BACHELOR THESIS 1:

EPIGENETIC REPROGRAMMING OF PDX1 GENE FOR THE GENERATION OF INSULIN-PRODUCING β CELLS

Name: Tamara Kotrba

Student ID 0810571026

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Institute: University of California, San Francisco

Department of Urology

Li Laboratory

Supervisor: Long-Cheng LI, M.D., Principal Investigator

Internal Supervisor: Gernot Schabbauer, PhD

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UCSF University of California San Francisco

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3 List of Abbreviations

Abbreviation	Description
AMV	Avian Myeloblastosis virus
APS	Ammoniumpersulfate
ATCC	American Type Culture Collection
BCA	bicinchonic acid
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic acid
DEAE	Diethylaminoethyl-dextran
DM	Diabetes melitus
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
ES	Embryonic stem cells
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehydes 3 phosphate dehydrogenase
HLA complex	Histocompatibility complex
iPSC	Induced pluripotent stem cells
MafA	v-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MMLV	Moloney murine leukemia virus
mRNA	Messenger RNA
MSC	Mesenchymal stem cells
Ngn3	Neurogenin 3
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PDX1	Pancreatic duodenal homeobox 1
PVDF	Polyvinylidine difluoride
RIPA	Radio immunoprecipitation assay
RNA	Ribonucleic acid
RNAa	RNA activation

RNAi	RNA interference
RPMI medium	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
saRNA	Small activating RNA
siRNA	Small interference RNA
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel
TBST	Tris buffered saline Tween-20
TEMED	tetrametylethylendiamine
UCSF	University of California, San Francisco
WHO	World Health Organisation

4 Background-Introduction:

4.1 Epigenetics

Epigenetics is the study of chemical reactions and other factors that influence gene expression and inherit changes in the phenotype, without changing the DNA sequence. Recent advances in cell fate reprogramming has revealed that the epigenome of a committed cell has great plasticity and can be altered by overexpressing certain transcriptional factors or by treatment of the cell with epigenetic modifying agents. This understanding offers tremendous opportunities for the generation of insulin-producing β cells by epigenetically modifying β cell specific transcription factor such PDX1 (pancreatic and duodenal homeobox 1) for cell based therapy of diabetes.

4.2 Diabetes

According to World Health Organization (WHO), diabetes mellitus (DM), which causes about 5 percent of all deaths globally each year, currently afflicts 246 million people worldwide and will affect 380 million by 2025. DM consists of two diseases, type 1 and type 2, with distinct etiology. Type1 diabetes accounts for only about 5-10% of all diabetes cases, usually beginning earlier in life than type 2 diabetes. However, its incidence continues to increase worldwide and it has serious short-term and long-term complications. The disorder has a strong genetic component, inherited mainly through the HLA complex, but the factors that trigger onset of clinical disease remain largely unknown. Complications consist of microvascular and macrovascular diseases, which account for the major morbidity and mortality associated with type 1 diabetes.

Type 1 diabetes is a condition in which pancreatic β-cell destruction usually leads to absolute insulin deficiency. Two forms are identified: type 1A results from a cell-mediated autoimmune attack on β cells, whereas type 1B is far less frequent, has no known cause, and occurs mostly in individuals of Asian or African descent, who have varying degrees of insulin deficiency between sporadic episodes of ketoacidosis.(Daneman 2006; Wagner, Lewis et al. 2010)

In type 2 diabetes the body continues to produce insulin, although insulin

production by the body may significantly decrease over time. Therefore the insulin secreted by the β cells is either not enough or it is not recognized as insulin and not used properly. Type 2 diabetes is the leading cause of diabetes; related complications are blindness, non traumatic amputations and chronic kidney failure. (http://diabetes.webmd.com/guide/understanding-diabetes-basics?page=2 5.10.2010).

Current treatments for diabetes type 1 such as insulin injections as well as islet transplantation are clinically unsatisfactory. In order to develop efficient cure for diabetes, a way to reverse the autoimmunity and β cell destruction must be found. Therefore an efficient strategy for generating new β cell has to be found. (Wagner, Lewis et al. 2010)

4.3 Cell based treatment for type 1 diabetes

Several experimental attempts to generate new β cells have been developed, such as reprogramming of non β cells to β cells using gene therapy in vivo or reprogramming of isolated non β cells to β cells in vitro. One approach is to generate β cells directly through differentiation/ transdifferentiation of pancreatic cells. Because pancreatic cells are either β cell precursor or developmentally related to β cells, the barrier for reprogramming into functional β cells might be lower than in other cell types. Pancreatic exocrine cell are a major cell type in the pancreas and also derived from PDX1 positive progenitors, therefore they could be reprogrammed to β cells. Under appropriate conditions, exocrine cells can be induced to assume phenotypic characteristics of β cells. It was recently shown, that β cells can be affected *in vivo* by transferring the genes for PDX1, Ngn3 and MafA into exocrine cells. Results confirmed the production of insulin in diabetic mice, but a concern about this is that there is a high risk of pancreatitis caused by direct injections of adenovirus particles into the pancreatic tissue.(Zhou, Brown et al. 2008; Wagner, Lewis et al. 2010)

Furthermore, α cells, which produce glucagon, can result in β cells in vivo, which were done by several researchers. The reprogramming of α cells to β cells using a transgenic animal approach shows currently some problems. Some mice

developed diabetes again after some weeks of treatment. (Collombat, Xu et al. 2009; Wagner, Lewis et al. 2010)

β cells have been shown to regenerate during normal growth, which has been explored as an option to obtain more β cells to rescue diabetes. Several studies already focused on determining whether β cells can regenerate themselves and at what rate. Unfortunately the regeneration rate is very slow and relatively rare. Maybe once we have better understanding of the important players in the regeneration process, regeneration can be a future option for treating diabetes.(Teta, Long et al. 2005; Teta, Rankin et al. 2007)

Furthermore, generation of β cells from embryonic and induced pluripotent stem (iPS) cells has been tested. One potential cell source which has been used is mesenchymal stem cells (MSC). It has been shown that this cell population can differentiate into adipocytes, chondrocytes, osteocytes both *in vitro* and *in vivo* as well as into insulin producing cells *in vitro*. (Vija, Farge et al. 2009; Wagner, Lewis et al. 2010) An alternative is to direct differentiation of embryonic stem cells (ESCs) or iPSC cells. A major benefit of using ESCs or iPSCs to generate β islet cells is the unique ability to divide indefinitely in culture while maintaining an undifferentiated fate in self renewal. In 2007 Jiang et al. showed the generation of insulin producing islet cluster from human embryonic stem cells by a 4 step culturing in RPMI containing glucose and epidermal growth factors. In stage 4 nicotinamide and insulin like growth factors were added to trigger cell cluster formation and maturation. (Jiang, Au et al. 2007). There were also several approaches to use iPSC to develop insulin producing β cells. In the future generation of iPSCs cells *in vitro* will be favored over using ESC, because iPSCs cells are patient specific, thereby minimizing the risk of immune rejection. In addition it does not present technical limitations that ESC fusion does. And as a major benefit no oocytes or the destruction of embryos are required. (Wagner, Lewis et al. 2010)

At last the transdetermination of liver progenitor cells into β cells is discussed. Liver and pancreas both originate from the gut endoderm and share common progenitors. In addition liver cells have natural regeneration capabilities and are an

accessible target for gene therapy. The liver was first targeted with first generation adenovirus and helper dependent adenovirus to deliver the PDX1 gene into liver cells of diabetic mice. The mice showed insulin production, but the viral vectors had a high toxicity and the mice developed hepatitis.(Kojima, Fujimiya et al. 2003; Wagner, Lewis et al. 2010)

Furthermore, transdifferentiation of liver cells into β cells *in vitro* has been shown by plasmid or recombinant adenovirus mediated over expression of PDX1 gene. (Li, Horb et al. 2005; Sapir, Shternhall et al. 2005)

4.4 Problems with viral vectors

As mentioned before several problems occurred in mice using viral vectors. Some developed, due to direct injection into the mouse tissue, pancreatitis or hepatitis. Furthermore, an immune response to the virus could occur, and might cause severe complications for the patients. In 1999 it was reported that a young man died after a clinical trial gene therapy, were he was injected with a adenoviral vector, because he developed a very strong immune response. This strong immune response led to organ failure and brain death. (Beardsley 2000) Another serious and sometimes detrimental side effect associated with viral vectors is insertional mutagenesis resulted from viral integration into the host genome.

Therefore another safer mechanism for gene therapy has to be established. A possible one is the usage of RNA activation.

4.5 RNAa

RNAa is a mechanism of gene induction triggered by synthetic short dsRNAs targeting promoter regions. RNAa represents a method for gene over expression and offers opposite effects as RNA interference (RNAi), which degrades complementary mRNA sequences or inhibits translation. By activating the expression of a particular target gene, RNAa induces predictable changes in phenotype and affects downstream gene expression in response to target gene induction. Like RNAi, RNAa could manipulate gene expression to alter cellular pathways and change the cell physiology. According to these benefits RNAa offers a new therapeutic application for diseases that can be treated by stimulating the

expression of a particular gene. (Li, Okino et al. 2006; Janowski, Younger et al. 2007; Place, Li et al. 2008; Place, Noonan et al. 2010)
RNAa has very unique kinetics. Effects of RNAa following transfection of cells with saRNA do not emerge for about 48 hours, but it lasts for 10-15 days following a single transfection. Such prolonged gene activation by RNAa has been attributed to epigenetic changes caused by promoter targeting with saRNA. (Li, Okino et al. 2006; Li 2008) This feature of RNAa makes it well suited for cell fate reprogramming.

A model for the RNAa mechanism in mammalian cells was described by Li in 2008 and is shown in figure 1. Exogenously introduced or naturally occurring dsRNAs form a complex with Ago proteins, where the passenger strand is cleaved and discarded. The RNA programmed complex is then transported to chromosomal DNA either through active transport in the nucleus or gains access during mitosis when the nuclear envelope disappears. After binding to target DNA, the RNA-Ago complex then acts as a reaction platform for recruiting histone-modifying activities such as histone demethylase as well as histone aceltyltransferases (HATs), which further results in a transcriptionally active chromatin. Another possibility is that the RNA-Ago protein complex is tied to a nascent transcript from the target promoter through base pairing and afterwards attracts active chromatin-modifying activities, which further lead to enhanced transcription.(Li 2008)

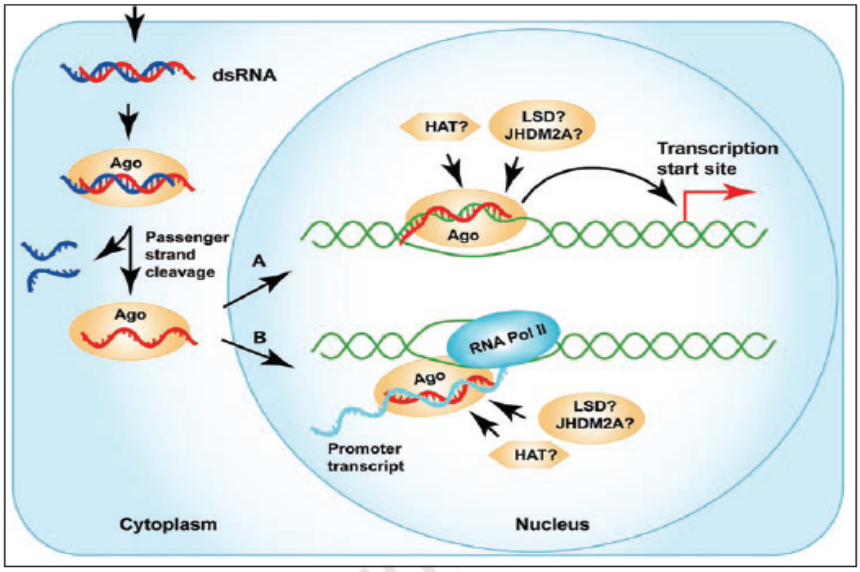

Figure 1: A proposed model for RNAa by Li 2008. (Li 2008) Exogenously introduced or naturally occurring dsRNA forms a complex with Ago proteins, where the passenger strand is cleaved off. The RNA–Ago protein complex can then be transported to DNA either by active transport or passive diffusion during mitosis when the nuclear envelope disappears. Then the complex binds to target DNA and attracts histone modifying activities. Another possible way is that the complex is tied through base pairing to a nascent transcript transcribed from the target promoter and afterwards attracts chromatin-modifying activities to enhance transcription.

5 Aims

5.1 Transdifferentiation of liver cells to insulin-producing β cells by RNAa-mediated PDX1 over expression

Expression of PDX1 gene will be induced in liver cells by RNAa. Such PDX1 programmed cells will be evaluated for β cell properties.

5.2 Differentiate ES and iPS cells to β cells by RNAa-mediated PDX1 overexpression

ES and iPS cells will be used to differentiate into functional β cells by RNAa-mediated PDX1 activation.

5.3 Treatment of a diabetes animal model with RNAa reprogrammed β cells.

Furthermore, after positive results from the first two aims, the reprogrammed β cells will be used to treat diabetes in an animal model.

6 Method

This section deals with the scientific methods that were used to transdifferentiate liver cells to β-cells and to confirm β-cell activity and pancreatic proteins in the transdifferentiated cells.

6.1 Cells and *in vitro* cell culture

The human hepatoma cell line HepG2 was used for the first transdifferentiation experiments. This cell line was obtained from a 15 years old adolescent male Caucasian. Furthermore, the hepatoma cell line has a modal chromosome number of 55 with a rearranged chromosome 1, is epithelial in morphology, and not tumorigenic in nude mice. (ATCC website, 11.10.2010)

HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium H-21, obtained from UCSF cell culture facility, containing 4,5g glucose/l, 10% Foetal Bovine Serum and 1% penicillin-streptomycin solution, all additional solutions for cell culture were also purchased from the UCSF cell culture facility). The cells were kept in a humidified atmosphere of 5% CO_2/95% air at 37ºC.

6.2 Introduction of saRNA into cells by liposome based transfection

Transfection is the delivery of foreign nucleic acid molecules, DNA or RNA, into mammalian cells by nonviral methods. In addition, transfection is a powerful tool to study gene function and protein expression in the context of a cell, by using various chemical, lipid or physical methods.

Transfection is a method that neutralizes or obviates the issue of introducing negatively charged molecules into cells with a negatively charged membrane. (Promega, Transfection Guide, 11.10.2010) Transfection can be classified in different ways. There is stable and transient transfection as well as reverse and forward transfection. The used liposome based transfection method is a chemical method. Therefore, a number of boundaries to deliver nucleic acids into the nucleus are overcome, such as the cell membrane, which is hydrophobic and negatively charged. DNA is often deposited in the cytoplasm and needs to be

transported to the nucleus via an intrinsic transport pathway. In contrast the complexes can be taken up by endocytosis, transported to an acidic endosome, deposited in lysosomes and degraded. For transfection with RNA the boundaries to overcome is the plasma membrane, and for RNA delivered via the endosomal pathway, escape to the cytoplasm. The final barrier is dissociation from the complex once within the cell, because only free DNA and RNA is able to interact with the host genome and can activate expression or interaction. (Richard M. Twyman 2005)

6.2.1 Lipofection

This method is widely used method, especially for gene therapy. The used liposomes are hydrophobic, unilaminar phospholipid vesicles into which the nucleic acid is packed to form a fusogenic particle. These vesicles are able to fuse with the cell membrane and deliver the nucleic acid directly into the cytoplasm. To prepare the lipid-nucleic acid the nucleic acid is mixed with the lipid preparation in a serum-free medium, and then added to the cells, which facilitates rapid and efficient DNA uptake and is therefore applicable to constructs of different sizes. Lipofection is highly efficient and reproducible for both transient and stable transfection. Moreover, it allows up to 90% off cells to be transiently transfected, and demonstrates stable transfection efficiencies up to 20-fold greater than standard chemical transfection procedures. It is very difficult to prepare the lipids in the laboratory; therefore they need to be purchased from a commercial source, which makes this procedure expensive.

Common structural principles in the most efficient cationic lipid-based transfection reagents include a positively charged head group, a linker and a hydrophobic anchor. In addition, the head group generally contains between one and four amine groups, which are joined either via a glycol linkage to an aliphatic hydrocarbon chain anchor, or through a variety of linkages to a cholesterol anchor. (Richard M. Twyman 2005)

6.2.1.1 One of the most efficient reagents is Lipofectamine® RNAimax

This reagent is used for RNAi transfection. RNAi can be used to turn gene expression off, or in case of RNAa on, or knock it down, to better understand the

function and role in a disease and to find treatments. Lipofectamine® RNAimax transfection reagent is a proprietary, siRNA-specific cationic lipid formulation that offer a high efficiency. The procedure for using this reagent consist of mixing it with siRNA, adding cells, incubating and then isolating of the needed product. (Lifetechnologies: ambion/invitrogen; 2010)

6.2.2 Protocol

The protocol for transfecting saRNA into cells is basically the same for RNA and Protein collection after transfection. The only difference is that for RNA collection the cells are transfected in 6-well plates and for protein in 60-mm dishes.

First 37 µl of Lipofectamine® RNAimax (Invitrogen, Carlsbad, CA) are mixed with 1850 µl of Opti-MEM (Invitrogen), a serum free medium, and left for 5 minutes. In between the samples are mixed according to following table

Table 1: Sample preparation example

code	Treatment	1x		4,5x	
		RNA	Opti-MEM	RNA	Opti-MEM
W7	MOCK				
W8	dsCONTROL	3 µl	100 µl	27 µl	450 µl
W9,W10	dsPDX-527	3 µl	100 µl	27 µl	450 µl
W11,W12	dsPDX-539	3 µl	100 µl	27 µl	450 µl

The samples are then combined 1:1 with the Lipofectamine® RNAimax/Opti-MEM mixture and left for 10-20 minutes. Meanwhile the medium of the cells is aspired, and cells are washed with PBS. Cells are then treated with trypsin. When the cells have detached 5 x 10^5 cells are seeded into the wells or dishes. 200 µl sample treatment/ ml of cell suspension are then added. The cells are then incubated at 37ºC in a humidified atmosphere containing 5% CO_2/95% air.

In order to obtain a higher activation rate and a higher protein yield sequential transfection was done. Therefore cells were transfected according to the described protocol and then incubated at 37ºC in a humidified atmosphere containing 5% CO_2/95% air for 3 days, on day 3 after the first transfection, the cells were

transfected a second time according to the above mentioned protocol. Then the cells were incubated at 37ºC in a humidified atmosphere containing 5% CO_2/95% air for 4 more days.

With this cell culture methods, the PDX1 promoter should be activated and the liver cells transdifferentiated into β-cells. In order to investigate transdifferentiation pancreatic markers and proteins, insulin, glucagon, amylase, somatostatin and pancreatic polypeptides need to be investigated. The main focus on this section is how to prove the pancreatic markers insulin and pdx1 in the transdifferentiated cells.

6.3 RNA isolation for mRNA expression analysis

RNA isolation is the first and often most critical step in performing molecular biology experiments High quality and intact RNA should be obtained for further experiments such as RT-PCR.

Different Kits are available for purifying and isolating RNA from cells.

RNeasy® Mini Kit, Quiagen
For purification of total RNA from animal cells the RNeasy® Mini Kit from Qiagen was used.
This Kit provides a silica based membrane with selective binding properties. Furthermore the speed of microspin technology is used. To optimize the binding capacitiy the system provides a specialized high-salt buffer system. Biological samples, such as animal cells, are first lysed and homogenized in the presence of a highly denaturating guanidine thiocyanate containing buffer. This buffer inactivates RNases to ensure purification of intact RNA. Next Ethanol is added, which supports the binding conditions. In the next step the sample is applied to the spin column and the RNA binds to the membrane in the column. The contaminants are not able to bind and get washed away. In the final step RNA is eluted in water.

6.3.1 Protocol

As RNA is collected from a cell line rich in RNases β-mercaptoethanol is added to the provided buffer RLT.

The medium is sucked of the cells, and 350 µl of RLT buffer containing 3.5 µl β-mercaptoethanol/ ml are added. Afterwards, the lysate is directly pipetted into a QIAshredder spin column and spinned for 2 minutes at full speed. Then 350 µl of 70% Ethanol are added to the lysate. Up to 700µl of the lysate are transferred to a RNeasy® spin column. The column is spinned for 15 sec. at 10000 rpm. Afterwards the flow through is discarded. 350 µl of buffer RW1 are applied to the spin column and centrifuged for 15 sec at 10000rpm. The flow through is again discarded. Next 10 µl of Dnase 1 stock solution are mixed with 70 µl Buffer RDD. Then 80 µl of the Dnase mix are added directly to the membrane of the spin column and placed at 20-30ºC for 15min. Afterwards 350 µl of RW1 are added and the column is again centrifuged for 15 sec at 10000 rpm. 500 µl of Buffer RPE are added and the column is centrifuged at 10000 rpm for 15 sec. In the next step again 500 µl of Buffer RPE are added and the column is centrifuged at 10000 rpm for 2 minutes. After discarding the flowthrough the column is spinned again at full speed for 1 minute to prevent carryover of the buffers. In the final step 50 µ of RNase free water are added to the column, which is now placed in a 1,5 ml collection tube, and centrifuged for 1 minute at 10000 rpm. (Qiagen, 2010)

6.4 mRNA expression analysis by RT-PCR

Reverse transcription Polymerase chain reaction is a two step procedure. In the first step the obtained RNA is transcribed to cDNA. The second Polymerase chain reaction step, where cDNA gets duplicated, can be either set up as a qualitative or quantitative-real time assay.

6.4.1 Reverse transcription

Because RNA is unstable and difficult to work with, it can be converted into complementary DNA.

Reverse transcription describes the synthesis of cDNA from mRNA. The used

reverse transcriptase enzymes is a RNA directed DNA polymerase produced and used by several RNA viruses to complete their life cycle with in a host. 2 reverse transcriptases from bird and mouse are the most commonly used: avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (MMLV). In addition DNA primers are required such as oligo dT primers, short random primers, used when not only mRNA but also rRNA should be transcribed or specific primers. Because mRNA has a poly (A) tail at it 3'end, an ideal primer for reverse transcription may be poly (T) oligonucleotide or short oligo dT.
The reverse transcription enzyme starts transcription at the 3'end.

An initial 70ºC heating is to remove secondary structures from the RNA, followed by the 42ºC incubation, which is the working temperature for the enzyme. Afterwards a 90-95ºC step is needed to inactivate the enzyme.
The resulting cDNA is used as a template for PCR and allows the study of genes expressed. (Dwight 2008)

6.4.2 PCR- Polymerase chain reaction

PCR is a fast, powerful and inexpensive DNA amplification technique. For PCR following components are required, a thermostable DNA polymerase, such as Taq polymerase, named after the thermophilic bacterium Thermus aquaticus from which it was isolated. In addition, two oligonucleotide primer, four deoxynucleotide triphosphates (adenosine triphosphate, guanosine triphosphate, thymine triphosphate and cytosine triphosphate), as well as a magnesium buffer are required. Amplification of DNA is achieved by repeating a three step cycle over and over. These steps are denaturation, annealing and extension. In the denaturation step the target double-stranded DNA is heated to a high temperature, usually 95^0C, in order to break the hydrogen bonds between nucleotide bases and opposite strands, leading to splitting into two single strands that are complementary to each other. The next step is the annealing step, where the reaction mix is cooled to 55-65^0C, allowing the oligonucleotide primers to bind to the specific target sequence on the single stranded DNA. The third and last step is called extension step. The temperature is raised to 72^0C, the working optimum of the Taq polymerase. Then the Taq polymerase recognizes the partially double

stranded DNAs and uses the forward and reverse primers as initiation points to begin extending the primers via polymerization in a 5'→3' direction along the two nascent strands, which is obtained by selecting high-energy deoxynucleotide triphosphates from the solution and placing them in the nascent strands directly across from their complementary base in the template strand. Both the genomic DNA and new amplicons can serve as templates in next PCR cycles. With subsequent cycles, the longer double stranded DNA PCR products are diluted out by the more numerous shorter PCR products. Because the primer recognizes only the target DNA they are designed for only a specific segment of DNA is amplified, even if it makes up only a fraction of all the different DNA sequences in the reaction solution.

The reaction is done in a thermocycler. A typical program starts with 5-10 minutes denaturation at 95^0C, followed by 30-50 cycles of denaturation, annealing and extension, and is concluded with a 5-10 minutes final polymerization step at 72^0C to ensure that nearly all amplicons are extended o their full length. (Dwight 2008)

6.4.2.1 Quantitative Polymerase Chain Reaction

Quantitative or real-time PCR assess quantitatively the level of RNA or DNA in a given sample. The detection and quantization of very small amounts of DNA, cDNA or RNA is achieved by fluorescence produced by a reporter probe. Therefore only during the exponential phase of the conventional PCR reaction, is it possible to extrapolate back in order to determine the quantity of initial template sequence. During the process a constant monitoring of fluorescence is necessary. There are two ways for detection, either by dyes that bind to the double stranded DNA or sequence specific probes.

One method for detection is the usage of double stranded DNA intercalating dyes. One of these is SYBR Green, which is simple and cost-efficient. SYBR Green only produces a strong fluorescence signal when intercalated in double stranded DNA. The intensity of the fluorescence signal is therefore dependent on the quantity of dsDNA present in the reaction. The Fluorescence signal of the intercalated SYBR Green is measured during the elongation step of each cycle. Advantages of using SYBR Green are that it is relatively inexpensive and can be used with any pair of

primer dimers for any target. A major disadvantage is that it binds to all dsDNAs non specifically, also to other products such as primer dimers formed during the reaction. To overcome this disadvantage a melting curve is generated and the generated peak is compared with the melting temperature of the amplicon. If there are more or brother peaks, not only the target amplicon was detected but also other side products such as primer dimers.
On the other hand there are hydrophobic probes, which require specific forward and reverse primers and a specific probe for usage. The oligonucleotide probe has a covalently bonded fluorescent reporter dye and a quencher dye. If the probe is intact, the proximity of the reporter and quencher dyes permits fluorescence resonance energy transfer (FRET), and no fluorescence is emitted. During amplification, the probe anneals to the target sequence and is cleaved by Taq polymerase, which allows an increase in fluorescence due to the separation of reporter and quencher dye. The fluorescence produced is proportional to the amplicon created in the reaction. This chemistry is the most widely used, because of its high specificity. (Patel, Arya and Shergill,2007)

6.4.3 Protocols

6.4.3.1 Reverse transcription

The gained RNA is diluted to a concentration of 100 ng/µl. 1 µl Oligo dT primers are added to 10 µl of RNA. The mixture is incubated for 10minutes at 70ºC. Afterwards the samples are immediately put on ice and cooled for 2 minutes. Then the Reverse transcription mixture is prepared, containing 5 µl RT Buffer, 1,25 µl 10 mM dNTP, 0,5 µl RNase inhibitor, 1 µl RT-MMLV, and 6,25 µl nuclease free water per 25 µl. 14 µl of this mixture are then added to the samples, shortly mixed and spinned down. Then the samples are incubated at 42ºC for 1hour and at 75ºC for 15 minutes, to inactivate the enzyme. Afterwards the samples are diluted with 75 µl nuclease free water and stored at -20ºC.

6.4.3.2 Regular PCR

First the Mastermix is prepared, containing 6,9 µl nuclease free water, 1 µl buffer, 0,2 µl dNTP, 0,2 µl forward primer (for PDX1, concentration 10 µM), 0,2 µl reverse

primer(for PDX1, concentration 10 µM) and 0,5 µl red Taq polymerase, per 9 µl of mixture. 16 µl of the master mix and 4 µl of cDNA are added to a 0,2 ml thermostable reaction tube, mixed and transferred to a thermocycler. In the same way, but with different primers a second PCR with the housekeeping gene GAPDH, for control purposes, is done.
Cycle conditions

PDX1

95ºC 2 min – preheating lid
95ºC 30 sec – denaturation step
60ºC 30 sec – annealing step
72ºC 30 sec – extension step
72ºC 10 min – final polymerization step

35 cycles

GAPDH
95ºC 2 min – preheating lid
95ºC 30 sec – denaturation step
60ºC 30 sec – annealing step
72ºC 30 sec – extension step
72ºC 10 min – final polymerization step

For 27 cycles

The obtained PCR product is loaded onto a 1,5% agarose gel and visualized on the Geldoc apparatus.

6.4.3.3 Quantitative PCR

The reaction is set up in a 96 well plate, where each sample should have at least 3 repeats.
5 µl SYBR green mix (AB Applied Biosystems), 2 µl of forward and reverse Primer mixture with a concentration of 1,67 µM, 2 µl of cDNA and 1 µl of nuclease free water are added to each well, for the target PDX1 and for GAPDH, which is used

for normalization. The plate is then transferred to the centrifuge for 3 minutes at 1000 rpm. Afterwards the plate is put into the apparatus containing a thermocycler and spectrometer.

Reaction conditions:

50°C 2 min

95°C 20 sec

95°C 3 sec

60°C 30 sec 40 cycles

Disassociation step:

95°C 15 sec

60°C 1 min

95°C 15 sec

60°C 15 sec

6.5 Protein lysis

In order to isolate protein cells need to be lysed. Then the protein content is freed and brought into the buffer solution.

6.5.1 Protocol

The media is sucked of and the cells are washed with cold PBS. Then 1 ml PBS is added to the cells. A scraper is used to detach the cells from the dish surface. The PBS/cell mixture is then transferred to a 1,5 ml Eppendorf tube and centrifuged for 5 minutes at 1000 rpm in the cold room. Meanwhile 980 µl RIPA buffer, 10 µl phosphatase inhibitor and 10 µl of protease inhibitor are mixed. The PBS is sucked of carefully and 70 µl of the prepared mix are added to the pellet. The samples are then mixed with a vortex for 2 minutes. Afterwards they are kept on ice for 15 minutes. Finally they are spinned down for 15 minutes at 3500 rpm.

6.6 Protein concentration assay

In order to prepare the samples for further analysis with Western Blotting, the concentration has to be measured. There are different systems available on the market.

6.6.1 BCA protein assay

The assay is based on the reduction of Cu^{2+} to Cu^{+} by the protein in an alkaline medium. In the first step the copper is in chelating with the protein in an alkaline environment to form a light blue complex. In the second step bicinchonic acid (BCA) reacts with the reduced Cu^{+} and results in an intense purple color, which exhibit a strong linear absorbance with increasing protein concentration. (Thermoscientific, 15.11.2010)

6.6.2 Protocol

First a BSA Protein standard is prepared according to the following table 2.

Table 2: BSA protein standard preparation table

Concentration[mg/ml]	ddH2O[µl]	BSA (2mg/ml) [µl]
0	20	0
0,2	18	2
0,4	16	4
0,6	14	6
0,8	12	8
1	10	10
1,2	8	12

Furthermore the samples are diluted 1:10. 2 µl of sample are mixed with 18 µl of water. The two solutions of the BCA assay are prepared in a dilution of 1:50. Therefore 5 ml of solution A are mixed with 100 µl of solution B. Afterwards 500 µl

of the BCA reagent mix is added to the before prepared standards and samples. 200 µl of each sample are plated in duplicates in a 96 well plate. After incubating at 37ºC for 30 minutes the plate is read at 492 nm.

6.7 Protein expression analysis by Western blotting assay

Western Blotting or Immunoblotting is a method for identifying antigens. The antigens are allowed to adhere to cellulose sheets and are identified by staining with labeled antibodies. Western blotting allows the transfer of proteins from a sodium dodecyl sulfate (SDS) polyacrylamide gel to an adsorbent membrane. Furthermore, Western offers specific advantages, the wet membranes are pliable and easy to handle. The proteins immobilized on the membrane are readily and equally accessible to different ligands. In addition, only small amount of reagents are required for transfer analysis, multiple replicates of a gel are possible, prolonged storage of transferred patterns, as well as the same protein transfer can be used for multiple successive analysis.

The transfer efficiency of the protein from the gel to a solid membrane depends on the nature of the gel, the molecular mass of the proteins and the used membrane. Therefore, the transfer is more complete and faster when thinner gels are used; whereas the thickness is limited to 0.4mm, due to handling problems.

6.7.1 Membranes

Different types of membranes can be used for blotting and binding protein, such as nitrocellulose, PVDF (polyvinylidine difluoride), activated paper or activated nylon. Among these membranes Nitrocellulose is the most commonly used, even though it has several disadvantages. The proteins are not covalently bound to the membrane, and Nitrocellulose becomes brittle when dry. In addition, small proteins tend to move through the membranes and only a small fraction of the total amount actually binds.

PDVF membranes have a high protein binding capacity, physical strength and chemical stability. Furthermore, proteins blotted onto PVDF membranes can be used for various purposes. In contrast to Nitrocellulose, PVDF membranes can also me stained with Coomassie brilliant blue.

Activated paper binds proteins covalently, but is incompatible with many gel electrophoresis systems, because the linkage is obtained via primary amines. In addition activated paper is expensive, and the reactive groups have a limited half life.
Nylon has excellent mechanical strength, but is only able to bind a small amount of protein and is not suitable for most applications. This problem can be solved by using activated nylon, which on the other hand has a higher non-specific binding.

6.7.2 Transfer technique

There are 2 different techniques to transfer proteins from native or sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) to membranes: simple diffusion, vacuum-assisted solvent flow and Western blotting or electrophoretic elution.

6.7.2.1 Electroblotting

Electroblotting is the most commonly used procedure, because the speed and completeness of the transfer are great advantages. There are two ways for electroblotting, electroelution can be either achieved by complete immersion of a gel membrane sandwich in a buffer tank, called wet blotting or by placing the gel-membrane sandwich between absorbent paper soaked in transfer buffer called semi-dry blotting. (Kurien and Scofield 2006)

6.7.3 Blocking

Further interaction between the membrane and the proteins and non-specific binding must be prevented, therefore the membrane needs to be blocked with nonspecific protein such as non-fat dry milk or BSA Bovine Serum Albumin. The non specific protein binds to the membrane, and the primary antibody just binds to the target protein specifically. This also prevents false positive results.

6.7.4 Detection

The membrane is probed with specific antibodies for target proteins. Detection is a multistep process. First a specific primary antibody is added. The cooler incubation temperature the more specific binding is obtained. After removing the unbound primary antibody, secondary antibody is added, which binds to the primary antibody. There are several methods for detecting proteins, following the addition

of primary antibody, most commonly radioactive, enzyme-linked or chemiluminescent reagents are used. (Kurien and Scofield 2006)

6.7.4.1 Chemiluminescent detection

The method proved inherent sensitivity and selectivity, and is used for detection of protein bands on X-ray film similar to radiolabeled probes. After the binding of the secondary antibody conjugated with either alkaline phosphatase or horseradish peroxidase the substrates are added. Therefore, alkaline phosphatases catalyze the dephosphorylation of the chemiluminescence substrate, resulting in the emission of light. Horseradish peroxidase catalyzes the oxidation of luminal in the presence of hydrogen peroxidase, which brings the luminol in an excited state immediately. The image of the blot is visualized by exposing the blot to film. Chemiluminescent detection allows re-exposing the membrane. However it requires careful optimization, since the band pattern obtained changes with time.(Kurien and Scofield 2006)

6.7.5 Protocol

6.7.5.1 Sample preparation

Cells are lysed and the concentration of the protein is measured. The calculated amount of protein for electrophoresis is then diluted with PBS (Phosphate Buffered Saline) and loading buffer containing β-mercaptoethanol is added. The samples are heated for denaturating 5 minutes at 100ºC.
The following protocol was used in the laboratory to identify expression of PDX1 protein.

First the SDS gel was prepared. As separation gel 10%gel containing 5 ml 30%acrylamide, 3,75 ml 4x (1,5M) Tris-Cl (trishydroxymethylamino methanel)/SDS pH8,8, 6,25 ml water, 50 µl 10% ammoniumpersulfate APS and 20 µl TEMED (Tetramethylethylenediamine) was used. The separation gel is then poured in the apparatus from Biorad, after it is solid the stacking gel containing 650 µl 30% acrylamide, 1,25 ml 4xTris-Cl/SDS pH 6,8, 3,05 ml water, 25 µl 10% ammoniumpersulfate and 5 µl TEMED (Tetramethylethylenediamine) is prepared and poured on top of the separation gel. A comb is inserted.

After the gel is solid the gel is put into a gel electrophoresis chamber (Biorad), which is filled with electrophoresis buffer(containing Tris base, Glycine and SDS), and 30 µl of the denatured protein samples are added to the slots, which were generated by the inserted comb. The gel is then run on 200 V for 1,5 h or until the separation is completed. The gel is then transferred onto a nylon and filter paper, which were previously soaked with transfer buffer (containing 48 mM Tris base, 39 mM Glycine, 0,037%SDS and 20%methanol). On top a nitrocellulose membrane and again filter paper and nylon are added. The sandwich is then put into the transfer chamber with the membrane to the red anode side and filled with transfer buffer. The transfer takes place at 20 mA for 1,5 h. After the transfer, the membrane is put into a dish and blocked with 6% milk containing Tween at room temperature for 1h. The primary antibody is diluted in 6% milk and then added to the membrane for an overnight incubation at 4ºC. The next day the membrane is washed 3 times with 1x TBST (pH 8,0 containing Trisbase, NaCl and 0,1%Tween 20) for 10 minutes each time. Then the secondary antibody goat anti rabbit, which was diluted 1:5000 is added, and the membrane is incubated at room temperature for 2 h. Afterwards the membrane is washed again with TBST 3 times 20 minutes. The membrane is then developed with the chemiluminescence substrate kit Supersignal West Femto provided by Thermoscientific. After development for 1minute the membrane is put into an autoradiography cassette and several films with different exposure time are developed in the dark room.

Afterwards, the membrane is washed 3 times with TBST. GAPDH (glyceraldehydes-3-phosphate dehydrogenase) antibody diluted in milk is added to the membrane for 2,5 h at room temperature. Then the membrane is again washed 3 times with TBST. Afterwards the membrane is incubated with secondary antibody goat anti mouse, prediluted in milk, for 1,5 h at room temperature. Again the membrane is washed with TBST and developed with the chemiluminescence substrate kit Supersignal West Pico provided by Thermoscientific. After development for 1 minute the membrane is put into an autoradiography cassette and several films with different exposure time are developed in the dark room.

6.8 Reprogramming of liver cells to β cells *in vitro*.

In order to reprogram liver cells to obtain functional insulin producing β cells *in vitro* sequential transfection was done.

First 37 µl of Lipofectamine® RNAimax (purchased from invitrogen) are mixed with 1850 µl of Optimem (purchased from invitrogen), a serum free medium, and left for 5 minutes. In between the samples are mixed according to following table

Table 3: Sample preparation example

code	name	1x		4,5x	
		RNA	Optimem	RNA	Optimem
W7	MOCK				
W8	CONTROL	3µl	100µl	27 µl	450µl
W9,W10	PDX 527	3µl	100µl	27 µl	450µl
W11,W12	PDX539	3µl	100µl	27 µl	450µl

The samples are then combined 1:1 with the Lipofectamine® RNAimax/Opti-MEM mixture and left for 10-20 minutes. Meanwhile the medium of the cells is aspired, and cells are washed with PBS. Cells are then treated with trypsin. When the cells have detached 5 x 10^5 cells are seeded into the wells or dishes. 200 µl sample treatment/ ml of cell suspension are then added. The cells are then incubated at 37ºC in a humidified atmosphere containing 5% CO_2/95% air for 3 days. On day 3 after the 1st transfection, the cells were transfected again with the same amount of RNA as in the 1st transfection.

On the second day after transfection high glucose medium was added to the cells. This medium was normal DMEM medium, supplemented with 10% glucose in order to force the cells to produce insulin.

The most important marker for functioning potential β cells is the production of insulin. In order to determine the insulin production qPCR and Western could be done and some further more specific assays, which are briefly described in the following paragraphs.

One option is to identify insulin by immunofluorescence staining. Therefore, the cells need to be fixed on slides and incubated with the primary insulin antibody. Next the cells are incubated with a fluorescence labeled secondary antibody.

Another possibility is to measure the insulin secretion. Therefore the media is collected and concentrated. Furthermore the concentration of the protein is measured. Then an insulin ELISA kit is used to identify insulin in the media.

7 Discussion

Much progress has been made in the reprogramming non β cells to functional β cells via vector-based overexpression of β cell specific transcription factors such as PDX1, although a definitive cure for diabetes has still not been achieved. A lot of cell types have been used for the reprogramming, but the most promising might be pancreatic cells or liver cells. Due to the fact that these cells could be isolated from the patient itself, no foreign cells would be needed, eliminating immune rejection concerns in patients. However, current method of inducing PDX1 expression requires the use of viral vectors which pose potential risk to patients including immune response and insertional mutagenesis. New approaches for generating virus-free β cells are necessary to advance β cell therapy from laboratory to clinic. In this regard, RNAa represents a promising approach due to its ability of inducing sequence-specific and long-lasting gene activation.

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