wingless-Type MMTV Integration Site Family, Member 3A</td>
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</tbody>
</table>
ABSTRACT

Emily Crafton Moorefield

STEM CELL-BASED REGENERATIVE PHARMACOLOGY FOR THE TREATMENT OF DIABETES MELLITUS

Dissertation under the direction of Colin Bishop, Ph.D., Professor, Wake Forest Institute for Regenerative Medicine

Stem cell-based regenerative pharmacology is aimed at establishing stem and progenitor cells as effective treatment for preventing disease and facilitating organ replacement or repair. Diabetes mellitus (DM) is a group of chronic metabolic disorders characterized by high blood glucose levels. Type 1 DM (T1DM) is a lack of insulin production caused by autoimmune destruction of the β-cells, while Type 2 DM (T2DM) is insulin resistance in peripheral tissue that leads to β-cell exhaustion. Chronic hyperglycemia leads to serious long term complications. Transplantation of whole pancreas or isolated islets are promising approaches to restore insulin production in these patients; however, the severe shortage of organ donors and risk of allogeneic graft rejection have limited this treatment. Stem cells are an attractive starting source for producing pancreatic lineage derivatives to be used in treatment of DM as a form of cell replacement therapy, immunotherapy and in attempts to model diabetic disease phenotypes in vitro for identification of novel drug targets. Amniotic fluid stem (AFS) cells are a long-lived, bankable cell source possessing extensive differentiation capacity. We found that by genetic manipulation and culture condition modifications, AFS cells can be induced to produce insulin, making them a potential source of renewable insulin-producing cells for cell-replacement therapy in DM patients. We also discovered that AFS cells possess immunomodulatory properties and are able to inhibit lymphocyte activation by release of soluble factors in vitro. This quality makes AFS cells an ideal candidate for cell-based immunomodulation in early stages of T1DM to prevent the autoimmune response or in combination with allogeneic islet transplantation to prevent rejection. Finally, we developed pluripotent stem cells, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, containing a deficiency in HNF4A expression that is known to cause a form of monogenic T2DM, maturity onset diabetes of the young 1 (MODY1). Pluripotent stem cells deficient in Hnf4a, a gene which has a known role in β-cell development and function, were used to model pancreatic development in vitro as a means to study the progression of disease pathology. We identified several genes potentially regulated by HNF4A in the gut tube endoderm stage of pancreatic lineage differentiation which may lead to identification of novel drug targets to treat DM.
INTRODUCTION

Regenerative Pharmacology

Degenerative diseases are a growing epidemic. Regenerative pharmacology is a form of therapy aimed at preventing and repairing the progressive cell destruction that is the cause of these degenerative diseases. This approach works to identify useful molecular targets for regenerative and therapeutic purposes. By restoring the development and function of tissues and organs, regenerative pharmacology has the ability to improve therapies for human disease. Stem cell-based regenerative pharmacology supports the use of stem cells for derivation of novel therapeutics. Ultimately, stem cell-based regenerative pharmacology aims to establish stem and progenitor cells as effective treatments for preventing disease and for facilitating organ replacement or repair.

The mission to maintain and restore β-cell mass through stem cell-based regenerative medicine led us to explore several possibilities. The possibilities outlined in this dissertation include (1) cell replacement therapy through isolation and in vitro differentiation of stem and progenitor cells, (2) exploitation of immunosuppressive and anti-inflammatory properties of stem cells and (3) development of an in vitro system using pluripotent stem cells, patient-specific iPS and knock-down ES containing a diabetic genotype for disease modeling and drug discovery.
Diabetes mellitus

Diabetes mellitus is a group of endocrine disorders characterized by hyperglycemia resulting from inadequate insulin production, release or action. Two main types of diabetes exist, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), each with distinct pathophysiology leading to elevated blood glucose levels. T1DM is diagnosed during childhood and is characterized by the complete lack of insulin production caused by autoimmune destruction of the insulin-producing β-cells in the endocrine pancreas [1, 2]. Patients with T1DM are dependent on exogenous insulin to maintain blood glucose homeostasis [3].

T2DM is the most common form of the disease and accounts for 90-95% of all diagnosed cases [4]. T2DM is the result of insulin resistance in peripheral tissue [5]. The pancreas increases insulin secretion to overcome impaired insulin action, a practice which eventually wears out the β-cells [6, 7]. As exhausted β-cells are no longer able to produce sufficient amounts of insulin, T2DM patients also become dependent on exogenous insulin delivery to maintain normoglycemia. The exact cause of T2DM remains unknown but several genes have been associated with the disease and environmental factors are also likely involved [8].

A subset of T2DM termed maturity onset diabetes of the young (MODY) is present in 2-5% of the T2DM population and is characterized by single gene defects of the pancreatic β-cell which alter its function [9]. This form of noninsulin dependent diabetes has autosomal dominant inheritance that develops before the age of 25 and presents primarily as a defect of insulin secretion in response to glucose. The primary
genes affected are those encoding the enzyme glucokinase (GCK/MODY2) [10] and the transcription factors hepatocyte nuclear factor-4α (HNF4A/MODY1) [11], hepatocyte nuclear factor 1-β (HNF1B/MODY3) [12], pancreatic and duodenal homeobox 1 (PDX1/MODY4) [13], hepatocyte nuclear factor 1-α (HNF1A/MODY5) [14], and neurogenic differentiation 1 (NEUROD1/MODY6) [15].

Treatment of diabetes and the associated complications involves great cost. The estimated healthcare expenditure associated with treating the 25.8 million Americans affected by the disease is 174 billion dollars per year [16]. Even in cases where relative normoglycemia is achieved through careful blood glucose monitoring, delivery of insulin analogs and strict diet control, numerous long-term complications arise including renal failure, cardiovascular disease, stroke, retinopathy, neuropathy and other vascular disorders [2, 17]. These facts make the search for a cure for diabetes more urgent than ever.

**Cell Replacement Therapy**

A critical goal in diabetes treatment is automatic maintenance of blood glucose homeostasis. Insulin replacement therapy has improved significantly since the advent of long and short acting insulin analogs. T1DM patients benefit from a range of insulin replacement options and T2DM patients also require exogenous insulin therapy as they reach β-cell exhaustion resulting from insulin resistance [18]. Unfortunately, normal glucose homeostasis remains difficult to achieve with current methods of exogenous insulin delivery [19]. A device capable of sensing blood glucose levels and releasing
appropriate levels of insulin in response would be an ideal therapy; however the development of such a device has proven difficult [20]. Cell-based therapy is another option that may close this gap in the treatment of diabetes. The focus of cell-based therapies must be on replacement of insulin-producing cells while taking the immune response into account.

Transplantation of the whole pancreas or of isolated insulin-producing cells is a promising approach in the treatment of DM. Currently pancreatic transplantation is most often performed on patients with insulin-dependent T1DM who also have resulting end-stage renal disease. In these cases, a simultaneous pancreas-kidney transplant, usually derived from a cadaver, is performed to stabilize both conditions. This approach results in maintenance of glucose homeostasis and some improvement in secondary complications but also carries with it some risk [21-23]. The immunosuppressive drug therapy required for allogeneic transplantation is associated with serious side-effects and up to 20 percent of transplantations fail within the first year due to technical problems or rejection [24].

Islet cell transplantation has gained attention since the successful transplantation of cadaver derived islets into T1DM patients [25]. Islets transplanted via the hepatic portal vein of DM patients led to achievement of improved metabolic control, insulin-independence and enhanced quality of life [26]. However, the severe shortage of islet donors and the need to combine pancreatic tissue from two or more donors to achieve the therapeutic dose of 10,000 islet equivalents/kg has limited the usefulness of this approach [27]. A follow-up study of patients who received islet transplantation also revealed that 76% reverted back to insulin-dependence within 2 years [28]. The primary reason for this relapse seems to be loss of transplanted islets due to engraftment failure. Possible
explanations of this type of failure include insufficient vascularization, immune destruction, toxicity of immunosuppressive drugs and/or insufficient transplanted islet mass [29].

The significant drawbacks in current treatment options have led to the investigation of alternative treatment methods. This dissertation will focus on (1) amniotic fluid stem cells as an alternative starting cell source for β-cell replacement, (2) amniotic fluid stem cell-based immunosuppression to mediate residual autoimmune processes and block rejection associated with allogeneic transplantation and (3) HNF4A deficient stem cell modeling of β-cell development in vitro for potential drug discovery assays.

Endocrine Pancreas Organogenesis

A critical factor in developing cell replacement therapies for DM is the ability to efficiently achieve glucose-responsive, insulin-producing cells for transplantation. The process of differentiating stem cells toward an endocrine pancreas cell fate is ideally based on signaling which occurs during normal development, therefore understanding pancreatic organogenesis is a critical step in differentiation of stem cells towards insulin-producing cells in vitro (Figure 1).

The first distinction in development occurs during gastrulation when the epiblast is separated into the three primary germ layers: ectoderm, mesoderm and endoderm. The endoderm is the inner-most layer and forms the lining of the gut, lung, liver and pancreas. Endoderm development is initiated by signaling with Nodal, a member of the TGF-β family of secreted growth factors. Nodal is expressed in the anterior regions of the
primitive streak and embryos containing Nodal defects do not develop mesoderm or endoderm [30]. Cells exposed to continuous high levels of Nodal signaling develop into endoderm and those exposed to lower levels become mesoderm [30]. Nodal signaling is critical in the anterior-posterior patterning of the embryo which establishes the body axis [31]. Also important in the development of definitive endoderm (DE) is Wnt3 signaling which stimulates and maintains elevated Nodal signaling [32]. Like Nodal, Wnt3 is expressed in the primitive streak and Wnt3 deficient embryos do not develop mesoderm or endoderm [33]. As a result of Nodal signaling, transcription factors such as Mix-like proteins FOXA2, SOX17 and GATA4-6 are turned on to define the cells and commit them to an endodermal fate [31].

Gut tube formation occurs after gastrulation when the pre-patterned endodermal layer elongates. The gut tube is subdivided into three main regions: foregut, midgut and hindgut. The pancreas eventually develops from the foregut region. Signaling pathways involved in establishing the gut tube include fibroblast growth factor (FGF), hedgehog (HH) and retinoic acid (RA). The notochord is in close contact with portions of the gut tube endoderm and is thought to release soluble factors that repress HH signaling and promote pancreatic development in those areas [34]. RA has been implicated in establishing the anterior-posterior (A-P) patterning of the gut which is crucial for normal development [35].

Following early endodermal patterning, the developing gut tube is further divided into organ specific areas. The pancreas arises from the foregut epithelium which also develops into esophagus, trachea, lungs, thyroid, stomach, liver and bile ducts. FGF works during this stage in a dose-dependent manner with low levels inducing PDX1
expression in the ventral pancreas and duodenum [36, 37]. BMP is another important signal at this stage originating from the septum transversum mesoderm to inhibit pancreatic development and induce hepatic development [38]. Repression of HH signaling in the notochord by FGF2 and activin allows for expression of PDX1 which causes pancreatic specification [39-42]. As development progresses and the distance between the notochord and dorsal gut tube extends, signals from the dorsal aorta, possibly VEGF, maintain PDX1 expression [43]. Expansion of the PDX1 pancreatic progenitors requires FGF10 mediated signaling which is provided in vivo by the mesenchymal cells [44].

The PDX1 expressing pancreatic progenitor cells within the gut tube endoderm proceed to branch out from the gut tube both dorsally or ventrally. These are the pancreatic buds and they contain precursor cells that can be specified to either endocrine or exocrine cell types. Interactions between the endoderm and mesoderm regulate the differentiation at this stage [45]. Following budding, the pancreatic progenitors begin to proliferate, form branches and eventually fuse to become a single organ. After bud formation and specification to the endocrine lineage, additional signals are necessary to define the type of hormone each cell will produce. NGN3 is a bHLH transcription factor that responds to Notch signaling and specifies endocrine fate. Detectable NGN3 expression is found only in those progenitor cells that will become insulin-producing β-cells [46]. However, not much is known about the extracellular signals that drive β-cell specification from the NGN3 positive pancreatic endoderm. As a result of this lack of knowledge, this final step of in vitro β-cell differentiation is the least efficient.
The adult pancreas consists of both exocrine and endocrine cell types. Exocrine cells make up about 95% of the total pancreatic mass. Clusters of endocrine cells, called the islets of Lagerhans, are embedded within the exocrine acinar cells. The function of the exocrine pancreas is to produce secretory products critical for digestion including the digestive enzymes trypsin, lipase, amylase, carboxypeptidase and bicarbonate. These enzymes are synthesized and secreted from the exocrine acinar cells and then drained to the duodenum through a network of pancreatic ducts. The islets of Lagerhans are typically comprised of 100-1000 endocrine cells, approximately 60% of which are the insulin-producing β-cells [47]. The islets also include α-cells which secrete glucagon, δ-cells which secrete somatostatin and PP-cells which secrete pancreatic polypeptide. The function of insulin is to decrease blood glucose levels by increasing glycogen synthesis in liver and muscle cells and increasing fatty acid synthesis in adipose tissue. Mature β-cells are characterized by their glucose responsive insulin release along with expression of other key factors including c-peptide, a byproduct of de novo insulin production, GLUT2, a cell membrane glucose transporter, and PDX1, a transcription factor required for β-cell function. Glucagon has complementary effects and functions to increase blood glucose levels. Somatostatin finely regulates the amount of insulin and glucagon that is released. The function of PP is unknown.

As mentioned above, the primary function of the pancreatic β-cells is production and controlled release of the hormone insulin. Insulin acts to stimulate glucose uptake and storage and primarily affecting cells of the muscle, liver and fat. In the muscle, insulin functions to enhance glucose uptake and stimulate the conversion of glucose to glycogen. In hepatocytes, insulin presence inhibits glycogenolysis and gluconeogenesis.
And in adipocytes, insulin promotes glucose conversion to glycerol which then forms triglycerides and prevents lypolysis. This process is highly regulated as β-cells are able to process and secrete insulin in response to varying amounts of circulating glucose. Glucose responsive insulin secretion is coordinated by the production of high levels of ATP with increasing glucose concentration. ATP induces potassium (K\textsubscript{ATP}) channel closure which leads to membrane depolarization, calcium channel opening and influx of calcium. High levels of intracellular calcium causes the release of insulin from the secretory vesicles in the β-cell. Chemical compounds have also been shown to have the ability to regulate insulin secretion and are currently being used clinically in the treatment of DM.

Figure 1. Endocrine pancreas organogenesis and \textit{in vitro} pluripotent stem cell differentiation. The endocrine pancreas develops in five distinct stages in response to a variety of growth factors. \textit{In vitro} pluripotent stem cell differentiation protocols aim to
recapitulate the sequence of developmental events that occur during in vivo pancreatic specification. Efficiency decreases over the course of in vitro differentiation and is represented by open circles. Reported efficiencies at the DE stage is about 90%, GTE 80%, PP 50%, PE 50% and B-cell 7-25%.

Cell replacement strategies for the treatment of diabetes mellitus

Current islet cell replacement strategies have revealed the benefit of increased β-cell numbers in the treatment of DM. However, the donor shortage prevents islet transplantation from being an option for every DM patient. Several additional cell replacement therapy strategies are being examined for potential treatment of DM.

Transplantation of stable insulin-producing cell lines is one option that has been considered a potential therapeutic cell source [48]. Attempts have been made to generate β-cell lines from several human pancreatic sources including islets, fetal pancreata and insulinomas. These attempts have been largely unsuccessful as the cells produced only low levels of insulin for very few passages [49-51]. Successful long-term culture insulin-secretion has been reported from one human insulinoma that is dependent on co-culture with trophic factors [52]. Additional attempts have been made to transduce human pancreatic tissue with oncogenes and pancreatic endocrine specific genes in attempts to achieve stable insulin-producing cell lines but have been met with varying degrees of success [53-55]. Electrofusion of human beta cells with human ductal epithelial PANC-1 cells has also reportedly resulted in development of several insulin-producing cell lines [56]. Although these insulin-producing cell lines show promise, their utility in cell-based
therapy is lacking as the extent of their insulin production and secretory responses is inferior to primary human β-cells.

An additional method of cell replacement therapy for DM involves β-cell regeneration including neogenesis, proliferation and transdifferentiation. To be a viable treatment for T1DM, β-cell regeneration would have to occur at a higher rate than the autoimmune breakdown of the cells to sustain a physiologically functional β-cell mass. Due to a lack of human pancreas regeneration models, conflicting evidence exists as to whether human β-cells have the capacity to regenerate [57-60]; however, rodent models clearly demonstrate this capacity [61]. Several studies have revealed pancreatic progenitors present in the pancreatic duct epithelium which have the ability to differentiate into β-cells [62-64]. There is also evidence for β-cell proliferation in injury models of the regenerating pancreas including partially pancreatectomized mice [65] and inducible diphtheria toxin expression in β-cells [66]. Cellular reprogramming studies reveal exocrine to endocrine transdifferentiation by overexpression of β-cell specific transcription factors PDX1, MAFA and NGN3 in the mouse pancreas [67]. More recently, forced overexpression of only PDX1 beginning at the stage of endocrine commitment was also shown to result in α-to-β cell conversion [68].

Stem cell sources for replacement insulin-producing cells

A promising alternative for the generation of transplantable insulin-producing cells includes stem and progenitor sources. Many different types of stem cells have been examined for their ability to form insulin-producing cells as a potential therapy for DM.
ES cells are derived from the inner cell mass of the blastocyst and are characterized by their self-renewal capacity and ability to differentiate into cells of all three germ layers [69]. Undifferentiated ES cells grow in tight colonies in vitro and express transcription factors OCT4, SOX2, NANOG, surface antigens TRA1-60, TRA1-81, SSEA4 and enzyme activities of alkaline phosphatase and telomerase [69]. Significant efforts by several laboratories have focused on generating insulin-producing cells from pluripotent embryonic stem (ES) cells using a multi-stage approach that mimics signaling pathways necessary for embryonic pancreatic development [70-72]. The sequential and combinatorial addition of growth factors and other small molecules replicate the stages of β-cell differentiation including definitive endoderm (DE) formation, pancreas and endocrine specification, culminating in β-cell maturation [73].

However, the usefulness of cell therapies derived from ES cells is limited. Although a great deal of progress has been made in the pursuit of developing insulin-producing β-cells from pluripotent stem cells, there is still work to be done. The differentiation process has proven more difficult than previously anticipated as a result of complex biochemical mechanisms and other cues critical in cell specification including mechanical force [74, 75], oxygenation levels [76, 77] and substrate composition [77]. Even the most efficient protocol reported to date achieves only 25% insulin positive cells with marginal glucose responsiveness [72]. One possible explanation for the lack of glucose responsiveness may be the derivation of a more primitive fetal cell rather than the mature homolog [70]. Inefficient differentiation protocols may also leave behind fully pluripotent stem cells which have the potential to form teratomas upon transplantation. Another concern of ES based cell replacement therapy is the accumulation of genomic
instabilities and chromosomal aberrations in long term culture of frequently studied ES cell lines [78]. In addition, it is likely that differentiated cells derived from allogeneic pluripotent stem cells will be subject to immune rejection due to their expression of major histocompatibility antigens [79-81].

Exciting advances in the stem cell field have demonstrated the ability to generate autologous, pluripotent stem cells from skin fibroblasts. By introducing genes important in pluripotency into the adult fibroblast it is possible to reprogram cells back to a pluripotent state [82, 83]. To date, (induced pluripotent stem) iPS cells appear to be identical to ES cells in their gene expression patterns, pluripotent differentiation capabilities and contribution to the germ line in chimeric animals [82, 83]. However, several differences in methylation patterns between iPS cells and ES cells as well as between iPS cells from different cell sources have been reported recently [84, 85]. iPS cells have the same capability to undergo in vitro differentiation to insulin-producing cells using similar developmentally based procedures [86, 87]. The advantages of this method of pluripotent stem cell generation include avoiding the ethical and moral controversy surrounding ES cell research and allowing for creation of patient specific stem cells which could potentially be modified and transplanted back into the donor without the fear of rejection. However, the relative novelty and lack of understanding surrounding the mechanisms of induced pluripotency combined with the ability of the pluripotent cells to form teratomas limits the potential clinical uses of iPS cells.

A prospective source of non-tumor forming, histocompatible stem cells capable of generating insulin-producing β-cells is adult stem and progenitor cells. Bone marrow mesenchymal stem cells (BM-MSCs) have been studied as a candidate for β-cell
replacement therapy thanks to their ease of extraction and potential to differentiate into mesenchymal, endodermal and ectodermal lineages [88-90]. Several reports have shown that BM-MSCs can produce insulin in vitro and restore normoglycemia in streptozotocin (STZ) treated mice [91-96]. Adipose-derived stromal cells have also been shown to produce insulin in vitro [97].

Methods used in attempts to enhance insulin production in adult stem or progenitor cell populations include over-expression of pancreatic genes. Ectopic expression of the pancreatic master transcription factor pancreatic duodenal homeobox 1 (PDX1) leads to in vitro insulin production in BM-MSCs and hepatic cells [54, 98-100]. PDX1 is indispensable in pancreatic development and maintenance of β-cell function and is expressed in all pancreatic precursors; however, its expression is limited to only β-cells in the mature pancreas [101, 102]. Forced expression of PDX1 in BM-MSCs from multiple patients resulted in glucose-stimulated insulin release in vitro and restored normoglycemia upon transplantation into STZ-treated diabetic mice [103]. Adult stem and progenitor cells circumvent problems identified in ES and iPS cell sources as they have low tumorigenic potential and they allow for the possibility of an autologous cell source which would not be rejected upon reimplantation. The ease of extraction and expansion of these cells is another advantage, creating a virtually unlimited cell source.

Fetal stem cell sources have also been of great interest as a result of their broad potency and lack of ethical concerns. For example, MSCs from the umbilical cord stroma [104], umbilical cord blood and Wharton’s jelly [105-107] and endometrial stroma [108] have all been reported to produce insulin without the need for genetic modification. Placenta-derived multipotent stem cells (PDMSCs) over-expressing MAFA, a
transcription factor also required for β-cell development and function, were shown to up-regulate expression of downstream genes involved in pancreatic development and could be further differentiated into glucose-responsive insulin-producing cells in vitro and in vivo [109]. Stem cells derived from amniotic fluid can also be induced to express insulin upon silencing of neuronal restrictive silencing factor (NRSF) and a two-step protocol including Activin A, bFGF and nicotinamide [110]. C-KIT selected amniotic fluid-derived stem (AFS) cells are a particularly attractive therapeutic cell source because of their broad differentiation capabilities, extensive capacity for self-renewal in culture and lack of teratoma formation [111]. Accumulating evidence reveals that AFS cells are less differentiated than adult somatic stem cells but are not as pluripotent as embryonic stem cells. AFS cells can be cultured and induced to differentiate into multiple lineages including hepatocytes (endodermal), adipocytes, chondrocytes and osteocytes (mesodermal) as well as neural (ectodermal) [111]. As a potential cell therapy, AFS cells possess two additional desired characteristics; they do not express cell surface markers associated with rejection, including CD80, CD86 and CD40 and they exhibit immunomodulatory activity [112, 113].

**Genetic modification**

Gene therapy is defined as the transfer of new genetic or therapeutic molecules into a cell to alter its function at the cellular or molecular level for therapeutic applications [114]. Some of the most successful cases of fetal derived stem cell differentiation towards insulin-producing cells have involved introduction of exogenous genes. Viral and non-viral methods have been examined to introduce genes important in
pancreatic development and function into stem cells to induce their differentiation down the pancreatic lineage.

Recombinant adenoviral vectors induce transient transgene expression and have the remarkable ability of transducing both dormant and dividing cells [115]. The complex structure of adenoviruses involves 11 individual structural proteins and over 50 serotypes [116]. Adenoviral vectors enter the cell by binding to the host cell receptor followed by clathrin mediated endocytosis and transport to the nucleus [117]. Successful adenoviral transduction of BM-MSCs and AFS cells has been reported in numerous studies [118-122]. There is some mild inflammation that may be associated with the introduction of viral vectors and non-viral gene delivery has been explored as an alternative to avoid these concerns [123].

Electroporation is a non-viral transfection technique that relies on a high voltage pulse to electropermeabilize the cell membrane. The permeable state lasts minutes after electric pulse application, allowing negatively charged DNA to be electrophoretically driven through the destabilized membrane [124]. Several factors can be changed to allow for optimal electroporation conditions including pulse strength, pulse length, amount of DNA and cell density [125]. Nucleofection is a commonly used technique based on electroporation in which DNA is delivered directly into the nucleus of the cell through a proprietary mechanism [126]. Nucleofection of BM-MSC has shown a transfection efficiency of up to 74% with cell survival of about 40% [127]; however, no reports currently exist on this method in AFS cells.
The focus of the first chapter of this dissertation will be genetic manipulation of AFS cells by nucleofection and adenoviral mediated PDX1 overexpression and subsequent *in vitro* differentiation towards insulin-producing β-cells for cell replacement therapy of DM.

**Immunomodulation**

Even with the rapid development of insulin-producing cell replacement therapies to treat DM, avoiding the immune response remains a challenge. In T1DM cases residual autoimmune reaction toward the β-cells remains, placing the transplanted insulin-producing cells in jeopardy. Additionally, if replacement insulin-producing cells are derived from an unrelated donor immunosuppressive therapy is required to prevent rejection. Immunosuppressive regimens are harsh and carry with them the risks of severe side effects including infection and malignancy. Stem cell-based immunosuppressive therapy offers the potential to mediate the autoimmune response and avoid rejection without the negative side effects associated with medication.

The autoimmune origin of T1DM results from autoantibodies directed against islet antigens including proinsulin, insulin, glutamic acid decarboxylase 65 (GAD65), islet tyrosine phosphatase II-2 (IA-2) [128]. The role of effector cells, T lymphocytes and antigen presenting cells is critical in the autoimmune process but the mechanisms causing the initiation and progression of DM lack understanding [129]. Drug-based immunosuppressive regimens have been shown to slow the loss of insulin production if administered on newly diagnosed T1DM patients [130-132]. These drugs work by interfering with cytokine production and/or T lymphocyte proliferation to mediate the
effector phase of the immune response; however, their use is limited because of toxic side effects [133]. Treatment using monoclonal antibodies against a T cell antigen (CD3) has also proven successful in reversing hyperglycemia and preventing recurring disease in these patients without the negative consequences [134, 135]. Other pharmacological approaches that have shown some level of effectiveness in improving metabolic control in recently diagnosed cases of T1DM include heat-shock protein peptide (DiaPep277) [136], polyclonal anti-T-cell therapy [137], alum-formulated glutamic acid descarboxilase (GAD-alum) [138] and the TNF inhibitor etanercept [139]. Cell-based therapies have also been examined in attempts to mediate the T1DM autoimmune response. Efforts using autologous hematopoietic stem cell transplantation to reconstitute the defective immune system and shift the balance between destructive immunity and tolerance have resulted in long-term insulin-independence with only minor adverse effects [140, 141]. Autologous umbilical cord infusion has also shown modest clinical improvements in newly diagnosed T1DM patients [142].

Another important immune response to consider in islet cell replacement therapy is the recognition and rejection of cells from a foreign donor. The rejection process can be characterized as hyperacute, acute or chronic based on the etiology, severity and timing [143]. It is the acute rejection process that occurs because of allelic differences between donor antigens and recipient antigens. Immunosuppressive regimens focused on suppressing the innate and adaptive immune response are the current standard of care and are accompanied by harmful side effects including infection and malignancy. As time and expense of personalized medicine is a current obstacle, it is likely that implanted insulin-producing cells will be from an HLA-matched donor; however, even with HLA matching
strong immunosuppressive medication is required. Stem cell-based immunomodulation may offer the ability to mediate the autoimmune response and improve graft implantation and survival without adverse side effects.

MSCs are becoming an increasingly popular form of cellular therapy for a range of autoimmune disorders and degenerative diseases due to their differentiation potential, capability to produce significant amounts of trophic factors, ability to modulate the activity of the immune cells, predisposition to home to sites of injury [144, 145]. This adherent cell population found in nearly every adult tissue, most often are obtained from bone marrow (BM-MSCs) or adipose tissue, and are being examined for multiple clinical purposes [146-150]. MSCs can give rise to differentiated cells of the mesodermal lineage including bone, fat, cartilage, tendon and muscle [151-153]. In addition, their ability to evade immunosurveillance after cell transplantation and to suppress the immune response has made BM-MSCs a particularly attractive candidate for clinical use [154, 155]. For example, it was observed that BM-MSCs could suppress lymphocyte proliferation and activation in response to allogeneic activation or chemical stimulation in vitro and in vivo [153, 156, 157].

Immunoregulation by BM-MSCs is thought to result from both direct interactions between the stromal and immune cells [158-160] and the actions of anti-inflammatory soluble factors released by the stromal cells [147, 161]. The secretion of these factors occurs in response to pro-inflammatory signals from the local environment, including IFN-γ, TNF-α, IL-1α and IL-1β [162-164]. Clinical applications for which the trophic action of BM-MSCs may prove valuable include support of hematopoietic transplantation and the treatment of graft versus host disease (GvHD), osteogenesis imperfect, and acute
myocardial infarction [165-169]. Possible mechanisms of MSC mediated immunomodulation include reducing generation and maturation of dendritic cells (DCs), increasing Treg production by producing TGF-β, promoting generation of regulatory DCs which produce IL10 and suppressing effector T cells by release of growth factors, inducible nitric oxide synthase (iNOS), heme oxygenase (HO)-1, prostaglandin or IDO. MSCs may also act by downregulating immunoglobulin production by B cells and by inhibiting NK cell cytotoxicity and proliferation [170].

The effects of human MSCs on development of DM have been examined in animal models. Studies in STZ-diabetic NOD/SCID mice revealed lower blood glucose and higher mouse insulin levels in MSC treated groups, suggesting that MSCs may be useful in enhancing insulin secretion [171]. More recently, syngenic BM-MSCs introduced into a rat model of STZ induced β-cell injury showed effective homing to the pancreas with enhanced PDX1 expression and insulin secretion from rat β-cells [172]. An effect was also seen in the host immune system with a shift towards the Th2 phenotype in peripheral T cells and increased amounts of FOXP3 positive cells. Together these results suggest anti-inflammatory and immunoregulatory effects of MSC secreted bioactive factors [172].

The first trials to show the therapeutic effect of immunomodulatory BM-MSCs was in the accelerated recovery in patients with acute GvHD [173]. A human clinical trial is currently underway to evaluate the immunomodulatory effects of Prochymal, a BM-MSC population derived by Osiris Therapeutics (Columbia, MD), in treatment of newly diagnosed T1DM. MSCs act to deplete activated T cell populations by blocking the maturation of dendritic cells and reversing the status of mature dendritic cells to
immature [174]. However, the relatively limited proliferation of BM-MSCs under standard conditions suitable for manufacture of a clinical product presents a potential drawback for their medical application [175]. For this reason, we sought to determine whether amniotic fluid-derived stem (AFS) cells, which display considerably greater expansion capacity and appear well suited to large-scale banking [111] possess comparable immunomodulatory capability.

The amniotic fluid contains multiple cell types derived mainly from exfoliating surfaces of the developing fetus [176]. These include cells from the fetal skin, respiratory system, urinary and gastrointestinal tracts, along with populations of MSCs [177]. De Coppi et al. (2007) described a novel population of multipotent stem cells from amniotic fluid obtained by immunoselection for c-Kit (CD117), the cell surface receptor for stem cell factor (SCF), and designated them amniotic fluid-derived stem (AFS) cells. AFS cells are characterized by their high capacity for self-renewal and their ability to differentiate toward lineages representative of all three germ layers including hepatocytes, osteocytes, chondrocytes and adipocytes [111, 178]. Some clonal AFS cell lines were shown to proliferate in vitro well past Hayflick’s limit (greater than 80 population doublings) with no signs of malignant transformation, chromosomal abnormalities, or loss of differentiation potential [111]. AFS cells and BM-MSCs share many characteristics including expression of the surface markers CD29, CD44, CD73, CD90 and CD105. However, AFS cells also express the more primitive stem cell marker SSEA4 [111]. The two cell types also share a similar immune antigen surface profile with positive MHC Class I expression but little to no MHC Class II expression. MSCs also have the ability to avoid allogeneic rejection [179]. We hypothesized that cells in the
amniotic fluid may have immunoprivileged status, as fetal cells must possess mechanisms to avoid destruction by the maternal immune system during development [180]. We further hypothesized that, like MSCs, AFS cells also possess immunosuppressive properties. There has been a single report that unselected mesenchymal stromal cells from amniotic fluid inhibit lymphocyte proliferation in vitro [181].

An additional attractive aspect of AFS cells is their ability to be expanded, fully characterized and stored in a cell bank. As derivation of stem cells specific to every patient may prove to be an overwhelming task, the creation of a bank of HLA-typed stem cells would reduce the chance of graft rejection upon transplantation. It has been shown that near perfect matches could be achieved with banks holding thousands of cell lines; however, a bank of this magnitude may remain out of reach [182]. Alternatively, a bank of around 150 cell lines would result in 8% of patients achieving a full match, 31% achieving a beneficial and up to 80% achieving an acceptable HLA match [182]. Even this type of partial matching will help reduce the need for immunosuppressive therapy and improve the survival of grafts in recipient patients.

The second chapter of this dissertation focuses on the ability and mechanisms of AFS cells to inhibit T-lymphocyte activation in vitro.

**Disease-specific iPS cells for drug development**

Disease modeling and the ability to study normal and pathogenic cellular processes is a critical factor in drug discovery and regenerative pharmacology. Recent developments in the stem cell field with the advance of iPS cells allows for the
opportunity to develop technologies enabling dissection of the relationship between genotype and complex disease phenotype. The ability to study the development of patient-specific pluripotent stem cells through stages of pancreatic development represents an invaluable tool to study genetic factors leading to β-cell destruction or dysfunction and an amazing platform for drug development aimed at correcting these deficiencies.

Methods of cellular reprogramming somatic cells back to pluripotent cells has been widely studied. Natural reprogramming occurs in mammalian cells immediately after fertilization when the sperm and oocyte come together and give rise to a totipotent embryo. DNA demethylation and remethylation along with histone modifications are essential for reprogramming and subsequent development however the mechanisms underlying these processes remain largely unclear [183]. Somatic cell nuclear transfer (SCNT) is one method of inducing pluripotency in which the nucleus of a somatic cell is transplanted into an enucleated zygote or an oocyte [184]. The outcome is reprogramming of the somatic nucleus resulting in a blastocyst that is a clone of the somatic nucleus donor. A major breakthrough in this technology was reported with the cloning of a sheep, ‘Dolly’, by transplanting the nucleus from a mammary gland cell into an enucleated sheep oocyte [185]. Since then other mammalian species have been cloned including cows, mice, goats, cats and pigs [186]. Pluripotent cells can also be derived through cell fusion by fusing a somatic cell with an embryonic carcinoma cell, an embryonic germ cell or an embryonic stem cell [187].

Most recently the ability to reprogram somatic cells by introduction of genes important in pluripotency has been evaluated. Human dermal fibroblasts transduced with
retroviruses containing human OCT4, SOX2, KLF4 and c-MYC were able to fully reprogram and appeared to be identical to ES cells [83]. Lentiviral delivery of the genes OCT4, SOX2, NANOG and LIN28 in addition to culture under embryonic stem (ES) cell conditions also resulted in dedifferentiation to an induced pluripotent stem (iPS) cell state [82]. These methods were shown to have the capability to reprogram a variety of adult and fetal somatic cell types including keratinocytes, hair follicle cells, and blood [188-190]. To date, iPS cells appear to be identical to ES cells in their gene expression patterns, pluripotent differentiation capabilities and contribution to the germ line in chimeric animals [82, 83]. However, it has been shown that integrating viral vectors such as retroviruses and lentiviruses randomly integrate into the genome and may induce mutagenesis at the site of integration or alter the function of surrounding genes [191].

More recent research in the iPS field has been aimed at new strategies of gene delivery that allow for excision of the integrated transgenes or that avoid integration of recombinant DNA into the host genome altogether. A Cre/loxP lentiviral vector system in which the transgenes can be excised has been used to generate iPS cells from patients with Parkinson’s disease [192]. The PiggyBac (PB) transposon system has also been used to deliver reprogramming genes only to remove them after reprogramming [193]. Successful attempts to create iPS cells have also been made with the use of adenoviral vectors [194], plasmids [195] and small molecules involved in the wnt signaling pathway [196]. The first iPS cells to be generated without genetic modification used recombinant proteins [197]. mRNAs have also been reported to successfully reprogram human foreskin fibroblasts [198].
As mentioned previously, iPS cells also have the same capability to undergo *in vitro* differentiation to insulin-producing cells, and cells of all other lineages, to the same degree as ES cells [86, 87]. However, it should be noted that, like ES cells, differences among iPS clones derived from the same donor have been reported, making this approach exceedingly technically challenging [199].

Another method to create targeted genetic deficiencies within a pluripotent cell is by RNA interference [200]. RNAi is a naturally occurring gene regulatory mechanism that is involved in regulation of gene expression. The development of the miRNA-based shRNA (shRNA mir) RNAi strategy exploits this process allowing for targeted, highly efficient long-term gene knockdown. In this system short hairpin RNAs are processed within the cells to produce siRNA which then bind to the RNA-induced silencing complex (RISC) [201]. RISC complex binding to the target site causes cleavage and degradation of the target gene, preventing translation. This powerful gene-knockdown technology has been applied in several ES studies examining the effect of gene loss of function on pluripotency and cell specification [202-204].

**MODY1/HNF4A**

The primary cause of MODY1 is impaired glucose-stimulated insulin release from the β-cells [205]. Clinically, MODY1 patients with mutations in the *Hnf4a* gene show progressive deterioration of glycemic control and fail to secrete adequate insulin in response to glucose challenge [206]. HNF4A is considered essential for normal pancreatic development and normal β-cell function and is the focus of this proposal. Mutations in HNF4A lead to a diabetes phenotype which demonstrates the essential role
of the gene in glucose homeostasis. However, even though the gene that is causing the phenotype is known, it is currently not possible to follow the development or examine the function of adult β-cells in the human patient.

HNF4A is a member of the steroid hormone receptor superfamily and is expressed in the liver, pancreatic islets, kidney and small intestine [206]. This transcription factor binds to DNA as a homodimer, recruits transcriptional coactivators and positively regulates the expression of target genes. HNF4A is made up of an N-terminal transactivation domain (AF-1), a DNA binding domain, and a complex C-terminal domain that forms a ligand-binding domain, a dimerization interface and a transactivation domain (AF-2). HNF4A has two distinct promoters which have been well characterized, P1 and P2. In the hepatocytes the P1 promoter is active, and in the mature islets the P2 promoter drives gene expression [207]. The P2 promoter contains binding sites for transcription factors important in pancreatic development, PDX1 and HNF1B, and mutations in either of these binding sites causes MODY, suggesting a role for these factors upstream of HNF4A [208, 209]. During organogenesis HNF4A is known to be expressed in the pancreatic buds of the gut tube endoderm which eventually form the pancreas, revealing a developmental function of HNF4A [210]. In the mature pancreatic islet, HNF4A is thought to function as a diverse regulator of many different transcription networks and recent studies have identified over 1000 promoter elements bound by HNF4A in islets [206]. One of these complex transcription factor networks is between HNF1B and HNF4A which are known to operate together in the mature β-cell to maintain normal function [208, 211, 212].
Several *in vitro* and *in vivo* systems have been developed to allow researchers to examine the function of HNF4A in cells with a mature β-cell like phenotype. Creation of a rat insulinoma cell line with the capacity for conditional expression of a dominant-negative mutant HNF4A revealed the importance of HNF4A expression in mitochondrial metabolism and insulin expression and resulted in down regulation of the genes mitochondrial 2-oxoglutarate dehydrogenase (OGDH) E1 subunit, glucose transporter-2 (GLUT2), aldolase B and L-pyruvate kinase (L-PK) [213]. Embryoid body (EB) formation from ES cells containing a deficiency in HNF4A expression confirms that HNF4A influences the expression of many of the same genes, including GLUT2, aldolase B and L-PK [214]. These findings suggest that HNF4A regulates multiple pathways within the mature β-cell. This is useful information detailing the effect of an HNF4A mutation on cells with a mature β-cell phenotype, however these systems have been unable to analyze the effect of HNF4A deficiency in the development of the β-cell.

Mouse models with HNF4A deficiencies have been developed to examine the *in vivo* effect of the gene. HNF4A null mice suffer embryonic death due to failure to undergo normal gastrulation which prevents analysis of the role HNF4A in mature β-cell function indicating a strong requirement for the gene in early development [215]. β-cell specific HNF4A knockout mice are able to survive to adulthood and demonstrate impaired glucose tolerance and defects in glucose-stimulated insulin-secretion [216]. Interestingly, these mice also show hyperinsulinemia, a condition which is also observed in fetal stages of the human disease [216, 217]. It is in the later stages of the human disease that hypoinsulinemia and hypoglycemia are observed. Also surprising was the finding that the levels of HNF1B were unaffected in the HNF4A knockout β-cells [216].
These results indicate that HNF4A may not be required for the continuation of HNF1B signaling in the mature β-cell but it is still very likely that HNF4A is required for the initiation of HNF1B at an earlier developmental stage. In these mice L-PK levels were also decreased in the mature β-cell, but GLUT2 and aldolase B levels were normal [216] contrary to the in vitro studies [215]. These discrepancies in gene expression may be the result of an overlap in transcriptional regulatory mechanisms that are present in the mature β-cell which can compensate for HNF1B, GLUT2 and aldolse B expression. Additional results from these studies identified KIR6.2, a potassium channel subunit that plays a role in the regulation of the K\textsubscript{ATP} channel-dependent pathway of glucose-stimulated insulin-secretion, as a target of HNF4A [216].

**Gene knock down pluripotent stem cells in disease modeling**

Due to the limited availability of human pancreatic β-cells, the study of disorders of the insulin-producing cells is limited to rodent insulinoma cell lines and animal models. However, these model systems are not ideal for adequately modeling human DM. The development of patient-specific iPS cells offers a unique model to study genetically complex disease in a background with a known phenotype. For genetic diseases such as MODY, iPS cells or knockdown ES cells, provide the opportunity to examine the pathways that lead to the disease pathogenesis based on a genetic mutation. By taking advantage of this new system, we can develop a system using pluripotent stem cells that allow us to study β-cell development and functionality in a MODY genotype.

The fully differentiated insulin-producing β-cells containing a mutation in the *Hnf4a* gene will facilitate the screening and discovery of new drugs for diabetes
treatment. The ability to produce HNF4A deficient pluripotent stem cells and fully differentiate them *in vitro* into insulin-producing β-cells would also ensure a continuous supply of pancreatic lineage cells and fully differentiated β-cells that could be used in drug discovery assays. Increasing evidence supports the theory that mutations in the genes involved in MODY are also directly involved in susceptibility to more common multifactorial forms of diabetes [218]. New gene targets, either in the mature β-cell or earlier in pancreatic development, that could be identified with this system would allow for improved understanding of the molecular defects that lead to chronic hyperglycemia and of the molecular mechanisms that maintain glucose homeostasis. This knowledge may lead to the development of more specific anti-diabetic drugs and the iPS system would allow for pharmacogenetic testing to determine the response of the subject to different drug types.

Induced pluripotent stem cells can be used to study the effects of a known genotype on the development and mature phenotype, but may prove even more useful in understanding the development of more complex genetic disorders, such as T2DM. We expect that mutations in additional genes expressed within differentiated β-cells would increase the risk of T2D and identification of these genes should lead to a better understanding of the disease as well as novel therapies (Figure 2). By identifying more genes that may be involved in the causation of DM we will be able to better identify high-risk individuals who may benefit from early interventions which may prevent the development of diabetes.

The focus of the third chapter of this dissertation is on the development of an HNF4A deficient pluripotent stem cell population to use as a model for β-cell
development. The highlight will be on gene regulation by HNF4A at the gut tube endoderm stage of development.

**Figure 2.** Medical applications of disease-specific iPS cells. Reprogramming technologies have the potential to be used to model human disease. Directed differentiation of DM patient iPS cells into the affected β-cell type allows disease modeling *in vitro*. Potential drugs can be screened on the developing and mature β-cells aiding in the discovery of novel therapeutic compounds.

**MATERIALS AND METHODS**
Cell culture. Human amniotic fluid stem (AFS) cell populations were isolated as previously described [111]. CD117 selected, cloned AFS cell lines A1 and H1 were used between passages 16 and 20 for these experiments. AFS cells were cultured on petri dishes in Minimum Essential Medium Alpha (MEM-α) (Invitrogen, Carlsbad, CA) containing 15% Embryonic Stem Cell Qualified Fetal Bovine Serum (ES-FBS), 2 mM L-glutamine and 5 units/ml Penicillin G and 5 units/ml Streptomycin sulfate (Invitrogen) and supplemented with 18% Chang B and 2% Chang C (Irvine Scientific, Irvine, CA). BM-MSC isolates were grown on tissue culture dishes Dulbecco’s Modified Eagle’s Medium (D-MEM) with 10% Fetal Calf Serum (FCS), 2 mM L-glutamine, 0.1 mM nonessential amino acids (NEAA), 5 units/ml Penicillin G and 5 units/ml Streptomycin sulfate (Invitrogen).

Primary human bone marrow mesenchymal stromal cell (BM-MSC) isolates were generously provided by Luis Solchaga (Case Western Reserve University, Cleveland, OH). Human BM-MSCs were derived and cultured as previously reported [219]. Peripheral blood mononuclear cells (PBMCs) were obtained from AllCell (Emeryville, CA). The human embryonal carcinoma cell line NCCIT, the human embryonic kidney cell line 293T, and the human hepatocellular carcinoma cell line HepG2 were acquired from American Type Culture Collection (Manassas, VA). Each of these cell lines were grown in D-MEM with 10% FCS, 2 mM L-glutamine, 5 units/ml Penicillin G and 5 units/ml Streptomycin sulfate (Invitrogen).

Primary fibroblasts from the MODY1 RW pedigree family 95 [220] were obtained from Coriell Cell Repositories (Camden, NJ). Banked fibroblasts from a 29 year old male with a MODY1 diagnosis were identified as GM01237. Fibroblasts from the
patient’s normal 42 year old sister, identification GM04422, served as a control. Primary fibroblasts were grown in Eagle’s MEM with 15% FBS, 1mM nonessential amino acids, 2mM L-glutamine, 5 units/ml Penicillin G and 5 units/ml Streptomycin sulfate.

   Human embryonic stem (ES) cell line H9 was acquired from WiCell Research Institute (Madison, WI). H9 cells were routinely cultured under feeder free conditions in on hESC qualified matrigel (BD Biosciences, San Jose, CA) coated dishes in mTeSR1 medium (Stem Cell Technologies, Vancouver, BC). Media was changed daily and cells were passaged at a 1:4 ratio every third day. To passage, cells were washed once with DMEM-F12, incubated in 1 mg/ml dispase (Stem Cell Technologies, Vancouver, BC) for 5 to 10 minutes or until the colony edges began to round, then washed 3 additional times with DMEM-F12. Colonies were then lifted off of the dish using a cell scraper, broken up into clusters containing about 100 cells each, and replated in feeder free conditions.

   **Selectable expression vector construction.** The pZL1 colonizing vector containing a mouse PDX1 gene was kindly provided by Chris Wright (Vanderbilt University, Nashville, TN). To create a plasmid expressing mouse PDX1 under the control of the SV40 promoter and the selectable mouse MHC class I surface marker H-2Kk under control of its own promoter, mouse PDX1 cDNA was excised from the cloning vector by sequential digestion with BamH1 and Sal1. pMACS Kk.II (Miltenyi Biotech) was opened by digestion with the same enzymes. Both DNAs were run on a 1% agarose gel, the 2.2 kb mouse PDX1 fragment and linearized pMACS Kk.II were excised. DNA was extracted from the gel using a Qiagen Gel Extraction kit following the
manufacturer’s instructions. DNAs were combined at a 4:1 insert:vector molar ratio and ligated overnight at 14 C using T4 DNA Ligase (New England Biolabs, Ipswitch, MA). (Figure 3).

![Selectable PDX1 expression vector](image)

**Figure 3. Selectable PDX1 expression vector.** The pMACS Kk.II vector containing mouse PDX1 cDNA was constructed, empty pMACS Kk.II vector was used as a control.

**Nucleofection.** The mouse PDX1 pMACS Kk.II or empty pMACS Kk.II vectors were introduced into AFS cells by the electroporation method, nucleofection (Lonza, Allendale, NJ). AFS cells were grown to 80% confluence, trypsinized and pelleted by centrifugation at 1,500 rpm for 5 minutes. AFS cells (5x10^6) were resuspended in 100 μl Human Epithelial Nucleofector Solution and endotoxin-free plasmid (20 μg) was added.
After gentle mixing, the solution was pipetted into an Amaxa certified cuvette. The cuvette was placed into the Nucleofector and nucleofected using a variety of test conditions. Warmed growth medium was gently added to the cells and they were cultured for 48 hours prior to analysis of transgene expression by FACS. Optimal results of high plasmid expression with low cell death were achieved with $5 \times 10^6$, 20 μg DNA and program T-23.

**Magnetic cell separation.** AFS cells were purified on the basis of H-2K$^k$ surface marker expression by immunomagnetic separation on an autoMACS separator (Miltenyi Biotech, Auburn, CA) according to the manufacturer’s instruction. Two days post nucleofection, AFS cells were trypsinized, washed with MACS buffer (PBS/0.5% BSA/2mM EDTA) and pooled. After resuspension in MACS buffer and incubation with FcR blocking reagent for 5 minutes at room temperature, AFS cells were incubated with a 1:100 dilution of anti-H-2K$^k$ MicroBeads (Miltenyi Biotech, Auburn, CA) for 45 min at 4°C. Labeled AFS cells were then washed, resuspended in 500 μl of MACS buffer and subjected to magnetic separation. The double column autoMACS program ‘Posseld’ was used to separate H-2K$^k$ positive populations from H-2K$^k$ negative populations. Cells were then placed in to culture under growth conditions or pancreatic lineage differentiation conditions.

**Adenoviral amplification.** Adenoviruses containing the mouse PDX1 (Ad-PDX1) or LacZ (Ad-LacZ) genes under the control of the cytomegalovirus (CMV)
promoter were kindly provided by Dr. Jeng-Shin Lee (Figure 4) (Harvard Gene Therapy Initiative, Harvard Univ., Boston, MA). Adenoviruses were amplified by infecting 70-80% confluent dishes of human embryonic kidney cell line 293 (HEK293) at a multiplicity of infection (MOI) of 10. After 3 to 4 days, when the majority of cells exhibited cytopathic effects, the cell suspension was collected and centrifuged at 1000 rpm for 5 minutes. The resulting pellets were lysed by three consecutive freeze/thaw cycles, the cellular debris was removed by centrifugation at 1000 rpm for 5 minutes and the viral lysate was collected and stored at -80°C.

![Diagram](image)

**Figure 4. Mouse PDX1 adenoviral vector diagram.** An adenoviral vector containing LacZ in place of PDX1 was used as a control.

**RNA extraction.** Cells grown in 35-mm tissue culture dishes were washed twice with PBS and 1 ml of RNA Bee was added to each well. Cells were incubated in RNAbee for 5 minutes on ice and then scraped with a cell scraper to disrupt the cell membranes. The lysate was then transferred to a 1.7 ml microcentrifuge tube. 200 μl of chloroform
was added and each tube was shaken vigorously. The homogenous mixture was incubated at 4°C for 10 minutes and allowed to separate into layers. The tubes were then centrifuged at 13000 rpm for 10 minutes at 4°C. The clear aqueous RNA containing layer (upper) was extracted and transferred to a new microcentrifuge tube containing an equal volume of isopropanol. This mixture was incubated at -20°C for 30 minutes and the tubes were centrifuged at 13000 rpm for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was gently washed once with 1 ml of 70% ethanol followed by centrifugation at 13000 rpm for 5 minutes at 4°C. The supernatant was removed and the RNA pellet allowed to air dry at for 10 minutes. The pellet was resuspended in 30 µl of nuclease free water. Contaminating genomic DNA was removed by treatment with DNase I (Roche Applied Science, Indianapolis, IN). To each RNA sample 3 µl of 10x DNase I buffer and 1 µl DNase I were added and incubated at 37°C for 30 minutes. After this incubation 5 µl of DNase inactivation reagent was added and incubated for 2 minutes. The sample was then centrifuged at 13,000 rpm for 5 minutes, the supernatant transferred to a new microcentrifuge tube and stored at -80°C.

**cDNA synthesis.** Supercript II (Life Technologies, Grand Island, NY) was used to perform reverse transcriptase polymerase chain reaction. 1 µg of RNA was added to a 0.2 ml tube and the volume brought up to 10 µl with nuclease free water. To prime the RNA for cDNA synthesis, 1 µl of 10 mM dNTPs and 1 µl of 150 ng/ml random primers was added. Samples were incubated at 65°C for 5 minutes and cooled on ice for 2 minutes. After cooling, 4 µl of 5X first strand buffer, 2 µl of 0.1 M DTT and 1µl of RNAse OUT was added. After incubation at room temperature for 1 minute, 1 µl of
Superscript II was added. Tubes were then transferred to a thermal cycler and incubated at 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes. Upon completion, 80 μl of nuclease free water was added to each cDNA sample and they were stored at -20°C.

**Quantitative and Semi-Quantitative PCR.** Taqman assays (Applied Biosystems, Carlsbad, CA) or SYBR Green assays and an ABI 7900 were used to quantify the absolute levels of a specific cDNA sequence by measuring fluorescent signal. Taqman assays are made up of 3 oligonucleotides, 2 primers which bind to the end of the sequence to be amplified and a labeled probe which hybridizes to the sequence between the primers. Taqman probes are covalently bonded to a 6-carboxyfluorescein (FAM) fluorophore at the 5’ end and a 6-carboxy-tetramethylrhodamine (TAMRA) quencher at the 3’ end. When the quencher is in close proximity to the fluorophore, the fluorescent activity is quenched. To complete quantitative PCR, 12.5 μl of 2x TaqMan Universal Master Mix (Applied Biosystems) was combined with 2.5 μl of TaqMan primer probe set, 10 μl of nuclease free water and 1 μl of cDNA. SYBR Green is a DNA binding dye which fluoresces upon binding. Quantitative PCR was completed by combining 12.5 μl of 2x SYBR Green Master Mix (Applied Biosystems), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 9.5 ul of nuclease free water and 1 μl of diluted cDNA.

After thorough mixing, samples were pipetted in duplicate into a 96-well reaction plate for a final reaction volume of 12.5 μl. This process was repeated for each gene and
sample to be tested. After loading, the plate was sealed and briefly centrifuged. It was then placed into the ABI Prism 7900 which performed the following cycles: 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. The ABI 7900 quantified the accumulation of the amplicon after each amplification cycle and levels of the housekeeping gene GAPDH were used as a control for normalization.

Semi-quantitative PCR was performed in buffer, 25 mM MgCl₂, 50 μM dNTP mix, 200 nM forward primer, 200 nM reverse primer along with 1 μl of diluted cDNA in a final volume of 25 μl. Following an initial denaturation step, the following steps were cycled 34 times: 30 seconds denaturing 95°C, 30 seconds annealing at 52-60°C (see Table), 1 min elongation at 72°C, followed by a final elongation step at 72°C for 10 minutes. Reactions were carried out in a Master Cycler Pro (Eppendorf, Hauppauge, NY). Amplification products were run on 1% agarose gels containing 0.5 μg/ml ethidium bromide and visualized using a Gel Logic 200 UV transilluminator imaging system (Kodak, Rochester, NY).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>TaqMan Probe</th>
<th>Relevance to Pancreas Development / Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Hs99999905_m1</td>
<td>Housekeeping gene</td>
</tr>
<tr>
<td>mPDX1</td>
<td>Mm00435565_m1</td>
<td>Transcription factor critical in β-cell development, maturation and function [221]</td>
</tr>
<tr>
<td>hPDX1</td>
<td>Hs00426216_m1</td>
<td>Transcription factor critical in β-cell development, maturation and function [221]</td>
</tr>
<tr>
<td>FOXA2 / HNF3B</td>
<td>Hs00232764_m1</td>
<td>Transcriptional activator with role in specification of definitive endoderm, regulates expression of genes important in glucose sensing [222]</td>
</tr>
<tr>
<td>SOX17</td>
<td>Hs00751752_s1</td>
<td>Critical for definitive endoderm formation, regulates Wnt signaling and activates endodermal genes including HNF1B [223]</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Hs00237052_m1</td>
<td>Chemokine receptor specific for stromal cell-derived</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Forward</td>
<td>Reverse</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>CD184</td>
<td>factor-1 (SDF-1), expressed in definitive endoderm [224, 225]</td>
<td></td>
</tr>
<tr>
<td>OCT-3/4</td>
<td>Hs00742896_s1</td>
<td>Transcription factor critical in stem cell self-renewal and maintenance of pluripotency [226]</td>
</tr>
<tr>
<td>HNF4A</td>
<td>Hs01023298_m1</td>
<td>Regulates pancreatic gene expression, known role in β-cell function, deficiency results in MODY1 [214]</td>
</tr>
<tr>
<td>HNF1B</td>
<td>Hs01001602_m1</td>
<td>Directs endodermal gene expression including HNF4A [227], deficiency results in MODY5 [228]</td>
</tr>
<tr>
<td>NGN3</td>
<td>Hs00360700_g1</td>
<td>Required for specification of the endocrine lineage [229], initiates islet differentiation [230]</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Hs00197884_m1</td>
<td>Transmembrane protein enabling bidirectional glucose transport across cell membranes, expressed in β-cells [231]</td>
</tr>
<tr>
<td>INSULIN</td>
<td>Hs02741908_m1</td>
<td>Hormone produced by the β-cells to regulate blood glucose levels</td>
</tr>
<tr>
<td>LGR5</td>
<td>Hs00173664_m1</td>
<td>Wnt target gene, marks intestinal stem cells [232]</td>
</tr>
<tr>
<td>NEUROD</td>
<td>Hs00159598_m1</td>
<td>Expression important in endocrine pancreas development [233], critical for achieving and maintaining β-cell maturity[234], deficiency results in MODY6</td>
</tr>
<tr>
<td>GCG</td>
<td>Hs00194967_m1</td>
<td>Hormone produced by the α-cells of the pancreas to increase blood glucose levels</td>
</tr>
<tr>
<td>PPY</td>
<td>Hs00237001_m1</td>
<td>Polypeptide secreted by the PP cells of the endocrine pancreas, inhibits pancreatic exocrine secretion [235]</td>
</tr>
<tr>
<td>HNF1A/TCF1</td>
<td>Hs00167041_m1</td>
<td>Transcription factor essential in maintenance of insulin storage and glucose mediated release, deficiency results in MODY3 [236]</td>
</tr>
</tbody>
</table>

Table 1. Primer/probe set of genes associated with pancreatic development and function screened by TaqMan quantitative PCR.
Table 2. Primer sequences for genes associated with pancreatic development and function screened by SYBR Green quantitative PCR.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CTGCTTCACCACCTTCTTG</td>
<td>CCACTGGCGTCTTACCAC</td>
<td>501 bp</td>
</tr>
<tr>
<td>mPDX1</td>
<td>TGTAGGCAGTACGGGTCTTC</td>
<td>CCACCCCATTTAACAGCCTC</td>
<td>325 bp</td>
</tr>
<tr>
<td>hPDX1</td>
<td>CCACGCAGCTTTACAAGGAC</td>
<td>TGTAGCCGCGGTGGGTCCGC</td>
<td>432 bp</td>
</tr>
<tr>
<td>H-2Kk</td>
<td>CCACGTTTTTCAGGTCTTCTG</td>
<td>TCATCTCTGTGGCTACGTG</td>
<td>335 bp</td>
</tr>
<tr>
<td>PAX6</td>
<td>GCCAAATGGAGAAGAGAAGAA</td>
<td>GTTGAAGTGGTGCGCGAGG</td>
<td>394 bp</td>
</tr>
<tr>
<td>LacZ</td>
<td>GAGAATCCGACGGGTGTTTA</td>
<td>CACGAGACAGACATTTTCAAC</td>
<td>681 bp</td>
</tr>
<tr>
<td>Adenoviral vector</td>
<td>GAGCGCCACTTCTTTTTGTCC</td>
<td>CCCAACTGCGACCTTACAGAT</td>
<td>330 bp</td>
</tr>
<tr>
<td>NANOG</td>
<td>GCTTGCGTGTGTTGAAGCA</td>
<td>TTCTTGACTGGGGCCTTGTC</td>
<td>256 bp</td>
</tr>
<tr>
<td>SOX2</td>
<td>AGTCTCAAACGGCAGCAAGAA</td>
<td>GGAAGTGGGTGATCGAACA</td>
<td>410 bp</td>
</tr>
<tr>
<td>C-KIT</td>
<td>GATGACGAGTGGCCCTAGA</td>
<td>CAGGTAAGTCGAGCGTCTCTC</td>
<td>223 bp</td>
</tr>
</tbody>
</table>

Table 3. Primer sequences for genes examined by semi-quantitative PCR.

Interferon-gamma ELISPOT analysis (Phytohaemagglutinin (PHA) activation assay). Elispot analysis was performed as described previously by Maitra et al. [157]. Briefly, Multiscreen filter 96-well plates for Elispot (Millipore, Billerica, MA).
were coated with human interferon - gamma (IFN-γ) capture antibody (2G1; Endogen, Rockford, IL). Peripheral blood mononuclear cells (PBMCs) were acquired from AllCells (Emeryville, CA), activated with 5 μg/ml PHA (L1668; Sigma, St. Louis, MO) and cultured in the IFN-γ coated wells with increasing stem cell densities or stem cell conditioned medium. Experiments examining cell contact included wells with 150,000 PBMCs, 5μg/ml PHA (L1668; Sigma, St. Louis, MO) and increasing amounts of AFS cells or BM-MSCs (“stem cells”) ranging from 4,688 (1:32 stem cell to PBMC ratio) to 75,000 stem cells (1:2 stem cell to PBMC ratio). Controls included unstimulated PBMCs and stimulated PBMCs with no stem cells. A minimum of quadruplicate wells for each condition was analyzed in at least 3 independent experiments and a single representative experiment is shown. After 24 hours, plates were washed and incubated with biotinylated detection antibody (B133.5; Endogen, Rockford, IL) for 2 hours at 37 C. Streptavidin-Horse Radish Peroxidase (HRP) (P0397; Dako, Carpinteria, CA) was then added and incubated for 1 hour at room temperature. IFN-γ spots were visualized after incubation with 3-amino-9-ethylcarbazole (34004; Pierce, Rockford, IL). An Elispot image analyzer was used to quantify the number of spots in each well (Immunospot Cellular Technology, Shaker Heights, OH). The percent activation for a given condition was derived as the ratio of the mean spot number to the mean spot number of the positive control, multiplied by 100.

**Transwell experiments.** Monolayers of AFS cells or BM-MSC were cultured in the presence of total blood monocytes separated by trans-well chambers (Corning, Corning, NY) or in the presence of IL-1β. In experiments examining the effect of
conditioned medium on the immune assay each well included 150,000 PBMCs, 5µg/ml PHA and 24 hour conditioned medium from one of the following sources: 1) Basal medium, 2) total blood monocytes, 3) AFS cells, 4) AFS cells cultured with total blood monocytes, 5) AFS cells cultured with IL-1β, 6) BM-MSCs, 7) BM-MSCs cultured with total blood monocytes, 8) BM-MSCs cultured with IL-1β. Controls included unstimulated PBMCs, which produced no IFN-γ, and stimulated PBMCs in growth medium, which produced the maximum amount of IFN-γ. A minimum of quadruplicate wells for each condition was analyzed in at least 3 independent experiments and a single representative experiment is shown. The Elispot plate was developed and analyzed as described previously.

**Cytokine secretion by antibody array.** Cell-free supernatants from cells cultured under the eight conditions outlined above were analyzed for cytokine secretion using Cytokine Antibody Array V, VI, VII (RayBiotech, Norcross, GA). Antibodies against each of 174 cytokines, chemokines and growth factors were spotted onto the array membrane in duplicate. After blocking for 30 minutes, the membranes were incubated with 200µl of supernatant at room temperature for 2 hours. Then primary biotin-conjugated antibody was added to each well and incubated at room temperature for 2 hours. Horseradish peroxidase-conjugated streptavidin was then added to each well and incubated at room temperature for 30 minutes. The wells were developed by addition of detection buffer and analyzed using a luminescent image analyzer system (GenePix4000B, Axon Instruments, Union City, CA).
**Semi-Quantification and analysis of cytokine array data.** Semi-quantification of cytokine levels was achieved using Axon software. The pixel density of each spot was measured and background levels from negative controls were subtracted. The intensity of positive control spots was used to normalize results between the three membranes. The intensity for each cytokine was then averaged over the duplicate spots. Controls of serum containing medium, total blood monocytes cultured alone or stem cells cultured alone were included in the array and these values were subtracted as background from the appropriate samples.

**Statistical analyses.** Results are expressed as mean +/- standard deviation. The statistical significance was determined by one way analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.

**Flow cytometry.** Cells were trypsinized, washed and resuspended in PBS/1% FBS. FcR Block (Miltenyi Biotech, Auburn, CA) was added and incubated for 5 minutes. Primary antibodies conjugated to FITC- or PE- were added in saturating amounts and incubated on ice for 30 minutes in the dark. Controls included unstained cells and corresponding isotype controls. Cells were washed once, resuspended in PBS/1%FBS and run on a Becton Dickinson FAC-Scan flow cytometric system (BD Biosciences, San Diego, CA). Analysis was completed using FlowJo v7.6.1 Software (FlowJo, Ashland, OR).
**Immunostaining.** Fluorescent antibody staining was used to visualize protein presence and localization in cell cultures. Cells cultured on glass slides were fixed with 4% PFA in PBS for 15 minutes at room temperature, washed 3 times with PBS and permeabilized by incubation with 0.1% TritonX-100 for 5 minutes. Following 3 additional PBS washes, cells were incubated with Dako Blocking Reagent (Dako, Carpinteria, CA) for 1 hour at room temperature to block nonspecific binding. Cells were then incubated with primary antibody (see Table) diluted in Dako Diluent (Dako, Carpinteria, CA) overnight at 4 C. After 3 PBS washes, fluorescently conjugated secondary antibody (see Table) diluted in Dako Diluent was incubated with the cells for 1 hour at room temperature in the dark. Cells were then washed 3 times with PBS, mounting medium containing DAPI was dropped onto the plates (Vector, Burlingame, CA) and coverslips were placed over the cells.

**Western blotting.** Cells were washed with PBS and a small amount of lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 1% NP-40, 10% glycerol) containing fresh Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN) was added to each dish. Plates were incubated on ice for 10 minutes, cells were scraped from the dish and transferred to a microcentrifuge tube. Lysates were passed through an 18-gauge needle several times and incubated on ice for 30 minutes. Lysates were then centrifuged at 13,000 rpm for 10 minutes to pellet the cellular debris. Supernatant was collected and stored at -80 C. Lysates were separated by SDS-PAGE using 4-15% Tris-HCl gradient gels (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred to a PVDF membrane using the iBLOT system (Bio-Rad Laboratories, Hercules, CA). Membranes
were blocked in blocking solution (5% milk in PBS/0.5% Tween-20). They were then incubated with primary antibody (see Table) diluted in blocking solution overnight at 4°C. Membranes were washed 3 times for at least 5 minutes each in PBS/0.5% Tween-20. Secondary antibody conjugated with horseradish peroxidase (HRP) was diluted in blocking solution (1:2000) and incubated with the membrane for 1 hour. Following three 5 minute washes in PBS/0.5% Tween-20, ECL Plus Western Blotting Substrate was added for detection (Pierce, Rockford, IL). Membranes were visualized using chemiluminescence on the Fujifilm LAS-3000 imaging system (GE Healthcare, Piscataway, NJ). Densitometry was performed using Multi Gauge 3.0 software (Fujifilm).

<table>
<thead>
<tr>
<th>Antibody</th>
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<tr>
<td>OCT4 (C-10)</td>
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<td>Mouse monoclonal</td>
</tr>
<tr>
<td>SOX2</td>
<td>Sigma, S7693</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>NANOG</td>
<td>RnD Systems, AF1997</td>
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</tr>
<tr>
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<td>Abcam, ab36175</td>
<td>Goat polyclonal</td>
</tr>
<tr>
<td>SSEA-4</td>
<td>BD, 560218</td>
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</tr>
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</table>

**Table 4. Antibody list.**

**shRNA subcloning.** HNF4A shRNAs were extracted from the GIPZ lentiviral vector and transferred to the TRIPZ inducible lentiviral vector. HNF4A GIPZ 239555, 377096 and 93152 and NeuroD TRIPZ 404996 were digested with Mlu I and Xho I. DNAs were run on a 1% agarose gel and HNF4A shRNAs (354 bp) and TRIPZ (13 kb) were gel extracted using the Qiagen Gel Extraction Kit and following the manufacturer’s instructions. HNF4A shRNA inserts were combined with the TRIPZ vector at a molar ration of 4:1 and incubated with T4 DNA Ligase (New England Biolabs) at 14°C overnight. Clones were chosen and verified by sequencing (Figure 5).
Figure 5. Human TRIPZ HNF4A shRNAmir lentiviral vector construction. Three individual plasmids were constructed, each containing a different HNF4A shRNA target sequence.
Table 5. Target sequences of HNF4A shRNAs.

**DNA sequencing.** DNA to be sequenced was combined with sequencing buffer, reaction mix and a single primer. The mixture was heated to 96 C for 3 min then cycled through 10 sec at 96 C, 6 sec at 50 C and 4 min at 60 C for 30 cycles. The PCR product was precipitated by addition of 125 mM EDTA, 3 M sodium acetate and 100% ethanol, centrifugation at 13,000 rpm for 15 min and a final 70% ethanol wash. The precipitated DNA was resuspended in formamide, heated for 2 min and loaded onto the ABI sequencer. Sequences were analyzed using the program.

Table 6. Sequences of primers used for DNA sequencing.

**Induced Pluripotent Stem (iPS) Cell Derivation**
**Cellular extract preparation.** Cell lysate was prepared from the embryonal carcinoma cell line, NCCIT. Cells were trypsinized, washed twice with PBS and resuspended in cold cell lysis buffer (50 mM NaCl, 5 mM MgCl2, 100 mM HEPES pH 8.2, 1 mM DTT, 0.1 mM PMSF) with fresh Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN) added. Cells were incubated on ice for 30 minutes, then sonicated 4 times 10 seconds each. Lysate was transferred to a microcentrifuge tube and centrifuged 13,000 rpm for 10 min at 4° C. Cleared supernatant was collected and stored at -80° C.

**Cellular extract reprogramming.** To reprogram, AFS and HEK293 cells were trypsinized, washed twice with PBS and once with HBSS. Cells were resuspended in cold HBSS, add 280 ng/ml streptolysin-O (SLO) and 50 μg/ml Texas Red to control tubes. Mix gently and incubate at 37° C for 50 minutes. Tubes were centrifuged, supernatant removed and cells resuspended in growth medium or NCCIT extract with ATP regenerating system (ATP:GTP:creatine kinase:phosphocreatine) and 25 mM NTPs.. Following a 1 hour incubation at 37° C, pre-warmed growth medium and 2 mM CaCl2 was added and cells were plated onto tissue culture dishes or mitomycin C (MMC) inactivated SNL (immortalized mouse 3T3 fibroblasts secreting LIF) feeder-layers. Medium was changed 4 hours after plating to fresh growth medium. Colonies began forming within one week and were assayed.
**Lentiviral preparation.** A lentiviral construct containing human OCT4, KLF4, SOX2 and c-MYC genes connected by linkers followed by IRES dTomato (Figure 31A) was kindly provided by Christopher Baum (Hannover Medical School, Germany). HEK293 cells were plated at a density of 6×10^6 into gelatin coated 10 cm tissue culture dishes. Two days after plating, cells were transfected with 6 μg psPax2 (Addgene 12260), 6 μg pMD2.G (Addgene 12259), 16 μg OSKM mixed with 1.35 ml OptiMEM and 56 μl FuGene HD (Roche Applied Science, Indianapolis, IN). The medium was changed 24 hours later to mTeSR1 and the viral supernatant collected after incubation for 24 hours and again at 48 hours. The viral supernatants were then pooled, filtered through a 0.45 μm syringe filters and concentrated using a 100,000 molecular weight cutoff spin device which was centrifuged at 3,500 rpm for 40 minutes at 4 C (Millipore, Billerica, MA). Virus was aliquoted and stored at -80° C.

**Lentiviral reprogramming.** Primary adult fibroblasts obtained from a MODY1 patient who has a Q268X mutation in the HNF4A gene (GM01237) and normal sibling control (GM02244) (Coriell Cell Repository, Camden, NJ) were plated at a density of 1e5 per well of a 6 well dish. The next day cells were transduced with 50 μl of concentrated OSKM lentivirus in 500 μl of growth medium containing 8 μg/ml polybrene. Following 3 hours of incubation with gentle rocking every 30 minutes, 1.5 ml of growth medium was added to each well. Growth medium was changed every other day and 5 days post-transduction cells were transferred to feeder layers, medium was changed to mTeSR1 and replaced daily. Approximately 20 days post-transduction single colonies were selected, cloned and expanded under feeder-free conditions.
**Alkaline (AP) staining.** Cloned iPS cell populations were assayed for AP expression after 5 or more passages. Media was aspirated and colonies were washed twice with PBS. Colonies were fixed with 100% ethanol for 5 minutes. Following 3 PBS washes, staining solution (0.25% Napthol AS-MX phosphate alkaline solution (Sigma), Fast Red TR salt (Sigma) in ultrapure water) was added. Following a 30 minute incubation, dishes were washed twice with PBS and AP positive colonies were visualized by light microscopy.

**Pancreatic lineage differentiation.** Induction of pancreatic lineage differentiation was achieved by addition of growth factors to pluripotent stem cells, ES and iPS cells, in a step-wise fashion mimicking the normal *in vivo* development of the pancreas. A modification of the original report from D’Amour et al.[70] with elements of the Jiang et al. [72] optimization included was used for this study (Figure 6). Pluripotent stem cells were induced through stages corresponding to definitive endoderm (DE), gut tube endoderm (GTE), pancreatic precursors (PP), pancreatic endoderm (PE) and finally to β-like cells. Recombinant human Activin A (AA) (100 ng/ml), Wnt3a (25 ng/ml), FGF7 (50 ng/ml), Noggin (50 ng/ml) were purchased from Peprotech (Rocky Hill, NJ). KAAD-Cyclopamine (0.25 uM) was purchased from Stemgent (San Diego, CA). Retinoic acid (RA) (2 uM) was purchased from Sigma-Aldrich (St. Louis, MO).
Epidermal growth factor (EGF) (50 ng/ml) and Exendin-4 (50 ng/ml) and DAPT (10 uM) were purchased from R&D Systems (Minneapolis, MN).

<table>
<thead>
<tr>
<th>Stage 1</th>
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<th>Stage 3</th>
<th>Stage 4</th>
<th>Stage 5</th>
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<td>RPMI 100ng/ml Activin A 25ng/ml Wnt3A</td>
<td>RPMI 0.2% FBS 100ng/ml Activin A</td>
<td>DMEM 1% B27 50ng/ml Noggin 50ng/ml FGF7</td>
<td>DMEM 1% B27 50ng/ml Noggin 50ng/ml FGF7 2uM RA 0.25uM KAAD</td>
<td>DMEM 1% B27 50ng/ml Exendin 10uM DAPT</td>
</tr>
<tr>
<td>1 day</td>
<td>2 days</td>
<td>2 days</td>
<td>4 days</td>
<td>5 days</td>
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![Figure 6. Schematic of step-wise differentiation procedure to achieve insulin-producing cells in vitro. Stage of differentiation, growth factors and medium components, and key markers expressed at each stage are represented.](image-url)
CHAPTER ONE

DEVELOPMENT OF INSULIN-PRODUCING CLUSTERS FROM PDX1 TRANSDUCED HUMAN AMNIOTIC FLUID STEM CELLS

The purpose of the experiments outlined in this chapter was to develop a novel source of renewable insulin-producing cells for cell-based therapy in the treatment of diabetes mellitus (DM). Amniotic fluid stem (AFS) cells were examined for their ability to respond to pancreatic developmental cues. We then developed methods to introduce the pancreatic regulatory gene, PDX1, into AFS cells using nucleofection and adenoviral transduction. In vitro differentiation of PDX1 expressing AFS cells was performed and cellular phenotype, gene expression and ability to produce insulin was examined.

Characterization of AFS cells

AFS cells were selected for C-KIT expression, cloned and cultured as a homogeneous, adherent cell population (Figure 7). Prior to experimentation, cells were analyzed by flow cytometry to verify the presence of surface markers characteristic of pluripotency and the absence of surface markers associated with lineage restriction (Figure 8). Pluripotent stem cells, including ES and iPS cells, express high levels of SSEA4, a surface marker which is also present on 91% of AFS cells. Analysis of additional markers revealed that 100% of AFS cells express the hyaluronic acid receptor
CD44 and 100% express the enzyme CD73. AFS cells also express high levels (100%) of the stem cell marker CD90 which is found on hematopoietic stem cells [237], hepatic progenitor cells [238] and human endometrial stem cells [239]. The TGF-β superfamily receptor CD105 is highly expressed on hematopoietic stem cells as well as AFS cells (100%). Other surface markers associated with pluripotency and multipotency were completely absent in the AFS cells including TRA1-60, TRA1-81. AFS cells were negative for the known hematopoietic marker CD45 (2%) and CD34 (2%). In their undifferentiated state, AFS cells demonstrated robust expression of embryonic and mesenchymal associated membrane-bound surface antigens.

![Figure 7. AFS cell morphology under growth conditions.](image)

**Figure 7. AFS cell morphology under growth conditions.** To maintain potency AFS cells are grown on petri dishes in Chang medium and passaged when they reach 70% confluency.
Figure 8. Human AFS cells express surface marker profiles consistent with stem and progenitor cell types. Flow cytometric analyses of AFS cells in growth conditions for up to 24 passages by fluorescently labeled antibodies to SSEA4, CD44, CD73, CD105, CD34 and CD45. Gate M1 was drawn based on expression of appropriately matched isotype controls.
Human AFS cell response to directed definitive endoderm differentiation

High levels of Activin A and low levels of serum, conditions which mimic in vivo signaling occurring during the endodermal stage of development, have proven to effectively differentiate pluripotent ES and iPS cells towards definitive endoderm [70, 240, 241]. Pluripotent AFS cells have have shown the ability to differentiate into cell types representative of all three germ layers in vitro [111]. The microenvironment is a critical factor in pancreatic development and the extracellular matrices that make up the pancreas include fibronectin, laminin and collagen IV [242, 243]. In an attempt to achieve efficient endodermal differentiation from AFS cells, a variety of extracellular matrix coatings were combined with Activin A addition.

The differentiation protocol applied to AFS cells is a slight modification of the protocol developed by D’Amour et al. (2007) which effectively induces insulin expression from pluripotent stem cells in a 5 stage process [70]. The first stage of our modification involves plating AFS cells onto extracellular matrices, cells are then cultured in medium with low serum (0.2-2%) and high levels of Activin A (100 ng/ml) for 4 days (Figure 9A). Compared to AFS cells under normal growth conditions, Activin A exposure resulted in modest upregulation of the endodermal transcription factor FOXA2 on each surface coating (Figure 9B). However, very little expression of other factors critical in this lineage, SOX17 and CXCR4, was induced (Figure 9B). As a definitive endoderm cell population was not achieved by exposing AFS cells to factors successful in pushing other pluripotent stem cells towards this lineage, methods of inserting the master pancreatic regulatory gene PDX1 to force pancreatic lineage differentiation were examined.
Figure 9. Treatment of AFS cells with Activin A induces low expression levels of some genes associated definitive endoderm. A. Schematic of definitive endoderm induction protocol applied to AFS cells. AFS cells were plated on a variety of extracellular matrices, exposed to high levels of Activin A in the presence of low levels of serum for 4 days. B. Quantitative PCR was performed to detect transcript levels of genes characteristic of definitive endoderm under each condition with normalization to undifferentiated AFS cells cultured under growth conditions. Gene expression levels
were normalized to GAPDH and undifferentiated AFS cells under growth conditions were used as a baseline for comparison.

Efficient introduction of a selectable PDX1 plasmid to AFS cells

*Pdx1* is a known master regulator gene that is required in pancreatic development from the gut tube [244-246]. Over-expression of *Pdx1* by plasmid transduction in to AFS cells may push them to differentiate toward the pancreatic lineage. To achieve a pure population of PDX1 expressing AFS cells, the vector also contained a truncated version of the cell surface antigen mouse H-2K<sup>k</sup> (murine MHC I) to allow for surface marker based magnetic cell sorting. Delivery of the PDX1-H-2K<sup>k</sup> or H-2K<sup>k</sup> control plasmids to AFS cells was performed by a form of electroporation called nucleofection. Cell-specific reagents and protocols provided by the manufacturer (Amaxa) do not include AFS cells. We therefore optimized the nucleofection conditions to achieve maximal viability and efficiency. Endothelial cell reagents were used and the nucleofection program controlling the pulse length and voltage, cell number and DNA concentration were varied.

Initial transfection experiments of nucleofecting PDX1 H-2K<sup>k</sup> or empty H-2K<sup>k</sup> constructs into human AFS cells and flow cytometry analysis confirmed that the H-2K<sup>k</sup> protein is expressed on the cell surface in both populations (data not shown). To determine the optimal nucleofection program, several were tested for their ability to effectively deliver the mPDX1-H-2K<sup>k</sup> vector into AFS cells. Flow cytometry for examination of H-2K<sup>k</sup> surface marker expression 48 hours after nucleofection revealed several programs that achieved around 50% efficiency including T20, T23, U21 and U23
(Figure 10). Under these conditions a considerable amount of cell death (>25%) was also observed immediately after the procedure, however 48 hours after the procedure light microscopy revealed normal cell morphology with no shrinkage or cell rounding which might be associated with continued cell death. Based on the overall AFS cell quality and continued growth as observed by light microscopy, program T23 was chosen for future experiments.

Further optimization by varying the AFS cell number and DNA concentration to be nucleofected was performed. As there was upwards of 25% AFS cell death, even under the most optimal nucleofection program, we increased the cell number to 5 million or 10 million cells with the goal of attaining a higher number of viable cells after nucleofection. Flow cytometry on the H-2K<sup>k</sup> surface antigen performed 48 hours after nucleofection revealed 70% efficiency in the condition with 5x10<sup>6</sup> and 20 μg of DNA (Figure 11). As this was the highest efficiency achieved we continued with these conditions for further experimentation.
Figure 10. Optimization of nucleofection program for introduction of a PDX1 expressing H-2K^k vector into AFS cells. Nucleofection programs with varying electrical pulse time and pulse strength were examined based on those recommended for primary epithelial cells to determine the most efficient for plasmid DNA delivery into AFS cells. Flow cytometry of fluorescently labeled antibodies against the H-2K^k surface antigen was performed 48 hours post nucleofection to quantify efficiency.
Figure 11. Optimization of cell number and DNA concentration for introduction of a PDX1 expressing H-2K\textsuperscript{k} plasmid into AFS cells. To achieve a large number of viable H-2K\textsuperscript{k} expressing AFS cells starting cell number and amount of DNA were varied while maintaining program T-23. Flow cytometry of a fluorescently labeled antibody against the H-2K\textsuperscript{k} surface antigen was performed 48 hours post nucleofection to quantify efficiency and viability was determined based on visual analysis of the number of cells attached and the rate of cell growth.

Enrichment of transfected, H-2K\textsuperscript{k} expressing AFS cells by immunoselection

To achieve pure population of AFS cells expressing either H-2K\textsuperscript{k} alone or PDX1 and H-2K\textsuperscript{k}, magnetic sorting was performed. A stringent sorting program consisting of a two column selection was chosen to achieve the highest amount of purification. The
percentage of AFS cells expressing the H-2K<sup>k</sup> surface antigen 48 hours post nucleofection was compared before and after magnetic purification. Nucleofected cells labeled with magnetic beads conjugated to the H-2K<sup>k</sup> surface marker were passed through the columns under a magnetic field and then labeled again with a fluorescently conjugated antibody against the magnetic bead and analyzed by flow cytometry. Prior to magnetic purification, the optimized nucleofection conditions were performed and a transfection efficiency of 67% was achieved for the H-2K<sup>k</sup> plasmid and 74% for the PDX1 H-2K<sup>k</sup> plasmid (Figure 12A). Upon stringent magnetic selection, the percentage of cells expressing the H-2K<sup>k</sup> transgene increased to 99% in both groups (Figure 12B). As a result of the rigorous sorting procedure a percentage of cells expressing low levels of the H-2K<sup>k</sup> surface marker, 33% H-2K<sup>k</sup> and 35% PDX1 H-2K<sup>k</sup>, were emitted in the flow-through portion (Figure 12C).
Figure 12. Purification of human AFS cells transfected with empty H-2K^k control or mPDX1 expressing H-2K^k vector. Flow cytometry analysis of H-2K^k expression. A. AFS cells transduced with the expression plasmid are 67-74% positive. B. AFS cells transduced with the expression plasmid and purified by magnetic immunoselection on autoMACS are 99% positive. C. The flow through fraction retains 33-35% of those AFS cells expressing low levels of H-2K^k.
Transfected AFS cells demonstrate transient transgene expression

Expression of PDX1 is not continuous throughout pancreatic development, but instead occurs in two distinct phases [247, 248]. The first phase of expression is in the dorsal and ventral pancreatic buds, PDX1 expression then decreases during endocrine lineage specification and reappears in endocrine cells possessing a β-cell fate. Our goal was to exploit ectopic PDX1 expression to induce pancreatic lineage commitment from AFS cells. We hypothesized that after this initial commitment, growth factors and medium components could take over to allow further differentiation to proceed as in normal pancreatic development. In this case, transient PDX1 expression is critical so that committed, partially differentiated AFS cells can regain transcriptional control to naturally undergo further differentiation and maturation.

After magnetically sorting AFS cells expressing high levels of the H-2K^k surface antigen with or without PDX1, cells were cultured under normal growth conditions and PDX1 expression was confirmed by semi-quantitative PCR and quantitative PCR. At 72 hours post transfection no PDX1 expression was detected in the cells receiving the control H-2K^k plasmid. However, high levels of PDX1 were detected in both the purified population and the flow-through population of AFS cells which had received the mPDX1 H-2K^k vector (Figure 13A). Quantitative PCR confirmed these results and also revealed that the amount of PDX1 expression in the flow-through is about half the level of the selected (Figure 13B).
Figure 13. Confirmation of PDX1 gene expression in transfected, selected AFS cells. AFS cells nucleofected with either PDX1 H-2Kk or empty H-2Kk were cultured under growth conditions for 48 hours and then immunoselected based on surface H-2Kk expression. The H-2Kk positive fractions as well as the flow through fractions were replated under growth conditions for an additional 24 hours. Semi-quantitative PCR (A) and quantitative PCR (B) were used to verify the expression of PDX1 from the transgene.
The highest levels of PDX1 expression was observed in the PDX1 H-2K^k nucleofected positive fraction at this stage.

Transfected cells remained viable in culture and expanded under normal growth conditions over the course of 16 days. To confirm the transient nature of transfection, expression of the transgenes, mouse PDX1 and H-2K^k, were examined in a single cell population at various time points throughout this expansion by semi-quantitative PCR. In the AFS cells transfected with mPDX1 H-2K^k, there was a marked drop in expression of both transgenes over the time period analyzed (Figure 14). PDX1 expression in these cells gradually decreased over time in culture and was undetectable by 16 days after selection (Figure 14A). H-2K^k expression in the same cell population declined more quickly and was undetectable by day 14 (Figure 14B).

Figure 14. Ectopic transgene expression in human AFS cells progressively decreases over a 16 day period. AFS cells nucleofected with the PDX1-H-2K^k plasmid using 5 million cells, 20 μg of DNA and program T-23 were cultured under normal growth conditions for 16 days with passage approximately every third day. Transgene expression was analyzed by semi-quantitative PCR at the time points indicated.
Pancreatic lineage induction of purified PDX1 positive AFS cells

The purified population of PDX1 expressing AFS cells was subjected to *in vitro* pancreatic lineage differentiation conditions including polyornithine coating and serum free medium containing bFGF (Figure 15). Partially differentiated stem cells expressing PDX1 have been shown to respond to a variety of growth factors including bFGF, EGF, ATRA, Noggin, IGFII and nicotinamide resulting in induction of insulin, c-peptide, glucagon and the glucose transporter (Glut2) expression [71, 72]. In particular, bFGF has been shown to promote proliferation of pancreatic lineage cells [249, 250]. We hypothesized that introduction of ectopic PDX1 into AFS cells would cause pancreatic lineage commitment and that bFGF would induce further differentiation and expansion.

AFS cells cultured under growth conditions maintain an elongated, fibroblast-like shape. However, within the first 24-48 hours after exposure to differentiation conditions the PDX1-AFS cells became rounded, aggregated together and formed large clusters (Figure 16). However, after several days the PDX1 transduced AFS cell clusters begin to shrink and by day 5 are nearly nonexistent. Interestingly, AFS cells that received the empty H-2K<sup>k</sup> control vector showed dramatically decreased ability to survive under the same differentiation conditions and were unable to form viable clusters at any time point (Figure 16).
Figure 15. Schematic of the procedure for attempted differentiation of AFS cells toward insulin-producing β-like cells. The differentiation protocol is divided into two distinct stages. The first stage is characterized by DNA transfection followed by cell recovery and expansion for 2 days. The second stage includes transfer of transfected AFS cells to poly-L-ornithine coated dishes in the presence of serum free medium and bFGF for up to 5 days. Effects of PDX1 expression in this system were analyzed.
Figure 16. AFS cells overexpressing PDX1 survive well and form clusters under differentiation conditions. AFS cells transfected with PDX1-H-2K^k or empty H-2K^k and cultured under the differentiation conditions outlined were observed by light microscopy for cluster formation at the time points indicated. AFS cells nucleofected with PDX1 survive well and form clusters in differentiation conditions. In contrast, AFS cells nucleofected with empty H-2K^k are unable to survive under these conditions.

**PDX1 over-expression induces PAX6 mRNA expression in pancreatic lineage differentiation from AFS cells**

Transcriptional regulation is a critical process throughout pancreatic development and activation of genes critical for each stage at the appropriate time and to the appropriate level is vital for normal development [251]. PAX6 is a transcription factor expressed in the developing pancreas and has been shown to be a marker of pancreatic progenitors which contribute to the islets and pancreatic ducts [252, 253]. All adult
pancreatic endocrine cells also express PAX6 and its inactivation causes significant reduction in the amount of insulin-producing cells in the pancreas [254, 255].

PAX6 expression was examined in PDX1 H-2K\textsuperscript{k} and empty H-2K\textsuperscript{k} transfected AFS cells and BJ fibroblasts after selection and induction of differentiation by culture on poly-L-ornithine with bFGF. RNA was extracted from cells prior to nucleofection, 3 days post-nucleofection and 5 days post nucleofection and semi-quantitative PCR was performed to examine PAX6 expression in each of these stages. Under normal growth conditions, AFS cells express small amounts of PAX6 but no PDX1 (Figure 17A). Immediately after H-2K\textsuperscript{k} based immunoselection 3 days post-nucleofection, both AFS cells and BJ fibroblast control cells nucleofected with PDX1 H-2K\textsuperscript{k} express high levels of PDX1 from the transgene, as expected (Figure 17B). At this stage AFS cells transduced with PDX1 H-2K\textsuperscript{k} but not the empty H-2K\textsuperscript{k} vector express low levels of PAX6 (Figure 17B). Purified cells were replated onto poly-l-ornithine coated dishes in serum free medium with bFGF and mRNA from clusters that were collected 2 days later (5 days post-nucleofection) showed continued PDX1 transgene expression and an increase in PAX6 expression in PDX1 H-2K\textsuperscript{k} AFS cells that was not seen under any other condition (Figure 17B). This stem cell specific, PDX1 dependent PAX6 expression reveals induction of pancreatic lineage differentiation from AFS cells.
Figure 17. Induction of PAX6 expression in transfected AFS cells is PDX1 dependent. A. AFS cells under normal growth conditions express no PDX1 and very little PAX6, BJ foreskin fibroblasts show no expression of either gene as analyzed by semi-quantitative PCR. B. AFS cells or BJ fibroblasts transfected with either Pdx-H-2K<sup>k</sup> or empty H-2K<sup>k</sup> were cultured in growth conditions for 3 days and transferred to poly-L-ornithine coated dishes with serum free medium and bFGF for an additional 2 days. RNA was collected at day 3 and day 5 post-nucleofection for semi-quantitative PCR analysis.

Adenoviral vector gene delivery

Adenoviral mediated gene delivery was also examined as an alternative method to over-express exogenous PDX1 and drive AFS cells toward the pancreatic lineage. Like nucleofection, adenoviral transduction is a non-integrating, transient approach for ectopic gene expression. However, nucleofection proved to be a harsh approach which killed many AFS cells and those cells that survived appeared to be fragile and unable to handle
manipulation without significant cell death for several days after the procedure. Adenoviral mediated gene introduction is a much more gentle approach that we believe may achieve high levels of PDX1 expression and allow manipulation of cell culture conditions required for pancreatic differentiation.

AFS cells were transduced with either a PDX1 expressing adenovirus (Ad-PDX1) or a control adenovirus expressing LacZ (Ad-LacZ). Transgene expression was confirmed 48 hours after transduction by semi-quantitative PCR which revealed high expression of the transgene as well as the presence of the adenoviral vector (Figure 18A). Immunostaining was performed at the same time point to determine protein expression and to confirm the transduction efficiency. In both Ad-PDX1 and Ad-LacZ, after transduction of 1 X 10^5 AFS cells with 1 X 10^6 IU Ad-PDX1 or Ad-LacZ (MOI=10), the efficiency was approximately 50% (Figure 18B). PDX1 over-expression was achieved by adenoviral vector; however, this vector did not include a form of selection so a pure population of PDX1-expressing AFS cells was not achieved.
**Figure 18. Adenovirus-mediated gene expression in AFS cells.** PDX1 and LacZ expression were visible by immunostaining (A) and semi-quantitative PCR (B) 48 hours after transduction with adenovirus.

Pancreatic lineage differentiation of transduced AFS cells

A similar protocol as previously described was used to differentiate Ad-PDX1 AFS cells toward the pancreatic lineage *in vitro* (Figure 19). After adenoviral transduction pancreatic differentiation was induced by including Activin A to stimulate endodermal differentiation followed by bFGF and nicotinamide to promote proliferation and β-cell maturation. Ad-PDX1 and Ad-LacZ transduced AFS cells exhibited no differences in survival and proliferation rates in the early stages of differentiation. However, upon addition of growth factors to stimulate pancreatic differentiation, a clear difference emerged between the groups. Ad-PDX1 AFS cells were able to proliferate under these differentiation conditions and self-assembled into rounded clusters while Ad-LacZ AFS cells exhibited poor survival and no colony formation (Figure 20).
Figure 19. Schematic of differentiation procedure for attempted differentiation of adenovirally transduced AFS cells toward insulin-producing β-like cells. Stage 1: AFS cells are transduced with adenovirus and cultured in growth medium. Stage 2: Culture with Activin A induces endodermal lineage commitment. Stage 3: Cells are replated on poly-L-ornithine coated dishes and cultured with bFGF to encourage progenitor proliferation. Stage 4: Nicotinamide is added to the culture long term to induce β-cell maturation.
**Figure 20. Effects of endocrine pancreas promoting culture conditions on AFS cells overexpressing PDX1.** AFS cells transduced with adenovirus containing PDX1 or LacZ control were cultured under the differentiation conditions outlined and observed by light microscopy for cluster formation at the time points indicated. AFS cells transduced with PDX1 adenovirus survive well and form islet-like clusters in differentiation conditions. In contrast, AFS cells transduced with LacZ control adenovirus are unable to survive under these conditions.

**Gene expression throughout differentiation**

Throughout the stages of differentiation the colonies were examined for expression of genes required to promote pancreatic differentiation by semi-quantitative and quantitative PCR. Endogenous expression of the pancreatic regulator PDX1 is critical as exogenous PDX1 expression is transient. NGN3 is a transcription factor involved in establishing neural precursor cells and in late stages of pancreatic development. In the
pancreas, NGN3 is critical in late stages of development as it has been shown to be expressed in endocrine progenitors and to be required for endocrine specification [256].

Quantitative PCR probes specific to either mouse or human PDX1 (Table 1) were used to distinguish between expression of exogenous, adenoviral gene expression and turn on of endogenous gene expression. Expression of adenoviral mouse PDX1 peaks 48 hours after transduction and steadily declines throughout the differentiation process until levels are undetectable by 14 days into differentiation (Figure 21A). Endogenous human PDX1 expression becomes upregulated during the differentiation process at day 10 and with declining expression at day 14 (Figure 21B). Low levels of human PDX1 expression were observed in the Ad-LacZ transduced cells at the latest stage of differentiation, suggesting that the culture conditions may have induced this slight upregulation (Figure 21B).

After adenoviral transduction and 48 hours of Activin A treatment, at the end of stage 2, a 3 fold upregulation of NGN3 expression was observed in AFS cells transduced with Ad-PDX1 compared to controls (Figure 22A). Interestingly, low levels of NGN3 expression were observed in Ad-LacZ but presence of PDX1 enhanced NGN3 expression by a factor of 3. After Activin A treatment, cells were replated onto poly-L-ornithine and cultured with bFGF for 2 additional days. At this time point, the end of stage 3, PDX1 dependent PAX6 turn on was observed, similar to that seen in the vector mediated AFS pancreatic differentiation (Figure 22B).
Figure 21. Effect of adenoviral transduction on PDX1 expression throughout the pancreatic differentiation procedure. Species-specific primer/probe sets were used to distinguish mouse PDX1 expression from human PDX1 expression throughout differentiation. A. Exogenous mouse PDX1 expression is down regulated over the course of the differentiation procedure in PDX1 transduced cells. No mouse PDX1 expression is present in LacZ and untransduced AFS cells at any time point. B. Endogenous human PDX1 expression is upregulated over the course of the differentiation procedure in PDX1
transduced cells. No human PDX1 expression is present in untransduced AFS cells and slight upregulation is observed at the latest time point in LacZ transduced AFS cells.

Figure 22. Pancreatic gene expression during pancreatic differentiation of PDX1 transduced AFS cells. After adenoviral transduction and pancreatic induction, quantatitive and semi-quantitative PCR was performed to examine pancreatic gene expression. PDX1 dependent induction of NGN3 expression was observed in stage 2 and PAX6 expression was observed in stage 3. A. PDX1 transduced AFS cells express levels of NGN3 3 fold higher than LacZ transduced and untransduced controls at stage 2 of
B. PDX1 transduced AFS cells express high levels of PAX6 at stage 3 of differentiation.

**PDX1 transduced AFS cell clusters express insulin**

To act as a cell replacement therapy for DM, fully differentiated AFS cells must release high levels of insulin. *In vitro* measurement of insulin production requires assay of insulin derivatives such as c-peptide to prove *de novo* insulin production and rule out the possibility of insulin uptake from the medium. Following transduction, Activin A treatment, transfer to poly-L-ornithine with bFGF treatment, cells were cultured for three weeks with nicotinamide to induce further differentiation and maturation of the β-cells. In the final stages of differentiation insulin expression and release from AFS cells was examined. Gene expression analysis by quantitative PCR revealed low levels of PDX1 specific insulin expression (Figure 23A). To confirm insulin production by cells at this stage a C-peptide specific ELISA was performed. The c-peptide ELISA revealed the presence of low levels of c-peptide in the Ad-PDX1 AFS clusters (13.5+/− 0.8 pmol per 300 clusters n=3), confirming de-novo insulin synthesis (Figure 23B).
Figure 23. AFS cell clusters transduced with PDX1 express insulin and release c-peptide after 3 weeks in differentiation conditions. After adenoviral transduction and 4 stages of differentiation, cell clusters were examined for insulin expression by quantitative PCR and c-peptide release by ELISA. A. AFS cell clusters transduced with PDX1 express levels of insulin 4-20 times greater than controls. B. AFS cell clusters transduced with PDX1 release high levels of c-peptide compared to controls.

In this chapter we have shown efficient plasmid transfection and magnetic immunoselection of AFS cells, resulting in a pure population of PDX1 expressing AFS cells. Culture with bFGF resulted in cluster formation and expression of the pancreatic lineage marker PAX6. In a similar system we induced ectopic PDX1 expression in AFS cells using adenoviral vectors. A three-step differentiation protocol involving sequential
culture with Activin A, bFGF and nicotanimide led to uniform cluster formation. Over the course of differentiation we documented exogenous PDX1 down regulation and endogenous PDX1 up regulation. At the end of step 2 pancreatic regulatory genes PAX6 and NGN3 were turned on. In the final stage of differentiation insulin expression was measured and c-peptide release was confirmed, suggesting that AFS cells have the potential to serve as a source of starting cells for cell replacement therapy in DM patients. To make this possibility a reality it would be necessary to verify that cells transduced with adenovirus pose no threat to the patient. In addition, it would be critical to achieve higher, clinically relevant levels of insulin production and to determine that the cells are able to release insulin in response to increasing glucose levels.
CHAPTER TWO

**IN VITRO IMMUNOMODULATORY ACTIVITY OF AFS CELLS COMPARED TO BM-MSCs**

The main purpose of the experiments in this chapter was to compare the immunomodulatory capacity of amniotic fluid stem (AFS) cells to bone marrow mesenchymal stromal cells (BM-MSCs). Experiments performed include stem cell immunophenotype, ability of stem cells to dampen T-lymphocyte activation *in vitro*, ability of conditioned medium from activated stem cells to dampen T-lymphocyte activation *in vitro* and protein array to assess soluble factors secreted by stem cells.

**Immunogenic characterization of amniotic fluid stem cells**

Expression of cell surface antigens can give an indication of the immunogenicity of a cell. T-cell activation occurs upon interaction of the T-cell receptor with the MHC peptide complex and interaction of the costimulatory molecules [257]. Cells expressing MHC molecules are capable of activating T cells only if they also express a costimulatory molecule such as CD80 or CD86 [258]. Upregulation of MHC molecule expression for antigen presentation to T cells can be induced on several cell types by culture with
cytokines such as IFN-γ, although the consequences of this induction remain unclear [259].

Flow cytometry was performed to directly compare the expression of immune-related surface markers between human AFS cells and bone marrow-derived MSCs (BM-MSCs) (Figure 24). Consistent with previous reports, the BM-MSCs were positive for major histocompatibility complex (MHC) – Class I but expressed no detectable MHC-Class II (Figure 24A). The BM-MSCs also showed no expression of the costimulatory molecules CD-80 and CD86. AFS cells likewise demonstrated positive expression of MHC – Class I and undetectable expression of MHC – Class II, CD-80 and CD-86 (Figure 24B). To compare their response to IFN-γ, AFS cells and BM-MSCs were cultured in the presence of the cytokine for 24 hours and flow cytometry was performed to examine the expression of the same immune-response related surface markers. We found that IFN-γ induced up-regulation of MHC I and II, but not CD40, CD80, or CD86, in both BM-MSCs (Figure 24A) and AFS cells (Figure 24B).
Figure 24. The effect of IFN-γ on the immunophenotype of AFS cells and BM-MSCs. BM-MSCs (A) or AFS cells (B) were cultured under growth conditions with (blue) and without (yellow) addition of IFN-γ stimulation for 24 hours. The cells were incubated with fluorescently labeled antibodies against the major histocompatibility complex molecules MHC-I and MHC-II as well as the costimulatory molecules CD40, CD80, and CD86.
CD80 and CD86. The red line shows appropriate isotype controls. **A.** BM-MSCs under growth conditions express high levels of MHC-I and no MHC-II, CD40, CD80 or CD86. Upon IFN-γ stimulation MSCs upregulate MHC-I and MHC-II while CD40, CD80 and CD86 remain unchanged. **B.** AFS cells show similar expression patterns to MSCs with high levels of MHC-I and no MHC-II, CD40, CD80 or CD86. AFS cells also upregulate MHC-I and MHC-II in response to IFN-γ stimulation. Before stimulation they express high levels of MHC-I but low levels of MHC-II, CD40, CD80 and CD86. After IFN-γ stimulation the levels of MHC-I and MHC-II expression increase but CD80 and CD86 expression remains unchanged.

**AFS cells suppress lymphocyte activation**

In allogeneic transplantation T-lymphocytes are activated upon exposure to non-self antigens presented by professional APCs such as DCs [260]. T-lymphocyte activation results in cytokine secretion, proliferation and maturation which induces effector function and leads to allograft rejection [261]. Interferon-gamma is one of the pro-inflammatory cytokines produced by activated T-lymphocytes in this response (Young 1995). T-cell activation and IFN-g production can be replicated in vitro by culture with stimulatory molecules such as PHA [262]. The ability of AFS cells to inhibit lymphocyte activation by PHA was examined in an Elispot assay measuring IFN-γ.

Two independent AFS cell lines, a BM-MSC isolate and a human dermal fibroblast were tested in this assay. Both AFS cell lines and the BM-MSC isolate induced exerted dose-dependent inhibition of lymphocyte activation (Figure 25). At low numbers of stem cells, the extent of inhibition of lymphocyte activation by the AFS cells was
essentially equivalent to that for BM-MSCs. At a ratio of 1 stem cell, either AFS or BM-MSC, to 32 lymphocytes, the percent activation was about 70 percent of the positive control wells which contained no stem cells. When the stem cell number was increased two-fold, lymphocyte activation was further inhibited to 60 percent. By increasing the number of stem cells again by two-fold we showed decreased activation of lymphocytes to about 50 percent of the positive control values. At the highest levels of stem cells, BM-MSCs appeared slightly more potent than AFS cells, and completely inhibited PHA-stimulated activation at the highest dose tested. By contrast, control dermal fibroblasts did not inhibit lymphocyte activation at any concentration.

**Figure 25.** Human AFS cells inhibit lymphocyte activation in a dose dependent manner similar to that of BM-MSCs. Immunoassays assessing lymphocyte activation were performed on two independent amniotic fluid stem cell lines (AFS1 and AFS2) or
bone marrow-mesenchymal stem cell (BM-MSC) isolates. T lymphocytes were activated with phytohemaaglutinin (PHA) and cultured in 96 well plates coated with IFN-γ capture antibody in the presence of increasing amounts of stem cells from 1:32 (4,688 stem cells cultured with 150,000 PBMCs) to 1:2 (75,000 stem cells cultured with 150,000 PBMCs) for 24 hours. Positive control wells contained lymphocytes activated with PHA and negative control wells included unactivated lymphocytes. Lymphocyte activation was assessed by counting the number of lymphocyte clones producing IFN-γ. Activation is expressed as a percentage of the positive control wells. Both AFS lines and BM-MSC inhibited T-cell compared to the PHA activated control to an approximately equal extent, and was dependant on the number of stem cells added. Inhibition varied from about 40% at a 1:32 ratio to 80-90% inhibition at the highest ratio of 1:2.

**Conditioned medium from AFS cells suppresses lymphocyte activation**

Cell-to-cell contact between stem cells and immune cells resulted in inhibition of the *in vitro* immune response as measured by IFN-g production by lymphocytes. Activation of MSCs with immune cells or cytokines such as IFN-g, TNFa, IL-1a or IL-1b results in cell-free supernatants which have also been shown to inhibit in vitro immune responses 5,6[162].

To examine the possibility that AFS cells secrete immunosuppressive factors, we assessed the effect of conditioned medium (CM) in the lymphocyte activation assay. CM was prepared from AFS cells and BM-MSCs with and without pre-treatment with the pro-inflammatory cytokine IL-1β. CM also was prepared from mixed cultures of AFS
cells or BM-MSCs with PBMCs. CM from unstimulated AFS cells or BM-MSC showed minimal suppression (less than 10% reduction) of lymphocyte activation (Figure 26). CM from AFS cells or BM-MSCs stimulated with IL-1β both showed significantly enhanced suppression of PBMC activation, to 60-70 percent of control levels (Figure 26). CM obtained after stimulation of AFS cells or BM-MSCs by co-culture with PBMCs also significantly suppressed lymphocyte activation to a similar extent compared to CM from IL-1β-stimulated cells.

![Figure 26. AFS mediated immunosuppression does not require cell-cell contact.](image)

Amniotic fluid stem (AFS) cells from two independent sources or bone marrow derived mesenchymal stem cells (BM-MSCs) were cultured under growth conditions (AFS 1, AFS 2, BM-MSC) or activated by co-culture with total blood monocytes (AFS 1 PBMC, AFS 2 PBMC, BM-MSC PBMC, BM-MSC IL-1)
AFS 2 PBMC, BM-MSC PBMC) or IL-1β (AFS 1 IL1, AFS 2 IL1, BM-MSC IL1) to release soluble factors. One way mixed lymphocyte reactions (MLR) were incubated in the presence of 24 hour conditioned medium from stem cells cultured with either peripheral blood mononuclear cells (PBMCs) or IL-1β. PHA activated lymphocytes were cultured in 96 well plates coated with IFN-γ capture antibody in the presence of conditioned mediums for 24 hours. Positive control wells contained lymphocytes activated with PHA and negative control wells contained unactivated lymphocytes. Lymphocyte activation was assessed by counting the number of clones producing IFN-γ. Percent activation was calculated by comparing wells containing stem cells to positive control wells. It can be seen that all supernatants were capable of inhibiting T-cell activation by PHA by approximately 20-25%. All conditions are statistically significant when compared to the positive control wells.

Identification of secreted cytokines and growth factors

Soluble factors have been implicated in MSC-mediated immunosuppression include IDO, iNOS, PGE2, IL-6, TGF-b and IL-10 among others [263]. These soluble factors have effects on immune cells which suppresses their activity including inhibition of proliferation [264], decreasing their pro-inflammatory cytokine production [265], and conversion toward anti-inflammatory Th2 phenotype [266]. Comparison of soluble factors secreted by MSCs and AFS cells will lead to identification of potential immunomodulatory mechanisms. Medium conditioned by activated AFS cells and BM-
MSCs were examined by protein array to determine factors important in immune response regulation.

Levels of 274 different cytokines, chemokines and growth factors in CM from AFS cells and BM-MSCs cultured under standard growth conditions or activated with either PBMCs (Figure 27A) or IL-1β (Figure 27B) were assessed. Controls to assess background levels of soluble factors included serum containing medium and PBMCs cultured alone. Two identical arrays were completed on different days, one including conditioned medium from 1) Cell free medium, 2) AFS cells, 3) AFS cells activated with PBMCs and 4) AFS cells activated with IL-1β and the other including 1) PBMC conditioned medium, 2) BM-MSCs, 3) BM-MSCs activated with PBMCs, 4) BM-MSCs activated with IL-1β.

Amniotic fluid stem cells and BM-MSCs under growth conditions released low levels of very few cytokines. However, upon activation by either PBMCs or IL-1β elevated levels of cytokines were released and a similar cytokine profile was seen from both AFS cells and BM-MSCs. Activation by PBMCs caused release of high levels of several cytokines by AFS cells and BM-MSCs including growth related cytokine – alpha (GRO-α), monocyte chemotactic protein-3 (MCP-3) and interleukin – 6 (IL-6). Granulocyte chemotactic protein -2 (GCP-2) and interleukin – 2 receptor alpha (IL-2Rα) were also released by both cell types, although to a lesser extent. While PBMC activated AFS cells released monocyte chemotactic protein-2 (MCP-2), macrophage inflammatory protein 1 – alpha and 3 - aplha (MIP-1α, MIP-3α) and Activin while BM-MSCs did not.
Culture with pro-inflammatory cytokine IL-1β resulted in a cytokine release profile similar to that seen with PBMC activation. Both AFS cells and BM-MSCs released detectable levels of GRO, GRO-α, MCP-1, IL-2, IL-6 and granulocyte macrophage colony stimulating factor (GM-CSF). Each stem cell type also released low levels of the cytokines MIP-3α, IL-2, regulated upon activation, normal T-cell expressed, and secreted (RANTES) and epithelial neutrophil-activating protein-78 (ENA-78). BM-MSCs released detectable levels of several additional cytokines upon IL-1β stimulation including GCP-2, Activin, IL-8 and insulin-like growth factor-binding protein 4 (IGF-BP4).
Figure 27. Soluble factors released from AFS cells and BM-MSCs in response to activation. Amniotic fluid stem (AFS) cells or bone marrow derived mesenchymal stem cells (BM-MSCs) were activated by culture with A. total blood monocytes or B. IL-1β and the cytokines released were measured by cytokine array. Background cytokine levels were subtracted to normalize samples and include cytokines released by PBMCs cultured alone and stem cells cultured alone.
The experiments in this chapter reveal that AFS cells share a similar surface marker profile to BM-MSCs with high expression of MHCI and little to no MHCII, CD80 and CD86 expression. AFS. We also present data to demonstrate that direct contact with AFS cells inhibits lymphocyte activation to a level similar to that seen with BM-MSCs. In addition, we show that cell-free supernatants derived from AFS cells primed with total blood monocytes or IL-1β, a cytokine released by monocytes and essential in mediation of the inflammatory response, also inhibited lymphocyte activation. Further investigation of AFS cell-free supernatants by protein array revealed secretion of multiple factors in common with MSCs that are known to be involved in immune regulation including growth related oncogene (GRO) and monocyte chemotactic protein (MCP) family members as well as interleukin-6 (IL-6). AFS cells activated by PBMCs released several additional cytokines as compared to BM-MSCs, including macrophage inflammatory protein-3α (MIP-3α), MIP-1α and Activin. AFS cells also released higher levels of MCP-1 and lower levels of MCP-2 compared to BM-MSCs in response to IL-1β activation. This suggests that there may be some AFS-specific mechanisms of inhibition of lymphocyte activation. Our results indicate that AFS cells are able to suppress inflammatory responses in vitro and that soluble factors are an essential component in the communication between lymphocytes and AFS cells (Figure 28). These facts make AFS cells a good source for cell-based immunotherapies to be used in autoimmune diseases such as T1DM and in co-transplantation with allogeneic grafts such as islets to avoid rejection.
Figure 28. Model of proposed mechanism by which human amniotic fluid stem cells interact within the confines of the immune response. Secretion of IL-1β and other cytokines from monocytes causes an activation of the immunodulatory properties in AFS cells. In response, AFS cells secrete a battery of soluble factors including IL-6, MCP-1 and others which inhibit T-lymphocyte activation.
CHAPTER THREE

EFFECTS OF HNF4A EXPRESSION ON PANCREATIC DEVELOPMENT

The purpose of these experiments was to examine the effects of a genetic deficiency known to cause a diabetes mellitus (DM) phenotype on \textit{in vitro} development and function of β-cells as a means to identify novel drug targets for the treatment of this disease. To this end we created pluripotent stem cells containing a deficiency in HNF4A found in the monogenic form of type 2 diabetes mellitus (T2DM), maturity onset diabetes of the young 1 (MODY1). MODY1 induced pluripotent stem (iPS) cells and inducible HNF4A knock down embryonic stem (ES) cells were developed and differentiated in a step wise process \textit{in vitro} toward the pancreatic lineage. Gene expression analysis was performed at each stage to identify effects of HNF4A knockdown on pancreatic differentiation.

**Cellular Reprogramming with Cell Extract**

Reprogramming without the need for ES chromosomes is critical for therapeutic use and has been shown to be possible using cell free extract. In the first of these experiments T cell nuclear and cytoplasmic extracts were used to reprogram 293T cells [267]. More recently 293T cells were reprogrammed by exposure to an extract of
undifferentiated human NCCIT carcinoma cells [268]. Derivation of patient specific pluripotent stem cells is critical for examining disease development in vitro so we examined the potential of cell-free NCCIT extract to reprogram human AFS to become induced pluripotent stem (iPS) cells.

AFS cells and 293T cells that had been reversibly permeabilized by streptolysin O were exposed to cell extracts from the embryonal carcinoma cell line NCCIT in an ATP regenerating reaction. After NCCIT extract exposure, the cells were cultured under ES maintenance conditions and the morphology observed by phase microscopy. Both AFS and 293T cells showed noticeable changes in cell morphology as early as 3 days after treatment, clustering together and forming colonies similar to those seen in pluripotent stem cell cultures (Figure 29A). Semi-quantitative mRNA expression of pluripotent cell specific transcripts Nanog, Sox2 and c-kit were induced in reprogrammed somatic cells and showed the highest expression in 293T cells cultured on a feeder layer (Figure 29B).

At the time of the completion of this experiment, the breakthrough of iPS cell creation by defined factors was announced [83]. The more defined nature of this method and the fact that it has proven to be successful in reprogramming somatic cells derived from adult patients led us to use this methodology for further reprogramming attempts.
Figure 29. Somatic cells reprogrammed in an embryonal carcinoma cell free extract exhibit stem cell like characteristics. AFS cells and 293T cells were examined 3 days after exposure to NCCIT embryonal carcinoma extract. A. Representative phase contrast images of stem-cell like colony formation with and without feeder layers. B. Semi-quantitative PCR analysis of the induction of pluripotency associated gene expression in reprogrammed cells.

Generation of iPS cell lines from patients with MODY1

Induced pluripotent stem cells are particularly useful in the investigation of monogenic diseases; here we chose to explore MODY1, a form of diabetes resulting from HNF4A haploinsufficiency [214]. Development of iPS cells containing an HNF4A mutation known to cause the MODY phenotype would allow us to follow the disease progression in vitro, analyze the mature β-cell function and gain valuable insight into the pathophysiology of the disease.
Fibroblasts derived from skin biopsies of the MODY1 patient and sibling control were cultured on gelatin-coated dishes (Figure 30) and transduced with a single lentiviral vector containing OCT4, SOX2, KLF4, c-MYC and RFP (Figure 31A). MODY1 dermal fibroblasts were transduced, allowed to recover in growth medium for 4 days, replated onto MEF feeder layers and cultured in growth medium for 1 day and then changed to ES growth medium for the remainder of the reprogramming process (Figure 31B0. Transduced fibroblasts were maintained in growth medium for 5 days and analyzed for fluorescent transgene 48 hours post-transduction revealing efficient transduction (Figure 31C). Within 4 days under growth conditions a morphology change was already beginning to emerge as many cells became smaller and rounded (Figure 31C). At day 5 post-transduction, cells were replated onto MEF feeder layers in ES cell maintenance conditions in medium containing the stem cell renewal growth factor bFGF. A dramatic change in morphology was observed 8 days post transduction as fibroblasts transformed from long, spindle-like cells into smaller, rounded cells which began to cluster together (Figure 31C). As expected, the fibroblasts which changed morphology expressed high levels of RFP and therefore high levels of the reprogramming factors. The RFP positive cell clusters formed tight colonies by 12 days after transduction which continued to proliferate until they were picked for clonal expansion (Figure 31C). At this stage around 50 colonies were observed per 35-mm dish from the initial 5x10⁴ cells seeded. The majority of these colonies exhibited ES-like morphology and stained positive for the enzyme alkaline phosphatase (Figure 32). Colonies were picked at day 25 post-transduction and transferred to feeder-free conditions of matrigel coated plates with
mTeSR1 medium. Five individual clones from the MODY1 patient and 5 clones from the normal sibling were expanded.

**Figure 30. MODY1 patient iPS cell morphology under growth conditions.** Dermal fibroblasts cultured in FBS containing medium appear elongated and spindle-like as viewed by light microscopy.
Figure 31. Derivation of MODY1 patient-specific iPS cell lines. A. Visual representation of lentiviral vector used to transduce patient-specific fibroblasts. The vector consists of an EF1a promoter which produces constant and robust expression of the polycistronic transcript. OCT4, SOX2, KLF4 and c-MYC are separated by 2A ribosome-slippage sites. An IRES site follows attached to RFP. B. Overview of patient-specific iPS cell line generation protocol. Fibroblast cell populations were transduced with the lentiviral vector and cultured under growth conditions for 4-5 days followed by transfer to feeder based ES culture conditions for 3-4 weeks before individual colony selection and expansion under feeder free ES culture conditions. Patient-specific iPS cell lines were expanded for several weeks to achieve sufficient cell numbers for characterization and cryopreservation. C. Representative phase contrast and corresponding red fluorescent protein images of time points indicated throughout the reprogramming process.

Figure 32. MODY1 patient-specific induced pluripotent stem cell clonies express alkaline phosphatase. During the final stages of colony formation in feeder-dependent
conditions many fully reprogrammed colonies expressed high levels of the enzyme alkaline phosphatase. Colonies demonstrating the most ES-like morphology, including a clear border between the colony and feeders, expressed the highest levels of alkaline phosphatase and were selected for further cloning and feeder-free expansion.

MODY Patient and Sibling iPS Cells Express hES Markers

To verify that the MODY1 patient fibroblasts had been fully reprogrammed by overexpression of the OKSM 4-in-1 lentiviral vector, we evaluated their similarity to ES cells. Evaluation of morphology (Figure 33), gene expression (Figure 34), surface antigen profile (Figure 35A) and transcription factor protein expression (Figure 35B) was completed for 2 individual MODY1 sibling iPS cell lines and 1 MODY1 patient iPS cell line. As similar results were obtained for each cell line, one MODY1 sibling control iPS cell line (GM44221-iPSA) is represented here.

The isolated iPS clones expanded under established feeder-free ES growth conditions and exhibited a morphology near identical to ES cells (Figure 33). Immediately upon transfer to the feeder-free expansion conditions, RFP expression ceased indicating promoter silencing resulting in loss of transgene expression (data not shown). However, there was noticeable variation in growth rate between the iPS clones. In particular, several clones from each fibroblast source exhibited a much slower growth rate when compared to ES cells grown under the same conditions. MODY1 patient clones and sibling clones which maintained an appropriate morphology and growth rate
comparable to that seen in ES cells for at least 5 passages under feeder-free conditions were selected for further pluripotency characterization.

Transcription factors were analyzed by quantitative PCR array and sibling iPS cells expressed high levels of each gene (Figure 34). In most cases pluripotency gene expression levels were much higher than ES cells potentially owing to the low passage of the iPS cell line. Pluripotency associated surface antigens Tra1-60, Tra1-81 and SSEA4 were analyzed by flow cytometry and all iPS clones revealed high levels of expression of these markers comparable to ES cell expression (Figure 35A). Protein expression of pluripotency associated markers was confirmed by immunostaining which demonstrated that nearly all iPS cells expressed OCT4, SOX2 and NANOG with nuclear localization and SSEA4 with cellular membrane localization (Figure 35B). As silencing of the transgene was confirmed by lack of RFP expression, it stands to reason that expression of these genes is the result of endogenous upregulation which indicates reprogrammed cells.
Figure 33. MODY1 patient-specific iPS colonies are morphologically identical to ES colonies. After cloning and culture under feeder-free conditions for at least 5 passages, MODY1 iPS cell colonies are indistinguishable from ES cell colonies cultured under the same feeder-free conditions based on visualization by light microscopy.
Figure 34. MODY1 patient-specific iPS clones express transcription factors associated with pluripotency. Gene expression analyses were performed by quantitative PCR pluripotent array after MODY1 iPS cell lines had undergone at least 5 passages under feeder free conditions. Results revealed a high similarity in pluripotent gene expression between ES cells and MODY1 iPS cell lines with both expressing high levels of pluripotency associated genes. Values were normalized to GAPDH and the starting MODY1 dermal fibroblast cells were used as a baseline.
Figure 35. MODY1 patient-specific iPS cell colonies express high levels of pluripotency associated proteins. MODY1 iPS colonies were expanded under feeder
free-conditions for at least 5 passages and protein expression was examined by flow
cytometry and immunostaining. A. Like ES cells, MODY1 iPS cell colonies expressed
high levels of pluripotency associated antigens including Tra 1-60, Tra1-81 and SSEA4
based on flow cytometric analysis. B. Immunostaining revealed the endogenous
upregulation of pluripotency associated transcription factors OCT4, SOX2, NANOG and
cell surface antigen SSEA4 in fully reprogrammed MODY1 iPS cell lines. Appropriate
protein localization was observed with transcription factors present in the nucleus and
cell surface antigens around the cell surface.

Directed differentiation of MODY Sibling iPS Cells into Pancreatic β-cells

Characterization of the functional capabilities of iPS cell clones was performed by
in vitro directed differentiation. Efficient production of pancreatic β-cells in vitro from
MODY1 patient-specific iPS cell lines is critical for the mechanistic studies and potential
for drug development applications. First we examined the ability of MODY1 sibling
control iPS cell lines to undergo pancreatic differentiation. We hypothesized that since
the starting fibroblasts possessed no known gene mutation and no known pancreatic
phenotype, they should differentiate toward pancreatic lineages at rates similar to those
seen in ES cells.

To achieve in vitro pancreatic lineage differentiation from pluripotent ES and
MODY1 sibling control iPS cells, an adaptation of a protocol that has proven to generate
insulin-producing cells from ES cells was used [70] (Figure 36A). The pluripotent cells
were exposed to the step-wise series of growth factors which mimic the in vivo pancreatic
organogenesis. At the end of each stage of this 5 step process, gene expression patterns were analyzed by qPCR to determine the relative success of the differentiation procedure.

As expected, there was a sharp decline in expression of the pluripotency marker OCT4 over the course of differentiation in both ES and normal iPS cells (Figure 36B). Definitive endoderm specific genes including SOX17 and CXCR4 are expressed at high levels at stage 1 immediately after Activin A treatment, however the normal iPS clone derived DE revealed much lower levels of SOX17 expression as compared to the ES cell derived DE (Figure 36C). Similar trends were seen throughout the differentiation with gene expression present at appropriate stages but with the MODY1 sibling control iPS cell derived intermediates expressing much lower levels of stage specific genes indicating their inability to undergo efficient pancreatic differentiation. This phenomenon was observed with gut tube endoderm specific genes HNF4A and HNF1B in stage 2 after treatment with FGF7 and Noggin (Figure 36D) and with pancreatic precursor marker PDX1 in stage 3 after treatment with KAAD CYC (Figure 36E).

In the final stage of differentiation ES cell derived β-cells expressed markers specific to the endocrine pancreas as measured by quantitative PCR including PDX1, high levels of the glucose transporter GLUT2, along with glucagon and PPY (Figure 36F). However, MODY1 sibling control iPS cell derived β-cells did not express these markers at this stage.
Figure 36. MODY1 normal sibling control iPS cell colonies show disparate capacity for directed pancreatic lineage differentiation compared to hES cell colonies. A.
Schematic of the protocol for step-wise directed differentiation of pluripotent stem cells into insulin-producing β-cells. ES and iPS cells were exposed to a succession of growth factors and other morphogens including Activin A, Wnt3a, FGF7, Noggin, RA, KAAD-CYC, EGF, EX-4 and DAPT. This protocol guides differentiation through five endodermal intermediates toward insulin-producing cells and genes characteristic of each stage are listed. Gene expression analysis was performed at the end of each stage of differentiation by quantitative PCR. **B.** In genes characteristic of pluripotency, including Oct 4, expression decreased throughout differentiation in ES and iPS cell lines. **C.** Genes characteristic of DE, including SOX17 and CXCR4, are upregulated at the end of stage 2 but are more highly expressed in DE derived from ES cells than MODY1 sibling control iPS cells. **D.** Genes characteristic of GTE, including HNF4A and HNF1B, are more highly upregulated during stage 3 in differentiation from ES cells compared to MODY1 sibling control iPS cells. **E.** PDX1 expression is highly upregulated in the pancreatic progenitor stage of differentiation from ES cells but very little is expressed in the same stage from MODY1 sibling control iPS cells. **F.** β-cells derived from ES cells express high levels of islet specific markers after pancreatic induction including GLUT2 while β-cells derived from iPS cells do not.

**Inducible Knockdown of HNF4A in Hepatic Carcinoma Cell Line**

Variability in iPS cell lines derived from the same fibroblasts is a looming concern associated with this relatively new technology. Line-to-line variability has been reported by several research teams investigating terminal differentiation of iPS cell lines;
the current methodology used to circumvent this issue is derivation and characterization of numerous (at least 8) iPS clones from each patient [269, 270]. These studies examined the potential of patient-specific iPS cells to reach a defined cell type; investigation of stages throughout differentiation in addition to the final, functional cell requires an even higher level of system control. With the current state of iPS technology we found that it would be very difficult to determine whether differences in differentiation efficiency are related to iPS line variability or whether they are, in fact, related to the genetic deficiency. To achieve our goal of examining the development and final β-cell function in an HNF4A deficient cell population, as is observed in MODY1, without the possibility for line to line variability or genetic variation, we developed an inducible HNF4A shRNA knock down ES cell population.

Three HNF4A specific shRNAmir plasmids (V2LHS_239555 / HNF4A shRNA 1, V2LHS_377096/HNF4A shRNA 2, V2LHS_93152/HNF4A shRNA 3, Open Biosystems) containing the shRNA and RFP under the control of a doxycycline inducible TRE promoter and puromycin resistance gene with transactivation domain under control of a ubiquitously expressed promoter were examined for their ability to knock down HNF4A expression in the hepatic carcinoma cell line HepG2. As HepG2 cells are derived from the liver, they are known to express high levels of HNF4A. After transduction of HepG2 cells and subsequent doxycycline induction, each HNF4A shRNA showed high transgene expression based on RFP analysis (Figure 37). The same population of transduced HepG2 cells that had not been exposed to doxycycline lacked expression of RFP, revealing tight control of the system (Figure 37). Upon quantification of the
percentage of RFP positive HepG2 cells by flow cytometry, around 80% of transduced cells in each group expressed the transgene (Figure 37).

After confirming expression of the transgene by visualization of RFP, we determined the ability of each HNF4A shRNA construct to knock down HNF4A gene and protein expression by quantitative PCR (Figure 38A) and western blot (Figure 38C), respectively. Examination of transcript levels by quantitative PCR proved high HNF4A mRNA knock down in doxycycline treated HepG2 cells transduced with constructs 1 (98%) and 2 (77%) as compared to no doxycycline treatment (Figure 38B). An inexplicable increase in HNF4A expression was seen in doxycycline treated HepG2 cells transduced with HNF4A shRNA 3 and although knock down of HNF4A expression was seen in comparison to the same cells not doxycycline treated (80%), there was no knock down when compared to untransduced HepG2 cells. Protein analysis by western blot confirmed this result with significant HNF4A knock down in constructs 1 and 2 but not 3 upon addition of doxycycline (Figure 38D). For further experimentation, HNF4A shRNA 1 and 2 were combined and used for transduction to knock down HNF4A expression level.
Figure 37. Doxycycline inducible expression of RFP HNF4A shRNA construct in hepatocellular carcinoma cells. A. Visual representation of inducible, selectable HNF4A shRNA lentiviral plasmid in HepG2 hepatocellular carcinoma cells. B. Two weeks after transduction and puromycin selection, HepG2 cells express high levels of doxycycline regulated RFP expression. C. Flow cytometric analysis reveals the efficiency of transduction of each shRNA construct based on RFP expression. Gates were constructed based on HNF4A shRNA transduced HepG2 cells that had not been exposed to doxycycline.

A  

% knock-down  
shRNA 1: 98%  
shRNA 2: 77%  
shRNA 3: 80%
Figure 38. Inducible HNF4A knockdown by lentiviral shRNA in HepG2 cells. ShRNAs to three individual target sequences of HNF4A were tested for their ability to knock down HNF4A gene and protein expression in HepG2 cells. A. Knockdown of HNF4A gene expression measured by quantitative PCR in transduced HepG2 cells expressing the shRNA transgene following exposure to doxycycline (shRNA dox) for 72 hours compared to transduced cells with no transgene expression (shRNA). Gene expression was normalized to GAPDH. C. Knockdown of HNF4A protein expression measured by western blot in transduced HepG2 cells expressing the shRNA transgene following exposure to doxycycline (shRNA dox) for 72 hours compared to transduced cells with no transgene expression (shRNA). D. Densitometry analysis revealed percent knockdown of shRNA dox compared to shRNA without dox, untransduced HepG2 cells were set as the baseline expression level. Comparison of HNF4A expression knockdown expressed as shRNA dox/shRNA x 100.
**HNF4A knock down in ES cells**

After confirmation of effective knock down of HNF4A expression, our goal was to transduce ES cells with HNF4A shRNA to further examine the effect of HNF4A deficiency on differentiation down the endocrine pancreas lineage. Based on the previous results, HNF4A shRNA 1 and HNF4A shRNA 2 were combined in equal proportion and used for ES transduction. A titration curve of the concentrated, combined HNF4A shRNA was performed on ES cells. Measurement of RFP expression by flow cytometry 4 days after transduction and doxycycline addition revealed a titration of $1 \times 10^9$ TU/ml (data not shown).

An MOI of 50 was used to transduce ES cells with the goal of creating a stable cell line by puromycin selection. One week after lentiviral transduction HNF4A shRNA ES cells exposed to doxycycline revealed high levels of RFP expression as examined by fluorescent microscopy (Figure 39A) which was quantified to be 54% positive by flow cytometry (Figure 39B). Transduced ES cells continued to highly express pluripotency associated surface antigens SSEA4, Tra1-60 and Tra1-80 (Figure 39C). However, after several passages the ES cells began to lose RFP expression which was quantified by FACS 25 days post transduction (Figure 40). This loss of RFP expression over time is likely attributable to the propensity of ES cells to silence gene expression by promoter methylation [271]. As a result of the technical difficulties encountered surrounding long term culture and selection of stable transfectants and to avoid the potential that clonal HNF4A shRNA ES populations may have an inherent deficiency, a polyclonal HNF4A shRNA ES cell population was used for differentiation experiments. ES cells transduced with the HNF4A shRNA lentivirus at an MOI of 50 which was observed by light
microscopy to cause significant cell stress combined with cell death. Transduced cells were allowed to recover for about 1 week in feeder-free ES maintenance conditions. The entire population of cells was then relpated under growth conditions with the addition of the doxycycline gradient and the induction of pancreatic differentiation was begun 48 hours later. At the start of pancreatic differentiation the amount of shRNA lentiviral construct being expressed was observed by fluorescent microscopy (Figure 41A). A high correlation between doxycycline concentration and percentage of RFP positive, and therefore HNF4A shRNA expressing, cells was revealed by flow cytometry analysis with 0 μg/ml expressing 0% RFP, 0.25 μg/ml expressing 17%, 0.5 μg/ml expressing 25%, 1 μg/ml expressing 28% and 2 μg/ml expressing 40% (Figure 41B).
**Figure 39. Inducible expression of RFP HNF4A shRNA construct in ES cells.** ES cells transduced with HNF4A shRNA were cultured under growth conditions for 3 days and upon passage were exposed to doxycycline. Analyses were completed 48 hours after the addition of doxycycline. **A.** Transduced ES cells exposed to doxycycline express high levels of the transgene as visualized by fluorescent microscopy. Transduced ES cells with no exposure to doxycycline expressed no transgene, revealing tight doxycycline control with no leakage present. **B.** Flow cytometric quantification of RFP transgene expression in ES 48 hours post-transduction. **C.** Flow cytometric analysis of pluripotency associated surface antigen expression in transduced ES cells under undifferentiated conditions.
Figure 40. Loss of inducible transgene expression after long term culture of undifferentiated ES cells. ES cells transduced with HNF4A shRNA were cultured under growth conditions for 3 days and upon passage were exposed to doxycycline. Transduced cells were cultured for an additional 15 days under undifferentiated expansion conditions with or without doxycycline and enzymatically passaged every 4 days. Flow cytometry analyses revealed a decrease in RFP transgene expression, likely as a result of promoter silencing through epigenetic modification.
Figure 41. Inducible expression of RFP HNF4A shRNA transgene in response to a doxycycline gradient prior to pancreatic lineage induction. ES cells were transduced, cultured under growth conditions for 3 days then replated at high concentration under growth conditions to prepare for pancreatic differentiation, in the presence of doxycycline. A. ES cells express increasing amounts of RFP transgene in response to increasing amounts of doxycycline. B. Flow cytometric quantification of RFP transgene expression in undifferentiated ES cells.
Differentiation of HNF4A shRNA expressing ES cells down the pancreatic lineage

To determine the effect of HNF4A knockdown on endodermal differentiation, ES cells expressing the HNF4A shRNA and untransduced ES control cells were exposed to the series of growth factors outline above. The focus of this differentiation was on the second stage, gut tube endoderm, a step where HNF4A is known to be expressed at high levels and likely regulates other genes [70]. The doxycycline gradient was maintained throughout the course of differentiation. HNF4A shRNA ES cells and ES cells without a knock down vector were examined by fluorescent microscopy to observe RFP and corresponding HNF4A shRNA expression during the first two stages of pancreatic differentiation (Figure 42). Upon culture with high levels of Activin A and Wnt3a, all ES cells showed a pronounced change in morphology and massive cell death was observed. Those cells transduced with HNF4A shRNA showed a dose dependent expression of RFP with the addition of increasing amounts of doxycycline (Figure 42A). The same dose dependent response to doxycycline proved to be true upon addition of FGF7 and NOGGIN to induce differentiation toward gut tube endoderm (Figure 42B). Although not quantified, the level of RFP expression appears to be consistent with levels observed in the transduced, undifferentiated ES cells.

Expression of stage specific genes as well as genes known to be regulated by HNF4A was examined at the definitive endoderm stage and the gut tube endoderm stage in differentiation. To confirm the knock-down of HNF4A expression, the gene was examined at the gut tube endoderm stage of differentiation. As expected, equally high levels of HNF4A were observed in the gut tube endoderm stage of HNF4A shRNA ES cells cultured without doxycycline and in untransduced ES cells (Figure 43). Upon
addition doxycycline and turn on of the shRNA at least a 50% decrease in HNF4A was observed in the ES cells transduced with the shRNA at every level of doxycycline (Figure 43).

To investigate the stability of housekeeping gene transcription under experimental conditions that include high levels of doxycycline, we compared GAPDH, 18s and cyclophilin A. Based on absolute Ct values, there is less GAPDH expression in samples as compared to 18s and cyclophilin A (Figure 44). However, there is no apparent effect of doxycycline treatment on any of the housekeeping genes examined. GAPDH was used as the endogenous quantitative reference gene in all experiments.

After confirmation of the knock down of HNF4A in this system, expression levels of genes critical in development prior to and within the gut tube endoderm stage were examined by quantitative PCR. Expression of HNF4A has been shown to be absent from definitive endoderm [202]. Consistent with this finding, we observed a complete lack of HNF4A expression in this stage of differentiation. Definitive endoderm specific genes including FOXA2, SOX17 and CXCR4 are expressed at high levels after Activin A treatment in ES cells and ES cells expressing HNF4A shRNA show no difference in expression levels of these genes (Figure 45). Representative graphs of the gene expression in the 2 μg/ml doxycycline condition which correlates to about 25% RFP and therefore shRNA expressing cells, are shown here.

To determine the role for HNF4A in regulating formation and gene expression within gut tube endoderm derived from human pluripotent stem cells, mRNA was extracted at this stage of differentiation and quantitative PCR was performed. Based on
previous studies, HNF1B has been shown to be regulated by HNF4A in the β-cells of the pancreas. Gene expression analysis of HNF1B in the gut tube endoderm confirmed about a 50% decrease in the cell population expressing the HNF4A shRNA compared to the untransduced control (Figure 46). Additional genes regulated by HNF4A include HNF4G, FOXA1 and FOXA3, each of which was examined in the gut tube endoderm. HNF4G and FOXA1 expression were slightly decreased in HNF4A shRNA populations and additional experimentation would be necessary to confirm their regulation by HNF4A at this stage (Figure 46). FOXA3 was expressed at high levels in gut tube endoderm expressing HNF4A shRNA and untransduced controls (Figure 46).
Figure 42. Maintenance of inducible RFP HNF4A shRNA transgene expression in response to a doxycycline gradient during first 2 stages of endocrine pancreas development. After exposure to a doxycycline gradient for 48 hours, pancreatic lineage differentiation was induced while continuing the doxycycline. A. Transduced ES cells exposed to high levels of Activin A with low serum for 3 days preserve RFP transgene expression as viewed by fluorescent microscopy. B. RFP transgene expression is
sustained through differentiation toward gut tube endoderm by culture with FGF7 and Noggin.

**Figure 43.** Confirmation of inducible HNF4A expression knock down in gut tube endoderm, the second stage of pancreatic differentiation. Gene expression analysis was performed on HNF4A shRNA transduced ES cells (red) and untransduced ES cells (blue) after induction of DE followed by GTE by addition of a series of growth facors in the presence of a doxycycline gradient. Quantative PCR was performed using an HNF4A specific primer/probe set.
Figure 44. RNA transcript levels of reference genes. Absolute Ct values are presented for each reference gene at levels of doxycycline from 0 to 2 μg/ml.
Figure 45. *In vitro* gene expression analysis of definitive endoderm, the first stage of pancreatic differentiation, derived from HNF4A shRNA ES cells and untransduced ES cells. Expression of genes characteristic of the DE stage of differentiation, FOXA2, SOX17 and CXCR4, were examined by quantitative PCR in ES cells transduced with HNF4A shRNA (red) and untransduced ES cells (blue). A doxycycline concentration of 2 µg/ml is represented. Expression levels were normalized to GAPDH and the baseline was set at undifferentiated ES cells.
Stage 2, GTE

DE Associated Gene Expression

GTE Associated Gene Expression
Figure 46. *In vitro* gene expression analysis gut tube endoderm, the second stage of pancreatic differentiation, derived from HNF4A shRNA transduced ES cells and untransduced ES cells. GTE derived from ES cells transduced with HNF4A shRNA (red) or untransduced ES cells (blue) were analyzed by quantitative PCR for gene expression of genes characteristic of DE (FOXA2, SOX17, CXCR4) and for a combination of genes characteristic of GTE and genes thought to be regulated by HNF4A (HNF1A, HNF1B, HNF4G, FOXA1, FOXA3, SOX9). A doxycycline concentration of 2 μg/ml is represented. Expression levels were normalized to GAPDH and the baseline was set at undifferentiated ES cells.

The data represented here demonstrate the ability to produce iPS cells from MODY1 patient and sibling dermal fibroblasts using a lentiviral vector expressing OCT4,
KLF4, SOX2 and c-MYC. Dedifferentiation occurred over a 25 day process and resulting colonies expressed genes associated with pluripotency at levels similar to ES cells. Differentiation of MODY1 sibling iPS clones toward the b-cell lineage revealed variation in efficiency of differentiation compared to ES cells. This variation led to a change in model system and creation of an inducible knock down HNF4A ES cell population using lentiviral vectors expressing HNF4A shRNA. We proved the ability of the HNF4A shRNAs to knock down HNF4A expression in the hepatic carcinoma cell line, HepG2. ES cells were then transduced and differentiated toward the pancreatic lineage. Gene expression analyses of transduced and untransduced ES cells at each stage of differentiation revealed no difference in expression at the definitive endoderm stage. However, a decrease in expression of HNF4A, HNF1B, HNF1A and SOX9 but not HNF4G was at the gut tube endoderm stage in the HNF4A transduced ES cells compared to the untransduced cells. This type of system will help to identify regulatory genes and pathways involved with pancreatic development and disease phenotypes which may lead to the discovery of novel drug targets.
DISCUSSION

The research presented here covers a wide array of topics related to development of stem cell-based therapeutics for diabetes mellitus. Projects include the ability of genetically modified AFS cells to become insulin-producing cells as an option for cell replacement therapy, the capacity of AFS cells to regulate the immune response as an option to mediate the onset of the autoimmune response present in T1DM or the immunorejection present upon allogeneic transplantation and finally, the ability of an HNF4A knock down pluripotent cell population to provide a model for pancreatic development leading to the potential for drug targeting. Here the results observed are discussed, potential pitfalls are addressed and future directions for each project are suggested.

Our results reveal that AFS cells have the potential to serve as a cell-based therapy for the treatment of DM. They can potentially be a source of replacement insulin-producing cells or may serve as immunomodulatory mediators to stop the autoimmune destruction of the β-cells in T1DM or be injected with additional allogeneic cell-based therapy to mediate the host immune response.

Evaluation of the ability of AFS cells to produce insulin

AFS cells may be an attractive source of self-renewing starting material to obtain insulin-producing cells for transplantation. Unlike their pluripotent counterparts, AFS cells do not form teratomas when transplanted \textit{in vivo}, demonstrating their safety for therapeutic applications. In addition their unlimited growth potential allows for the
creation of fully characterized, bankable cell lines. However, as AFS cells are not extracted early in embryonic development like ES cells, their ability to respond to developmental cues driving pancreatic differentiation is compromised. However, breakthroughs in cellular reprogramming prove that cell fate can be modified by the introduction of one or a combination of genes essential in the desired lineage.

In our attempts to differentiate AFS cells to functional β-cells in vitro, we discovered that AFS are incapable of upregulating genes essential in definitive endoderm, including Sox17, in response to culture with the Nodal mimetic Activin A. This result is not completely unexpected as we recognize that fetal AFS cells lie somewhere between ES cells and adult stem cells on the continuum of pluripotency. Like AFS cells, adult stem cells do not generally respond to culture with Activin A, although several reports have described forced differentiation toward insulin-producing β-like cells by genetic modification [106, 108, 272].

To drive pancreatic differentiation in AFS cells, we developed an efficient method for gene delivery and subsequent cell purification. By nucleofecting AFS cells with a dual reporter construct containing the pancreatic regulator gene PDX1 and the cell surface molecule H-2K^k we were able to effectively immunoselect the positive population. We verified that transgene levels were initially high and decreased progressively with passage, indicating the transient nature of the transfection. Upon promoting further pancreatic lineage differentiation using bFGF-containing medium we observed cell cluster formation and PDX1-dependent PAX6 expression. Overexpression of PDX1 by adenoviral transduction was also examined similar transient transgene expression was demonstrated. Similarly, upon pancreatic induction by culture with
Activin A followed by nicotinamide cluster formation and upregulation of genes critical for endocrine pancreas development, including PAX6 and NGN3, were observed. Moreover, the timing of gene expression was consistent with that seen in pancreatic development. PAX6 expression was detected early in the differentiation process and NGN3 expression in the late stages, suggesting that it may mark endocrine pancreas progenitors as in the developing pancreas [273].

In addition insulin message, c-peptide release were measured using ELISA from PDX1 transduced AFS cell clusters after long term culture under differentiation conditions. It is critical to evaluate c-peptide release from insulin-producing cells as a confirmation of *de novo* insulin production. Serum and other supplements used in pancreatic differentiation protocols contain high levels of insulin [274]. To ensure that insulin detected at the final stages of pancreatic differentiation is in fact made by the cell and not simply absorbed from the medium, detection by-products of insulin biosynthesis is essential. Although AFS cell derived clusters produced some insulin, the levels are far below those necessary for therapeutic applications. As a result, the *in vitro* differentiation protocol to achieve β-like cells from AFS cells must be modified to achieve effective insulin production. Opportunities to adjust current strategies for AFS differentiation to insulin-producing cells include changing culture conditions including differentiation medium factors and additional genetic modification.

Strategies to modify culture medium to encourage pancreatic differentiation from AFS cells may be difficult to consider as the cells do not respond to developmental cues found in the pancreas. One possibility may be addition of the chromatin remodeling agent, trichostatin A (TSA). The acetylation status of the histone mediates chromatin
structure and exerts epigenetic control over gene expression. Addition of the histone deacetylase inhibitor TSA may be used to loosen the chromatin structure to allow differentiation to occur through epigenetic modification. HDACis are also useful in modification of pancreatic cell fate determination and lead to an enhancement of endocrine and ductal cell types [275].

Another possibility to enhance AFS cell differentiation toward insulin-producing cells is added genetic modification. The creation of induced pluripotent stem cells changed our idea of lineage commitment and proved that fully differentiated somatic cells could be dedifferentiated to become stem cells by introducing expression of 4 transcription factors [82, 83]. The ability of fully differentiated cells to change fate into another functional adult cell type by genetic modification, or transdifferentiate, has also been explored. In the pancreas, exocrine to endocrine transdifferentiation is possible by ectopic expression of 3 transcription factors critical to endocrine pancreas development: PDX1, MafA and NGN3 [67]. Injection of adenoviral vectors expressing these genes into the adult mouse pancreas led to direct conversion of abundant pancreatic exocrine cells to fully functional insulin-producing cells with the ability to rescue STZ-induced hyperglycemia [67]. The same strategy has also proven successful in an in vitro model of exocrine cell transdifferentiation, although the resulting β-cells were not fully glucose responsive suggesting the need for further optimization of the culture system [276]. This type of approach may prove useful in vitro to convert AFS cells toward insulin-producing cells. Pancreatic exocrine and endocrine cells are derived from common progenitors and therefore may require less epigenetic changes for conversion. However, AFS cells are a more primitive cell type and may require addition of a different complement of
transcription factors or culture conditions to induce insulin production. Examination of other transcription factors critical in pancreatic specification and endocrine function such as Pax4 may prove useful in this effort.

Pax4 is a member of the paired box (PAX) family of transcription factors with a critical role in regulating commitment of pancreatic islets and late-stage proliferation, maturation and survival of β-cells [277-279]. Pax4 mutation and misregulation has been associated with T2DM and results in a newly defined form of MODY highlighting the importance of the gene in the endocrine pancreas [280-282]. Studies have demonstrated that ectopic expression of PAX4 promotes β-cell differentiation in a variety of cell types. Over expression of PAX4 induces differentiation of endocrine precursor cells and mature α-cells to take on a β-cell fate in vivo that are capable of restoring chemically induced hyperglycemia [248]. Co-expression of Pax4 in PDX1 expressing hepatic stem-like cells also resulted in fully functional β-cell transdifferentiation in vitro [283]. Ectopic expression of PAX4 alone or in combination with other critical pancreatic transcription factors may facilitate pancreatic lineage differentiation from AFS cells in vitro.

Another approach to force differentiation by genetic modification is reduction of genes that inhibit the pathway of interest. Recent reports show that lentiviral-mediated knock down of neuronal restrictive silencing factor (NRSF) induces differentiation of unselected amniotic fluid cells toward insulin-producing cells [110]. NRSF is a transcription factor involved in self-renewal and neuronal development [284] and has more recently been shown to target genes which regulate endocrine pancreas development including PAX4, NEUROD, HNF4A and NGN3 [285, 286]. Knock down of NRSF and culture with Activin A and bFGF to promote initiation of pancreatic gene
expression resulted in cells with a β-like cell phenotype [287]. Additionally, knock down of Foxo-1, a transcription factor critical in regulating β-cell mass [288, 289], in enteroendocrine progenitor cell of the gut has been reported to give rise to glucose-responsive insulin-producing β-like cells in vivo [290].

In another in vivo model, sophisticated cell tracking after chemical destruction of β-cells revealed α-cell to β-cell conversion without genetic manipulation [291]. Further examination of signaling processes involved in this naturally induced change in cell fate may reveal potential gene targets for forced differentiation. A tightly controlled transcriptional network is required for development and function of the endocrine pancreas. By taking advantage of what we know about pancreatic transcriptional regulation and artificially manipulating the system we may achieve functional insulin-producing cells from AFS cells.

In conclusion, we have presented evidence to suggest that AFS cells over expressing PDX1 can be differentiated in vitro to form insulin-producing clusters. Although further optimization is necessary, AFS cells present a promising resource as a renewable source of β-cells for diabetes therapy.

Evaluation of the immunomodulatory functions of AFS cells

AFS cells appear to be better suited for immunomodulatory applications rather than differentiation toward fully functional insulin-producing cells. In developing cell-replacement therapeutics for diabetes patients it is important to consider two distinct
immune responses. The first is the autoimmune response to the insulin-producing β-cells in T1DM and the second is the potential for rejection of implanted allogeneic cells.

Human amniotic fluid stem (AFS) cells are broadly multipotent, derived from an immunoprivileged site, can be expanded extensively in culture, and do not form tumors when implanted in immune deficient mice. We assessed AFS cells for immunomodulatory properties, compared to bone marrow-derived mesenchymal stromal cells (BM-MSCs). Characterization of AFS cell surface markers revealed high expression of major histocompatibility (MHC) Class I and lack of MHC Class II or co-stimulatory molecules (CD80, CD86, CD40) consistent with previous reports [111, 153, 161]. Interferon-gamma (IFN-γ) greatly increased expression of both MHC Class I and MHC Class II. This indicates that AFS cells, like MSCs, may not strongly activate rejection responses in allogeneic hosts.

We further observed that AFS cells, again similar to MSCs, inhibit phytohemagglutinin (PHA)-induced activation of lymphocytes in a dose-dependent manner. The mechanism of immunosuppression by AFS cells involves both direct cell-cell contact between the stem cells and the immune cells and indirect interaction through immunosuppressive factors secreted by the stem cells in response to activation. Analysis of cell-free supernatants demonstrated a substantial overlap in the cytokine release profiles of AFS cells and BM-MSCs, either at rest or when activated with PBMCs or interleukin 1-beta (IL-1β).

In response to activation, the most highly upregulated protein in both cell types was interleukin 6 (IL-6). IL-6 is a broad-acting cytokine involved in the control of the
immune response as well as stem cell development and regulation [165, 292]. Mesenchymal stem cells derived from cord blood (CB-MSCs) and BM-MSCs have also been reported to secrete high amounts of IL-6 when activated with IL-1β [293]. The biological relevance of this IL-6 response likely lies in both local and systemic protection against inflammation [294, 295]. BM-MSCs and AFS cells also released high levels of growth related oncogene family members GRO and GRO-α as well as monocyte chemotactic protein-1 (MCP-1) upon IL-1β activation. These chemokines have well known effects on cells of the immune system and are important in inflammation and wound healing. GRO acts on neutrophils and MCP-1 acts mainly on macrophages, recruiting them to sites of inflammation [296].

Compared to BM-MSCs, activated AFS cells released significantly higher levels of several cytokines including MCP-2, MIP-3α and MIP-1α whereas the activated BM-MSCs released significantly higher levels of GRO-α, MCP-3, GCP-2 and IL-8. These data suggest that AFS cells may possess some alternative molecular mechanisms to modulate the immune response. In this regard, we have recently compared the effect of rat AFS (rAFS) and rat BM-MSC (rBM-MSC) in a rat model of experimental necrotising enterocolitis (NEC). We were able to show that rAFS cells significantly improved the survival and enhanced the repair of the damaged intestine, whereas rBM-MSC had no effect (Paolo De Coppi, personal communication). Similar results have been achieved using human AFS cells to repair the kidney and restore its function in an immunodeficient mouse model of acute tubular necrosis (ATN) [112]. In this model AFS cells appear to possess immunomodulatory function, initiating the release of several
murine anti-inflammatory cytokines and reducing the release of pro-inflammatory cytokines [112].

Current stem cell therapies using MSCs require large cell doses and as a result of the limited growth potential of MSCs, cells from several donors must be pooled for a single treatment, a practice resulting in treatment variation. AFS cells have the unique advantage of extensive self-renewal in vitro allowing for long-term expansion, full characterization and cryopreservation. These factors make AFS cells an ideal source for cell banking and standardized ‘off-the-shelf’ therapeutic use, allowing them to serve as a reproducible immunomodulatory cell therapy source especially for autoimmune diseases such as T1DM.

The first immune response of concern when treating diabetes is the autoimmune response which recognizes and destroys β-cells in T1DM patients. Trials aimed at early intervention and prevention of disease progression are currently underway in humans and some are stem cell-based. For example, autologous hematopoietic stem cell transplantation (HSCT) in newly diagnosed T1DM patients achieved insulin independence for up to 5 years [297]. AFS cells have the potential to be used in the same capacity to inhibit the autoimmune attack and preserve existing β-cells in T1DM patients. The autoimmune response is active 3-5 years before the onset of symptoms and diagnosis of T1DM. Significant efforts are also being made to identify biomarkers of an ongoing autoimmune response to diagnose and treat T1DM prior to destruction of β-cell mass. Persistence of autoimmunity has also been observed in islet transplantation recipients and is associated with decreased rates of insulin independence and shorter graft survival time [298]. Autoantibodies including anti-GAD65 and anti-IA2 along with β-cell specific
auto-reactive cytotoxic and memory T cells have been observed in transplantation recipients [299]. Great strides have been made in the development of targeted therapies, in particular CD-3 specific antibodies, to hinder the continuing autoimmune response [300].

The host immune response to implanted foreign islets or β-like cells is also of significant concern. The loss of transplanted islets mass and function is the result of the alloimmune response [301, 302]. Current immunosuppression regimens perform well at keeping the acute host immune response at bay but as previously mentioned carry significant risks. Studies of co-transplantation of MSCs with islet grafts in diabetic mouse models suggest that MSCs may be capable of facilitating the survival of insulin-producing β-cell grafts through graft remodeling as well as immunomodulation [303]. Several reports prove that MSCs promote islet revascularization [304-306], enhance β-cell proliferation [307, 308] and decrease β-cell apoptosis [306, 309] when transplanted with islet grafts. In addition, examination of the immune response in co-transplanted animals has revealed a decrease in Th1 and increase in Th2 cells [310, 311], a decrease in naive and memory T cells [311] and a decrease in pro-inflammatory cytokines [310-312]. Co-transplantation of MSCs and islets in STZ-induced diabetic mice resulted in improved capacity to reverse hyperglycemia, better maintenance of islet morphology and increased graft vascularization [313]. AFS cells, like MSCs, have the potential to be transplanted alongside allogeneic insulin-producing cells to mediate the host immune response, improving long-term engraftment and preventing rejection.

In the future, additional efforts could be made to classify the immune response to AFS cells. In the results presented here, we cultured AFS cells or cell-free supernatant
with total peripheral blood mononuclear cells and examined T-lymphocyte activation by IFN-γ release. However, T-cells represent only one part of the immune response; many more cell types and responses make up the complete immune system. Dendritic cells (DCs) are an antigen-presenting cell (APC) type critical in the immune response. Co-culture of lipopolysaccharide (LPS) stimulated monocytes with AFS cells would likely inhibit monocyte maturation into DCs and could be measured by examining phenotypic and functional changes in the monocyte population [314]. We could expect to see inhibition of upregulation of costimulatory molecules and chemokine receptors assessed by flow cytometry and a decrease in the formation of dendrites by confocal microscopy in cultures with AFS cells. B-cells and natural killer (NK)-cells are also important in the immune response and AFS cells may have the ability to inhibit their proliferation [265, 315]. Co-culture of activated and CFSE labeled B-cells or NK-cells with AFS cells would be likely to show a decrease in proliferation by flow cytometry compared to activated B or NK-cells cultured without AFS cells or with a fibroblast cell control. Identification of immune cell/stem cell interactions and further characterizing the mechanisms of AFS-mediated immunomodulation would be useful in determining their ideal therapeutic use.

AFS cells also have the potential to serve as an amazing research tool for identification and isolation of soluble factors involved in immunomodulation for use in cell-free therapeutics. We discovered that cell-free supernatant derived from IL-1β stimulated AFS cells has the ability to suppress T lymphocyte activation. Characterization of the soluble factors secreted by AFS cells in response to stimulation with IL-1β was performed by protein array. Application of the factors identified including IL-6, MCP-1, GRO and GRO-α, individually or in combination, to systems of
inflammation would advance knowledge on the effects of these morphogens. Further characterization of the complex profile of soluble molecules secreted by AFS cells is critical and may lead to the development of novel strategies for direct application of these factors for therapeutic approaches.

Evaluation of HNF4A deficient pluripotent stem cells as an in vitro disease model

Somatic cell reprogramming with defined factors allows for the generation of patient-specific iPS cells that have the potential to be a model for complex genetic disorders, drug and toxicology screens and autologous cell replacement therapy. The ability to generate patient-specific pluripotent stem cells using iPS technology has the potential to revolutionize our understanding of diabetes mellitus. This approach has been reported to be successful in several model systems of diseases of metabolic function including T1DM [86]. MODY is a useful model for genetic studies of diabetes because of its well-defined monogenic inheritance, high penetrance and early age of onset which allows for the collection of pedigrees including several generations of subjects [316].

Our results on this subject have indicated that we are able to produce induced pluripotent stem cells from MODY1 patients and an unaffected relative of the patient using an integrating lentiviral vector. Characterization of the reprogrammed pluripotent stem cells reveal gene and protein expression of several pluripotency associated factors including OCT4, SOX2, NANOG, TRA1-60, TRA1-81 and SSEA4. However, upon in vitro differentiation toward the endocrine pancreas lineage using defined factors, we observed variation in differentiation efficiency in iPS cell lines compared to ES cells.
To circumvent this issue we created an inducible, selectable knock down HNF4A ES cell population using a lentiviral vector. As a result of loss of transgene expression observed over time in transduced ES cells, a heterogeneous HNF4A shRNA transduced ES population was examined by quantitative PCR in differentiation studies down the pancreatic lineage. As expected, culture with high levels of Activin A pushed the stem cells toward a definitive endoderm phenotype. There was no difference in gene expression in the definitive endoderm stage between HNF4A shRNA expressing ES cells and ES cells that had been cultured under the same conditions. Both HNF4A shRNA transduced and untransduced ES cells expressed equally high levels of FOXA2, SOX17 and CXCR4. Upon investigation of the gut tube endoderm stage of differentiation, a stage where HNF4A is known to be expressed, some interesting differences were seen between the HNF4A shRNA transduced and the untransduced ES cells. First, we analyzed the expression of the definitive endoderm associated genes in the gut tube endoderm stage of differentiation. As expected, we saw a down regulation of FOXA2, SOX17 and CXCR4 expression to similar levels in both cell populations suggesting the transition away from a definitive endoderm phenotype. We also investigated the expression of genes known to be associated with the gut tube endoderm and genes known to be regulated by HNF4A at this stage. Gene expression studies revealed that several genes were downregulated in the HNF4A shRNA expressing gut tube endoderm compared to normal gut tube endoderm including HNF1A, HNF1B, FOXA1 and SOX9 but not HNF4G or FOXA3. To ensure that the gene regulation that has occurred is the result of HNF4A deficiency, we should continue with experiments to replace HNF4A expression and observe a rescue of the
phenotype. These results suggest that HNF4A deficiency has an effect on many genes in the gut tube endoderm stage of pancreatic development.

In these experiments we observed that knock down of HNF4A resulted in decrease of HNF1A expression in the gut tube endoderm stage of differentiation. Previous studies indicate that HNF4A regulates HNF1A expression in mature β-cells and that a functional binding site for HNF4A exists in the HNF1A promoter [211, 317]. Interestingly, HNF1A haploinsufficiency causes MODY3, the most common form of the disease [12]. Evidence suggests that HNF1A plays a role in maintaining β-cell mass as it has been shown to regulate genes involved in cell cycle control including cyclin E and IGF1 [318-321]. We would then hypothesize that β-cells deficient in HNF1A as a result of HNF4A knock down would have less replicative potential. To test this hypothesis examination of cell replicative potential can be detected by proliferation assays incorporating the synthetic nucleoside bromodeoxyuridine (BrdU). However, quantification of stem cell derived β-cell proliferative potential in vitro may be difficult as attempts to culture β-cells have resulted in rapid deterioration of β-cell phenotype following only a few population doublings [322-325]. Induction of β-cell expansion is a critical area of research and several small molecules have been identified in this pursuit. Activation of Wnt signaling has been shown to promote β-cell survival and replication [326-328]. Small molecule screens led to the finding that treatment with L-type calcium channel activator agonist 2A and Glp-1 receptor agonist Exendin-4 also induced β-cell replication [329]. Closer examination of signaling pathways occurring during natural events which result in β-cell growth, including pregnancy, may lead to the discovery of additional β-cell proliferative agents. It would be expected that HNF4A deficient β-cells
would be less responsive to these proliferative agents compared to their normal counterparts.

HNF1B expression was also decreased in the gut tube endoderm stage of HNF4A knock down cells. Expression of HNF4A and HNF1B is characteristic of the gut tube endoderm stage in pancreatic development [70]. Haploinsufficiency of HNF1B results in MODY5, a form of the disease leading to impaired insulin secretion and decrease in β-cell mass [330, 331]. HNF1B is expressed during pancreatic development and not in the mature b-cell, suggesting that disease phenotypes can occur as a result of gene dysregulation in pancreatic progenitors. Expression of HNF1B is found in primitive pancreatic ductal cells that are direct precursors of NGN3 positive endocrine progenitor cells [332]. HNF1B and HNF4A have been linked as the genes have reciprocal binding sites within their promoters, indicating a direct regulatory relationship. Previous studies in β-cell specific HNF1B knockout mice reveal a decrease in HNF4A expression in mature β-cells [333]. The decrease of HNF1B expression in HNF4A-depleted gut tube endoderm would likely result in a decrease of NGN3 expressing progenitors and therefore a decrease in β-cell mass in vitro.

We observed a decrease in SOX9 expression in the gut tube endoderm stage of differentiation upon HNF4A knock down. Studies have revealed SOX9 as a marker of pancreatic progenitors with a critical role in initiation of endocrine development [334]. Along these lines, Sox9 has been shown to regulate a network of endocrine differentiation genes including NGN3 and PDX1 [334, 335]. Although HNF4A and SOX9 have demonstrated expression during the same developmental stage, until now, HNF4A has not been associated with SOX9 regulation. Interestingly, both HNF4A and
SOX9 have a biphasic expression pattern in pancreatic development. HNF4A and SOX9 are expressed early in the gut tube endoderm stage, both are down regulated and in the mature pancreas HNF4A is expressed in β-cells while SOX9 is expressed in ductal cells. As we have yet to perform antibody staining, it is unclear whether the decrease in SOX9 expression observed is the result of a smaller number of cells expressing the same level of transcript or the same number of cells expressing lower levels of transcript. Either way, the decrease in SOX9 expression resulting from HNF4A knock down would likely lead to a decrease in the pancreatic progenitor pool and a decrease in functional pancreatic endocrine cells. The results presented here suggest that HNF4A expression is critical for normal pancreatic development. The type of system developed here has great potential to identify novel regulatory genes in pancreatic lineage specification.

The system developed allows for inducible expression of an HNF4A shRNA construct in pluripotent stem cells. Future experiments could examine the effect of HNF4A deficiency on each stage of in vitro pancreatic lineage differentiation. Addition of doxycycline to specific stage(s) in the differentiation protocol and analysis of resulting β-cells may help to pinpoint the time of pancreatic development in which HNF4A is critical for normal β-cell function. This type of experiment could be performed with a variety of pancreatic regulatory genes and information gained from the results would give us more information on the signaling and gene regulatory pathways important in later stages of endocrine pancreas development. This kind of information would likely lead to improvements in current pancreatic differentiation protocols and may allow achievement of large numbers of functional β-cells which could then be used as cell-replacement therapy in DM patients.
Clinical considerations

Many issues must be addressed before patient specific iPS cells or gene knock down ES cells are used for cell replacement therapy or to model pancreatic development \textit{in vitro} to screen for potential therapeutics. The biggest hurdle we faced in the development of patient specific iPS cells was the line-to-line variation; this known issue must be addressed before iPS cells are a viable option for the majority of labs. In the alternative HNF4A knock down ES cell population approach, loss of transgene expression created an obstacle. Both pluripotent stem cell lines have the potential to form teratomas \textit{in vivo}, a potential problem for clinical translation. However, the chief concern in these efforts is the inability to obtain pure, functional β-cells from pluripotent stem cells. Significant efforts should be placed toward understanding pancreatic cell signaling and manipulating \textit{in vitro} culture conditions to achieve this essential goal.

Variability in the ability of pluripotent stem cell lines to achieve lineage-specific differentiation has been reported previously in numerous studies [336-338]. Stem cell populations in general are not a homologous population, \textit{in vitro} or \textit{in vivo} [339], but are likely inherently heterogeneous at the molecular level which leads to these differences in differentiation capabilities [340, 341]. Identification of a molecular phenotype which could further characterize and classify pluripotent stem cells may aid in prediction of their differentiation capabilities.

Introduction of the transcription factors OCT4, SOX2, KLF4 and c-MYC by integrating lentiviruses into MODY1 patient and MODY1 normal sibling fibroblasts
followed by culture in ES conditions led to dedifferentiation. Morphological criteria were used to identify reprogrammed iPS colonies and selected colonies appeared to be identical to ES cells with regards to pluripotency-associated gene and protein expression. However, attempts at directed differentiation down the pancreatic lineage revealed significant variation between ES and iPS colonies. Variation has been reported by several researchers in the field and the current method to circumvent the issue is creation and characterization of a large number of iPS lines from each patient [270, 342, 343]. To make the iPS system a viable option for examination of genetic disease development and cell function *in vitro*, it is critical to address the issue of line-to-line variation.

Although insulin production was not achieved from pancreatic lineage differentiation from normal iPS clones, a large amount of variation between iPS cell lines derived from the same donor as well as with the iPS cell lines compared to the ES cell line was observed throughout differentiation. In our current system, possible reasons for the variation observed include the use of an integrating lentiviral vector for reprogramming, the potential for heterogeneity within individual clones and the presence of copy number variations and point mutations.

Use of an integrating lentiviral vector that brings with it the issues of integration site, position effects and copy number, is one potential cause of the observed variation. Lentiviral integration sites are located randomly throughout the genome and are different in each cell. The location of the integration sites in individual cells, which went on to form colonies and then iPS cell lines, may cause differential expression or regulation of the transgene and surrounding endogenous genes. In addition, viral copy number, which determines the number of integration sites, may have been different from fibroblast to
fibroblast and as a result from iPS line to iPS line. Non-integrating methods of iPS production including recombinant protein [197] and mRNA [198], are the current focus in the field to avoid these issues.

Cellular reprogramming involves gene expression transformation and change in the epigenetic state of pluripotency promoters [344]. Although individual iPS colonies were selected and expanded, true clonal selection of iPS cells is technically challenging as clonal growth efficiency is extremely low when pluripotent cell types are plated as single cells. As a result, it is possible that heterogeneity may exist within the iPS cell lines created here. In addition, cells in the same culture may not respond identically to ectopic expression of pluripotency associated transcription factors at the epigenetic level. Differing epigenetic states may also lead to heterogeneity and altered response to differentiation protocols. Recent studies have also found that iPS cells carry copy number variations including deletions and duplications [345-347] and point mutations [348], potential sources of additional variances which could cause altered differentiation capacity.

As a result of the line to line variability observed in iPS cells, our focus shifted toward gene knockdown in pluripotent ES cells using a lentiviral shRNA. Interestingly, ES cells transduced with HNF4A shRNA gradually lost RFP expression in response to doxycycline exposure, presumably as a result of transgene silencing. The fading of RFP expression occurred quite rapidly with only 11% remaining after just 4 passages. Pluripotent stem cells are known to silence several commonly used promoters through epigenetic modification [271]. The promoter driving the expression of RFP and HNF4A shRNA is a tetracycline response element (TRE) fused to a minimal CMV promoter. The
minimal CMV lacks the enhancer of the complete CMV promoter and as a result is inactive in the absence of doxycycline binding TRE.

To verify that gene silencing was taking place, it would be necessary to perform bisulfite sequencing on the promoter to reveal its methylation state which would allow extrapolation of the promoter activity level. An additional experiment to assess transgene silencing would be to detect transcription and translation of the HNF4A shRNA transgene by PCR and western blot, respectively. As there is very little RFP expression observed by fluorescence microscopy we would expect there to also be a progressive loss of HNF4A shRNA expression as measured by these methods as well. An important consideration of potential transgene silencing is whether silencing occurs in all cells equally or whether there is selective pressure for silenced ES cells which enriched RFP negative cells throughout passaging.

Even with a stable pluripotent stem cell source, the inability to attain large numbers of functional β-cells in vitro draws attention to a lack of understanding of pancreatic organogenesis and β-cell maturation. Directed differentiation from pluripotent stem cells towards the pancreatic lineage in vitro is a currently largely two-dimensional process which results in a small percentage of insulin-producing β-cells classified as being immature. Insulin-producing cells derived using current differentiation protocols more closely resemble fetal b-cells rather than mature b-cells as they are unable to respond to increasing glucose levels and they express multiple hormones within the same cell [71, 72]. However, pancreatic progenitors derived from similar protocols and subsequently implanted in vivo are able to mature to produce high quality, functional β-cells [349]. Recent attempts have been made to identify markers of functional b-cell
maturation to identify and potentially isolate mature b-cells derived in vitro. Comparison of gene expression of in vitro and in vivo derived b-cells revealed the presence of ucnortin 3 (UCN3) only in the functional in vivo derived insulin-producing cells [350]. Defining mature, adult b-cell markers is an important step in screening culture conditions to induce functional b-cell differentiation in vitro.

The complex in vivo environment directing pancreatic development is made up of signaling molecules, growth factors, extracellular matrix interactions and three-dimensional structure. In comparison, the in vitro system used in these experiments and in most other reports of β-cell differentiation from pluripotent stem cells is lacking. Current in vitro directed differentiation protocols demand extensive revision to achieve the pure numbers of mature β-cells or β-cell progenitors required for cell replacement therapy and for in vitro drug development assays. Obstacles worth investigating in existing directed differentiation practices include the less than perfect emulation of signaling pathways from pancreatic organogenesis at earlier stages, the potential inability of current systems to support β-cell maturation and finally, absence of three-dimensional niches including extracellular matrix components.

Throughout pancreatic organogenesis the developing organ is responding to signals released from cells of each of the other germ layers [351]. Although efficient in vitro differentiation to definitive endoderm and gut tube endoderm is achievable, it is possible that current in vitro differentiation protocols are lacking essential early-stage signals that may be secreted from mesodermal or ectodermal cells in vivo. As a result, the intermediates or later stage progenitors may be unable to effectively respond to the appropriate growth factors. Further investigation and clarification of early developmental
signals is warranted, although this seems less likely to be an issue as β-cells are able to be derived from pancreatic progenitors that have been developed in vivo, suggesting that progenitors have the ability to respond to maturation signals if placed in the appropriate environment [349].

More likely is the inability of the current system to fully support late stage pancreatic development and maturation. The largest decrease in differentiation efficiency of in vitro cultures is observed in the last two stages, from pancreatic precursors to pancreatic endoderm and finally to insulin-producing β-cells, suggesting that modification of these stages is the key to efficient β-cell production. Revisiting in vivo pancreatic development and inspecting critical signaling pathways and their origins is a critical step in perfecting in vitro β-cell differentiation. Manipulating signaling pathways by including additional growth factors, incorporating co-culture with other cell types, integrating extracellular matrix molecules and use of three-dimensional culture techniques are potential ways to increase differentiation efficiency and induce mature β-cell function.

As cell-cell and cell-matrix interactions are critical signaling initiators in development of functional β-cells, incorporation of co-culture with cells and/or extracellular matrix components will likely prove to be an essential factor in efficient in vitro differentiation. Key interactions between mesenchymal and epithelial cells that occur during pancreatic development are currently not recapitulated in vitro. Introducing co-culture with pancreatic mesenchyme or endothelial cells may lead to increased differentiation and maturation efficiency. Examination of gene expression and soluble
factor release from pancreatic mesenchyme may also lead to the identification of additional medium components.

Mature β-cells developed in vivo reside in a three-dimensional structure in which interaction with other β-cells, other endocrine lineage cells, neuronal cells and vascular endothelial cells are critical for advanced function [352, 353]. Employing three-dimensional scaffolds loaded with pancreatic endocrine derived extracellular matrix to promote aggregation in late stages of differentiation will likely facilitate β-cell maturation and in turn will enhance in vitro derived β-cell function. These steps are critical for the development of a suitable, transplantable source of β-cells for cell replacement therapy and for pancreatic lineage intermediates or mature β-cells to be tested in drug development assays.

Once functional β-cells are able to be derived from pluripotent stem cells, a major cause for concern involves the potential for remaining undifferentiated stem cells within the population. A chief characteristic of undifferentiated pluripotent stem cells is their ability to form teratomas upon transplantation [69]. Teratoma formation after pancreatic progenitors derived from ES cells were implanted in the fat pads of mice for maturation to functional insulin-producing cells has been observed previously [349]. To avoid the risk of teratoma formation the cell population to be transplanted must be purified and homogeneous to be sure that it is free of undifferentiated pluripotent stem cells. This type of purification may be achieved by fluorescence activated cell sorting or magnetic cell sorting upon identification of a cell surface antigen characteristic for a pancreatic progenitor or fully differentiated β-cell. One cell surface antigen that has the potential to be useful in this effort is CD24, a marker that has been shown to be present on the cell
surface in the pancreatic progenitor stage of *in vitro* differentiation and to be associated with PDX1 at this stage [354]. Additional efforts are focused on insertion of a suicide vector which may be activated to specifically destroy undifferentiated pluripotent cells expressing a stem cell specific gene such as OCT4 [355]. Incorporating these methods into differentiation protocols would likely result in a pure and transplantation ready source of pancreatic progenitors or β-cells for cell replacement therapy.

Prior to beginning use of stem cell derived β-cells in cell-replacement therapy applications, the patient immune response must also be addressed. However, with advancement of patient-specific stem cells may make host immune response suppression an unfounded concern in β-cell replacement therapy. Induced pluripotency in somatic cell types using defined factors allows for the creation of immune matched pluripotent stem cells that can be differentiated toward any lineage, including endocrine pancreas, *in vitro* for reimplantation *in vivo*. Patient specific iPS cells derived from patients diagnosed with T1DM have been successfully differentiated toward β-cells *in vitro* [86]. Although these iPS cells were derived with integrating retrovirus, several successful attempts have been made to create iPS cells through nonintegrating methods making them safe for transplantation. These results indicate that when methods to eliminate pluripotent stem cells from mature cultures have been solidified and when the differentiation procedure is perfected to achieve large numbers of highly functional β-cells, β-cell replacement therapy will become a reality.
Conclusions

The ultimate goal of stem cell research is to develop therapeutics to cure patients with diseases caused by the loss of functional tissue as in diabetes. Stem cells are an attractive starting source for producing pancreatic lineage derivatives to be used in cell replacement therapy, immunotherapy and for attempts to model disease phenotypes in vitro as a means to screen potential drug targets. Stem cells offer a renewable and in some cases bankable source of starting cells from which to achieve insulin-producing cells or immunomodulatory cells. Stem cells also offer the ability for cost-effective drug screening on human cells to test for therapeutic targets as well as in toxicity studies.

Although there is great promise for stem cells to advance therapeutics for diabetes patients, several challenges remain to be overcome. The primary concern with stem cell derived cell therapy is the inability to achieve efficient functional β-cell differentiation in vitro. Even the most effective β-cell differentiation protocols yield a mixed population consisting of a small percentage of insulin-producing cells combined with other pancreatic progenitors and non-pancreatic cells. For therapeutic applications including cell-replacement therapy, examining the pathogenesis of disease or for testing potential drug targets, cell types should be tested individually or combined in known proportions. Pancreatic differentiation protocols capable of increasing efficiency, sorting out insulin-producing cells and/or replicating resulting β-cells are necessary to supply the large numbers of homogeneous β-cells required for therapeutic applications.

Stem cell derived insulin-producing cells are not yet at a stage where they are suitable for clinical application in β-cell-replacement therapy and obstacles must also be
overcome before their use in drug discovery assays. However, stem cells present a tremendous resource for use in basic research, the pharmaceutical industry and regenerative medicine. Stem cell-based regenerative pharmacology will likely lead to cures for many diseases, including diabetes.


151. Bruder, S.P., N. Jaiswal, and S.E. Haynesworth, Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during


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ABSTRACTS


