EXPLORING MOLECULAR MECHANISMS OF LIVER TO PANCREATIC BETA CELL REPROGRAMMING

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To my financial advisors and best friends, Grandma Jane and Grandpa Billy
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EXPLORING MOLECULAR MECHANISMS OF LIVER TO PANCREATIC BETA CELL REPROGRAMMING

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Ectopic expression of pancreatic transcription factors (PTFs) reprograms hepatic cells toward pancreatic beta cells. However, molecular mechanisms underlying this process have not been well studied, particularly regarding suppression of the hepatic phenotype. To study the mechanisms involved in hepatic to pancreatic beta cell reprogramming, we generated lentiviral vectors expressing PTFs (Pdx1, Pdx1-VP16, and Ngn3), transduced hepatocellular carcinoma Huh7 cells, and examined the early effects on expression of key pancreatic and hepatic genes. We demonstrated that Pdx1 not only activates beta cell-specific genes but also suppresses the key hepatic transcription factor HNF1alpha and hepatic functional gene ALB by RT-PCR and western blotting. Luciferase reporter assays further confirmed Pdx1-mediated suppression of HNF1alpha in hepatic cells. HNF1alpha suppression may be, in part, due to up-regulation of HNF4alpha P2 isoforms, which was also observed following ectopic Pdx1 expression, because these isoforms are weaker than P1-driven isoforms and they may compete for binding the HNF1alpha proximal promoter. We also cloned a 3kb proximal HNF1alpha promoter element into the pGL3 luciferase reporter to further study its regulation. We found that NK6 homeodomain 1 (Nkx6.1) binds to a cis
regulatory element in the HNF1α promoter and is a major regulator of this gene in beta cells. We identified an Nkx6.1 recognition sequence in the distal region of the HNF1α promoter and demonstrated specific binding of Nkx6.1 in beta cells by EMSAs and ChIP assays. Site directed mutagenesis of the Nkx6.1 core binding sequence eliminates Nkx6.1 mediated activation and substantially decreases activity of the HNF1α promoter in beta cells. Over-expression or siRNA-mediated knockdown of Nkx6.1 gene expression results in increased or diminished HNF1α gene expression, respectively, in beta cells. We conclude that Nkx6.1 is a novel regulator of HNF1α in pancreatic beta cells.

In addition, following our lab’s discovery of Pdx1 autoantibodies (PAA), we developed a sensitive, specific, and non-radioactive liquid-phase luciferase immunoprecipitation systems (LIPS) assay for detection of PAA. We screened sera from patients with type 1 diabetes, systemic lupus erythematosus, rheumatoid arthritis, and various forms of cancer (including pancreatic cancer) and detected positive PAA sera from all groups.
Autoimmune (type 1) diabetes is a disease in which the immune system targets and destroys the insulin-producing beta cells within the pancreatic islets of Langerhans, resulting in the inability to produce sufficient insulin in response to elevated glucose levels\textsuperscript{1,2}. Insulin replacement is the most widely used therapy for controlling blood glucose concentration to prevent acute disease. Although this therapy provides short-term benefit to patients with type 1 diabetes, euglycemia is extremely difficult to maintain. The serious acute complications of hypoglycemia, such as diabetic coma, and the difficulty of maintaining euglycemia lead type 1 diabetic patients to frequently have blood glucose levels that are elevated from the physiological norm. Unfortunately, long-term hyperglycemia can damage the integrity of the vasculature and is commonly associated with disease of the heart, kidneys, and retinas, and extremities.

**Tissue Pathology and Cellular Pathology**

Type 1 diabetes is an autoimmune disease characterized by the immunological destruction of beta cells within the pancreatic islets of Langerhans\textsuperscript{1}. Islets are clusters of endocrine cells consisting of insulin secreting beta cells, glucagon secreting alpha cells, somatostatin secreting delta cells, pancreatic peptide secreting PP cells, and ghrelin producing epsilon cells. Beta cells account for about 70\% of cells within the islet and are responsible for synthesizing and releasing insulin in order to maintain physiological blood glucose concentration. Insulin release allows for the cellular uptake and utilization of glucose for energy. Clinical diagnosis of type 1 diabetes usually occurs after 80\% - 90\% of pancreatic beta cell mass has been destroyed\textsuperscript{3}. At this time,
remaining beta cells often have enlarged nuclei and degranulated cytoplasm and islets become abnormally small due to the destruction of the majority of their cells.\(^1\)

The mechanism by which beta cell destruction occurs is through autoimmune attack.\(^1\) This autoimmunity is associated with a chronic inflammatory infiltrate known as insulitis, consisting primarily of CD8+ T lymphocytes, although CD4+ T lymphocytes, B lymphocytes, macrophages, and natural killer cells are also involved in the response. The T cell mediated destruction of beta cells is due to autoreactive T cells for specific beta cell proteins, including insulin, glutamic acid decarboxylase (GAD), insulinoma 2 associated protein (IA-2), and zinc transporter 8 protein (ZnT8)\(^4-6\). Autoantibodies can serve as an early indicator for increased susceptibility to type 1 diabetes because they are present in 70% - 80% of newly diagnosed patients as opposed to their presence in only 0.5% of the general population.\(^1\)

**Molecular Pathology**

Genes provide both susceptibility and protection for type 1 diabetes\(^1-3,5\). The greatest contributing genetic component is the major histocompatibility complex (MHC), located on human chromosome 6. The region containing the highly polymorphic human leukocyte antigen (HLA) genes has been determined to have the greatest influence on disease susceptibility. There are three classes of HLA genes differing in their function. HLA class I molecules form dimeric proteins composed of alpha chains in association with \(\beta2\) microglobulin. These molecules are present on the surface of all nucleated cells and platelets and their function is to present antigenic peptide fragments to T cell receptors on CD8+ T lymphocytes. The HLA class II molecules consist of three subclasses (HLA-DR, HLA-DQ, and HLA-DP) and are also dimeric proteins composed of alpha and beta chains. They are constitutively expressed in antigen presenting
dendritic cells and B lymphocytes, while expression can be induced in macrophages and endothelial cells. These molecules contain a binding site for peptide fragments of antigens and present these antigenic peptides to T cell receptors on CD4+ T lymphocytes. The HLA class III molecules form a variety of structures with many differing functions; examples include complement components, tumor necrosis factor, and heat shock protein, Hsp70.

Although the functions of HLA genes are well characterized, their specific contribution to disease pathogenesis is poorly understood\(^3,\,5\). It is obvious that the structural differences between susceptible and protective HLA proteins will functionally affect their interaction with antigens and autoreactive T lymphocytes. Several hypothesized mechanisms have been proposed. It is possible that defective HLA molecules bind to beta cell specific antigens in the periphery and induce an autoreactive T cell response. Another explanation is that unstable HLA/self antigen complexes in the thymus fail to induce self tolerance and release auto reactive T cells to the periphery. Yet a third hypothesis is that defective HLA molecules alter T cell activity by their interactions with T cell receptors and modulate their phenotype from regulatory to inflammatory.

**Susceptibility: Genetic and Environmental**

It is clear that there are both environmental and genetic components related to type 1 diabetes susceptibility\(^3\). This is evident from its familial occurrence; however, it does not follow a Mendelian pattern of inheritance, likely because it is a multifactorial disease. In the United States there is increased lifetime susceptibility for first degree relatives of diabetics when compared to members of the general population (5% vs. 0.3%). Twin studies also demonstrate a higher concordance rate for monozygotic twins.
when compared to dizygotic twins (30-50% vs. 6-10%)\(^3\). Although there is a clear genetic relationship to type 1 diabetes susceptibility, the concordance rate for identical twins is very low relative to other genetic diseases. Nutrition, viral infections, exposure to toxins, vaccinations, climate, and stress are all commonly implicated in the manifestation of the disease; however, extensive research has been unable to determine that any environmental factor actually causes the disease\(^2,3,5\). It is therefore likely that genetic susceptibility for type 1 diabetes is inherited and that environmental factors influence or modify its development.

**Therapy and Treatment**

Currently, insulin replacement therapy is required to maintain euglycemia and to prevent serious acute disease. Unfortunately, this therapy is incapable of maintaining the equivalent stringent control of blood glucose levels provided by healthy beta cells. Without proper blood glucose regulation, several complications due to vasculature degradation can occur. New therapies for the treatment or cure of type 1 diabetes must focus on two elements: beta cell replacement and recurrent autoimmunity\(^2,7\).

Transplantation therapies are an option, but donor organs are difficult to acquire and require the use of long term immunosuppression in order to prevent allograft rejection and recurrent autoimmune destruction\(^2\). In addition, average transplantation fails after five years. Islet transplantation is perhaps a safer treatment option, but requires several donor pancreata for each patient\(^8-10\). In-vitro differentiation of beta cells, however, could theoretically supply an indefinite source of islet tissue\(^8\).

Gene therapy for the generation of beta cells (or insulin producing beta-like cells) may provide a source of cells for transplantation or a method of in vivo beta cell reprogramming\(^11\). Many studies\(^12-28\) have demonstrated that beta-like cell differentiation
is inducible by modulating external conditions or delivering pancreatic transcriptional factors. Exposing beta cell precursors to a high glucose environment alone has the ability to shift the cells towards a beta cell gene expression profile\textsuperscript{22, 25}. It is also clear, from reprogramming studies, that various transcriptional factors exhibit a primary role in the induction of beta cell-like differentiation and insulin gene regulation, most importantly the pancreatic and duodenal homeobox gene-1 (Pdx1). Depending on the conditions and transcriptional factors used, beta cell precursors can differentiate into glucose-responsive insulin-producing cells that are morphologically indistinguishable from true beta cells. Several sources for cells have been successfully utilized including hepatic cells,\textsuperscript{12-21, 23-28} bone marrow cells,\textsuperscript{22} and pancreatic exocrine cells\textsuperscript{29}. Transplantation of beta-like cells into chemically-induced diabetic mice has been shown to restore euglycemia\textsuperscript{12-14, 17, 19, 20, 22-25, 27, 28}.

Several methods have been employed in successful gene therapy including direct protein delivery, lentiviral mediated delivery, and protein transduction technology. Protein transduction technology is of specific interest due to its relative safety and efficacy of transduction\textsuperscript{30-36}. Some of the transcriptional factors involved in beta cell-like differentiation (Pdx1\textsuperscript{31, 35, 36} and NeuroD\textsuperscript{30, 32}) contain protein transduction domains (PTDs) which are specific amino acid sequences, enriched with positively charged arginine and lysine residues, allowing for their transduction across cellular membranes by lipid raft-mediated macropinocytosis. Molecules can also be engineered to utilize the same mechanism by addition of a PTD, as has been done for TAT-mediated Ngn3\textsuperscript{37}.

Following beta cell transplantation or regeneration requires either long term immunosuppression or an immune system modulation to eliminate the possibility of
recurrent autoimmunity. Studies have been focusing on modulation of the T cell repertoire toward a regulatory status, with the goal of establishing a T cell profile that consists of a majority of regulatory cells in order to establish long-term tolerance\textsuperscript{7, 38}.

**Diabetic Complications**

The majority of the morbidity and mortality associated with type 1 diabetes is due to complications arising from poor glycemic control\textsuperscript{39}. In theory, maintaining euglycemia would effectively eliminate the complications related to diabetes, but this is nearly impossible to achieve. Hypoglycemia results in serious acute disease that can quickly lead to diabetic coma and possible death. Recurrent or long lasting episodes of hypoglycemia can lead to cognitive impairment and predisposes individuals to future hypoglycemic occurrences.

The fear of hypoglycemia often leads patients to maintain hyperglycemia because the short term problems are much less severe. However, long term hyperglycemia leads to several complications as the result of microvascular and macrovascular disorders including nephropathy, retinopathy, neuropathy, and cardiovascular disease\textsuperscript{39}. Vascular dysfunction plays a major role in the aforementioned complications related to long term hyperglycemia. The damage to endothelial tissue results in organ and tissue failure. Hyperglycemia leads to the accumulation of reactive oxygen species such as superoxide, hydrogen peroxide, and hypochlorous acid, and also reactive nitrogen species such as nitric oxide\textsuperscript{40}. The reactive oxygen species initiate lipid peroxidation leading to inflammation. The increase in oxidative stress is due to advanced glycation end product accumulation which activates vascular NADPH oxidase. Mitochondrial uncoupling and activation of poly ADP-ribose polymerase also plays a role resulting in endothelial damage.
Endothelial progenitor cells play a major role in protecting the integrity of the vasculature system as well as promoting angiogenesis\textsuperscript{41}. They are derived from the bone marrow and can be mobilized through release of growth factors and cytokines to sites of ischemia and endothelial injury. At the site of injury endothelial progenitor cells adhere to the vessel walls where they begin to migrate through the extracellular matrix. Their expansion leads to the formation of new vasculature structures. Most type 1 diabetic patients have decreased levels of circulating endothelial progenitor cells and increased dysfunction of these cells. The accumulation of vascular ischemic sites prevents adequate blood supply to several tissues and can lead to organ failures and critical limb ischemia requiring amputation.

**MODY**

Monogenetic diabetes accounts for 1-2\% of all diabetes cases, and because it is caused by mutation in a single gene, it can be diagnosed by genetic testing\textsuperscript{42-45}. Maturity onset diabetes of the young (MODY) is caused by autosomal dominant mutation in one of at least six beta cell specific genes including the enzyme glucokinase (GCK) and the transcription factors hepatocyte nuclear factor 4 alpha (HNF4\alpha), HNF1\alpha, Pdx1, HNF1\beta, and NeuroD1. Mutations in HNF4\alpha, GCK, HNF1\alpha, Pdx1, HNF1\beta, and NeuroD1 that result in diabetes are respectively termed MODY1-6\textsuperscript{46-49}. MODY2 is characterized by hyperglycemia onset at birth that does not progress with age and is most often controlled by diet. MODY2 patients rarely develop complications due to hyperglycemia. MODY3 is the most prominent form of MODY and accounts for about 70\% of the disease. MODY1 is less common, accounting for about 5\% of MODY but has similar disease progression as MODY3. These forms are characterized by adolescent onset of hyperglycemia that progressively worsens with age and most often
requires pharmacological treatment. Patients with MODY1 or MODY3 often suffer from diabetic complications of the microvasculature and macrovasculature. Because HNF1β is an important developmental transcription factor, MODY4 is characterized by a wide range of developmental pathologies that affect several systems and accounts for about 2% of MODY. The other transcription factor forms of MODY have only been found in a few families and account for less than 2% of MODY. In general, they progress similarly to MODY1 and MODY3 but they are too rare to be well characterized. In more than 10% of patients who are considered to have MODY, the cause is unknown and suggests that other genes or unknown regulatory elements for the known MODY genes are likely to contribute to disease. The group comprising unknown MODY may also be higher than estimated due to misdiagnosis of type 1 or type 2 diabetes in these patients.

Defining the etiology of MODY is essential for proper pharmacogenetic treatment of the disease. MODY1 and MODY3 genes regulate the expression of a plethora of genes involved in glucose stimulated insulin secretion. HNF4α and HNF1α mutations mainly result in defective glucose metabolism and insulin secretion in the beta cell. For this reason, it is not surprising that MODY1 and MODY3 patients respond well to treatment with oral sulfonylureas, given that these drugs bind the K\textsubscript{ATP} channel which is downstream of glucose metabolism in the insulin secretion pathway. Sulfonylurea therapy can close the K\textsubscript{ATP} channel which increases intracellular Ca\textsuperscript{2+} and stimulates insulin secretion by an ATP independent pathway. MODY1 and MODY 3 patients, who are initially misdiagnosed with type 1 or type 2 diabetes, have successfully transferred to sulfonylurea treatment without deterioration in glycemic control.
treatment often leads to better glycemic control than insulin especially for children and adolescents. Sulfonylurea therapy has been shown to be ineffective in treating other forms of MODY\textsuperscript{42-45}. Sulfonylurea treatment reduces the serious risk of hypoglycemia compared to insulin treatment, and patients who have switched from insulin to sulfonylurea report a great increase in their quality of life\textsuperscript{44}. However, current treatments for MODY3 are not sufficient because patients with MODY3 have hyperglycemia that often worsens with time, and up to 40\% require the use of insulin\textsuperscript{46}. Long term hyperglycemia in this disease can lead to the full scale of diabetic complications, especially neuropathy and retinopathy\textsuperscript{46}.

**Transcription Factors**

DNA binding proteins have long been studied for their important roles in a variety of cellular processes including transcription\textsuperscript{55}. (For Reviews regarding transcription see Refs\textsuperscript{56-59}). Transcription factors play a crucial role during development and tissue differentiation,\textsuperscript{21,60-63} and their role in the reprogramming of mature adult tissues into alternative tissue lineages has become an important topic of study in the field of regenerative medicine\textsuperscript{64-66}. Several transcription factors have been discovered that act as master switch genes and regulate a cascade of downstream gene expression leading to cellular differentiation, such as MyoD\textsuperscript{67} for muscle cell differentiation and Pdx1\textsuperscript{21,60,61} for pancreatic beta cells. Induced expression of MyoD alone in many differentiated cell types including primary fibroblasts, melanoma, adipocytes, and hepatocytes induces expression of muscle cell markers and adoption of the muscle cell phenotype\textsuperscript{67}. More recently, it has been found that transcription factors can lead to the reprogramming of both mouse\textsuperscript{68-70} and human\textsuperscript{71-73} fibroblasts into induced pluripotent cells that closely resemble embryonic stem cells with the capacity to differentiate into all
three germ layers. Transdifferentiation of cells occurs by shifting their gene expression and phenotype from one differentiated cell type to another\textsuperscript{64-66}. (Note: Originally, transdifferentiation was defined as direct conversion of one cell type to another without acquiring an intermediate state, whereas reprogramming required cellular dedifferentiation before converting to another cell type. However, current literature often uses these terms synonymously.) Transcription factors maintain cellular phenotype by working with epigenetic modulators\textsuperscript{74-77} and by regulating cell specific gene expression\textsuperscript{21} and are involved in many diseases including MODY\textsuperscript{42-45}.

**Pdx1**

Pancreatic and duodenal homeobox 1 (Pdx1), also known as STF1, IDX1, IUF1, and IPF1, is a transcription factor necessary for pancreatic development and beta cell maturation\textsuperscript{78}. The Pdx1 gene\textsuperscript{79} has been cloned independently by several labs\textsuperscript{80-82} and, in humans, encodes a 283 amino acid protein that is part of the ParaHox gene cluster\textsuperscript{83}. Pdx1 expression is first detected in the primitive foregut endoderm as the ventral and dorsal pancreatic buds develop\textsuperscript{84,85}, which later merge to form the pancreas. Pdx1 expression later shifts to the endocrine compartment as islets form and the exocrine cells begin to appear in the pancreas. Later in development, Pdx1 becomes mostly restricted to the mature beta cells, with limited expression in other endocrine cells\textsuperscript{80-82,84}. Homozygous mutations to the Pdx1 gene in mice lead to pancreatic agenesis\textsuperscript{85,86} (while all other internal organs appear normal including the gastrointestinal tract) leading to death shortly after birth\textsuperscript{86}. No pancreatic tissue or insulin gene expression was detected in these neonates. It has also been reported that a human with a homozygous inactivating mutation to the Pdx1 gene was born without a pancreas\textsuperscript{87}. 
Regulation of the Pdx1 gene has been well characterized by several reports, some of the major positive regulators include HNF3β, HNF1α, MafA, Pax6, NeuroD, Nkx2.2, USF, and Pdx1 itself. Pdx1 is also positively regulated by nutritional and hormonal factors such as glucose, GLP-1, insulin, T3, HB-EGF, and TNF-α. Pdx1 is involved in the expression of many genes (including insulin, somatostatin, Glut2, islet amyloid peptide, and glucokinase) and is considered the master transcriptional regulator of pancreatic beta cells. Pdx1 also inhibits expression of glucagon. Ectopic expression of Pdx1 has been shown to induce hepatic dedifferentiation by suppressing the expression of CCAAT/enhancer binding protein beta (CEBPβ).

Pdx1 contains an intrinsic protein transduction domain (PTD) which is a specific amino acid sequence, enriched with positively charged arginine and lysine residues, allowing for transduction across cellular membranes by lipid raft-mediated macropinocytosis. It is also interesting to note that Pdx1 has been previously implicated in immune modulation by preventing development of hyperglycemia in non-obese diabetic (NOD) mice following intraperitoneal injection of Pdx1 protein.

Ngn3

Neurogenin-3 (Ngn3), also known as Atoh5, bHLHa7, and Math4B, is expressed by all pancreatic endocrine cell precursors and regulates islet formation. It peaks in expression during endocrine cell development and is only detectable at low levels in adult pancreas. Ngn3 has been cloned and encodes a 214 amino protein that is a member of a family of basic helix-loop-helix transcription factors. Mice with homozygous mutations to the Ngn3 gene fail to develop endocrine cells or precursors and die from diabetes shortly after birth. Over-expression of Ngn3 also leads to a
reduced endocrine mass demonstrating the importance of stringent regulation of this gene during development.

Ngn3 is positively regulated by HNF-1, HNF-3, and HNF-6 while Hes-1 represses Ngn3 expression through the Notch signaling pathway by binding to silencing elements in its proximal promoter. Ngn3 is involved in the expression of many endocrine genes and is a direct regulator of NeuroD and Pax4.

**Nkx6.1**

NK6 homeobox 1 (Nkx6.1), also known as NKX6A, is a homeodomain transcription factor involved in pancreatic differentiation and beta cell homeostasis. It has been cloned and encodes a 367 amino acid protein. In the mature human islet, it is exclusively expressed in the beta cell and is required for normal glucose stimulated insulin secretion. Embryonic expression of Nkx6.1 is dependent on Nkx2.2 and in the mature beta cell it is regulated by Pdx1. Nkx6.1 maintains beta cell phenotype in part by direct interaction with the glucagon promoter suppressing its activity. Nkx6.1 inhibits glucagon expression by competing with Pax6 (glucagon activator) for occupancy of the G1 element on the glucagon promoter. Nkx6.1 has also been linked to beta cell proliferation by up regulating cyclins A, B, and E, as well as many regulatory kinases. Over expression of Nkx6.1 has been shown to increase glucose stimulated insulin secretion in rat islets while in human islets it caused beta cell replication and maintained normal glucose stimulated insulin secretion. Nkx6.1 homeodomain constructs have been shown to bind sequences containing the core homeodomain binding site (5' TAAT 3' or 5' ATTA 3') and direct both gene repression and gene activation. In its own promoter, it has been shown to bind a similar sequence (5' ATTT 3') to positively regulate its own expression. Nkx6.1 has the ability to function
as both a transcriptional activator and repressor which may be sequence dependent.\textsuperscript{125} The transcriptional repression domain has been isolated to the N-terminus\textsuperscript{124} while the transcriptional activation domain has been shown to be dependent on the C-terminus\textsuperscript{125}. The C-terminus has also been observed to interfere with DNA binding but greatly enhance specificity for homeodomain core containing sequences.\textsuperscript{126} It is also of interest that type 2 diabetic islets have been shown to have altered Nkx6.1 expression.\textsuperscript{127} The specific function of Nkx6.1 in glucose stimulated insulin secretion of the mature beta cell remains elusive.

**HNF1α**

Hepatic nuclear factor 1 alpha (HNF1α) (also known as HNF1, LF-B1, TCF1, APF, and HP1) is an important gene involved in the pancreatic beta cell transcriptional regulatory network.\textsuperscript{128} It is also involved in the regulation of important beta cell specific genes such as Pdx1,\textsuperscript{90} IGF-1,\textsuperscript{129} and insulin.\textsuperscript{130} Loss of function mutations\textsuperscript{131, 132} in HNF1α lead to maturity onset diabetes of the young (MODY) 3, the most prevalent form of monogenic diabetes in humans.\textsuperscript{46, 47, 49, 133, 134} MODY3 is characterized by impaired beta cell function caused by defective insulin secretion in response to glucose stimulation. While clinical presentation of the disease is often mild, hyperglycemia worsens with time and up to 40% of patients require treatment with insulin. Long term hyperglycemia can lead to the full range of diabetic complications, especially microvascular complications of the retinas and kidneys. Since HNF1α is expressed in an incomplete dominant manner, disease occurs with the loss of function of a single allele. The haploinsufficiency of this gene demonstrates the critical importance of gene dosage to maintain normal beta cell function. Abnormal regulation of gene transcription by HNF1α leads to a defective insulin secretory pathway, loss of beta cell mass, or both.
Therefore, precise regulation of this gene is necessary to prevent a prominent form of diabetes.

Double immunofluorescent staining shows that HNF1α protein is present in both exocrine and endocrine cells and all islet cells of the murine pancreas\textsuperscript{135, 136}. (For brief reviews regarding HNF1α structure and liver specific function see Refs\textsuperscript{137, 138}). The structure of the gene encoding HNF1α is highly conserved between human, mouse, and rat species\textsuperscript{139-141}. The human and mouse HNF1α genes are located on chromosomes 12q24.3 and 5F respectively\textsuperscript{139, 141}. Human and rat nucleotide sequences show an overall 94\% homology in coding regions and overall amino acid homology of 95\%\textsuperscript{139, 140}. Rat and mouse amino acid sequence homology was found to be 99\%\textsuperscript{141}. There is even considerable homology within the intronic gene sequences, especially in the flanking regions of exons, between species\textsuperscript{139-141}. This demonstrates high conservation of genomic structure and suggests that this protein acts in a very specific manner and contains critical structures required for natural function. The DNA sequence consists of a long open reading frame of 1884 bp and the sequence surrounding the first ATG codon (5’-GGAGCCATGG-3’) has high homology to the translation initiation site consensus sequence\textsuperscript{142} (5’GCC\textsubscript{A/G}CCATGG3’)\textsuperscript{143}. There is also a TATA like box (5’-GATAAATA-3’) in close proximity to the transcription initiation site\textsuperscript{144} with very close homology to the TATA box consensus sequence\textsuperscript{145} (5’-TATA\textsubscript{A/G}AA\textsubscript{A/G}-3’). HNF1α transcription in the liver is largely due to proximal promoter binding of HNF4α\textsuperscript{144, 146-148} and this complex has been crystallized\textsuperscript{149}. One study\textsuperscript{150}, using a -497 bp proximal HNF1α promoter luciferase reporter (containing the HNF4α binding site), demonstrated that this reporter was activate in hepatocytes, but not in rat insulinoma (INS1) cells.
The rat and mouse gene both contain 9 exons and 8 introns and the rat full length coding region is less than 40kb\textsuperscript{140}. The full length cDNA encodes a 628 amino acid 88kd dimeric protein and was first cloned from rat liver\textsuperscript{142}. HNF1α is capable of forming dimers in the presence or absence of DNA\textsuperscript{151, 152}. The N-terminus contains a DNA binding site that is structurally, though distantly, related to the homeobox domain\textsuperscript{142, 151} (See Refs\textsuperscript{153-155} for comprehensive reviews regarding the homeodomain and Ref\textsuperscript{153} regarding its role in gene expression). HNF1α is a unique homeoprotein that contains an additional 21 amino acid sequence loop connecting helix 2 and helix 3 with 100% homology between mouse and rat\textsuperscript{141}. The protein also shares some similarity to the POU family of transcriptional activators\textsuperscript{156, 157}. HNF1α binds as a homodimer to the consensus sequence 5’-GTTAATNATTAAC-3’\textsuperscript{137}.

Alternative isoforms of HNF1α are generated by post translational modification\textsuperscript{158}. Three separate isoforms have been identified (HNF1αA, HNF1αB, and HNF1αC) by real-time PCR and no evidence for additional transcripts was found\textsuperscript{159}. The different isoforms are generated through differential selection of polyadenylation and alternative splicing, generating distinct 3’ ends\textsuperscript{159}. The fact that the different HNF1α isoforms are distributed differently between tissues and during different time points in development suggests that they may control gene expression in a temporal and tissue specific manner\textsuperscript{159}. The alternate isoform ratios may also contribute to differential gene regulation between cell types. Alternative splicing of transcripts increases versatility of function and several documented proteins act as activators and repressors from the same gene\textsuperscript{160}. 
Electron mobility shift assays (EMSA) demonstrate that the HNF1α isoforms can dimerize and bind DNA with similar affinity\textsuperscript{159}. Transient CAT transfection assays in C33 human cervical carcinoma cells (HNF1α null) showed that HNF1αB and HNF1αC have a five-fold higher transactivation potential than HNF1αA\textsuperscript{159}. It is noteworthy that addition of DCoH (an HNF1α coactivating molecule) to the transfection mixture did not significantly increase the activation potential of HNF1α, suggesting that this coactivator was not a limiting step in this experimental procedure.

A few studies demonstrate the biochemical characterization of the full length HNF1α protein\textsuperscript{152, 161}. The DNA binding domain is a tripartite homeodomain composed of 81 amino acids and contains an N-terminal helix similar to part of myosin\textsuperscript{152}. The N-terminal 31 amino acids mediate dimerization with these helical regions essential for DNA binding\textsuperscript{152}. The A domain (amino acids 1-33) is responsible for DNA binding which can be abolished by substitutions\textsuperscript{152}. The B domain (amino acids 100-184) shows weak similarity to the subregion of A of the POU domain\textsuperscript{157} and is important for efficient DNA binding. (See Refs\textsuperscript{157, 162, 163} for brief reviews or Ref\textsuperscript{164} for a comprehensive review of the POU homeodomain). Mutations in the POU-like region of HNF1α can prevent DNA binding\textsuperscript{152}. The highly divergent homeodomain is located in domain C (amino acids 198-281), and substitutions here can lead to loss of binding activity, even when using conserved substitutions\textsuperscript{152}. The C-terminal 150 amino acids are rich in serine and threonine\textsuperscript{159} and deletion of the C-terminus portion of HNF1α has no measurable effect on DNA binding\textsuperscript{142, 152}.

HNF1α is an important molecule involved in cell specific transcriptional regulatory networks\textsuperscript{128}. (See Ref\textsuperscript{58} for review of tissue specific gene regulation). It is involved in
the development of the pancreas and critical for insulin and glucose regulation in the adult\textsuperscript{46, 49, 165}. There are several excellent reviews and studies that document these transcriptional regulatory networks in liver\textsuperscript{128, 163, 166} and pancreas\textsuperscript{60, 128, 167, 168}. HNF1α tissue distribution is predominantly endodermal\textsuperscript{141, 156}. In the mouse\textsuperscript{141} HNF1α transcripts have been found in the liver, kidney, stomach, and intestine, but not in the ovary, brain, heart, or lung. In rats\textsuperscript{156} transcripts have been found in the liver\textsuperscript{142}, kidney, and at low level in the intestine, spleen and thymus, but not in the skin, lung, heart, or brain. In humans HNF1α transcripts have been found in the kidney\textsuperscript{159}, liver\textsuperscript{159, 169}, intestine\textsuperscript{159}, thymus\textsuperscript{159}, adult and fetal pancreas\textsuperscript{169}, and isolated islets\textsuperscript{169}, but at almost null levels in skeletal muscle\textsuperscript{169} and visceral adipose\textsuperscript{169}.

There is differential tissue distribution of HNF1α isoforms. One study which examined mRNAs in certain fetal and adult human tissue cell lines by a semi-quantitative RT-PCR method did not attempt to quantify absolute amounts of each mRNA, but rather compared the ratios between each isoform at the RNA level\textsuperscript{159}. HNF1αA was found at significantly higher ratios in all tissues studied (liver, kidney, intestine, and thymus) except the fetal liver, which was significantly higher for HNF1αC. HNF1αA was also found at significantly higher ratios in a variety of cell lines. Pancreas was not examined in this study. The RNAse protection method was not sensitive enough to detect the low amounts of HNF1αB mRNAs. Using RT-PCR another group\textsuperscript{169} demonstrated that high levels of HNF1αA are found in the kidney and liver and lower levels in the pancreas and islets. Conversely, HNF1αB was found at higher levels in pancreas and islets. This study found HNF1αC to have the lowest expression level in all tissues examined (fetal liver was not examined). Almost no HNF1α expression was
determined in skeletal muscle or visceral adipose as expected. It is interesting to note
that high levels of HNF1αA are present in the fetal pancreas\textsuperscript{60, 61}.

The role of miRNA in post-translational gene regulation has been shown to be
highly important in the regulation of gene expression. Traditionally, miRNA has been
shown to be a translational repressor but in some cases it can function to increase gene
expression\textsuperscript{170}. (See Ref\textsuperscript{171} for a review of miRNA function). During intestinal epithelial
differentiation in Caco-2 cells, HNF1α has been found to regulate expression of pri-miR-
194-2\textsuperscript{172, 173}. This indicates that HNF1α is able to control gene expression by regulation
of miRNAs, and regulation may be cell type specific. There are likely HNF1α target
miRNAs that are involved in beta cell regulation, and HNF1α itself, may be controlled by
miRNAs. Therefore, future studies regarding miRNA interaction with HNF1α would
provide greater understanding of gene regulation in beta cells.

Several studies in mice demonstrate the role and importance of HNF1α. HNF1α
null pups display no obvious phenotypic abnormalities, suggesting that HNF1α is not
required for embryonic development\textsuperscript{174}. Additionally, HNF1α mRNA cannot be detected
in dedifferentiated hepatoma cell lines and hepatic extinguishing somatic hybrids\textsuperscript{156, 175-
178}. Although HNF1α is expressed as early as embryonic day 10.5 in most cells of the
developing murine pancreas\textsuperscript{135}, its transcriptional activation becomes dependent only in
terminally differentiated cells\textsuperscript{167}. This may be due to the reciprocal expression of a
highly homologous gene HNF1β (also called vHNF1, 58% identity relative to HNF1β)
that share dimerization domains and homeodomains, but not activation domains\textsuperscript{179, 180}.
HNF1β is found in undifferentiated or dedifferentiated cells while HNF1α becomes
prominent in differentiated cells\textsuperscript{156, 175-178, 180-182}. Tissue distribution of HNF1β resembles
that of HNF1α in adult mouse and the two proteins have been shown to form heterodimers in vitro\textsuperscript{180, 183}.

HNF1α null mice, generated by standard gene disruption methodology, show no obvious abnormalities during embryonic development but following birth they fail to thrive and suffer from a progressive wasting syndrome\textsuperscript{174}. These mutant mice suffer from hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome\textsuperscript{174}. HNF1α expression is important for beta cell function and maintaining glucose homeostasis. Beta cell dysfunction occurs in HNF1α null mice marked by hyperglycemia and impaired glucose tolerance without loss of beta cell mass\textsuperscript{184}. Genes involved in beta cell regulation and metabolism are expressed abnormally in HNF1α null mice\textsuperscript{185}. Decreased steady state mRNA levels were observed for genes encoding glucose transporter 2 (Glut2), neutral and basic amino acid transporter, liver pyruvate kinase (L-Pk), and insulin, as well as the islet enriched transcription factors Pdx1, HNF4α, and NeuroD\textsuperscript{185}. HNF1α null mice, generated by the Cre-loxP recombination system, develop Laron dwarfism and non-insulin dependent diabetes\textsuperscript{186}.

HNF1α cellular concentration is critical for beta cell homeostasis. Homozygous HNF1α null mice have abnormal beta cell gene expression, impaired glucose tolerance, and fasting hyperglycemia, leading to the development of diabetes\textsuperscript{174, 184-186}. Transgenic mice in which an HNF1α transgene is under control of the tetracycline dependent operon specifically in beta cells demonstrates that over expression also leads to a diabetic phenotype\textsuperscript{187}. HNF1α over expression compromises islet morphology and reduces beta cell mass. These mice show a threefold reduction in beta cells and many appear scattered near ductal structures. Over expression also
suppresses beta cell cycle activity and induces cell death. Ki67 staining shows that beta cell proliferation is inhibited in HNF1α deficient mice with an increase in the expression of cell cycle inhibitor p27. Apoptosis is induced in a significant density of beta cells as demonstrated by activated caspase 3. HNF1α over expression causes hyperglycemia at birth that progressively worsens with age. Other studies in mice show that overexpression of dominant negative mutant HNF1α leads to diabetes with a MODY3-like phenotype\textsuperscript{188, 189}. HNF1α has been shown to regulate expression of collectrin which facilitates SNARE complex formation and controls insulin exocytosis\textsuperscript{190, 191}. Studies in INS1 insulinoma cells over expressing a dominant-negative mutant under control of a reverse tetracycline dependent transactivator (or wild type HNF1α) also show the key importance of HNF1α gene dosage for beta cell homeostasis\textsuperscript{192}. Cells over expressing wild type HNF1α remained viable while dominant-negative over expressing cells activated caspase-3 leading to apoptosis. Apoptosis was accompanied by mitochondrial hyperpolarization, decreased expression of anti-apoptotic Bcl-xL, and mitochondrial release of cytochrome c and caspase-9. Apoptosis could be rescued with transient over expression of Bcl-xL. Another study using the same dominant negative conditions showed pronounced decrease in insulin expression and other genes involved in glucose stimulated insulin secretion\textsuperscript{193}. Other interesting studies in INS-1 insulinoma cells overexpress HNF1α-P291fsinC, with the most common MODY3 mutation, under control of the reverse tetracycline dependent transactivator\textsuperscript{129, 194}. This forces the endogenous HNF1α to compete with the mutant protein and led to the reduced
expression of several genes important for beta cell function. Insulin secretory responses to glucose were also impaired in these cells.

HNF1α cellular concentration is critical for epigenetic maintenance. Studies of HNF1α null murine tissues demonstrate that loss of HNF1α leads to modification of chromatin structure and alters gene expression. In the liver, the phenylalanine hydroxylase (PAH) gene is activated prenatal and is regulated by several transcription factors including HNF1α. However, this gene is completely silent in HNF1α null murine livers. Hepatic PAH expression could not be induced by traditional activation-enhancing hormones in these mice. Liver specific DNase I hypersensitivity sites of the PAH gene in normal mice are absent in HNF1α null animals. This hypersensitivity region contains several HNF1α binding sites. PAH silencing was also associated with promoter hypermethylation. Another study found that HNF1α and HNF4α control chromatin structure and gene expression of alpha 1 antitrypsin (AAT) and corticosteroid binding globulin (CBG) in hepatocytes. Other studies in HNF1α null mice also found that HNF1α can direct nucleosomal hyperacetylation in a tissue specific manner. HNF1α and several other factors regulate the promoters of GLUT2 glucose transporter and L-type pyruvate kinase (pklr) in both pancreatic islets and liver cells of normal mice. HNF1α null mice fail to express these genes in pancreatic insulin producing cells while expression is maintained in liver cells. These expression patterns correlate directly with hyperacetylation of GLUT2 and pklr promoter nucleosomes in pancreatic insulin producing cells, but not in liver cells. Studies in dedifferentiated M29 cells show that several genes fail to reactivate upon ectopic expression of HNF1α. These studies provide evidence that HNF1α can regulate gene expression by recruiting mediators of
epigenetic modification and that its cellular concentration may determine chromatin architecture.

HNF1α is an essential member of a transcriptional network that regulates cell type-specific global gene expression and maintains normal cellular phenotype and function. Liver and pancreas share common embryonic lineage\(^\text{196}\) and both are derived from the same endoderm tissue mass\(^\text{61-63}\). They also share expression of several HNF transcription factors\(^\text{60, 128, 163, 166-168, 197}\). An alignment of HNF1α binding sites in liver specific genes from several organisms gives an HNF1α recognition consensus sequence of \((5' - G^A/GTTA^A/C TNNT^C/T NNC^A/C-3')\)\(^\text{177}\). One study identified several HNF1α target genes by computer assisted analysis and subsequent binding assays\(^\text{198}\). In a genome-scale promoter analysis in human liver and pancreatic islets\(^\text{128}\), HNF1α was found to occupy the promoters of at least 222 genes in liver and 106 in pancreas analyzed on a micro array of 13,000 genes (Hu13K array). The targeted promoter regions span -700 to +200 from the transcription start site, where most identified transcription factor binding site occur. Analysis of distal promoters would likely reveal additional genes targeted by HNF1α. The micro array results were confirmed with individual ChIP experiments and stringent threshold criteria to have a high accuracy. Many of the HNF1α targeted genes encode important biochemical proteins related to normal cellular function as well as important transcriptional regulators of gene expression. Transient transfection assays in HepG2 cells have demonstrated that an indirect negative autoregulatory feedback mechanism is involved in HNF1α regulation\(^\text{146}\). HNF1α is also capable of indirectly inhibiting expression of HNF4α.
dependent genes\textsuperscript{146} by a direct interaction with the AF-2 activation domain of HNF4α\textsuperscript{199, 200}.

Transcriptional activation by HNF1α often functions in synergistic association with several coactivating proteins. Dimerization cofactor of HNF1α (DCoH), also known as PHS or PCD, is an 11kDa homotetrameric protein (45kDA) that selectively stabilizes HNF1α dimers and enhances transcriptional activity\textsuperscript{201-204}. The cDNAs from human, mouse, and rat encode a 104 amino acid protein\textsuperscript{204} but transcriptional modification has been demonstrated in the liver of humans and rats\textsuperscript{206}. DCoH binds as a dimer to the helical dimerization domain of dimeric HNF1α forming a heterotetrameric complex\textsuperscript{206}. It has been purified from human and displays identical protein primary structures to rat\textsuperscript{205}. Its crystal structure has been determined alone\textsuperscript{202, 206} and in complex with HNF1α\textsuperscript{206, 207} leading to the biochemical characterization of some MODY3 mutations\textsuperscript{207}. Interestingly, mice that are DCoH null display mild glucose intolerance, but do not develop diabetes\textsuperscript{208}. In these mice, HNF1α levels and DNA binding activity in liver nuclear extracts was not affected; however, mRNA levels of several HNF1α dependent genes were reduced. It was also observed that low levels of a second DCoH-related gene (DCoH2) may have had a complementing activity. The crystal structure of DCoH2 has been determined and biochemical studies have found it to be partially redundant in enzymatic and transcriptional functions\textsuperscript{209}.

HNF1α has been shown to recruit the transcriptional coactivator p300 to the GLUT2 promoter in HIT-T15 cells\textsuperscript{210}. Coimmunoprecipitation assays demonstrate that histone acetyltransferases (HATs), CREB-binding protein (CPB), p300/CPB-associated factor (P/CAF0), Src-1, and RAC3 interact with HNF1α in vivo\textsuperscript{77}. CBP was shown to
interact with the N-terminal region and P/CAF, Src-1, and RAC3 interacted with the C-terminal activation domain. The diverse functions of these coactivators suggest that HNF1α directs transcription by coupling chromatin modification and transcriptional machinery recruitment. All of the aforementioned coactivators have been shown to acetylate surrounding nucleosomes, allowing greater access for other transcription factors to their promoters. (For excellent reviews regarding transcriptional regulation by nucleosomal histone acetylation see Refs211,212). Increased transcriptional activation potential was demonstrated for these HNF1α coactivators using an HNF1α dependent CAT reporter assay in NIH3T3 cells. HNF1α alone could activate transcription 5-fold above basal levels, and cotransfection with CBP, P/CAF, Src-1, and RAC3 expression vectors increased activation by 20-fold, 44-fold, 26-fold, and 19-fold respectively. Coexpression of HNF1α and CBP with P/CAF, Src-1, and RAC3 increased activation respectively by 135-fold, 121-fold, and 89-fold. Coexpression of HNF1α and P/CAF with Src-1 and RAC3 increased activation 132-fold and 152-fold respectively. The dramatic increase in activation after coactivator recruitment is clearly a synergistic reaction. The transcription factor HNF4α in cooperation with p300 has also been show to behave as a coactivator for transcription199.

**HNF4α**

Several studies show that the relationship between HNF1α and HNF4α is integral for cell specific regulation of gene expression and that these factors can positively regulate each other’s expression128,134,167,168,213,214. HNF4α is expressed in all cells of the pancreas in mice,136 and in murine beta cells it is essential for the regulation of gene expression and for glucose metabolism215-218. HNF4α was originally purified from rat liver nuclear extracts219 and has been well characterized structurally and functionally by
several reports. The A/B region contains an AF-1 strong terminal transactivation domain. The DNA binding region is in the C domain and the E region contains a ligand-binding domain, dimerization domain, and AF-2 transactivation domain. The F region is variable and some isoforms contain a coactivator recruiting domain. HNF4α is a member of the steroid hormone receptor superfamily and binds DNA as a homodimer. The human HNF4α gene contains 12 exons while the mouse gene contains only 10 exons. The 1365 base pair open reading frame of human HNF4α encodes a 50.6kDa 455 amino acid homodimeric protein with six different structural regions.

Almost half of protein coding genes function by use of alternative promoters, and the functional consequences of alternative promoter use in mammalian genomes have been reviewed. A total of nine HNF4α isoforms are generated by alternative promoter usage and alternative transcript splicing and they can be organized into two categories. HNF4α isoforms generated from the P1 promoter (HNF4α1-3) contain exon 1A with the strong AF-1 activation domain, whereas isoforms generated from the P2 promoter (HNF4α7-9) contain exon 1D, or exon 1D and 1E, and lack this activation domain. Note: The initial reports concerning HNF4α4-6 isoforms may be unreliable and these isoforms are unlikely to exist as suggested by more recent reports. The two promoters are separated by approximately 46 kilobases. Both the P1 and P2 HNF4α promoters contain binding sites for HNF1α/β and the P2 promoter also contains Pdx1 and HNF6 (also known as OC-2) binding sites. Similar to mice, HNF4α expression in human pancreatic islets is driven predominantly from the P2 promoter producing HNF4α7-9 isoforms (and potentially
HNF4α10-12\textsuperscript{238}) in similar concentrations as demonstrated by RT-PCR analysis of transcripts. Liver expression is predominantly driven by the P1 promoter\textsuperscript{229, 236, 240, 241}. In concordance with these findings, murine nucleosomal histones of the P2 region are hyperacetylated in pancreatic islets; P1 regions are hyperacetylated in liver\textsuperscript{167}. Another group demonstrated that HNF4α1, HNF4α2, HNF4α7, and HNF4α8 were the predominant transcripts in human beta cells\textsuperscript{224}, but also found very low levels of HNF4α3 and HNF4α9 and were unable to detect HNF4α4-6. It was previously shown that HNF4α8 has significantly weaker transcriptional activity in beta cells than HNF4α2\textsuperscript{224, 229} and that HNF4α7 has significantly weaker transcriptional activity in liver cells than HNF4α1\textsuperscript{232}. To generalize, the expression of HNF4α in hepatocytes is most prominently expressed through the P1 promoter, while in beta cells, most reports indicate that HNF4α P1 transcripts cannot be detected\textsuperscript{238}. Primarily P2 isoforms have been detected in adult beta cells, and they have been associated with reduced transcriptional activation potential when compared to P1 isoforms\textsuperscript{224, 229, 232}.

HNF4α1 and HNF4α7 are respectively similar to HNF4α2 and HNF4α8 but lack a 10 amino acid sequence in the F domain. Studies show deletion of the F domain increases transcriptional activity of HNF4α\textsuperscript{229, 242} and the 10 amino acid insert can help to abrogate this inhibitory effect\textsuperscript{233, 234}. The inhibitory effect caused by the F domain has been localized to a 14 amino acid residue (428-441) that is sufficient to repress activity of the AF-2 activation domain\textsuperscript{230}.

mRNA analysis of adult HNF1α null mice show no loss of HNF4α in the liver; however, 10 fold decreases were found in the pancreas\textsuperscript{167}. This suggests that HNF4α may act as a downstream target of HNF1α in pancreatic cells\textsuperscript{167} establishing a different
regulatory network than in hepatocytes. HNF4α1 has also been shown to repress P2 activity in transfection assays in rodent liver. HNF4α1 only and HNF4α7 only knock-in mice were generated to examine the in vivo role of the AF-1 activation domain. Both mouse types show no obvious phenotypic abnormalities although HNF4α1 only mice displayed slight glucose intolerance. This is in contrast to HNF4α total knockouts which are embryonic lethal and also demonstrates the embryonic functional redundancy of the two isoforms.

Transient transfection assays have shown that HNF1α is a negative regulator of its own transcription and that it can also repress the transcription of HNF4α dependent genes by disrupting HNF4α activity. HNF1α exerts repression of HNF4α dependent genes by directly interacting with the AF2 activation domain of HNF4α, thereby functioning as both an activator and a repressor to regulate gene expression.

**HNF Regulatory Circuit**

HNF transcription factors are part of a complex regulatory circuit that is known to maintain hepatocyte phenotype and maintain insulin secretion in response to glucose stimulation in the beta cell. In hepatocytes, HNF3β is known to be a master regulator of HNF4α and HNF1α and it is well established that HNF4α and HNF1α regulate each other as a feedback loop. A genome-scale promoter analysis in human liver and pancreatic islets using a micro array of 13,000 genes (Hu13K array) demonstrated that HNF4α occupied the promoters of at least 1575 genes in hepatocytes and 1428 in pancreatic islets, and that HNF1α occupied the promoters of at least 222 genes in hepatocytes and 106 in the pancreatic islets. Promoter occupancy of HNF4α overlapped with RNA polymerase II for 80% of hepatic genes and 73% of islet genes, while HNF1α overlapped with RNA polymerase II for 6% of hepatic genes and
2% of islet genes, suggests that a large number of genes are actively transcribed by HNF4α and HNF1α.
CHAPTER 2
PDX1-MEDIATED SUPPRESSION OF HNF1α DURING REPROGRAMMING OF HUMAN HEPATIC CELLS TOWARDS PANCREATIC BETA CELLS

Introduction

Reprogramming of hepatocytes into pancreatic beta cell-like insulin-producing cells (IPCs) by ectopic expression of pancreatic transcription factors (PTFs) has been well established through detailed in vitro characterization of pancreatic beta cell gene expression\textsuperscript{12, 15, 16, 18, 19, 23-25, 27, 28} and in vivo restoration of normoglycemia in chemically-induced diabetic mice\textsuperscript{12, 13, 17, 19, 20, 23-25, 27, 28}. This has significant clinical implications due to its therapeutic implication for type 1 diabetes (T1D). Transdifferentiation studies aimed at the treatment of T1D have mainly focused on using the liver\textsuperscript{12, 13, 15-20, 23-25, 27, 28} as a tissue source due to its tissue abundance and high regenerative capacity\textsuperscript{247}, common developmental kinship with the pancreas\textsuperscript{196}, and the glucose sensing system shared by hepatocytes and pancreatic beta cells\textsuperscript{248, 249}. Since the liver and pancreas share a remarkably similar gene expression profile, including the expression of many specific transcription factors\textsuperscript{128, 245} and both tissues are responsive to glucose\textsuperscript{248, 249}, hepatocytes are an excellent target for reprogramming into pancreatic beta-like IPCs. Although much attention has been given to understanding how to activate genes related to pancreatic beta cell development, understanding of the molecular mechanisms involved in shutting down the hepatocyte phenotype during PTF-mediated hepatocyte-to-beta-like IPC conversion remains quite limited\textsuperscript{64-66}.

Hepatocytes make up 70-80% of the mass of the liver and perform an astonishing number of metabolic, endocrine, and secretory functions\textsuperscript{63, 250}. The liver plays a major role in metabolism, glycogen storage, plasma protein synthesis, hormone production, and detoxification. Ectopic expression of Pdx1 triggers hepatocyte
dedifferentiation by down-regulating several liver-specific functional genes including albumin (ALB), alcohol dehydrogenase-1β (ADH1β), glucose 6-phosphatase (G6PC), glutamate synthase (GLUL), α-1-antitrypsin (AAT), and hexokinase 2 (Hxk 2)\textsuperscript{105, 251}. However, the molecular links between Pdx1 over-expression and down-regulation of these hepatic genes are not entirely understood.

Several transcription factors are necessary for the differentiation and maintenance of hepatocyte phenotype\textsuperscript{128, 163, 166, 252, 253}. In particular, hepatocyte nuclear factor-1 alpha (HNF1α) and -4 alpha (HNF4α) are important liver-enriched transcription factors that play an important role in establishment and maintenance of the hepatocyte phenotype\textsuperscript{128, 185, 245}. HNF1α and HNF4α regulate the expression of hundreds of downstream target genes in both hepatocytes and beta cells\textsuperscript{128} and the regulation of specific isoforms and levels of expression by alternative splicing differ significantly between hepatocytes and beta cells\textsuperscript{128, 240, 245}. A developmental switch in the relative isoform expression was demonstrated previously for HNF1α and HNF4α between the fetal and adult pancreas\textsuperscript{169, 240}. The human HNF4α gene contains two promoters (P1 and P2) that drive the expression of P1-derived isoforms (HNF4α 1-3) or P2 -derived isoforms (HNF4α 7-9) by alternative splicing and alternate usage of the promoters\textsuperscript{236}. The different promoters are used in different tissues and at different times during development. HNF4α P2-isoforms are exclusively detected in adult pancreatic islets and positively regulated by HNF1α, Pdx1, and HNF1β\textsuperscript{236, 240, 241}, whereas, HNF4α P1-isoforms are present in developing human fetal pancreas\textsuperscript{240}. In contrast, P1-derived isoforms are most abundant in adult hepatic tissues with relatively low levels of P2 isoforms\textsuperscript{222, 240, 241}. Due to the tissue-specific expression of HNF4α, in hepatocytes,
HNF1α is mainly regulated by HNF4α P1 isoforms; in contrast it is regulated by P2 isoforms in beta cells. Previous studies indicate that HNF1α P2 isoforms have weaker transactivation capacities than P1 isoforms\textsuperscript{224, 229, 232}. Pdx1, which is not normally expressed in hepatocytes, activates HNF4α P2 isoforms by binding directly to the P2 promoter\textsuperscript{236}. The difference in the relative expression of the HNF1α and HNF4α isoforms following ectopic expression of Pdx1 may be responsible for hepatic cell dedifferentiation and transdifferentiation toward pancreatic beta cells. Here, we tested the hypothesis that ectopically expressed Pdx1 protein in hepatocytes activates HNF4α P2 isoforms that competitively suppress HNF1α activity, contributing to Pdx1-mediated hepatic dedifferentiation.

In this study, we constructed lentiviral vectors to deliver PTFs into human hepatocellular carcinoma cells (Huh7 cell line), examined early molecular events related to the activation of pancreatic endocrine genes and suppression of hepatic genes, and explored how Pdx1 alters the expression levels of specific HNF4α isoforms, which in turn suppresses HNF1α and its downstream targets by competitive inhibition. This work provides some insights into the mechanism of reprogramming from hepatocytes into pancreatic beta-like IPCs. Understanding the molecular events during cell type conversion may help to elucidate the mechanisms underlying tissue regeneration and plasticity.

**Materials and Methods**

**Cell Lines and Cell Culture**

**Human hepatocellular carcinoma cell line Huh7, 3T3 mouse fibroblast cells, and 293 human embryonic kidney cells were purchased from ATCC and were cultured in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin in a 37°C incubator**
with 100% humidity and 5% CO$_2$. Rat insulinoma (INS-1) cells were cultured in RPMI 1640 supplemented with 200mM L-glutamine, 100mM sodium pyruvate, 2-mercaptoethanol, and 1% Penicillin/Streptomycin in the same incubator.

**Construction of Plasmids**

The human Pdx1 expression plasmid (pCMV6-XL5) was purchased from Origene. The mouse Pdx1 expression plasmid was constructed by insertion of mouse Pdx1 cDNA into the BamHI/XbaI sites of the pCDNA3 vector (Invitrogen). The truncated mouse Pdx1 expression vectors were constructed as previously described.$^{254}$ The HNF4α2 expression plasmid (pCMV-Sport6) was purchased from Open Biosystems. HNF4α8 expression plasmid was made by removing exon 1A from HNF4α2 plasmid and replaced it with exon 1D. The HNF4α3 and HNF4α9 expression plasmids were constructed by modifying the HNF4α2 and HNF4α8 plasmids respectively by introducing a stop codon at the 3’ end of exon 8 (note: The HNF4α3 and HNF4α9 expression plasmids code for proteins lacking the 41 amino acids from the extended exon 8). The pRL-TK expression vector was purchased from Promega. The mouse HNF1α luciferase reporter was constructed as previously described.$^{255}$ The rat insulin I promoter (RIP) luciferase reporter was constructed by removing the TK promoter from the pRL-TK plasmid and cloning the RIP promoter into this site using the BglII/HindIII restriction sites. The Pax4, NeuroD, Ngn3, and Nkx2.2 luciferase reporters (pFOXluc) were generous gifts from Michael German, the University of California, San Francisco, CA.

**Lentiviral Vector (LV) Preparation, Titration, and Transduction**

The RIP driving the green fluorescence protein (RIP-GFP) reporter was constructed as previously described.$^{12}$ The LV containing the mouse Pdx1-VP16 (PV)
fusion gene was constructed as previously published\textsuperscript{12,22}. The LV containing rhGFP and mouse Pdx1 were constructed as previously described\textsuperscript{24}. The LV containing mouse Ngn3 was constructed by inserting the cDNA of mouse Ngn3 into the pTYF vector cassette under the control of elongation factor-1 alpha (EF-1α) promoter. LVs were generated and titrated as previously described\textsuperscript{256-258}. For lentiviral transduction, Huh7 cells were transduced with different LVs as indicated at a multiplicity of infection (MOI) of 10 in the presence of 10μg/ml polybrene for 24 hours. The transduction efficiency was monitored by transducing Huh7 cells with LV encoding rhGFP. The insulin 1 promoter activities in Huh7 cells were detected by observing RIP-GFP expression under a fluorescence microscope.

**RT-PCR and Real Time RT-PCR Analysis**

Total RNA was extracted from Huh7 cells transduced with different combinations of transcription factors or from human islets (generous gift from Dr. Xiaoping Deng at The University of Pennsylvania) using Trizol according to the manufacturer's protocol. Gene expression was detected by RT-PCR. The forward and reverse PCR primers were designed (IDT Technologies) to be located in different exons. Amplification was performed for 35 cycles at 94°C for 30s, 56°C for 30s, and 72°C for 30s, and followed by 72°C for 7 min. The PCR products were separated in 2% agarose gels by electrophoresis in TAE buffer. Digital images were captured and analyzed with a Quantity One Imager (BioRad). All of the PCR products were confirmed by Big-Dye DNA sequence analysis in an ABI 377 sequencer (Global Medical Instrumentation, Inc.) following the manufacturer's protocol. Real Time RT-PCR was performed on selected samples, collected as described above, using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Primers were designed to detect
HNF4α P1 products (exon 1A) or HNF4α P2 products (exon 1D) as well as the C-terminal modifications by detecting exon 9, exon9+, or exon 8+. Primer sequences are available upon request.

**Western Blotting and Immunocytochemistry (ICC)**

Huh7 cell lysates were harvested at day 4 post-LV-Pdx1, LV-PV, LV-Ngn3, or LV-GFP treatment. The cellular proteins were quantified and standardized. Pdx1, PV, alpha 1 anti-trypsin (AAT), albumin (ALB), and HNF1α proteins were detected according to our previously published methods\(^{12,24}\). In brief, cellular proteins were separated by SDS-PAGE using 12% Tris-HCl gels (Bio-Rad) and transferred to the filter membrane. Proteins were blotted with rabbit anti-Pdx1 serum (1:2000, made in LiJun Yang’s Lab), anti-VP16 (1:200, BD Pharmagen), anti-HNF1α (1:200, Santa Cruz), anti-ALB (1:200, Santa Cruz), and anti-AAT (1:200, Santa Cruz) followed by HRP-conjugated secondary antibody (1:20 000). All proteins were visualized by enhanced chemiluminescence (ECL) using a western blotting detection kit (Amersham Bioscience). For ICC, cytospin slides were prepared from Huh7 cells and Ngn3 protein was detected by rabbit anti-serum against mouse Ngn3 (a generous gift from Michael S. German, University of California, San Francisco) at a dilution of 1:3000 according to our previously published methods\(^{12,24}\).

**Transfections and Luciferase Assay**

Cells were plated in 12-well plates and transfected with 0.1-1.0μg/well plasmid DNA (as indicated) using Lipofectamine 2000 Reagent (Invitrogen) according to manufacturer’s protocol. 0.02μg/well TK-Luc plasmid was used as a transfection efficiency control in all experiments. Cell lysates were harvested and measured 24 hours post transfection using the Dual Luciferase Reporter Kit (Promega) according to
manufacturer’s protocol except that only 50µl of each substrate reagent was used (as optimized by our lab) to read samples using a Lumat LB 9507 Luminometer (Berthold Technologies). All luciferase assays were done in duplicate or triplicate as indicated. All results are expressed as fold/control using pcDNA3 null expression vector following standardization by TK-Luc for transfection efficiency.

**Statistical Analysis**

The statistical significance of our experimental findings was analyzed by using Student’s t-test (2-tailed, assuming equal variance). P values represent comparison to controls unless otherwise indicated. * = p<0.05 and ** = p<0.001

**Results**

**Pdx1-VP16 (PV) and Ngn3 Together Strongly Induced Insulin Promoter Activity**

We first assessed if our LVs designed to express PTFs were functional. We confirmed the transgene expressions of Pdx1, PV, and Ngn3 at the mRNA level by RT-PCR (Fig. 2-1) and protein levels by western blotting for Pdx1 and PV (Fig. 2-2) and ICC for Ngn3 (Fig. 2-3). In order to monitor the activation of the insulin gene during the reprogramming process from hepatic cells toward pancreatic beta-like IPCs, we constructed and produced LV-RIP-GFP as a reporter for monitoring RIP activity. The transduction efficiency of Huh7 cells was determined by LV-GFP and more than 99% of transduced Huh7 cell expressing GFP was observed at day 2 (data not shown). RIP-GFP was co-transduced into Huh7 cells with Pdx1, PV, Ngn3, or with combinations of Pdx1/Ngn3 or PV/Ngn3 as indicated. At 96 hours of transduction, the expression of GFP was examined (Fig. 2-4). Green fluorescence was observed in rare Huh7 cells transduced with single gene Pdx1, PV, or Ngn3, therefore Pdx1, PV, or Ngn3 alone cannot effectively activate the insulin promoter RIP-GFP at day 4 post-transduction.
Green fluorescence was observed in nearly 50% of Huh7 cells transduced with Pdx1/Ngn3 and in more than 90% of Huh7 cells transduced with PV/Ngn3, indicating a synergistic effect of Ngn3 and Pdx1 in activating RIP-GFP. This result is consistent with previous studies demonstrating that the insulin promoter can be more effectively activated by PV, a super active form of Pdx1.\textsuperscript{12, 16}

To further compare the effect of Pdx1 and PV, we consecutively observed GFP expression at 48, 72, and 96 hours after Huh7 cells were transduced with Pdx1/Ngn3 or PV/Ngn3 (Fig. 2-5). The results show that PV is much more effective than Pdx1 to activate RIP-GFP. Green fluorescence was observed in 50% of Huh7 cells transduced with PV/Ngn3 at day 2 compared with only 2% of cells treated with Pdx1/Ngn3.

**Pdx1 and Ngn3 Induced Expression of Genes Related to Endocrine Pancreas**

Given the limitations imposed by the chromatin structure on endogenous genes, we next asked whether the activation of the RIP-GFP reporter might be applicable to the endogenous human insulin gene. As shown in Fig. 2-5, although transduction of PV/Ngn3 led to activation of the ectopic promoter RIP-GFP reporter as early as 24 hours, the activation of the endogenous human insulin gene could only be detected at 96 hours by RT-PCR (Fig. 2-6). Notably, the absolute level of activation of the human insulin gene in the cells still remains low, implying that limitations may be due to the inaccessible chromatin structure of the endogenous gene.

To investigate the gene expression profile of Huh7 cells with different treatment after 96 hours, cellular RNA samples were collected and RT-PCR analysis was performed (Fig. 2-6 and 2-7). We screened several endocrine pancreas functional genes and found that endogenous expression of Pdx1 and Nkx6.1 could be activated by all treatments. Cells treated with Ngn3, Pdx1/Ngn3, and PV/Ngn3 activated NeuroD,
Pax4, and Isl-1 genes which are involved in pancreatic endocrine and beta cell
development, and this is consistent with previous findings that NeuroD\textsuperscript{113} and Pax4\textsuperscript{114} are direct targets of Ngn3\textsuperscript{112}. All treatments using Ngn3 activated insulin gene expression. PV, not Pdx1, alone or combined with Ngn3 induced expression of somatostatin (SS). Pancreatic type glucokinase (P-GK) was only expressed in cells transduced with Pdx1/Ngn3 or PV/Ngn3. No glucagon (GLUC) was detected in any samples. Huh7 cells did have basal expression of Pax6, GLUT-2, and β2 microglobulin (β2-M). Huh7 cells continued to express hepatic marker genes such as HNF1β, HNF4α, HGFR, and AFP following all treatments. Significantly down-regulated expression of HNF1α, and to a lesser extent its direct target gene albumin (ALB), was observed in cells with over-expression of Pdx1 or PV.

**Quantitative Analysis of PTF Promoter Activities**

Using the dual-luciferase assay we quantitatively analyzed the promoter activity of PTFs in the Huh7 cells undergoing reprogramming. Huh7 cells were treated with LVs carrying Pdx1, PV, Ngn3, Pdx1/Ngn3 or PV/Ngn3 for 72 hours and then transfected separately with PTF-luciferase reporter plasmids (Pax4, NeuroD, INS1, Ngn3, or Nkx2.2). The relative luciferase activities are shown in Fig. 2-(8-12). Consistent with published data and our own gene expression results, Ngn3 alone can directly activate Pax4, NeuroD, INS1, and Nkx2.2 genes, and suppress its own activation. Ngn3 combined with Pdx1 or PV did not show a synergistic effect on the Ngn3-mediated activation of Pax4, NeuroD, and Nkx2.2 genes, but showed a significantly synergistic activation of INS1 (6-fold in Pdx1/Ngn3, and 16-fold in PV/Ngn3). In addition, Pdx1, but not PV, could effectively activate Pax4, Nkx2.2, and Ngn3 promoter reporters, indicating
that PV, the super active form of Pdx1, has no activation effect in this experimental setting.

**Pdx1 Suppresses HNF1α in Hepatic Cells**

Previous studies have shown that several hepatic genes are down-regulated following over-expression of Pdx1 in hepatic cells\(^{105,251}\). In our current study, we have shown a down-regulation of endogenous HNF1α and its target gene, ALB, in Huh7 cells following treatment with Pdx1 or PV by RT-PCR (Fig. 2-7). To confirm this finding, proteins of cell lysates harvested from day-4 LV-Pdx1- or LV-GFP-treated Huh7 cells were separated by SDS/PAGE and probed by western blotting using antibodies against Pdx1, HNF1α, ALB, and alpha 1 antitrypsin (AAT) proteins (the latter two are direct down-stream target genes of HNF1α). Following Pdx1 expression, a dramatic down-regulation of HNF1α, ALB, and AAT proteins was observed in the Pdx1-treated Huh7 cells (Fig. 2-13). This prompted us to evaluate the effect of Pdx1 on the expression of HNF1α in hepatic cells. Huh7 cells were cotransfected with the HNF1α-luciferase reporter and various concentrations of human Pdx1 or mouse Pdx1 expression plasmid as indicated for 24 hours. Transfection of both Pdx1 plasmids suppresses HNF1α promoter activity in a concentration dependent manner (Fig. 2-14). With 0.8ug Pdx1 expression plasmid, approximately 60% of the activity of the HNF1α promoter is lost. The specificity of inhibition of HNF1α promoter activity by Pdx1 was supported by the fact that C-terminal truncated Pdx1 constructs did not show any inhibitory activity (Fig. 2-15).

**Pdx1 Increases HNF4α P2 Transcripts in Hepatic Cells**

It is well known that the major regulator of HNF1α is HNF4α\(^{147}\). HNF4α is a complex gene regulated by two distinct promoters (P1 and P2) and alternative
splicing\textsuperscript{232, 236-238} that shows tissue specific expression\textsuperscript{222, 240, 241}. In the adult human liver, HNF4α isoforms are primarily expressed from the P1 promoter, whereas in the adult human pancreas and islets, P2 driven isoforms are predominant\textsuperscript{222, 240, 241}. In addition, Pdx1 is a known positive regulator of HNF4α P2 driven isoforms and has a binding site in the P2 proximal promoter\textsuperscript{236}. To test our hypothesis, we performed real time RT-PCR in Huh7 cells following treatment with LV-PV to examine the effects on HNF4α P1 (α1-3) and P2 (α7-9) isoform expression. As expected, we observed a pronounced increase in HNF4α P2 transcripts compared to P1 transcripts (Fig. 2-16). Using primers to identify specific HNF4α P2 driven isoforms, we observed an increase in HNF4α7 and HNF4α8, and a decrease in HNF4α9 (Fig. 2-16).

**Pdx1-Mediated Increase of HNF4α P2 Isoforms Suppress HNF1α Via a Competitive Inhibition**

HNF4α P2 driven isoforms have been previously reported to be weaker transactivators of their target genes\textsuperscript{224, 229, 232}. We therefore investigated the transactivation potential of various HNF4α isoforms from both the P1 and P2 promoters in Huh7, INS-1, and 293 cells using the HNF1α-luciferase reporter (Fig. 2-17). In all of these cell lines it is clear that HNF4α P1 driven isoforms are stronger activators than their corresponding (same c-terminal modification) P2 driven isoforms. However, HNF4α mediated regulation of HNF1α is different between Huh7 and INS-1 cells. In Huh7 cells, both HNF4α P1 isoforms function as stronger activators whereas in INS-1 cells, the strongest activators are the isoforms containing the truncated C-terminal end (HNF4α3 and HNF4α9).

To further investigate the mechanism by which Pdx1 influences down-regulation of HNF1α, we set up competition assays in 3T3 cells because they don’t express any
transcription factors related to our system (data not shown). We first demonstrated that Pdx1 has no direct effect on the HNF1α-luciferase reporter (Fig. 2-18). We then activated this reporter using the liver specific HNF4α P1-isoform, HNF4α2, and demonstrate that Pdx1 can then suppress this activation in a concentration dependent manner (Fig. 2-18). Since Pdx1 is not known to interact directly with the HNF1α promoter, we hypothesized that it may function indirectly by altering the regulation of its major activator HNF4α. Since Pdx1 can increase the expression of HNF4α P2 driven isoforms, we set up a competition assay in 3T3 cells to examine the effect of expressing multiple HNF4α isoforms (Fig. 2-19). In this case, we activated the HNF1α-luciferase reporter using the liver specific P1 isoform, HNF4α2, and then examined the effect of competition with the corresponding beta cell specific P2 isoform, HNF4α8 (both have the same C-terminal modification). As expected and similar to the effect of Pdx1, HNF4α8 can suppress the activity of the HNF1α-luciferase reporter in a concentration dependent manner. With a 1:1 ration of HNF4α2/HNF4α8, the activity of the HNF1α-luciferase reporter is almost the same as the HNF4α8 base level. Therefore, ectopically expressed Pdx1 in hepatic cells enhances the expression of HNF4α P2 driven isoforms that are able to compete with P1 driven isoforms for the same binding site on the HNF1α promoter, which results in suppression of HNF1α and its target genes and contributes to the early events of dedifferentiation of hepatic phenotype during liver-to-beta cell reprogramming.

**Discussion**

In the present study, we have established an effective model by LV expression of Pdx1, PV, Ngn3, and combinations of Pdx1/Ngn3 or PV/Ngn3 in Huh7 cells in which to study the early events of hepatic gene down-regulation and pancreatic gene up-
regulation during the process of Pdx1-mediated hepatic reprogramming into beta-like IPCs. We have demonstrated that coexpression of Ngn3 with Pdx1 is important for the activation of several endocrine pancreas genes such as Pax4, NeuroD, Isl-1, and Nkx2.2. We also examined the role of a Pdx1 fusion protein (PV) that is known to strongly activate insulin gene expression and increase the efficiency of hepatic to pancreatic reprogramming\textsuperscript{12,16,23}. Previous studies have shown that this modified form of Pdx1 carrying the VP16 transcriptional activation domain from the herpes simplex virus more efficiently induces insulin gene expression in the human HepG2 cell line and the rat WB cell line\textsuperscript{12,16}, but whether this attribute was a general characteristic of other PTF genes or limited only to insulin gene was not explored. Our results show that insulin promoter activity was about 3-fold higher in cells treated with PV/Ngn3 than in those treated with Pdx1/Ngn3. However, the Pdx1 modified with VP16 showed lower activation on Pax4, Ngn3, and Nkx2.2 promoters when compared to Pdx1 alone. Therefore, the addition of VP16 restricts Pdx1 transactivation in some contexts, suggesting the mechanism of activation of insulin by Pdx1 may be different from activation of the Pax4, Ngn3, and Nkx2.2 genes.

The liver is largely composed of hepatocytes, which occupy 70-80\% of parenchymal liver volume in the rat\textsuperscript{63,250}. Hepatocytes carry out the primary functions of the liver such as metabolism, detoxification, and protein synthesis of several essential compounds including serum ALB, fibrinogen, and transferrin. It has been suggested that the dominant mechanism for controlling the expression of hepatocyte specific genes is at the transcriptional level\textsuperscript{259}, however, the molecular mechanism by which Pdx1 regulates the expression of these hepatic genes is not well established. Using our
reprogramming model, we have demonstrated that over-expression of Pdx1 can down-regulate expression of HNF1α and its target genes (ALB and AAT) in hepatic cells by RT-PCR and western blotting. Because HNF1α is so important in the regulation of a wide array of hepatic genes\textsuperscript{128,252}, it may play a fundamental role in the process of dedifferentiation of hepatocyte phenotype during the reprogramming toward IPCs. Our findings are consistent with a previous study\textsuperscript{105} where adenovirus-mediated expression of Pdx1 led to down-regulation of several mature hepatocyte-specific genes including ALB, ADH1B, G6PC, GLUL, and AAT. Several key hepatic genes are direct downstream transcriptional targets of HNF1α including ALB, AAT, AFP, α- and β-fibrinogen, transthyretin, and pyruvate kinase\textsuperscript{260-266}. Therefore, Pdx1 induced down-regulation of HNF1α expression can affect these downstream target genes and may be important in the process of dedifferentiation of hepatic cells by down-regulating the expression of an array of genes that determine hepatocyte phenotype.

Our results also suggest that the mechanism for down-regulation of HNF1α is by affecting the expression of its major activator HNF4α. (Note: See The Sladek Lab webpage for detailed information about HNF4α structure and function at http://www.sladeklab.ucr.edu/HNF4.shtml). We demonstrated that specific HNF4α isoforms function differently in hepatocytes and in beta cells (Fig. 2-17). In hepatic cells, the activity of specific HNF4α isoforms appears to rely on the N-terminal modification that results from using different promoters (P1 vs. P2). HNF4α isoforms that are expressed using the P1 promoter contain exon 1A and are the strongest activators in hepatic cells. The C-terminal modifications that result from alternative splicing don’t significantly alter the function of HNF4α in hepatic cells in our
experimental setting. In pancreatic beta cells the C-terminal modifications appear to play a major role in the function of HNF4α (Fig. 2-17). P1 driven isoforms are still stronger activators in beta cells when compared to their corresponding P2 driven isoforms (with similar C-terminal ends) but having the truncated C-terminal end that stops in exon 8 increases the activity independent of the first exon. The isoform driven from the P2 promoter that terminates in exon 8 (HNF4α9) is a stronger activator than the P1 driven isoform that include exon 9 and 10 (HNF4α2). This suggests that the C-terminal modifications of HNF4α due to alternative exon splicing may have cell-specific function.

According to our real time RT-PCR data (Fig. 2-16), HNF4α isoforms that are stronger transcriptional activators containing exon 1A (AF-1 activation domain) or terminating in exon 8 (HNF4α2, HNF4α3, or HNF4α9) are suppressed by treatment with Pdx1 while HNF4α isoforms that are weaker transcriptional activators that contain exon 1D and exons 9 and 10 (HNF4α8) are up-regulated. Exons 9 and 10 comprise the inhibitory F domain which inhibits transactivation potential by blocking coactivator binding\textsuperscript{234}. It appears that HNF4α dependent regulation of HNF1α in hepatocytes requires stronger activators (P1 driven) than in beta cells which predominantly rely on P2 driven isoforms and this may be due to cell specific mechanisms for regulating this gene. For example, the PTF Nkx6.1, which is expressed in beta cells but not in hepatocytes\textsuperscript{14}, has been shown to regulate HNF1α by binding to its distal promoter\textsuperscript{255}. We also show evidence (Fig. 2-19) that promoter specific isoforms can compete with each other for activation of their target genes. It is possible that induced expression of HNF4α from the P2 promoter may suppress P1 expression during the reprogramming
process of hepatocyte toward IPCs, as the opposite has been shown during rodent liver development\textsuperscript{221}. These findings are similar to the competitive inhibition of HNF3β, by NF3α, that has been previously demonstrated for the hepatic regulation of both HNF1α and HNF4α\textsuperscript{246}.

Taken together, we propose the following molecular mechanism that may be in part responsible for Pdx1-mediated dedifferentiation of hepatocyte phenotype in transition toward pancreatic beta-like IPCs (Fig. 2-20). In normal hepatocytes, HNF4α P1-derived isoforms are mainly responsible for activation of HNF1α that in turn activates its downstream target genes and maintains hepatic phenotype. Following ectopic expression of Pdx1, Pdx1 binds to the HNF4α P2 promoter, resulting in up-regulation of P2 isoforms. The increased amount of HNF4α P2 isoforms then compete with P1 isoforms for the same DNA binding site on the HNF1α promoter, resulting in an overall effect of HNF1α down-regulation. The competitive inhibition of HNF4α P1 isoforms, by P2 isoforms, may be a driving force in the dedifferentiation of the hepatic phenotype by suppressing downstream target genes.
Figure 2-1. Confirmation of LV-PTF gene expression by RT-PCR. Total RNA was collected from Huh7 cells following transduction with expression vectors for *Pdx1*, *PV*, *Ngn3*, or combinations as indicated. Indicated gene expression was measured by RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pdx1</th>
<th>PV</th>
<th>Ngn3</th>
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<tr>
<td>P-Con</td>
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<tr>
<td>Pdx1</td>
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<td>PV</td>
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<tr>
<td>Ngn3</td>
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![Gene expression bands](image)
Figure 2-2. Confirmation of LV-PTF gene expression by western blotting. Huh7 cells were transduced with expression vectors for Pdx1, PV, Ngn3, or combinations as indicated. Following transduction, Huh7 cells were scraped off and lysed in RIPA buffer. Equal amounts of cell lysates were separated on 12% polyacrylamide gels by SDS-PAGE and immunoblotted with polyclonal antibody against Pdx1 or PV as indicated. INS-1 cell lysates were used as a positive control for Pdx1.
Figure 2-3. Confirmation of LV-PTF gene expression by immunocytochemistry. Huh7 cells were transduced with expression vectors for *Pdx1*, *PV*, *Ngn3*, or combinations as indicated. Following transduction, cytospin slides made from Huh7 cells were air-dried, fixed and stained with Anti-*Ngn3* antibodies using ICC.
Figure 2-4. Activation of rat insulin I gene in Huh7 cells with *Pdx1*, *PV*, *Ngn3* and combinations. Huh7 cells were transduced with *RIP-GFP* reporter and *Pdx1*, *PV*, *Ngn3*, or combinations as indicated for 96 h. The expression of *GFP* was observed under a fluorescence microscope.
Figure 2-5. Time course activation of rat insulin I gene in Huh7 cells with \textit{Pdx1/Ngn3} or \textit{PV/Ngn3}. Huh7 cells were transduced with \textit{LV-Pdx1/Ngn3} or \textit{LV-PV/Ngn3} in LVs in the presence of LV-\textit{RIP-GFP} reporter. The expression of \textit{GFP} at 48h, 72h, and 96h was recorded by fluorescence microscopy.
Figure 2-6. RT-PCR analysis of pancreatic gene expression. Huh7 cells were treated with LV-transgenes (Pdx1, PV, Ngn3, or combinations as indicated) for 96h. Equal amounts of total RNA/cDNA sample were used for RT-PCR analysis with gene specific primers.
Figure 2-7. RT-PCR analysis of liver specific gene expression. Huh7 cells were treated with LV-transgenes (*Pdx1*, *PV*, *Ngn3*, or combinations as indicated) for 96h. Equal amounts of total RNA/cDNA sample were used for RT-PCR analysis with gene specific primers.
Figure 2-8. *Pax4*-luciferase reporter gene analysis during reprogramming. Relative activity was measured for the *Pax4*-luciferase reporter following Huh7 cell transduction with LVs for *Pdx1*, *PV*, *Ngn3*, or combinations as indicated for 72h. Huh7 cells transduced with LV-GFP were used as a control. All experiments were done in triplicate and repeated independently at least three times. All p values are compared to non-treated controls. ** = p<0.001
Figure 2-9. **NeuroD-luciferase reporter gene analysis during reprogramming.** Relative activity was measured for the **NeuroD-luciferase reporter** following Huh7 cell transduction with LVs for **Pdx1, PV, Ngn3**, or combinations as indicated for 72h. Huh7 cells transduced with LV-GFP were used as a control. All experiments were done in triplicate and repeated independently at least three times. All p values are compared to non-treated controls. ** = p<0.001
Figure 2-10. *INS1*-luciferase reporter gene analysis during reprogramming. Relative activity was measured for the *INS1*-luciferase reporter following Huh7 cell transduction with LVs for *Pdx1*, *PV*, *Ngn3*, or combinations as indicated for 72h. Huh7 cells transduced with LV-*GFP* were used as a control. All experiments were done in triplicate and repeated independently at least three times. All p values are compared to non-treated controls. ** = p<0.001
Figure 2-11. Ngn3-luciferase reporter gene analysis during reprogramming. Relative activity was measured for the Ngn3-luciferase reporter following Huh7 cell transduction with LVs for Pdx1, PV, Ngn3, or combinations as indicated for 72h. Huh7 cells transduced with LV-GFP were used as a control. All experiments were done in triplicate and repeated independently at least three times. All p values are compared to non-treated controls. ** = p<0.001
Figure 2-12. *Nkx2.2*-luciferase reporter gene analysis during reprogramming. Relative activity was measured for the *Nkx2.2*-luciferase reporter following Huh7 cell transduction with LVs for *Pdx1, PV, Ngn3*, or combinations as indicated for 72h. Huh7 cells transduced with LV-*GFP* were used as a control. All experiments were done in triplicate and repeated independently at least three times. All p values are compared to non-treated controls. ** = p<0.001
Figure 2-13. Down-regulation of HNF1α, ALB, and ATT in Huh7 cells over-expressing Pdx1 by western blotting. Huh7 cells were transduced with or without LV-Pdx1 or LV-rhGFP for 96h. Following transduction, Huh7 cells were lysed in RIPA buffer. Equal amount of cell lysates were separated on 12% SDS-polyacrylamide gels and immunoblotted with antibodies against Pdx1, HNF1α, ALB, ATT, or actin.
Figure 2-14. Down-regulation of *HNF1α-luciferase reporter* in Huh7 cells over-expressing *Pdx1*. Huh7 cells were transfected with 1.0µg of *HNF1α-luciferase* reporter plasmid and 0.2-0.8µg *Pdx1* expression plasmid as indicated. pcDNA3 was used as a DNA quantity control. All values are significant (p<0.05) compared to mock transfection controls.
Figure 2.15. Down-regulation of $HNF1\alpha$ by full length Pdx1. Huh7 cells were transfected with 1.0µg of $HNF1\alpha$-luciferase reporter plasmid and 0.8µg of each Pdx1 truncated (120, 160, or 200 amino acids) or full length (283 amino acids) plasmid with pcDNA3 as a DNA quantity control. ** = $p<0.001$
Figure 2-16. *Pdx1* increases *HNF4α* P2 transcripts that compete with P1 transcripts. Total RNA was collected from Huh7 cells following transduction with LV-*PV* or control (LV-*GFP*). Indicated gene expression was measured by real time RT-PCR. Primers were designed to detect *HNF4α* expression from the P1 promoter (Exon 1A) or the P2 promoter (Exon 1D) as well as the three *HNF4α* C-terminal modifications. This experiment was done in triplicate and repeated independently at least 3 times.
Figure 2-17. Cell-type specific effect of HNF4α isoforms on activation of HNF1α-luc. Relative activity was measured for the mouse HNF1α-luciferase reporter (1µg/well) following transduction of various HNF4α isoforms (1µg/well) in Huh7, INS-1, and 293 cells. Activity was normalized in each cell type to the pcDNA3 empty vector control. This experiment was done in triplicate and repeated independently at least 3 times. All values are significant (p<0.05) compared to mock transfection controls.
Figure 2-18. Down-regulation of HNF1α-luciferase reporter by Pdx1 in 3T3 cells. Relative activity was measured for the mouse HNF1α-luciferase reporter (1µg/well) following transduction of HNF4α2 and Pdx1 alone, and in combination. + indicates 1µg/well and the concentration gradient shows three concentrations (0.1, 0.5 and 1.0 µg) per well. All activation and suppression values are significant (p<0.05) except for activation by Pdx1 alone. Experiments were done in triplicate and repeated independently at least three times.
Figure 2-19. Down-regulation of HNF1α-luciferase reporter by HNF4α8 in 3T3 cells. Relative activity was measured for the mouse HNF1α-luciferase reporter (1µg/well) following transduction of HNF4α2 and HNF4α8 alone, and in combination. + indicates 1µg/well and the concentration gradient shows three concentrations (0.1, 0.5, and 1.0µg)/well. All activation and suppression values are significant (p<0.05) except for suppression by 0.1µg HNF4α. Experiments were done in triplicate and repeated independently at least three times.
Figure 2-20. A proposed mechanism of *Pdx1*-mediated hepatocyte-toward-IPC reprogramming. This cartoon depicts our proposed molecular mechanism for *Pdx1*-mediated down-regulation of *HNF1α* during hepatocyte-to-IPC reprogramming. The left side shows the molecular events involved in normal hepatocytes and the right side shows how these molecular events may be altered following ectopic expression of *Pdx1* (based on our cumulative data). The *HNF1α* and *HNF4α* genes and promoters are shown in order to understand how their relationship and downstream gene targeting is affected by the expression of *Pdx1* during reprogramming from hepatocytes toward IPCs. We propose that the competitive inhibition of *HNF4α* P1 isoforms, by P2 isoforms, suppresses *HNF1α* and its downstream target gene expression and promotes hepatocyte dedifferentiation.
CHAPTER 3
DISTINCT REGULATION OF HNF1α BY Nkx6.1 IN PANCREATIC BETA CELLS

Introduction

Hepatic nuclear factor 1 alpha (HNF1α) is a key transcription factor involved in glucose stimulated insulin secretion (GSIS) and is the major factor involved in most cases of maturity onset diabetes of the young (MODY) which accounts for about 1% of worldwide diabetes cases. Precise cellular concentrations of HNF1α are required to maintain normal beta cell function and both under expression and over expression have been shown to lead to diabetes.

HNF1α is an important gene involved in the developmental regulation of the liver, pancreas, kidneys, stomach, and intestines. This transcription factor is essential for control of mature cellular phenotype in these tissues. In pancreatic beta cells, HNF1α is involved in regulating the transcription of several genes that are involved in glycolysis and glucose stimulated insulin secretion such as insulin, Glut2 glucose transporter, pyruvate kinase, aldolase B, HNF3γ, and HNF4γ. Although the regulation of HNF1α has been well characterized in hepatocytes, its regulation in beta cells has not been studied in detail, and this gene is assumed to be regulated based on mechanistic studies from hepatocytes.

HNF1α has an HNF4α binding site in its proximal promoter that has been shown to be sufficient for its activation in hepatocytes, but evidence exists to suggest that the mechanism for regulation of HNF1α may different in beta cells. First, HNF1α is expressed as three isoforms (A, B, and C) that have tissue specific distribution ratios.

HNF1αA is the predominant form in hepatocytes while HNF1αB is predominant in pancreatic islets. It has been shown that HNF1αB/C have greater transactivation potential than HNF1αA\textsuperscript{159}. Second, the major regulator of this gene, HNF4α, is predominantly expressed from the P1 promoter in hepatocytes, whereas in the pancreas only transcripts from the P2 promoter can be detected according to most reports\textsuperscript{238, 240, 241}. HNF4α transcripts expressed in the pancreas from the P2 promoter have a truncated N-terminal region containing the transactivation domain and these isoforms have been shown to have lower transactivation potential when compared to isoforms expressed from the P1 promoter\textsuperscript{224, 229, 232}. This suggests that these HNF4α isoforms expressed from the P2 promoter may be insufficient to fully activate HNF1α in the pancreas. Third, Huang et al.\textsuperscript{150} show that while a -497 bp proximal HNF1α luciferase reporter was shown to be activated in hepatocytes, it failed to be activated in rat insulinoma (INS1) beta cells and this promoter contains the proximal HNF4α binding site used for activation in hepatocytes. The aforementioned data suggests that HNF1α may utilize a different mechanism for gene transcription in beta cells than previously identified for hepatocytes.

NK6 homeodomain 1 (Nkx6.1) is a homeodomain transcription factor involved in pancreatic differentiation and beta cell homeostasis\textsuperscript{115}. In mature human islets, it is exclusively expressed in beta cells\textsuperscript{117} and is required for normal GSIS\textsuperscript{118}. Over expression of Nkx6.1 has been shown to increase GSIS in rat islets\textsuperscript{122}. It is also of interest that islets isolated from type 2 diabetic patients have altered Nkx6.1 expression\textsuperscript{127}. However, the specific function of Nkx6.1 in GSIS of mature beta cells remains elusive.
In this work, we hypothesized that a distinct regulatory mechanism for HNF1α gene expression exists in pancreatic beta cells. Here we report a novel finding that Nkx6.1 is a key regulator of HNF1α expression in beta cells, which may provide insight into the understanding of the regulation of GSIS in beta cells.

**Materials and Methods**

**Cell Culture**

NIH 3T3 mouse fibroblast cells, rodent insulinoma cell lines (βTC3, NIT1, INS1) and Huh7 human hepatocarcinoma cells were cultured in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 0.1% Kanamycin in a 37° incubator with 100% humidity and 5% CO₂.

**Plasmid Construction**

HNF1α-Luc: 2772 bp of the mouse HNF1α proximal promoter was cloned into the pGL3 vector (Promega) using the EcoRI and BglII restriction sites. pGL-TK: The pGL-TK expression vector was purchased from Promega. pcDNA3: The pcDNA3 vector was purchased from Invitrogen. CMV-Pdx1: The human Pdx1 expression plasmid (pCMV6-XL5) was purchased from Origene. Nkx6.1: The human Nkx6.1 expression plasmid (pBAT12) was a generous gift from Dr. Michael German from the University of California, San Francisco, CA. Ngn3: The mouse Ngn3 expression plasmid was a generous gift from Dr. Marko Horb at the Institute de Recherches Cliniques de Montréal, Montréal, QC Canada. Mouse Ngn3 cDNA was cloned into the pcDNA3 vector using the BamHI restriction site. MafA: Human MafA cDNA was cloned into the pTYF lentiviral vector cassette under control of the elongation factor 1 alpha promoter using the BamHI and SpeI restriction sites. NeuroD1: Mouse NeuroD1 cDNA was cloned into the pcDNA3.1 CT-GFP-TOPO vector. Pax6: The xenopus Pax6 expression plasmid (a
generous gift from Dr. Marko Horb) was derived by inserting Pax6 cDNA into the pcDNA3 vector using the HindIII and XhoI restriction sites. HNF1α: The human HNF1α expression plasmid was a generous gift from Dr. Michael German. Human HNF1α cDNA was cloned into the pCMV-Sport6 vector. HNF1β: The human HNF1β expression plasmid (pCMV-Sport6) was purchased from Open Biosystems. HNF4α: The mouse HNF4α expression plasmid (pCMV-Sport6) was purchased from Open Biosystems. Pbx1: Mouse Pbx1 was cloned into the pcDNA3 vector using the BamHI and XbaI restriction sites.

**Transient Transfection and Luciferase Assays**

Cells were cultured as previously indicated and transfected with 0.1-1.0μg DNA (as indicated) using Lipofectamine 2000 Reagent (Invitrogen) according to manufacturer’s protocol. 0.02μg TK-Luc plasmid was used as a transfection control in all experiments. Cell lysates were harvested and measured 24 hours post transfection using the Dual Luciferase Reporter Kit (Promega) according to manufacturer’s protocol except that only 50μl of each substrate reagent was used (as optimized by our lab) to read samples using a Lumat LB 9507 Luminometer (Berthold Technologies). All luciferase assays were done in triplicate.

**Site Directed Mutagenesis**

Primers were designed (IDT Technologies) to induce a block mutation of 4-6 base pairs as indicated at the core sequence inducing a restriction enzyme site for easy confirmation of mutation. Primer sequences are as follows: Nkx6.1, (F:5’-GGACCTGTTCCTCGAGGAAATGTGACACTTTAC-3’) and (R:5’-GTAAAGTGTCACATTTCCTCGAG-GAACAGGTCC-3’), HNHF4α, (F:5’-CTTGCAAGGCTGAAGTCCGGCCGTCAGTCCCTTC-3’) and (R:5’-GTAAAGTGTCACATTTCCTCGAG-GAACAGGTCC-3’)
GCTTAGGAAG-GGACTGACGGCCGGACTTCAGCCTTGCAAGTGCAG-3'). Mutations were induced using PCR on relevant plasmid with Pfu polymerase enzyme. PCR products were incubated for 1 hour with DpnI (New England Biolabs) methylation sensitive restriction enzyme to remove template plasmid and transformed into competent E. coli. Positive colonies were inoculated in LB medium (MP Biomedicals) and plasmid was purified using a Qiagen plasmid purification maxi kit.

**Electrophoretic Mobility Shift Assay (EMSA)**

Biotin labeled probes were designed (IDT Technologies) spanning the cis regulatory element identified. Sequences are as follows:

(F:5'-GAAGGATGGACCTGTTCCTAATGG-AAATGTGACACTTTA-3') and (R:5'-TAAA-GTGTCACATTTCATTAGGAACAGGTCCATCCTTC-3'). Nuclear lysate from beta cell lines (INS1, βTC3, or NIT1) was prepared using the NE-PER nuclear extraction reagents (Peirce) according to manufacturer's protocol. INS1 cells were a generous gift from Dr. Christopher Newgard at the Duke University Medical Center, Durham, NC. Anti-Nkx6.1 polyclonal antibodies (Santa Cruz) were used. Binding reactions were performed using the LightShift Chemiluminescent EMSA Kit (Promega) according to manufacturer's protocol. Binding reactions were resolved by polyacrylamide gel electrophoresis using a 7.5% Tris-HCl polyacrylamide gel (Biorad). Complexes were detected with the Chemiluminescent Nucleic Acid Detection Module (Pierce) according to manufacturer’s protocol.

**Chromatin Immunoprecipitation (ChIP) Assay**

ChIP assay was performed using the chromatin immunoprecipitation assay kit (Millipore) according to manufacturer's protocol. Beta cells cultured in 10cm culture dishes were used both with and without indicated transfection. Specific polyclonal
antibodies to Nkx6.1 or HNF4α (Santa Cruz), were used for immunoprecipitation. Following DNA isolation, sequences were evaluated by PCR using primers (IDT Technologies) flanking respective cis regulatory elements as indicated. Amplification primer sequences are as follows: Nkx6.1, (F:5′-CCCATCCAGGATGAAGTGAG-3′) and (R:5′-GACAAGGAGTTCTGGGCTAG-3′) HNF4α, (F:5′-TCACTCCCAATTGCAAGCCATG-3′) and (F:5′-TGCTGCTCTGTATTACATTGG-3′).

Gene Expression and Quantitative RT-PCR

Cells were cultured as previously indicated and transfected with DNA or siRNA (as indicated) using Lipofectamine 2000 Reagent (Invitrogen) according to manufacturer’s protocol. Nkx6.1-specific siRNA and control siRNA was purchased from Santa Cruz. Total RNA was extracted from cells using Trizol Reagent (Invitrogen) and RT-PCR and real-time RT-PCR were performed as previously described(38). All sequences of primer pairs are available upon request.

Statistical Analysis

Statistical analysis was carried out using the two-sample Student’s t-test. A P value < 0.01 was considered significant. All indicated significant values are in comparison to controls.

Results

HNF1α Employs a Distinct Regulatory Element in Beta Cells

Regulation of the HNF1α promoter has been extensively studied in hepatocytes. Transcription is regulated through a TATA-like box (-21 to -15) and proximal HNF4α binding site (-47)144,147,148. The HNF1α -497bp promoter (containing the HNF4α binding site) is fully active in hepatocytes but not in rat insulinoma INS1 cells150 providing evidence that its regulation may be controlled by alternative elements in other
tissue types. To test this hypothesis, we have cloned a 2772 base pair (bp) region of the mouse HNF1α proximal promoter upstream of the firefly luciferase gene in a pGL3 vector to study its regulation in pancreatic beta cells. This 2772 bp HNF1α promoter has full activity in both beta cells (Fig. 3-1) and hepatic cells (Fig. 3-2). We created several truncated HNF1α promoter constructs by 5’ deletion analysis (Fig. 3-1) using restriction enzymes and have determined that the major regulation of HNF1α in beta cells is conferred through a more distal regulatory element (-2772/-1820). Loss of this regulatory region significantly diminishes transactivation of the HNF1α reporter in beta cells (Fig. 3-1) but not in hepatic cells (Fig. 3-2). NIH 3T3 cells serve as a control in which the HNF1α promoter is inactive. This result shows that there is indeed a distinct regulatory region employed by beta cells for the regulation of HNF1α.

**HNF4α Binding Site is Only Partially Responsible for HNF1α Activation in Beta Cells**

Next, we evaluated the role of HNF4α regulation of HNF1α in beta cells. Using site directed mutagenesis, we constructed an HNF1α reporter with a mutated HNF4α binding site (Fig. 3-6). As previously reported\(^{144, 147, 148}\), HNF4α is required for transactivation in hepatic cells (Fig. 3-3); however, mutation of the HNF4α binding site only partially diminished transactivation in βTC3 cells (Fig. 3-3). This demonstrates that beta cells are regulated by a separate response element that is different from hepatic cells.

**HNF1α Can Be Activated by Nkx6.1**

After finding an HNF1α regulatory response element (-2772/-1820) that is beta cell specific, we were interested in discovering which factors regulate transcription at this site. Several beta cell specific transcription factors were screened (Pdx1, Ngn3, MafA,
NeuroD1, Pax6, Nkx6.1, HNF1α, HNF1β, HNF4α, and Pbx1) using transient transfection assays in 3T3 cells with cotransfection of the HNF1α luciferase reporter (Fig. 3-4). Our results confirm previous reports that HNF1α can be activated by HNF4α$^{144,147,148}$ and we have found that Nkx6.1 is also a strong activator. Nkx6.1 can activate HNF1α in a concentration dependent manner (Fig. 3-5) and is likely to be the regulator of the cis regulatory element that is unique to beta cells.

**Mutational Analysis of HNF1α Promoter**

The literature pertaining to Nkx6.1 binding suggests that the most common core DNA sequence utilized by Nkx6.1 for binding is: (5' TAAT 3')(49-54) or its compliment (5' ATTA 3') and it has also been shown to bind a similar sequence (5' ATTT 3')(55).

We visually examined the HNF1α promoter sequence conferring strong transactivation in beta cells (-2772/-1820) and found there is a single (5' TAAT 3') DNA sequence in this entire region (Fig. 3-6). An alignment of this promoter region between mouse and rat shows a very high level of homology similar to alignment of the promoter region near the known HNF4α binding site (Fig. 3-7). This prompted us to use site directed mutagenesis to induce a mutation (Fig. 3-6) at this potential Nkx6.1 binding site in order to examine the effect on transactivation by Nkx6.1. We also generated a double mutant HNF1α luciferase reporter with both the HNF4α binding site mutation and the potential Nkx6.1 binding site mutation

Using our mutant plasmids we examined the ability to activate the HNF1α luciferase reporter with HNF4α or Nkx6.1 in 3T3 cells (Fig. 3-8). Results were as expected and demonstrated that the normal HNF1α promoter can be activated by either HNF4α or Nkx6.1. The HNF4α binding site mutant failed to respond to HNF4α but can still be activated by Nkx6.1. Similarly, the Nkx6.1 binding site mutant failed to respond
to Nkx6.1 but can still be activated by HNF4α. The double mutant failed to respond to either HNF4α or Nkx6.1. This result suggests that our mutated site (5' TAAT 3') is a real Nkx6.1 binding site that is used for initiating HNF1α transcription.

The Nkx6.1 Binding Site Mutation Reduces Activation of the HNF1α Promoter in Beta Cells

Since the Nkx6.1 binding site mutation was sufficient to prevent Nkx6.1 mediated activation of the HNF1α luciferase reporter in 3T3 cells, we next assessed its function in beta cells. Transfection of the Nkx6.1 binding site mutant construct shows approximately 50% reduced activity when compared to the normal HNF1α luciferase reporter in βTC3 beta cells (Fig. 3-9). Four other random (5' TAAT 3') mutant constructs were generated and had no change in activity when compared to the normal promoter construct in similar assays (data not shown). Activity was not completely diminished in the Nkx6.1 binding site mutant construct suggesting that other beta cell specific transcription factors, such as HNF4α or Pax6, may play a role in regulation of this gene. This result suggests that Nkx6.1 is a major beta cell specific activator of HNF1α in beta cells.

Nkx6.1 Can Bind to the HNF1α Distal Regulatory Element

To further evaluate DNA-protein interaction between Nkx6.1 and the HNF1α distal promoter element (-2772/-1820) we designed biotin labeled oligonucleotide probes spanning the potential Nkx6.1 binding site (5' TAAT 3'). Using EMSA, we have demonstrated that Nkx6.1 protein from beta cell lysate can bind to our probe (Fig. 3-10, Lane 2). Addition of Nkx6.1 polyclonal antibody caused a disruption and supershift of the DNA-protein band (Lane 3) confirming the protein is indeed Nkx6.1. Unlabeled cold probe is able to compete for binding Nkx6.1 (Lanes 4 & 5) while nonspecific probe is
unable to compete (Lane 6). This result confirms that Nkx6.1 protein is capable of binding the HNF1α distal promoter element.

**Nkx6.1 Occupies the HNF1α Distal Regulatory Element in Beta Cells**

To confirm that Nkx6.1 actually occupies the HNF1α promoter endogenously in beta cells, βTC3 cells were grown normally (-) or transfected with the Nkx6.1 expression plasmid (+). Following chromatin immunoprecipitation with Nkx6.1 polyclonal antibody, we were able to amplify this DNA sequence from normal βTC3 cells (Fig. 3-11). The βTC3 cells transfected with the Nkx6.1 expression plasmid show an increase in the intensity of the band confirming Nkx6.1 binding at this site. Our cumulative data demonstrates that Nkx6.1 occupies the endogenous HNF1α promoter cis regulatory element in βTC3 cells and it is able to positively regulate its expression.

We also evaluated the occupancy of HNF4α at the proximal binding site of the HNF1α promoter as previously reported for hepatocytes. We performed a similar ChIP assay (Fig. 3-12) with βTC3 cells that were grown normally (-) or transfected with the HNF4α expression plasmid (+). Following chromatin immunoprecipitation with HNF4α polyclonal antibody, we were unable to amplify this DNA sequence from normal βTC3 cells indicating that HNF4α may not be a major regulator of HNF1α in beta cells. However, the βTC3 cells transfected with the HNF4α expression plasmid (hepatocyte specific isoform) shows a weak amplification band indicating that the binding site may still have function to allow its detection. It may also be that HNF4α binding cannot be detected due to the relatively lower binding affinity of beta cell specific HNF4α isoforms, therefore we cannot exclude the possibility that HNF4α is involved in HNF1α regulation.
**Nkx6.1 Regulates Gene Expression of HNF1α in Pancreatic Beta Cells**

To determine a role of Nkx6.1 in regulating HNF1α in beta cells, mouse beta cell lines (βTC3 and NIT cells) were used for this study. First, we confirmed that Nkx6.1 indeed can bind to the promoter probe of HNF1α by EMSA using βTC3 (Fig. 3-13) and NIT (Fig. 3-14) cell lysate. Next, we confirmed that both beta cell lines expressed key pancreatic transcription factors (including insulin, Pdx1, Nkx6.1, and HNF1α) that are important for maintaining beta cell function (Fig. 3-15). To determine whether Nkx6.1 regulates HNF1α in beta cells, the effects of Nkx6.1 over expression or knockdown by its siRNA on mRNA levels of HNF1α were investigated. Over expression of Nkx6.1 in NIT1 beta cells markedly increased endogenous HNF1α expression 3-fold over control (Fig. 3-16), while Nkx6.1-specific siRNA knocked down endogenous expression of HNF1α by ~80% of original level (Fig. 3-17), providing further support that Nkx6.1 is a regulator of HNF1α. Cyclin B expression is used as an internal control because it has been previously shown to be regulated by Nkx6.1 through distal promoter binding\textsuperscript{122}. Our data confirm that Nkx6.1 is a major activator of HNF1α in pancreatic beta cells.

**Discussion**

In this study, we have shown a distinct mechanism of regulation of HNF1α in beta cells. Different from hepatocytes, we demonstrate that Nkx6.1 is a key regulator for HNF1α by binding to the distal region of the HNF1α promoter in beta cells. We confirmed a specific physical binding of HNF1α promoter to Nkx6.1 protein in beta cells by EMSA and ChIP assays. Furthermore, we also demonstrated that the endogenous HNF1α gene transcription is indeed regulated by NKx6.1 in beta cells. To the best of our knowledge, this is the first time Nkx6.1 is reported as a key regulator of HNF1α in pancreatic beta cells.
Nkx6.1 is known to be involved in pancreatic differentiation and beta cell function\textsuperscript{115, 117, 118}. Embryonic expression of Nkx6.1 is dependent on Nkx2.2\textsuperscript{119, 120} and in mature beta cells it is regulated by Pdx1\textsuperscript{120}. Nkx6.1 maintains beta cell phenotype in part by direct interaction with the glucagon promoter, suppressing its activity\textsuperscript{118, 121}. Nkx6.1 inhibits glucagon expression by competing with Pax6 (glucagon activator) for occupancy of the G1 element on the glucagon promoter. Nkx6.1 has also been linked to beta cell proliferation by up regulating cyclins A, B, and E as well as many regulatory kinases\textsuperscript{122}.

Studies in knockout mice reveal that Nkx6.1 gene is required for beta cell development, terminal differentiation, and biological function\textsuperscript{119}. Over expression of Nkx6.1 has been shown to increase GSIS in rat islets\textsuperscript{122}. To address what happens to gene expression in beta cells when Nkx6.1 expression is altered, we performed functional studies in beta cell lines to show the effects of Nkx6.1 over expression (Fig. 3-16) and knockdown (Fig. 3-17). As expected, Nkx6.1 over expression led to an increase in HNF1α expression as measured by real-time PCR while Nkx6.1 knockdown by siRNA led to decreased expression. In addition, Uchizono et al. 2009\textsuperscript{270} shows that deficiency of HNF1α severely constrains the extent of beta cell proliferation in mice leading to significant changes in blood glucose levels. The same paper, by exploring gene expression profile using immortalized beta cells generated from HNF1α homozygous deficient mice, reveals decreased Nkx6.1 gene expression. It also shows changes in expression of genes involved in beta cell growth and proliferation, providing insight into the mechanism whereby HNF1α affects beta cell function. In terms of beta cell functional studies using islets, Newgard's group has published a study\textsuperscript{122} showing...
that over expression of Nkx6.1 increases GSIS in rat and human islets by inducing beta
cell replication. In contrast, knockdown of Nkx6.1 in human islets leads to impaired GSIS\textsuperscript{122}.

A recent report indicates that mutations affecting the expression of B-lymphocyte kinase (BLK) gene was found to be responsible for some patients with MODY symptoms but without mutations in known MODY genes\textsuperscript{271}. BLK is a previously unidentified modulator of insulin synthesis and GSIS in beta cells by the mechanism of enhancing the expression of Nkx6.1, providing further evidence that Nkx6.1 may be a new candidate gene involved in MODY.

Nkx6.1 homeodomain constructs have been shown to bind sequences containing the core homeodomain binding site (5' TAAT 3' or 5' ATTA 3') and direct both gene repression and gene activation\textsuperscript{123, 124}. In its own promoter, it has been shown to bind a similar sequence (5' ATTT 3') to positively regulate its own expression\textsuperscript{125}. Nkx6.1 has the ability to function as both a transcriptional activator and repressor which may be sequence dependent\textsuperscript{125}. The transcriptional repression domain has been isolated to the N-terminus\textsuperscript{124} while the transcriptional activation domain has been shown to be dependent on the C-terminus\textsuperscript{125}. The C-terminus has also been observed to interfere with DNA binding but greatly enhance specificity for homeodomain core containing sequences\textsuperscript{126}.

Previous work on HNF1α expression has established that alternative isoforms of HNF1α are generated by post translational modification\textsuperscript{159, 169}. Three separate isoforms have been identified: HNF1αA, HNF1αB, and HNF1αC. These isoforms have been confirmed by real-time PCR and no evidence for additional forms was found\textsuperscript{159}. The
different isoforms are generated through differential selection of polyadenylation and alternative splicing generating distinct 3’ ends\textsuperscript{159}. Furthermore, HNF1\(\alpha\) transcription does not use an alternative promoter as demonstrated by 5’ RACE analysis\textsuperscript{159}. The different HNF1\(\alpha\) isoforms are distributed differently between tissues and during different time points in development suggesting that they may control gene expression in a temporal and tissue specific manner. The alternate isoform ratios may also contribute to differential gene regulation between cell types. Alternative splicing of transcripts increases versatility of function of products and several documented proteins act as activators and repressors from the same gene\textsuperscript{160}.

Several studies in mice demonstrate the importance of HNF1\(\alpha\). HNF1\(\alpha\) knockout mice develop many symptoms of diabetes\textsuperscript{186}. Beta cell dysfunction occurs in HNF1\(\alpha\) knockout mice marked by hyperglycemia and impaired glucose tolerance without loss of beta cell mass\textsuperscript{184}. Genes involved in beta cell regulation and metabolism are expressed abnormally in HNF1\(\alpha\) null mice such as Glut2, pyruvate kinase, and insulin as well as the islet enriched transcription factors Pdx1, HNF4\(\alpha\), and NeuroD1\textsuperscript{185}. HNF1\(\alpha\) cellular concentration is critical for beta cell homeostasis. HNF1\(\alpha\) knockout mice have abnormal beta cell gene expression, impaired glucose tolerance, and fasting hyperglycemia leading to the development of diabetes\textsuperscript{174,184-186}. Mice engineered to over express HNF1\(\alpha\) also develop a diabetic phenotype\textsuperscript{187} marked by compromised islet morphology and reduced beta cell mass. HNF1\(\alpha\) cellular concentration is also critical for epigenetic maintenance. Studies of HNF1\(\alpha\) null murine tissues demonstrate that loss of HNF1\(\alpha\) leads to modification of chromatin structure and alters gene expression\textsuperscript{75}. 

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MODY is a form of monogenetic diabetes caused by an autosomal dominant mutation in one of several genes. The most prominent form of this disease (>70%) is MODY3, caused by mutations affecting expression of HNF1α\(^{42, 48, 50, 272}\). MODY3 is characterized by adolescent onset of hyperglycemia that progressively worsens with age and most often requires pharmacological treatment. Patients with MODY3 often suffer from diabetic complications.

Defining the etiology of MODY is essential for proper pharmacogenetic treatment of the disease\(^{42-45}\). HNF1α regulates the expression of a plethora of genes involved in GSIS\(^{48}\) and mutations mainly result in defective glucose metabolism and insulin secretion in beta cells\(^{48}\). For this reason, it is not surprising that MODY3 patients respond well to treatment with oral sulfonylureas given that these drugs bind the K\(_{\text{ATP}}\) channel which is downstream of glucose metabolism in the insulin secretion pathway\(^{44}\). Sulfonylurea therapy can close the K\(_{\text{ATP}}\) channel which increases intracellular Ca\(^{2+}\) and stimulates insulin secretion by an ATP independent pathway. Patients who are misdiagnosed with type 1 or type 2 diabetes have successfully transferred to sulfonylurea treatment without deterioration in glycemic control\(^{50, 53, 54}\). Following etiologic identification of MODY3, patients have been transferred from insulin to oral sulfonylurea treatment and show improved glycemic control, reduced risks of hypoglycemia, and delay or prevention of diabetic complications\(^{50, 53, 54}\). However, etiologic identification is essential to alter treatment in MODY patients because sulfonylureas are not effective for all other forms of this disease\(^{42-45}\). Current screening for HNF1α mutations is restricted to the coding sequence and proximal promoter\(^{43, 272}\).
In up to 20% of MODY patients, the etiology of the disease is unknown, making it very difficult to predict the clinical course of the disease or provide the most effective treatment. We have demonstrated that Nkx6.1 is a major regulator of HNF1α and that this regulatory system is unique to beta cells. Our work provides potential novel regulatory elements that should be included when screening for mutations that effect HNF1α expression. Nkx6.1 is uniquely expressed in mature beta cells of the pancreas but not expressed in the liver. This novel regulatory network may provide potential new targets for diagnosis and MODY and possibly implicate a new gene (Nkx6.1) involved in this disease. Identification of novel molecular targets that cause MODY has the potential to greatly improve treatment and the quality of life for a great deal of diabetic patients.
Figure 3-1. Deletion analysis of the HNF1α promoter in 3T3 and βTC3 cells. Cells (3T3 and βTC3) were transfected with various lengths of HNF1α promoter luciferase plasmids (1μg) as indicated. Luciferase values are relative to the full length constructs that were arbitrarily set to 1.0. For transactivation control the shortest construct value for 3T3 and βTC3 cells was set equal. Bracket indicates beta cell specific cis response element. Samples were measured 24 hours following transfection and experiments were repeated independently at least three times. (*** p<0.001)
Figure 3-2. Deletion analysis of the HNF1α promoter in Huh7 cells. Huh7 cells were transfected with various lengths of HNF1α promoter luciferase plasmids (1μg) as indicated. Luciferase values are relative to the full length constructs that were arbitrarily set to 1.0. Samples were measured 24 hours following transfection and experiments were repeated independently at least three times.
Figure 3-3. Luciferase activity of HNF4α binding site mutant HNF1α promoter. βTC3 and Huh7 cells were transfected with 1μg of indicated normal (open box) and mutant HNF4α binding site (filled box) HNF1α promoter constructs. Luciferase value of normal HNF1α promoter construct was arbitrarily set to 1.0. Samples were measured 24 hours following transfection and experiments were repeated independently at least three times.
Figure 3-4. HNF1α promoter screening. Full length (-2772) HNF1α promoter (1μg) was cotransfected with various beta cell specific transcription factor expression plasmids (1μg) in 3T3 cells. Samples were measured 24 hours following transfection and experiments were repeated independently at least three times.
Figure 3-5. Nkx6.1 activation of HNF1α promoter in a concentration dependent manner. Full length (-2772) HNF1α promoter (1μg) was cotransfection in 3T3 cells with increasing amounts of Nkx6.1 expression plasmid as indicated. pcDNA3 was used as a control to equalize DNA quantity used for each transfection. Samples were measured 24 hours following transfection and experiments were repeated independently at least three times.
Figure 3-6. HNF1α promoter mutations. Locations of the Nkx6.1 and HNF4α binding sites are indicated in boxes on the promoter. Sequences below the shaded boxes show the induced mutations.
Figure 3-7. HNF1α promoter species alignment. Mouse and rat (GenBank accession #: X67649) HNF1α promoter sequences surrounding the Nkx6.1 (upper alignment) and HNF4α (lower alignment) binding sites are shown. Vertical lines indicate the close homology between species. The shaded boxes indicate core binding sequences.
Figure 3-8. Activation of mutant HNF1α promoter constructs. Normal (top left panel), mutant HNF4α binding site (top right panel), mutant Nkx6.1 binding site (bottom left panel), or double mutant (bottom right panel) HNF1α promoter constructs (1μg) were cotransfected into 3T3 cells with Nkx6.1 (1μg) or HNF4α (1μg) expression plasmids as indicated. Filled boxes indicate mutation and open boxes indicate normal sequence. pcDNA3 was used as a control and the relative value obtained was arbitrarily set to 1.0. Samples were measured 24 hours following transfection and experiments were repeated independently at least three times.
Figure 3-9. Luciferase activity of Nkx6.1 binding site mutant HNF1α promoter in beta cells. βTC3 cells were transfected with 1μg of indicated normal (open box) and mutant Nkx6.1 binding site (filled box) HNF1α promoter constructs. Luciferase value of normal HNF1α promoter construct was arbitrarily set to 1.0. Samples were measured 24 hours following transfection and experiments were repeated independently at least three times. (*** p<0.001)
Figure 3-10. Nkx6.1 binding to HNF1α promoter by EMSA with INS1 cell lysate. EMSAs were conducted with INS1 cell lysate and biotin labeled HNF1α promoter oligonucleotide (probe) as indicated. Arrows indicate Nkx6.1 binding and supershift. In competition assays, DNA-binding reactions were preincubated with 10-fold (10x) or 100-fold (100x) unlabeled HNF1α oligonucleotide or non specific (NS) oligonucleotide as indicated.
Figure 3-11. Nkx6.1 ChIP assay. Nkx6.1 binding experiments are shown with (+) or without (-) Nkx6.1 over expression in βTC3 cells. Representative images are shown following agarose gel electrophoresis of PCR products. Quantification of bands was done with Microsoft Photoshop quantification tool.
Figure 3-12. HNF4α ChIP assay. HNF4α binding experiments are shown with (+) or without (-) HNF4α over expression in βTC3 cells. Representative images are shown following agarose gel electrophoresis of PCR products. Quantification of bands was done with Microsoft Photoshop quantification tool.
Figure 3-13. Nkx6.1 binding to HNF1α promoter by EMSA with βTC3 cell lysate. EMSAs were conducted with βTC3 cell lysate and biotin labeled HNF1α promoter oligonucleotide (probe) as indicated. Arrows indicate Nkx6.1 binding and supershift. In competition assays, DNA-binding reactions were preincubated with 10-fold (10x) or 100-fold (100x) unlabeled HNF1α oligonucleotide or non specific (NS) oligonucleotide as indicated.
Figure 3-14. Nkx6.1 binding to HNF1α promoter by EMSA with NIT1 cell lysate. EMSAs were conducted with NIT1 cell lysate and biotin labeled HNF1α promoter oligonucleotide (probe) as indicated. Arrows indicate Nkx6.1 binding and supershift. In competition assays, DNA-binding reactions were preincubated with 10-fold (10x) or 100-fold (100x) unlabeled HNF1α oligonucleotide or non specific (NS) oligonucleotide as indicated.
Figure 3-15. RT-PCR for determination of gene expression. Total RNA was isolated from indicated cell lines and expression of pancreatic genes was measured by RT-PCR.
Figure 3-16. Effect of Nkx6.1 over expression on mRNA levels. NIT1 mouse beta cells were transfected with CMV-Nkx6.1 expression plasmid for 48 hours. Total RNA was isolated and subjected to reverse transcription. Target gene expression was quantified by real-time PCR and expressed as fold over control as indicated. Empty vector plasmid (pcDNA3) transfection was used for a negative control for all tested genes, and arbitrarily set to 1.0.
Figure 3-17. Effect of Nkx6.1 knockdown on mRNA levels. NIT1 mouse beta cells were transfected with Nkx6.1 siRNA for 48 hours. Total RNA was isolated and subjected to reverse transcription. Target gene expression was quantified by real-time PCR and expressed as fold over control as indicated. Empty vector plasmid (pcDNA3) transfection was used for a negative control for all tested genes, and arbitrarily set to 1.0.
CHAPTER 4
NOVEL DETECTION OF PANCREATIC AND DUODENAL HOMEBOX 1 (Pdx1) AUTOANTIBODIES (PAA) IN HUMAN SERA USING LUCIFERASE IMMUNOPRECIPITATION SYSTEMS (LIPS) ASSAY

Introduction

Pancreatic and duodenal homeobox 1 (Pdx1) is a key transcription factor for pancreatic development and beta cell maturation and function, and it also plays important roles pancreatic beta cell survival and regeneration\(^{273}\). Pdx1 autoantibodies (PAA) have recently been identified in serum from both non-obese diabetic (NOD) mice and human type 1 diabetes (T1D) patients\(^{254}\). Other autoantibodies in T1D such as insulin autoantibodies (IAA)\(^{274}\), islet cell autoantibodies (ICA)\(^{275}\), glutamic acid decarboxylase (GAD) autoantibodies (GADA)\(^{276}\), and insulinoma 2 (IA-2)–associated autoantibodies (IA-2A)\(^{277}\) are useful markers for diagnosis as well as for predicting disease onset and may have a role for the timing of interventions\(^{6}\). Clinical liquid-phase radioimmunoprecipitation assays (RIPA) for these autoantibodies are available. However, a non-radioactive alternative assay has been developed for detection of GADA\(^{278, 279}\) and IA-2A\(^{279, 280}\) with similar sensitivity and specificity know as the luminescence immunoprecipitation system (LIPS) assay\(^{281, 282}\). Here, we report a LIPS assay for detecting PAA in human sera using a Pdx1-luciferase fusion protein produced in mammalian cells. This new liquid-phase assay provides a non-radioactive means of detecting PAA in human sera.

Materials and Methods

Plasmid Construction

The human Pdx1-renilla luciferase fusion plasmid was constructed by cloning the renilla luciferase gene (from the pRen2 plasmid) upstream of the Pdx1 gene in pCMV-
XL5 (Open Biosystems) using HindIII/BamHI restriction sites for expression in mammalian cells. A stop codon was introduced at the 3’ end of the luciferase gene by site-directed mutagenesis to construct the luciferase-only expression control. Renilla luciferase-GAD65\(^{278}\) and renilla luciferase-IA2\(^{280}\) fusion plasmids were generous gifts from Dr. Peter Burbelo (NIH, Bethesda, MD). All plasmids were purified using the Plasmid Maxi Kit (Qiagen).

**Fusion Protein Lysate**

Mammalian fusion protein lysates were prepared by transfecting human embryonic kidney (293) cells with each plasmid using Lipofectamine 2000 Reagent (Invitrogen) according to manufacturer’s protocol in 10cm\(^2\) culture dishes. Forty-eight hours following transfection, lysates were harvested using 1ml Passive Lysis Buffer (Promega) per dish and supernatant (lysate) was collected following centrifugation. 293 cells were cultured at 37°C in DMEM containing 10% fetal bovine serum and 1% Penicillin/Streptomycin.

**Sera**

Healthy human donor sera (n = 10) with no known history of autoimmune diseases were used to establish the cut-off between positive and negative. Fifty-four serum samples from the University of Florida Pathology Laboratories, Endocrine Autoantibody Laboratory were used to validate the LIPS assay, 29 of which are clinically tested triple-positive for ICA (by indirect immunofluorescence), GADA, and IA-2A (by RIPA). The ICA assay has been validated previously and was the basis for the Diabetes Prevention Trial-1 study\(^{283}\) and is used in the current T1D TrialNet studies\(^{284}\). The GADA and IA-2A assays were manufactured by Kronus, Inc. (Star, Indiana) and validated previously. In addition, four sera from T1D patients awaiting renal transplants (that were previously
identified as PAA positive) were used for determination of PAA antigenic specificity. All sera were measured blindly.

**LIPS Assay**

LIPS assays were performed similarly to the previously published protocols\(^{281, 282}\) with minor modifications. Pdx1-luciferase fusion protein cell lysate (≥ 2 X 10\(^7\) RLU in Berthold Lumat LB9507) was incubated with 10μl human serum for PAA (or 1μl serum for GADA and IA-2A) in 96-well round-bottom plates at a total volume of 100μl in PBS overnight with agitation. Healthy normal donor control sera were used to establish a normal range. Samples were then transferred to 96-well filter plates containing 10μl Immobilized Protein A/G plus Beads (Pierce) of 50% concentration (beads/volume) and incubated at 4°C for 2hrs with agitation. All samples were washed 8 times with Buffer A as previously described\(^{281, 282}\). 20μl PBS was added to each well before reading in a LUMIstar Omega plate reader (BMG Labtech). Competition assays used purified recombinant Pdx1 protein (rPdx1)\(^{285}\) or BSA at indicated concentrations and were incubated with Pdx1-luciferase fusion cell lysate overnight. Due to limited sera volumes, all assays were performed in singlet. We obtained extra sera for negative controls and some strong PAA positives that were used for competition assays to determine antigenic specificity.

**Results**

Because PAA are a recent discovery, there is no standard assay for detecting these antibodies in the clinical setting. To validate the LIPS assay, we first compared our detection of GADA (Fig. 4-1) and IA-2A (Fig. 4-2) by LIPS assay to clinical data previously determined by RIPA. Sera from patients (n = 54) and control subjects (n = 10) were screened for GADA or IA-2A by LIPS assay and a positive cutoff was set at 3
standard deviations (SD) above the mean of control subjects. For GADA, LIPS identified 28 out of 29 (97%) RIPA-positive sera and 6 additional LIPS positive sera that were RIPA-negative. For IA-2A, LIPS identified all 29 (100%) RIPA-positive sera and one additional LIPS positive serum that was RIPA-negative. However, the degree of positivity for individual samples varied greatly between RIPA and LIPS assay without a linear correlation. For example, a particular sample may yield a high positive value by RIPA but a low to moderate value by LIPS assay, or vice versa. Compared to clinical RIPA, LIPS assay shows 97% sensitivity and 76% specificity for GADA detection and 100% sensitivity and 96% specificity for IA-2A detection (Fig. 4-3). However, since the accuracy of the reference assay (RIPA) is unknown, the low specificity could alternatively indicate that the LIPS assay is actually more sensitive than RIPA. Taken together, these results suggest that the LIPS assay is comparable to the clinical RIPA for GADA and IA-2A in our hands.

Next, we tested the above serum samples (54 patients and 10 healthy controls) for PAA by LIPS assay (Fig. 4-4). We arbitrarily set the positive cutoff as the mean +3 SD of 10 healthy control sera and defined any sample above this cutoff as positive for PAA. Using this cutoff, 7 out of 54 sera were positive for PAA (13%). Among the PAA positive samples, 6 out of 29 triple-positive serum samples were PAA positive (21%) and 1 out of 25 triple-negative serum samples was PAA positive (4%). Despite the abundance, PAA did not reach statistical significance in association with triple-positive T1D-related autoantibodies (p = 0.10) according to Fisher’s exact test.

To validate the antigenic specificity of the LIPS assay for PAA, we selected serum samples from T1D patients awaiting renal transplants that previously tested negative,
medium, and high positive for PAA by LIPS assay. To exclude the possibility of antibody binding to luciferase, immunoreactivity with both Pdx1-luciferase antigen and with luciferase antigen alone was tested, demonstrating that the PAA-positive sera bound only to Pdx1-luciferase (Fig. 4-5). We next confirmed Pdx1 antigenic specificity using a competition assay with purified rPdx1 (Fig. 4-6). We selected our highest-PAA positive sample for LIPS assay to detect PAA by incubating serum with various concentrations of rPdx1 or bovine serum albumin (BSA) as control. The PAA signal was reduced with increasing concentrations of rPdx1 but not with BSA, confirming that the PAA signal is specific for Pdx1 protein. BSA non-specifically reduced the signal but was unable to block detection of PAA even at high concentrations (0.125mg/ml).

Discussion

We have developed a liquid-phase, non-radioactive assay to detect PAA in human sera and confirmed the presence of PAA in 7 out of 54 samples. While developing our assay, we first performed LIPS assays to detect GADA (Fig. 4-1) and IA-2A (Fig. 4-2) from samples that had been previously evaluated clinically by RIPA. If RIPA is held as the reference standard, one would conclude that our LIPS assay has nearly identical sensitivity but a reduced specificity for detection of these autoantibodies. This would suggest that we have identified several false positive sera. However, another interpretation of the data is that our assay is more sensitive than RIPA and that these “false positive” samples are actually true positive samples that are undetectable by RIPA. We conclude that the LIPS assay has similar sensitivity to RIPA, and because it does not involve the use of radioisotopes, it is safer and more convenient. Thus, the LIPS assay may be valuable in the clinical setting for detecting autoantibodies.
Insulin is considered to be an early autoantigen in T1D and it has been proposed to be the primary antigen related to T1D\textsuperscript{286-288}. This is based on the early detection of IAA in people later developing T1D and by the fact that insulin is uniquely expressed and secreted by the beta cell whereas other autoantigens are not unique to the beta cell (e.g., GAD or IA-2A). Pdx1 could also be an early autoantigen with respect to T1D development and some evidence suggests that Pdx1 may be a primary autoantigen related to T1D. In our previous report\textsuperscript{254}, we detected PAA in NOD mice by ELISA, western blotting, and RIPA. We also followed the PAA titer of several female NOD mice during the development of diabetes and found that PAA titers tended to peak prior to the onset of hyperglycemia and then dropped to undetectable levels after several weeks. This may be due to a lack of Pdx1 antigen stimulation following destruction of beta cells. To determine whether this is the case for humans will require evaluation of PAA in human T1D patients throughout disease development. Serum from individual patients should be measured for PAA at various time points during disease development, from pre-diabetic to clinical manifestation of diabetes.

According to Fisher’s exact test, PAA were not significantly associated with the presence of triple-positive (ICA, GADA, and IA-2A) T1D-related autoantibodies (p = 0.10). Since our study only examined sera that was triple-positive (or negative) for other T1D-related autoantibodies, and since the p value was relatively low, PAA association with other T1D-related autoantibodies merits further investigation. Future studies should examine whether PAA are significantly associated with sera from patients with only one or two T1D-related autoantibodies and in patients with other T1D-related autoantibodies such as zinc transporter 8 protein (ZnT8) islet autoantibodies.
(ZnT8A)\textsuperscript{289} or IAA\textsuperscript{274}. In addition, T1D patients that are negative for all other known T1D-related autoantibodies could be examined.

Due to our limited resources, several questions remain unanswered regarding PAA. Large scale systematic, longitudinal studies will be required to determine the prevalence of PAA in the normal population, T1D patients, high-risk or prediabetic populations, and in other diseases as well as to identify if there exists any clinical value of PAA for prediction, diagnosis, or monitoring of T1D patients.
Figure 4-1. Detection of GADA in human sera by LIPS assay. Sera (n = 54) that were tested for ICA, GADA, and IA-2A clinically by RIPA or healthy normal donor control sera (n = 10) were used and the transverse line is 3SD above control mean. RLU (relative light units) are expressed as fold-increase over control mean (left y-axis). Standard LIPS assay (1μl serum) was performed with renilla luciferase-GAD65 fusion protein lysate and compared to clinical RIPA data (right y-axis).
Figure 4-2. Detection of IA-2A in human sera by LIPS assay. Sera (n = 54) that were tested for ICA, GADA, and IA-2A clinically by RIPA or healthy normal donor control sera (n = 10) were used and the transverse line is 3SD above control mean. RLU (relative light units) are expressed as fold-increase over control mean (left y-axis). Standard LIPS assay (1μl serum) was performed with renilla luciferase-IA-2 fusion protein lysate and compared to clinical RIPA data (right y-axis).
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Figure 4-3. Sensitivity and specificity of LIPS assay vs. RIPA for GADA and IA-2A. Sera (n = 54) that were tested for ICA, GADA, and IA-2A clinically by RIPA or healthy normal donor control sera (n = 10) were used. Standard LIPS assays (1μl serum) were performed with renilla luciferase-GAD65 fusion protein lysate or renilla luciferase-IA-2 fusion protein lysate. Sensitivity and specificity were calculated using RIPA as a reference assay.
Figure 4-4. Detection of PAA in human sera by LIPS assay. Sera (n = 54) that were tested for ICA, GADA, and IA-2A clinically by RIPA or healthy normal donor control sera (n = 10) were used to detect PAA using a standard LIPS assay (10μl serum) with Pdx1-luciferase fusion protein lysate. The transverse line is 3SD above control mean. RLU (relative light units) are expressed as fold-increase over control mean. AAb = autoantibody.
Figure 4-5. Antigenic specificity of PAA using luciferase only control. Sera (10μl) that were negative- (Ctrl), medium- (A & B), or high-signal positive (C) for PAA (from T1D patients awaiting renal transplants) were used in a standard LIPS assay using either Pdx1-luciferase antigen or luciferase-only antigen for detection.
Figure 4-6. Antigenic specificity of PAA by competition with purified rPdx1 protein. A standard LIPS assay was performed to detect PAA from our highest signal positive serum (10μl) incubated with indicated concentrations of rPdx1 or BSA.
PANCREATIC AND DUODENAL HOMEobox 1 (PDX1) AUTOantibodies (PAA) FROM HUMAN SERA DETECTED IN AUTOIMMUNE DISEASES AND CANCER

Introduction

In our previous report, we developed a liquid-phase, non-radioactive luciferase immunoprecipitation systems (LIPS) assay\textsuperscript{281, 290} for detection of pancreatic and duodenal homeobox 1 (Pdx1) autoantibodies (PAA)\textsuperscript{254} in human sera and we determined that PAA are not significantly associated with the presence of triple-positive T1D autoantibodies (to ICA, GADA, and IA2A). In the present report we have assessed the presence of PAA in patient sera with recent-onset (RO)-T1D and age-matched non-T1D controls, and longstanding (LS)-T1D in order to determine the relationship of PAA with T1D. We also assessed the presence of PAA in patient sera with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and various forms of cancer (including pancreatic cancer) in order to determine if PAA are unique to T1D.

Materials and Methods

The human Pdx1-renilla luciferase fusion plasmid was constructed and purified and mammalian fusion protein lysates were prepared and used for LIPS assay as previously described. All sera were measured blindly and consisted of samples from patients with RO-T1D (n = 100) and age-matched non-T1D controls (n = 100), LS-T1D (N = 50), SLE (n = 48), RA (n = 30), or various forms of cancer (n = 70). Healthy human donor sera (n = 10) with no known history of autoimmune diseases were used as normal controls for setting up the cut off between positive and negative (3SD above control mean).
Results

Using a cutoff of three standard deviations above the mean of norm human control sera, we detected positive PAA sera (Fig. 5-1 and 5-2) as follows: 7% from RO-T1D patients (n = 100) and 13% from age-matched non-T1D controls (n = 100), 20% from LS-T1D patients (N = 50), 48% from SLE patients (n=48), 3% from RA patients (n=30), and 16% from cancer patients (n=70). Sera that produced high positive signal for detection were assayed several times whenever possible (based on serum volume) and consistent results were produced. It is interesting to note that a high PAA positive signal was detectable in a patient with pancreatic cancer (Fig. 5-2, cancer plot).

Discussion

Based on our data, the relationship of PAA to T1D is unclear. Although PAA were found in 7% of RO-T1D patients, we surprisingly found PAA in 13% of age-matched non-T1D controls and also identified PAA in 20% of LS-T1D patients. The high prevalence of PAA in the non-T1D age matched controls is confounding. One possibility for this data is that the age-matched control group may not actually be a normal population, and this is supported by the fact that our control group (used to establish PAA positive cutoff) was completely negative for PAA. Another possibility is that this is an anomaly due to small sample size and larger scale studies may be required to identify the true prevalence of PAA in T1D vs normal patient sera. Finally, PAA could be the result of cross-reactive autoantibodies to another antigen291.

This is the first report of PAA association with diseases other than T1D. We have observed a high prevalence (48%) of PAA in patients with SLE. Since double stranded DNA autoantibodies are common among SLE patients292, and because Pdx1 is a DNA binding protein293, we have assessed whether detection of our PAA positive signal from
SLE patient samples was actually due to double stranded DNA autoantibodies. A standard radioimmunoprecipitation assay was used to assess the presence of double stranded DNA autoantibodies in the SLE patient sera. Double stranded DNA autoantibodies were only detected in PAA negative sera (data not shown) confirming that PAA and double stranded DNA autoantibodies are distinct and have no association.

One of the most intriguing pieces of data we found was that one of the highest signals of detection for PAA was from a pancreatic cancer patient. We hypothesize that the resulting lesion from pancreatic cancer could cause leaking of Pdx1 protein and subsequent presentation to the immune system leading to production of PAA. If this is true, over production of Pdx1 from pancreatic cancer cells could lead to high titer PAA production by B-cells. If PAA is directly associated with pancreatic cancer, detection of PAA could be useful for screening and diagnosing pancreatic cancer which remains one of the most elusive forms of cancer with a poor prognosis and 5 year survival rate less than 5\%\textsuperscript{294}.

PAA are detected in human sera from patients with autoimmune diseases or cancer by LIPS assay, but whether there is an association of PAA with each disease is unknown. Large scale, systematic, and longitudinal studies are still required to determine the clinical value of PAA for prediction, diagnosis, or monitoring of T1D patients or patients with other diseases.
Figure 5-1. PAA detected in human T1D patient sera. Sera from recent-onset (RO)-T1D patients (n = 100) and non-T1D age-matched controls (n = 100), long-standing (LS)-T1D patients (n = 50), or healthy normal donor controls (Ctrl, n = 10) were screened for PAA by standard LIPS assay using mammalian produced Pdx1-luciferase fusion protein lysate. The transverse line is 3SD above control mean. RLU (relative light units) are expressed as fold-increase over control mean.
Figure 5-2. PAA detected in human autoimmune disease and cancer patient sera. Sera from healthy normal donor controls (Ctrl, n = 10) and patients with systemic lupus erythematosus (SLE, n = 48), rheumatoid arthritis (RA, n = 30), and various forms of cancer (Cancer, n = 70) were screened for PAA by standard LIPS assay using mammalian produced Pdx1-luciferase fusion protein lysate. The transverse line is 3SD above control mean. RLU = relative light units and are expressed as fold over control mean.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Before the discovery of insulin by Banting and Best in 1921\textsuperscript{295} T1D was a fatal disease. Insulin replacement therapy is not a cure for T1D because the difficulty of maintaining normal blood glucose concentration causes most T1D patients to have a hyperglycemic condition leading to vascular degradation and subsequent tissue damage and organ failure\textsuperscript{1}. However, in 2012, insulin replacement therapy continues to be the standard treatment for T1D. This provides the rationale for the work in this dissertation, with the ultimate goal of providing adequate control of blood glucose to T1D patients without any harmful side effects. Successful pancreas\textsuperscript{296, 297} and islet transplantations\textsuperscript{8, 298, 299} have occurred but are not suitable for the entire T1D population because they require use of immunosuppressants that cause harmful side effects and they are not permanent cures. For these reasons, investigators have focused on alternative methods for generating beta cells. Success in these attempts may still not be sufficient to provide a cure for T1D. As mentioned before, the pathogenesis of diabetes is two-fold, and surrogate beta cells will face possible destruction from recurrent autoimmunity\textsuperscript{297}. Enhanced knowledge of the mechanisms involved in T1D pathogenesis and beta cell differentiation will be important in developing a cure.

Previous studies aimed at beta cell generation have used many different tissue sources and many different factors to investigate the reprogramming process. We have focused our work on studying the mechanisms involved in reprogramming liver to pancreatic beta cells. We chose this system based on previous reports that demonstrate the extraordinary ability of hepatic cells to transform into insulin producing beta cells. We investigated the reprogramming of liver into beta cells by ectopic
expression of pancreatic beta cell specific transcription factors Pdx1 and Ngn3 because they are fundamental factors that are shown to be essential for beta cell development. Most of the other factors used in similar reprogramming studies are downstream targets of Pdx1 and Ngn3, or expressed during later stages of development\textsuperscript{60,293}. Using these factors allowed us to study the fundamental early events involved in reprogramming liver to beta cells.

Discovering suppression of HNF1α in Huh7 cells, following ectopic expression of Pdx1, was a key finding in our early studies and prompted us to further investigate the mechanism as to how this occurred. Although we cannot rule out the possibility that many mechanisms account for Pdx1-mediated HNF1α suppression, we did discover major changes in the regulation and expression of its major activator HNF4α. Since we saw a shift in HNF4α expression from P1 promoter to P2 promoter use, and since liver and beta cells used primarily P1 and P2 promoter respectively for HNF4α expression\textsuperscript{240}, we believe our data strongly supports this shift in HNF4α promoter usage as an early event in Pdx1 mediated liver to beta cell reprogramming. The fact that Pdx1 is a known regulator of the HNF4α P2 promoter, specifically in beta cells\textsuperscript{236}, further supports this notion.

In order to study this process in greater detail, we cloned approximately 3kb of the proximal mouse HNF1α promoter into a luciferase reporter. Since reprogramming is a major focus of our lab, we have produced or acquired many transcription factor expression plasmids related to beta cells. Having these tools available prompted us to screen our HNF1α-luciferase construct for potential transcriptional activators that are beta cell specific. Another driving factor in this exploration was the fact that, previously,
the longest HNF1α reporter construct (less than 500 bp proximal promoter) was found to be active in hepatic cells but completely inactive in rodent INS1 beta cells\textsuperscript{150}, and this reporter contains the binding site for HNF4α. This single piece of data strongly suggests that HNF1α may require beta cell specific transcription regulators. And indeed, we found a novel activator of HNF1α in beta cells, Nkx6.1, which is not expressed in hepatic cells.

Future studies should investigate the beta cell specific regulation of HNF1α in greater detail. One of the main points left unresolved from our work is the role of HNF4α in the regulation of HNF1α in pancreatic beta cells. The previously reported short HNF1α reporter (with HNF4α binding site) was not active in the INS1 beta cell line\textsuperscript{150}, and we were unable to identify HNF4α binding endogenous HNF1α in our βTC3 cell line\textsuperscript{255}. However, much of our HNF1α-luciferase reporter data obtained from transfections in beta cell lines demonstrates activation by HNF4α, including both liver specific isoforms generated from the P1 promoter and beta cell specific isoforms generated from the P2 promoter. Although all beta cell specific HNF4α isoforms contain the same DNA binding domain as liver specific HNF4α isoforms, their structural differences may allow for interactions with other molecules that prevent them from functioning in a similar manner. The N-terminal domain of liver specific HNF4α P1 isoforms contain exon 1A, whereas the beta cell specific HNF4α P2 isoforms contain exon 1D or exon 1D and exon 1E. These unrelated domains (1A vs 1D) have the potential to interact with other proteins in a cell-type specific manner and form protein complexes that function in gene regulation. This could explain why the short HNF1α reporter was not active in the INS1 beta cell line, even considering that the cell line
expressed the major activator, HNF4α. It is possible that exon 1D is not sufficient alone to recruit the necessary molecules for transcription, while exon 1A may be sufficient. It is possible that protein-DNA complexes (normally present in the endogenous context) are not available to interact with beta cell specific HNF4α isoforms due to the architecture of the short HNF1α luciferase reporter. To further explain, HNF4α isoforms expressed in beta cells may not confer transactivation alone, but may require the participation from proteins or protein complexes that bind the distal regions of the HNF1α promoter. These potential protein-DNA complexes may not be present or functional in hepatic cells but may be required for proper regulation in beta cells.

Another factor to consider is how alternative splicing of HNF4α affects the regulation of target genes, such as HNF1α. The specific mechanisms that govern regulation of alternative splicing of HNF4α are not known, although alternative splicing can lead to three potential C-terminal domains. Both P1 and P2 driven HNF4α isoforms are believed to have the potential to acquire any of the three possible C-terminal domains. Regulation of alternative splicing of HNF4α may have significant consequences for its regulation of target genes because it affects the interactions with other proteins and complexes. Since Pdx1 is a known regulator of the HNF4α P2 promoter in beta cells, it is possible that Pdx1 has influence on the specific mechanisms of alternative splicing, and thus Pdx1 may play a role in the regulation of specific isoform expression of HNF4α.

It is also possible that, in general, the data from luciferase transfection assays is not representative of endogenous regulatory events since the gene architecture and epigenetic regulation contrasts so greatly. The complexity of HNF1α and HNF4α gene
expression will make these studies difficult, but understanding cell specific mechanisms of regulation will certainly enhance our understanding of beta cell function and may provide additional markers useful for following liver to beta cell reprogramming.

The discovery of PAA was very interesting within the context of my work, considering that Pdx1 is the primary transcription factor used during the reprogramming of hepatic cells into beta cells and because Pdx1 expression in hepatic cells leads to suppression of the HNF1α promoter. Our lab initially discovered PAA by accident following our development of a system to produce rPdx1 in *Pichia pastoris*. Pdx1 is a transcription factor that contains a protein transduction domain (PTD) which is a specific amino acid sequence, enriched with positively charged arginine and lysine residues, allowing for its transduction across cellular membranes by lipid raft-mediated macropinocytosis. For this reason, our lab injected NOD mice directly with Pdx1 protein in order to stimulate beta cell expansion and hepatic reprogramming into insulin producing cells to prevent the onset of diabetes. As a control, we generated a mutant non-functional rPdx1 protein with the PTD (as well as DNA binding domain) removed. Although injecting NOD mice with mutant rPdx1 did not stimulate beta cell expansion or hepatic reprogramming, it prevented the onset of diabetes similarly to normal Pdx1 (unpublished data). We hypothesized that this protection was induced by antigen specific immunotherapy following rPdx1 or mutant rPdx1 injection, prompting us to examine the presence of Pdx1 antibodies in the serum of these mice. We also examined the serum of NOD mice for the presence of Pdx1 antibodies before treatment, leading to the discovery of PAA in NOD mice (which spontaneously develop autoimmune diabetes) but not in other strains examined, including NOD-scid, C57/B6,
and BALB/c. It is interesting to note that, in general, the appearance of PAA in NOD mice peaked before the onset of hyperglycemia and then tapered off to very low or undetectable levels by ELISA. In this report, we also discovered the presence of PAA in human serum by western blotting, but were unable to detect human PAA from the same samples by ELISA, which is possibly due to distortion of the PAA specific epitope. For this reason, I developed the liquid-phase luciferase immunoprecipitation systems (LIPS) assay for detection of PAA in human sera.

In regard to our work on PAA, autoantibodies related to T1D are useful markers for diagnosis as well as for predicting disease onset. Although we have demonstrated that PAA are detected in human sera, it is unclear what their relationship is to T1D, if any. While a search of the scientific literature for autoantibodies in T1D patient sera will yield hundreds of results, few studies are able to demonstrate any association with disease and most fail to have any clinical utility. While our follow-up study demonstrated more PAA positive sera from non-T1D patients compared to T1D patients, our initial work did find 6 out of 29 triple-positive serum (ICA, GADA, and IA-2A) samples were PAA positive (21%) and 1 out of 25 triple-negative serum samples was PAA positive (4%). Although this finding is not statistically significant (p = 0.10) according to Fisher’s exact test, the relatively low p-value for a limited sample number merits further investigation of PAA in order to determine their clinical value for prediction, diagnosis, or monitoring of T1D patients or patients with other diseases. Perhaps the most interesting data from this study is the discovery of PAA from a patient with pancreatic cancer (5 year survival rate less than 5%). Pdx1 and pancreatic cancer are colocalized to the pancreas and it is reasonable to hypothesize that the molecular instability within the cancer tissue
could lead to presentation of Pdx1 to the immune system, resulting in PAA production by B cells. Due to the severity of pancreatic cancer, this single discovery merits further investigation to determine if PAA are a marker for pancreatic cancer, as it could lead to earlier detection and improve prognosis for patients.

In conclusion, this work sheds further insight into the molecular mechanisms involved in liver to pancreatic beta cell reprogramming and also presents a system that will be useful for determining the clinical utility of PAA. Future studies will be required to determine and employ other molecular mechanisms involved in the reprogramming process in order to successfully restore beta cell mass for the growing diabetic population.
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

William LePage Donelan was born in Adams, Massachusetts in the United States of America on May 10, 1982. Early in life, he moved to New York and graduated from Saratoga Springs High School in 2000. He attended the Crane School of Music and the State University of New York (SUNY) at Potsdam until 2006 and received a B.A. in music and a B.S. in biology. Will then moved to Gainesville Florida to attend graduate school at the University of Florida College of Medicine. He was accepted to the Interdisciplinary Program in Biomedical Sciences and worked in the laboratory of Dr. Li-Jun Yang until graduating in 2012 with a Ph.D. in medical sciences.