Abstract

Diabetes Mellitus is a pandemic disease that affects 250 million people worldwide, and is characterized by defects in insulin secretion and insulin action, resulting in chronic hyperglycemia. Islet Transplantation has been viewed as a potential curative treatment to replace traditional insulin injections in addressing Type 1 diabetes. However, a shortage in the supply of islets from donors remains a setback in the widespread implementation of this treatment. Pluripotent human embryonic stem cells have been viewed as a potential solution to this cell scarcity given their ability to differentiate into insulin producing beta-cells. Protein Transduction, a novel technology that allows for the insertion of a biomolecule into the cellular cytoplasm through an 11 amino acid Protein Transduction Domain (PTD), is seen as a viable mechanism for initiating cell differentiation. In this study, a novel protocol for recombinant protein purification was developed to transduce a known transcription factor, Paired Box Gene 4 (Pax-4), into pancreatic buds of embryonic Mus musculus in vivo. When compared to controls, our results show that treatment with PTD-Pax-4 induced an increased expression of key genes that regulate pancreatic endoderm maturation. These outcomes suggest that this treatment may be used to increase production of insulin producing cells in vivo.

Diabetes Mellitus is a pandemic disease that affects 250 million people worldwide, a number expected to reach 380 million by the year 2025 (1). Diabetes manifests itself in two major forms, known as Type 1 and Type 2 diabetes. The latter is the most common form of the disease defined by insulin resistance and insulin hyposecretion that inhibits glucose regulation in the body (2). Type 2 diabetes is known to be caused by poor health standards; fortunately healthy diet and exercise have been proven to help reverse the mentioned symptoms in many instances (3). Type 1 diabetes, involves an idiopathic autoimmune response of the body that targets insulin producing beta-cells in the Islets of Langerhans, resulting in chronic hyperglycemia in humans (4).

Management of Type 1 diabetes through insulin injections is the current standard of care. However, the necessity for daily injections and daily glucose monitoring prohibits a normal lifestyle for diabetics (5). Recently, an alternative treatment, islet transplantation, has emerged as a potential curative treatment for diabetes, shown to reverse the symptoms of the disease, and potentially ensure insulin independence for more than 5 years (6). In the past three decades scientists have witnessed tremendous progress in islet transplantation technology, due to inventions such as the Ricordi chamber, which enables the automated isolation of transplantable islets (14). Furthermore, the discovery of new immunosuppressive drug therapies has reduced post-transplantation complications and increased treatment efficacy. Despite these achievements, the literature suggests that still only 10% of islet recipients are able to maintain insulin independence for a period greater than 5 years, warranting the need for additional transfusions and a greater supply of transplantable islets (13).

Given their ability to differentiate into any cell type and propagate infinitely in cell cultures, human embryonic stem cells are an excellent candidate to provide an unrestricted supply of islets. Additionally, recent research has uncovered the major transcription factors involved in the differentiation of a stem cell into an insulin producing beta-cell (7). However, due to the extreme physiological and biological complexity of stem cells, current technology is unable to reproduce, in vitro, the environmental factors necessary to initiate differentiation (16).

This research focuses on a novel approach to induce differentiation through protein transduction, a powerful technology based on the fusion of a protein of interest to a small cationic 11 amino acid PTD. Proteins attached to a PTD can penetrate cell walls and be “dropped off” within the cell cytoplasm, serving as transcription factor promoters within the cell (11). It is believed that one can induce differentiation of a certain cell fate, by introducing the correct set of transcription factors in stem cells. Studies in a diabetic model have demonstrated the ability of PTDs to transduce anti-apoptotic proteins into physiologically stressed islets thus preventing apoptosis (11). Furthermore, many known transcription factors associated with endocrine tissue formation have been transduced into embryonic stem cells resulting in increased endocrine differentiation (8).

The transcription factor Pax-4 is a key gene in endocrine development. This study observes the effects of PTD-mediated Pax-4, a known beta-cell transcription factor, on cell differentiation in vivo. Pax-4 has been shown to be an influential transcription factor in the final stages of beta-cell differentiation (10). This study utilized the Trans Activator of Transcription PTD (TAT), which is derived from the HIV virus and has been shown in previous studies to successfully transduce mammalian cells (9). In this study Pax-4 was purified and injected into embryonic C57/BL6 Mus musculus. This report describes advancements made in the purification of transducible Pax-4 in addition to elucidating its function as an endocrine transcription factor in vivo.

Materials and Methods

Protein Purification

Positive TAT-Pax-4 transformants were cultured in LB broth.
for 24 hours and then induced with β-D-1-thiogalactopyranoside (IPTG). After incubation, the culture was harvested, pelleted, and washed with 10mL of PBS.

Starting and elution buffers at 30mM and 400mM respective imidazole concentrations were prepared in PBS containing 2mM β-Mercaptoethanol. The bacterial sample was incubated with a Protease Inhibitor Cocktail in addition to Lysozyme and Benzonase (Sigma-Aldrich). The treated sample was incubated on ice for 40 minutes. After incubation the sample was sonicated at 49% amplitude for 2 minutes and 55 seconds. Following sonication, the sample was pelleted, and the remaining supernatant containing the protein was injected into an AKTA+ FPLC™ chromatogram using a 6-His Tag affinity column (Amersham/GE Healthcare). The pumps and column were washed with water and ethanol, and then equilibrated with the 5mM imidazole starting buffer. The protein was injected into the affinity column at 0.7ml/min. Four washes were performed before eluting the protein with the 400mM imidazole elution buffer at a speed gradient of 0.5-3.5ml/min. The protein fractions were then passed through a PD-10 desalting column to remove all remaining reagents. Sample concentrations were then determined using electronic electrophoresis.

**Protein Labeling**

Purified TAT-Pax-4 was labeled using the Alexa Fluor 568 Protein Labeling Kit (Invitrogen) as per manufacturer instructions. The labeled protein was then added at a concentration of 10ug/mL to a cell culture of human cord blood cells for 3 hours at 37°C.

**Electric Mobility Shift Assay (EMSA)**

An EMSA was performed using the Pierce LightShift Chemiluminescent EMSA Kit according to manufacturer’s instruction. The Pax-4 Probe Set used in the experiment was provided by Panomics and is commercially available. Control reactions were carried out according to manufacturer recommendations. Reactions containing Pax-4 substrate (1.5ug/ul) were prepared with the provided 10X Binding Buffer and 1ug/ul Poly (Dl*dC).

Western Blot

Protein samples were run on a 10% Lonza SDS-PAGE Gold Tris-Glycine Precast gel (150V, 230mA, 45mins) at different volumes and transferred onto a nitrocellulose membrane. Rabbit Polyclonal IgG Pax-4 antibodies (Santa Cruz Biotechnology) were applied and used at a 1:250 dilution. The membrane was developed using the Fast Western Blot Kit (Pierce Thermo Scientific).

**Protein injection and analysis**

TAT-Pax-4 was injected into two sets of C57/BL6 mice. Embryos were injected at e15.5 with 2.18mg of protein. Two sets of controls were prepared and injected with 10ul of PBS containing 200uM imidazole. The weight of the mice used ranged from 31-35g. The mice were sedated and using an ultrasonic all embryos were located and injected with the protein. Immediately after birth, the pancreases of the neonatal mice were extracted and prepared for RT-PCR.

RNA extraction was done using the mirVANA Kit (Ambion) following the manufacturer's instructions, and cDNA was prepared at a final RNA concentration of 5ng/µl using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI) following the manufacturer’s instructions. Real Time RT-PCR was done on a 7500 Fast Real-Time PCR System using Fast Universal Master Mix and primers from ABI.

**Results**

**HPLC protein purification results in highly pure protein sample**

Protein purification is a very complex procedure and protocols must be optimized individually for every protein. Proteins can be expressed by bacteria in two main ways: in the cytosol as a soluble polypeptide or in particle crystal-like structures known as inclusion bodies (12). Each physiological form requires a different approach in regards to the purification and refolding of the protein. It was discovered that recombinant Pax-4 is located in the cellular cytoplasm and can be extracted without the use of harmful denaturants that are typically used for inclusion bodies. Following the injection of the sample into the column, multiple peaks corresponding to the amount of eluted protein were observed. A wash containing 10% elution buffer was performed eluting non-specifically bound biomaterial. Upon elution, 100% of elution buffer was pumped into the column resulting in a peak exceeding 2000 mAU (milli-absorbance unit) signaling the elution of TAT-Pax-4.

Samples were studied on an automated electrophoresis chip to observe sample purities. Results showed a gradual increase in purity with the most contamination present in wash samples (lanes 1-4). Following the elution of non-specifically bound material, 100% of the elution buffer was pumped into the column eluting a more pure and concentrated sample of TAT-Pax-4. This is evident in the chip reading, as lane 10 displays a single dense band along the 40kD marker that represents Pax-4.

**Immunoblot analysis confirms the identity of the protein**

A Western Blot was performed to confirm the protein’s identity. For this purpose, a specific Pax-4 antibody (see Methods) was utilized. A band near the 40 kD marker would indicate a positive result.

Pax-4 sample was added to four wells at decreasing volumes of
20uL, 15uL, 10uL and 5uL, respectively from left to right. The protein immunoblot results show the presence of a strong band at the 40kD marker, confirming that the purified protein is indeed Pax-4 (Figure 2).

**EMSA assay confirms protein functionality**

To characterize the protein-DNA binding interaction of purified TAT-Pax-4, an EMSA assay was performed. Based on protein interaction with probe DNA sequences, one can determine functionality based on a band shift that indicates a proper reaction on an electrophoresis gel.

In this experiment a band “shift” in lane 5 was observed confirming the successful binding of the Pax-4 Probe to the protein substrate (Figure 3). The sixth lane containing the Cold Pax-4 Probe successfully competed with the target Pax-4 Probe inhibiting the intended reaction. These results confirm functionality of purified Pax-4.

**Protein labeling exhibits transduction capacity**

To address the capacity of the purified TAT-Pax-4 to permeate cellular membranes, the protein was labeled with a fluorescent marker and culture images were acquired after incubating for two hours. The treated cells displayed a strong fluorescence at a wavelength of 568 nm. Almost 100% of the cells demonstrated an uptake of the TAT-Pax-4. This confirms the functionality of the TAT-Pax-4 in permeating cord blood cell membranes (Figure 4).

**Pax-4 treatment increases the expression of pancreatic endoderm maturation genes**

Despite several recently published reports on pancreas development and maturation, the roles of certain transcription factors remain to be fully identified. Pax-4 has been identified as one of the key transcription factors in pancreatic development. However, a detailed study evaluating how the protein will affect the genetic profile of pancreatic development and function genes (e.g. insulin release) has yet to be determined.

To address these questions, an in vivo set of experiments was performed by injecting lab mice with a pure, concentrated sample of recombinant TAT-Pax-4. Control animals were treated with equivalent volumes of an imidazole buffer devoid of protein. A total of 40 genes were studied using RT-PCR. TAT-Pax-4 treated embryonic Mus musculus RNA was compared to buffer injected controls. 18S rRNA was used as an endogenous control for the experiment. The 40 gene array was analyzed and up-regulation and down-regulation of multiple genes was observed. Insulin-1 and Insulin-2 (Ins1&2), two marker genes related to beta-cell differentiation were up-regulated approximately 1.5 fold (Figure 5), although this increase was not statistically significant. Glucose blood-level raising hormones, particularly Glucagon (GCG), displayed an even greater expressional increase, approximately 1.75 fold. Glucose monitoring enzymes such as Glucokinase (GcK) also exhibited a moderate increase in expression. Gata-4 a protein related to endoderm differentiation was also up-regulated, indicating greater genetic expression for pancreatic development (data not shown). Additional genes such as Slc2a2, which has been shown to be directly related to proper insulin secretion and beta-cell plasma membrane development, increased in expression as a result of Pax-4 injection substantially, approximately 8 fold. Pcsk1,
a protein involved in insulin biosynthesis, was also observed to have increased expression.

In spite of these results, three genes considered to be integral transcription factors in beta-cell development, Pdx-1, MafA, and MafB, were down-regulated. Another gene, Arx, known to inhibit Pax-4 function, was up-regulated in injected mice.

Discussion

Protein purification requires repeated trial and error (results discussed and analyzed in this paper constitute only a small portion of a total number of trials) to find the adequate buffer concentrations, pH, and elution rate. The results obtained in this study mark significant progress in the purification of TAT-Pax-4 and culminate with a successful isolation of a relatively pure protein at a workable concentration. FPLC protocols were optimized and all toxic reagents were removed successfully using PD-10 desalting columns. Furthermore all biological and functional assays were performed successfully indicating proper function of both the protein and the TAT-PTD.

Additionally 15 embryos were successfully treated with TAT-Pax-4 and were processed for genetic profiling. The analyzed genes are associated with final maturation and fully differentiated pancreatic tissue. A total of 40 genes were successfully analyzed to determine the effects of Pax-4 on genetic expression in embryonic C57/BL6 Mus musculus. While multiple beta-cell related genes were up-regulated, others were down-regulated. Additionally multiple genes associated with alpha-cell development were up-regulated. A larger n will be necessary to draw meaningful, statistically significant conclusions. Thus the experiments were not definitive and will be repeated for further genetic and functional analysis. Studies of glucose stimulated insulin secretion will be performed to assess the actual pancreatic activity of the embryos in vivo. However, notwithstanding certain irregularities, the data does indicate an increase in pancreatic endocrine tissue maturation as multiple genes associated with cell structure development and islet cell hormones were expressed in all cases.

This paper marks the first experiment in a set of experiments to be performed using Pax-4 as a beta-cell differentiation transcription factor. In the future, the effects of Pax-4 in conjunction with other assisting proteins will be analyzed. Additionally, the effect of protein amount and time of injection will be observed in order to gain a better understanding of the effects of Pax-4 on beta-cell differentiation in C57/BL6 Mus musculus. Finally, although preliminary, these results are very promising. They suggest that a transcription factor can be successfully delivered into embryos and that the treatment leads to up-regulation of several key genes to the maturation of the pancreas. This suggests a window of opportunity to intervene and increase both alpha and beta-cell maturation despite down-regulation of certain genes. The results obtained from this set of experiments shed light on many unanswered questions related to pancreatic development and beta-cell differentiation.

![Fig. 5. RT-PCR results. Pax-4 vs. Control](image-url)
Noam Rosenthal is a sophomore who intends to declare a major in Atmosphere/Energy. In his free time he sings with his a cappella group Mixed Company and gives tours of Stanford to campus visitors. He is also a proud resident of Columbae, where a fellow resident encouraged him to submit his work to SURJ. He is excited to share his findings with the Stanford community and hopes it helps to raise awareness about exciting new diabetes treatments currently in development.