Suitability of Laser-Capture-Micro-Dissection for Microarray Analysis:

A Study of Beta Cell Maturation

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This Thesis was performed at the Joslin Diabetes Center in the Division of Islet transplantation and Cell Biology in the research group of Susan Bonner-Weir (PhD).

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Statutory declaration "I declare in lieu of an oath that I have written this bachelor thesis myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This master thesis has not been submitted elsewhere for examination purposes."

Date: 03.03.2013

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Abstract

Small groups of beta cells are distributed across the pancreas and produce exquisitely calibrated amounts of insulin, a hormone needed to maintain proper glucose levels in the blood. These insulin producing cells must go through several steps which eventually result in a mature glucose responsive beta cell. New-born rodent islets lack key phenotypic beta cell genes and can serve as a model for the induction of the maturation process. Previous reports could elucidate sharp increases of transcription factors associated with beta cell maturation occurring between day 7 and day 10 after birth. This thesis will deal with the characterisation of the genetic environment accompanying these effects and provide an overview of the pitfalls and requirements of the laboratory techniques involved.

Beta cell rich cores of rat pancreatic islets were excised using laser capture micro dissection from day 7 and day 10 neonatal Sprague-Dawley rats after birth to compare their gene expression profiles using Affymetrix microarrays.

Six qualified RNA samples could be extracted from neonatal pancreatic beta cells. RNA was amplified and labelled using biotin. After labelling purification RNA was greatly compromised with regard to integrity and quality.

Laser Capture Microdissection from frozen sections greatly reduces the quality and quantity of extracted RNA samples and might impair its suitability for Microarray analysis.

Zusammenfassung

Verteilt über den Pankreas befinden sich kleine Gruppen von Beta Zellen und produzieren genau kalibrierte Mengen von Insulin, ein Hormon das der Normalisierung des Blutzuckerspiegels dient. Die Beta Zellen müssen mehrere Entwicklungsschritte durchlaufen um vollständig einsatzbereit zu sein.

Beta Zellen neugeborener Ratten weisen gewisse Beta -Zell-Gene nicht auf und können deshalb als Modell dienen um die Entwicklung der Beta Zellen zu studieren.Vorhergehende Studien haben gezeigt das bestimmte transkriptionsfaktoren zwischen Tag 7 und Tag 10 nach der Geburt ansteigen. Diese Arbeit wird sich mit der Charakterisierung des genetischen Umfeldes dieser Veränderungen näher beschäftigen und auf Probleme der involvierten Labortechniken hinweisen.

Beta Zellen wurden aus dem Pankreas ausgeschnitten mit Laser-microdissection von 7 und 10 Tage alten Ratten und anschliessend wurde die Gen Expressierung mithilfe von Microarray Technologie untersucht.

Sechs qualifizierte RNA Proben konnten extrahiert, verfielfacht und mit Biotin markiert werden. Aufgrund des Verlustes von RNA bei der Biotin Markierung war eine Analyse mit Microarray Gen Chips nicht möglich.

Die Laser Microdissection sorgt für eine Einbuße der Menge und Qualität der RNA und könnte so die Microarray Analyse erschweren.

List of Abbreviations

cDNA   Complementary DNA

aRNA   amplified RNA

cRNA   Compementary RNA

David   Database for Annotation, Visualisation, and Integrated
                      Discovery

GDP   Glutamic Acid Decarboxylase

H & E Stain   Haematoxylin-Eosin Stain

LCM   Laser Capture Microdissection

RIN   RNA integrity Number

1. Introduction

1.1. Background and Significance

Diabetes is a life-threatening condition and its frequency is drastically rising all over the world. At least 171 million people worldwide have diabetes. This figure is likely to more than double by 2030. In developing countries the number of people with diabetes will increase by 170% in the next 17 years. [1]

Prevalence of diabetes

Europe
2000: 33.3 million
2030: 48 million

Middle East
2000: 15.2 million
2030: 42.6 million

Africa
2000: 7 million
2030: 18.2 million

The Americas
2000: 33 million
2030: 66.8 million

Asia and
Australasia
2000: 82.7 million
2030: 190.5 million

Fig.1 Prevalence of Diabetes The numbers of people with diabetes will more than double over the next 17 years. Most of this increase will occur as a result of a 170% rise in developing countries.

Prevalence of diabetes (%) in persons 35 - 64 years

< 3   3 - 5   6 - 8   > 8

2000 = number of people with diabetes in 2000
2030 = number of people with diabetes in 2030

Source: Wild et al, 2004

Many studies support predictions of the epidemic nature of diabetes in the world during the first quarter of the 21st century as a result of population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. Worldwide surveillance of diabetes is a necessary first step toward its prevention and control, which is now recognized as an urgent priority. [2]

Diabetes is a predominant cause of complications such as blindness, kidney failure and various amputations. These complications lead to the severe economic burden caused by diabetes due to the monetary value associated with the disabilities and loss of lives that occur as a result of the disease itself and its related complications. [3]

National healthcare expenditures on diabetes accounted for 11.6% of the total healthcare spending in the world in 2010. Despite widespread beliefs that diabetes is essentially a first world issue it is increasingly the developing countries that have to struggles with diabetes. The developing countries are likely to experience a strong increase in people suffering from diabetes, from 84 to 228 million, by 2030 across all developing countries of the World Health Organization. In developing countries, the majority of people with diabetes are in the age range of 45–64 years in contrast to the developed countries where the majority of people with diabetes are aged 65 years and above. Thus people with diabetes in the developing countries are in the midlife and productive phase of their lives which suggests that diabetes is more a lifestyle related disease rather than a sickness that can be associated with old age. [2]

The urban areas of the world are expected to absorb all the population growth expected over the next four decades while at the same time drawing in some of the rural population. [4]

As more people move from bucolic areas to cities the number of people who are likely to develop diabetes increases since people in cities have a tendency to be less physical active and to be obese. The number of patients with diabetes rises worldwide. Therefore there has never been a more urgent need for social and therapeutic measures that might arrest the growth of the disease and alleviate its secondary manifestations. [1]

1.2. Definition of the Disease

Two disorders of the endocrine system, or the glands that produce hormones, are given the name diabetes. The diseases are not related, but they both cause excessive thirst and urination. [5]

Diabetes insipidus is rare and is caused either by a failure to produce or the blocked action of antidiuretic hormone (also called vasopressin), which regulates urine production in the kidneys. *Diabetes insipidus* is so named because the large volume of urine that is excreted is tasteless, or "insipid," rather than sweet, which is the case in diabetes mellitus, in where the urine may contain large quantities of glucose. [6]

Diabetes mellitus is caused by the body's inability to produce or respond to insulin and is characterized by abnormal glucose level in the blood. For clarity's sake diabetes mellitus will be referred to as diabetes in this thesis.

Diabetes is in general diagnosed by measuring patients plasma glucose levels and prognoses varies with already existing extend of glucose control applied. Treatments involve diet changes, exercise, and drugs that reduce glucose levels, including insulin and oral anti-hyperglycaemic drugs. Diabetes can occur in anyone but people who have close relatives with the disease are more likely to develop it. [7]

Risk factors include obesity, high cholesterol, high blood pressure and physical inactivity. In addition, people who develop diabetes while pregnant (a condition called gestational diabetes) are more likely to develop full-blown diabetes later in life. Although no cure for diabetes exists currently, it can be managed and controlled.

It is critical for patients with diabetes to keep their glucose levels in as normal ranges as possible because poorly managed diabetes can lead to serious, long term complications such as heart attacks and strokes, as well as diseases of the eyes kidneys and nerves. A nationwide study (USA) completed over a 10 year period showed that if people keep their blood glucose levels as close to normal as possible, they can reduce their risk of developing some of these complications by 50 percent or more. [7]

1.3. The "Diabetic Diseases"

Diabetes is not a single disease but several disorders characterized by the body's inability to produce or properly use and store blood glucose. For patients with diabetes, glucose backs up in the bloodstream, causing hyperglycaemia (excessive blood glucose levels), and leading to a host of symptoms which if not addressed, can lead to serious complications. Early symptoms are typically related to *hyperglycaemia* and include *polydipsia*, *polyphagia*, and *polyuria* while later complications involve vascular disease, peripheral neuropathy, and predisposition to infection. [8]

There are two major forms of the disease. Type I diabetes, also referred to as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes, and Type II diabetes, also called non-insulin-dependent diabetes mellitus (NIDDM) and adult-onset diabetes which usually occurs after age 40 and becomes more common with increasing age. Type I diabetes is less common (about 10% of all cases) than Type II diabetes (about 90% of all cases). Type I diabetes is thought to be caused by an autoimmune destruction of the beta cells pertaining to the islets of Langerhans on the pancreas. The auto islet antigens insulin and GAD65 (glutamate decarboxylase) have been demonstrated to activated naïve islet-reactive T cells and promote thymic selection of islet-reactive T- cells. People may develop type I diabetes at any age, but it is frequently diagnosed before adulthood. [9] [10]

Type II diabetes is characterized by a state of reduced responsiveness to normal circulating levels of insulin which is often referred to as insulin resistance. Key rate-controlling steps in insulin-stimulated glucose disposal are impaired due to defects in insulin-stimulated glucose transport in skeletal muscles and an excess supply of fatty acids, beyond that required for energy needs. [11] [12]

Type II diabetes is strongly associated with obesity. According to recent studies on obesity a protein called PEDF (pigment epithelium-derived factor) is released into the bloodstream where it causes the muscle and liver to become desensitised to insulin. The pancreas then produces more insulin to counteract these negative effects and eventually becomes overworked, eventually slowing or stopping insulin release from the pancreas, leading to Type II diabetes. The more fat tissue a person has the less sensitive they become to insulin therefore the number one risc factor to develop Type II diabetes is overweight. Despite their former classifications as juvenile or adult, either type of diabetes can occur at any age. [13] [14]
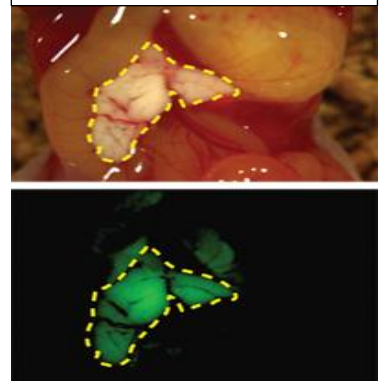
1.4. The Pancreas

The pancreas is a jelly like gland, attached to the back of the abdomen behind and below the stomach. It is long and narrow and thin, irregular in size, but in humans usually measuring about 20 x 6 x 1 centimetres and weighing about 95 grams. The main function of the pancreas is to produce digestive enzymes. These are secreted through the pancreatic ducts into the duodenum (or small intestine), where they become important constituents of the juices working to break down food passing down the alimentary canal. [15]

Fig. 2 The Pancreas as it appears just before the dissection in the rat

These enzymes help in the breakdown of the carbohydrates, proteins, and fats in the chyme. It is both an exocrine gland, secreting pancreatic juice containing digestive enzymes into the small intestine, as well as an endocrine gland producing several important hormones, including insulin, glucagon, and somatostatin. [16]

The cells in the pancreas that produce digestive enzymes are called acinar cells (from Latin acinus, meaning "grape"), which is due to the resemblance of the cell aggregates to clusters of grapes. Additionally patches of another cell type can be found on the pancreas: the islets of Langerhans. The islets are a class of secretory tissue and carry out the endocrine functions of the pancreas, though they account for only 1 to 2 percent of pancreatic tissue.

A large main duct, the duct of Wirsung, collects pancreatic juice and empties into the duodenum. The Enzymes active in the digestion of carbohydrates, fats, and proteins continuously flow from the pancreas through this duct. The flow of the enzymes is controlled by the hormones secretin and
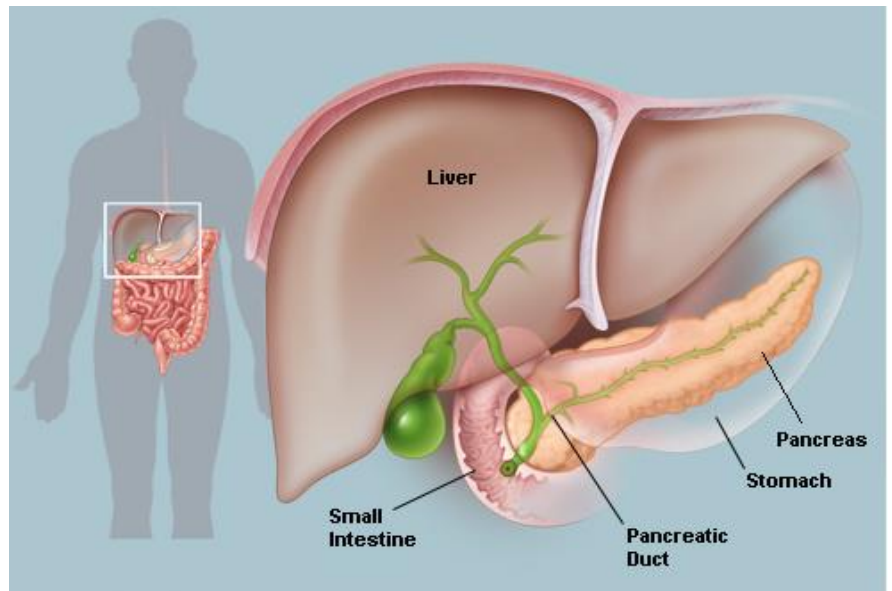
cholecystokinin, which are produced in the intestinal mucosa and the vagus nerve. As soon as food enters the duodenum, secretin and

Fig.3 The pancreas is about 15 cm long and sits across the back of the abdomen, behind the stomach. The head of the pancreas is on the right side of the abdomen and is connected to the duodenum through a small tube called the pancreatic duct. The narrow end of the pancreas, called the tail of the pancreas, extends to the left side of the body.
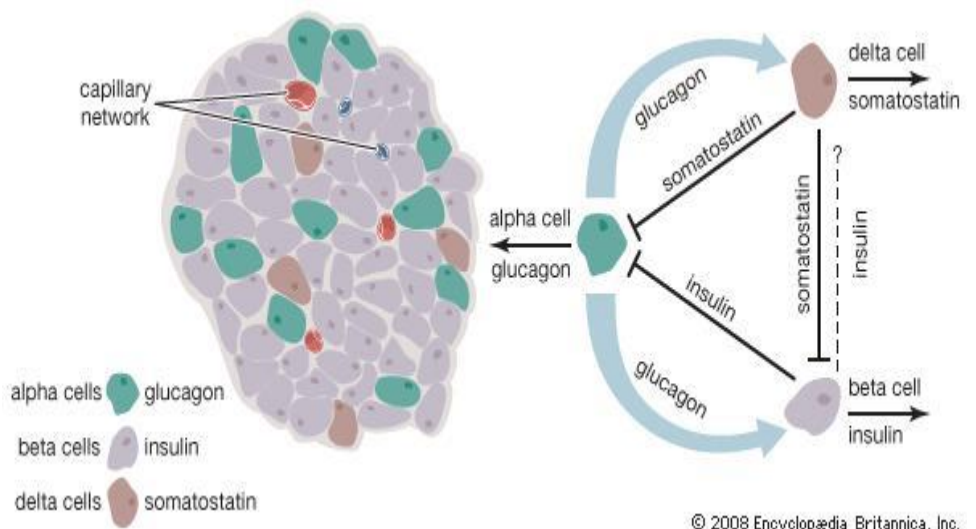
cholecystokinin are released into the bloodstream by secretory cells of the duodenum. When these hormones reach the pancreas, the pancreatic cells are stimulated to produce and release large amounts of bicarbonate, water and digestive enzymes (e.g. Trypsin protease), which then flow into the intestine. [17]

1.5 Anatomy of the Islets of Langerhans

The islets of Langerhans are round shaped spots of endocrine tissue located within the pancreas and consist of five distinct cell types, three of which (alpha, beta, and delta cells) are producing vital hormones. The alpha cell produces glucagon, the beta cell insulin and the delta cell somatostatin. To this day no data on the function of the fourth cell type (C cells) exists.

The impact of PP-cells (pancreatic polypeptide producing cells) is not well established as of yet but since they only make up a minor part of the islet cell population their influence on the behaviour of the islet might not be as significant compared to the alpha, beta and delta cells. [18]

Fig.4 The islets of Langerhans contain alpha, beta, and delta cells that produce glucagon, insulin, and somatostatin, respectively. A fourth type of islet cell, the F (or PP) cell, is located at the periphery of the islets and secretes pancreatic polypeptide. These hormones regulate one another's secretion through paracrine cell-cell interactions.

The various islet cell types are likely to communicate with one another in many different ways. The glucagon that the alpha cell secretes causes the beta cells to secrete insulin and the delta cells to secrete somatostatin. Somatostatin in turn inhibits both insulin and glucagon secretion. Insulin has a suppressive effect on glucagon secretion and most likely on somatostation secretion as well.

These concepts suggest that the islets of Langerhans are highly differentiated and intricate structures in which the alpha, beta and delta cells instead on functioning independently actually interact and regulate one other. The fact that islets contain neuronal elements and autonomic nerve terminals indicates that they have many important physiology effects on the body. [19]

1.6. Autoimmune Destruction of Beta Cells

Type 1 diabetes occurs when the insulin secreting pancreatic beta cells are destroyed by a beta cell directed autoimmune response that involves autoantigens, macrophages, dendritic cells, B- lymphocytes, and T lymphocytes. Many different beta cell autoantigens have been proposed to cause Type I diabetes. One of the strongest candidates is the glutamic acid decarboxylase (GAD) which has been shown in beta cells of none obese diabetic mice to induce the earliest T- cell related immune response. [20]
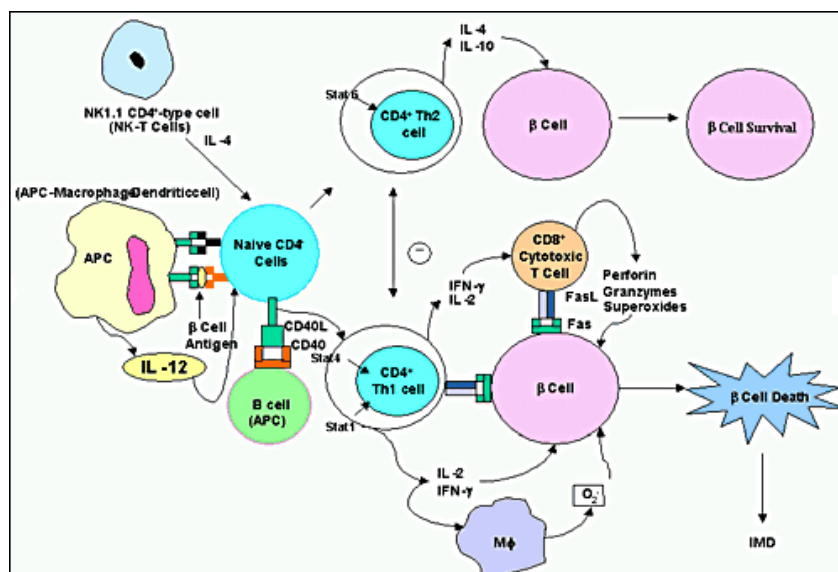

Fig.5 Beta cell specific auto antigens are recognized by macrophages, dendritic cells, B lymphocytes, and T lymphocytes, a process that has been shown to be involved in the pathogenesis of autoimmune diabetes

The exact procedure of the initiation of beta cell destruction is still poorly understood. The first immune cells to enter the islet are the dendritic cells

and the macrophages. Naïve CD4+ T cells that are circulating in the bloodstream and lymphoid tissue including the pancreatic lymph nodes are recognizing major histocompatibility complexes formed from beta cell autoantigens which are presented by dendritic cells and macrophages. The naïve CD4+ T cells and are then activated by the lymphatic cytokine interleukin (IL)-12 which is released from macrophages and dendritic cells. Once the CD4+ T cells are activated they release IL-2 which activates beta cell antigen-specific CD8+ T cells which differentiate into cytotoxic T cells and migrate into the pancreatic islets. Cytotoxic CD8+ T cells release granzyme

Fig.6 Destruction of insulin producing beta cells. Activated macrophages, CD4+ T cells, and cytotoxic CD8+ T cells act synergistically to destroy beta cells, resulting in autoimmune Type I diabetes

and perforin and destroy the beta cells. Macrophages produce reactive oxygen molecules and cytokines to further damage the beta cells. Thus activated macrophages, CD4+ T cells, and cytotoxic CD8+ T cells act synergistically to destroy beta cells, resulting in autoimmune type 1 diabetes. [21]
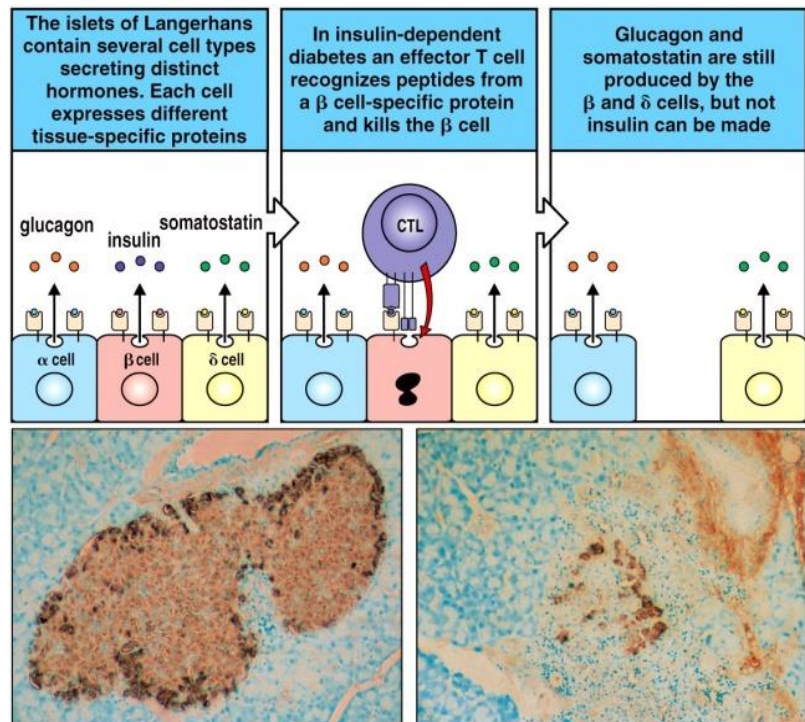
1.7. Insulin- The Key to Diabetes

Insulin is synthesized in the pancreas within the beta cells of the islets of Langerhans. One to three million pancreatic islets form the endocrine part

of the pancreas which is the endocrine portion of the organ and accounts for only 2% of the total mass of the pancreas. The beta cells in the islets release insulin in two phases: The first release is rapidly triggered in response to increased blood glucose levels. The second phase is a sustained, slow release independently of sugar.
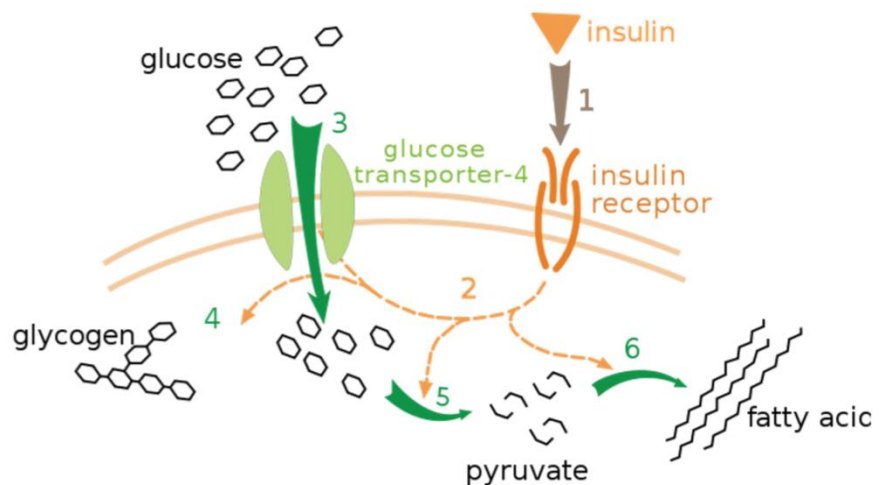
Fig. 7 Insulin binds to its receptor (1), which starts many protein activation cascades (2). These include translocation and influx of glucose through the plasma membrane (3), glycogen (storage form of glucose) synthesis (4), glycolysis (5) and fatty acid synthesis (6), i.e. increased lipid synthesis**.**

When the glucose level comes down to the usual physiologic value, insulin release from the beta cells slows down or stops entirely. Two types of tissues are most strongly influenced by insulin, as far as the stimulation of glucose uptake is concerned: muscle cells and fat cells. [8]

 The former are important because of their central role in movement, breathing, circulation, etc., and the latter because they accumulate excess food energy in order to store it for future needs. Together, they account for about two thirds of all cells in a typical human body. Insulin is not required for the transport of glucose into liver cells, but it has a significant impact on glucose metabolism in these cells. It stimulates the formation of glycogen, and it inhibits the breakdown of glycogen (glycogenolysis) and the

synthesis of glucose from amino acids and glycerol (gluconeogenesis).

Therefore, the overall effect of insulin is to increase glucose storage and to decrease glucose production and release by the liver. These actions of insulin are opposed by glucagon, another pancreatic hormone produced by alpha cells in the islets of Langerhans. [22]

1.8. Preliminary data

While protocols have been effective in producing in vitro insulin- positive cells from human embryonic stem cells, these insulin positive cells fail to become glucose responsive in vitro. Since rat beta cells are functionally immature (lacking glucose responsiveness) at birth and mature only gradually over the first postnatal month, they can provide a model to

Fig.8 Glucose responsive insulin production of beta cells: A Major remaining challenge of beta cell replacement

determine the mechanisms of maturation of beta cells. It has been previously shown that increasing expression of the transcription factor MafA in early neonatal rat islets can induce glucose responsiveness. [23]

The goal of current research is to understand how the major physiological changes occurring during the neonatal period regulate dramatic changes in expression of key phenotypic beta cell genes and lead to functional maturation of beta cells. The identification of physiological triggers capable of enhancing the genes necessary for this process could lead to the development of an in vitro maturation protocol that could be translated to immature human tissue. This would be an important step forward to meet one of the challenges of beta cell replacement for type 1 diabetes. [24]

A major gap in recent knowledge is how to obtain in vitro functional beta cells from human embryonic stem cells or induced pluripotent stem cells, which could be used for beta cell transplantation. Novel approaches dissect the mechanisms of maturation of functional beta cells in the neonatal rat model, to mimic this process in vitro initially with neonatal rat islets and then translate the
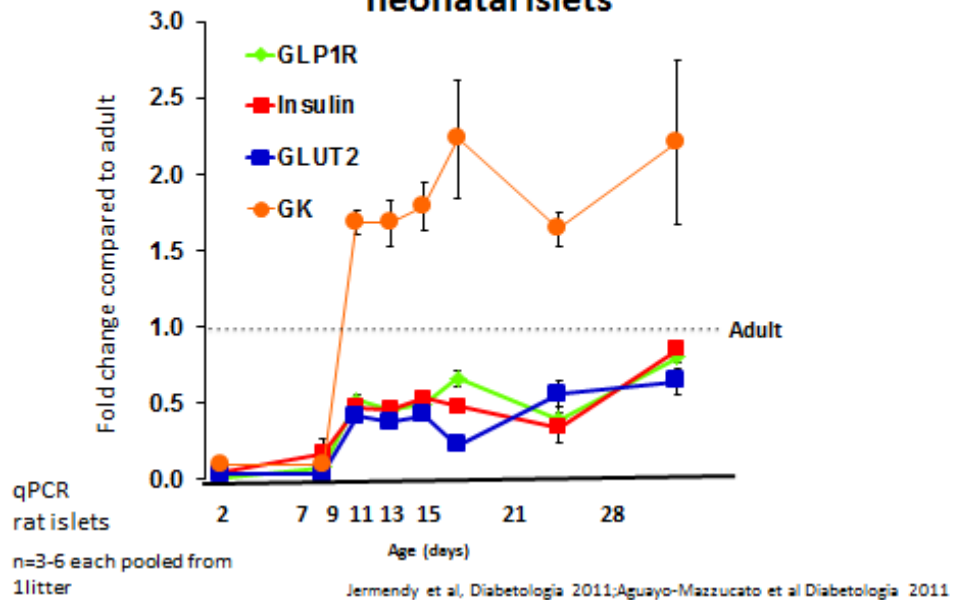
Fig.9 Beta cell Genes GLP1R, Insulin, GLUT2 and GK show different expression patterns over the neonatal period. Data expressed as fold change compared to adult levels; Major changes are happening between day 7 and day 10 after birth.

process to human stem cell- derived progenitors, operationally first tested and explored using islet cell clusters (ICC) from human fetal pancreas. This research approach is combining an understanding of the physiology with gene expression and the functional readout of secretion to dissect the mechanisms of functional maturation of the beta cell. [25]

The acquisition of glucose responsiveness in neonatal beta cells is gradual and not obtained fully until after weaning. Using laser capture micro-dissection (LCM) and Affymetrix microarrays, genes were identified that were differentially expressed in neonatal 1 day old and adult beta cells that suggested that the known lack of glucose-induced insulin secretion of neonatal beta cells is due to a generalized immaturity of phenotype, i.e. that the specialized metabolism of the mature beta cell is lacking. During the first postnatal month as the glucose-stimulated insulin secretion gradually improves, multiple patterns of islet gene expression were found with common infliction points between day 7 and day 10 after birth and again about day 15, times at which there are major plasma hormone surges and nutrient changes. [26]

Fig.10 Beta cell transcription factors: Neuro D, Pdx1, MafA and Nkx 6.1 show different patterns of expression over the neonatal period. Notice the dramatic surge of transcription factors between day 7 and day 10 after birth. Whatever happens in this time period it is likely to be critical for beta cell maturation Data expressed as fold change with respect to adult using S25 as internal control gene. Mean ± SEM, n=4-6 isolated samples per age, each pooled from 3-10 animals, qPCR

Several key beta cell transcription factors (Pdx1, MafA, NeuroD1) have very low expression in neonatal beta cells, being only less than 20% than that of the adult. Importantly, adenoviral mediated-MafA expression in day 2 islets induced glucose-responsive insulin secretion to levels approaching that of adult islets within 5 days of culture, however similar experiments with

adenoviral Pdx1 did not. Thus MafA must be considered a key transcription factor driving the functional maturation of beta cells. Previous research focused on how physiological triggers drive the immature insulin-expressing cell to become a fully functional mature beta cell. The abrupt changes in expression of most genes in neonates that were examined (particularly MafA, its target genes and transcription factors NeuroD1, Pdx1, and MafB) between day 7 and day 10 and then again at day 15 after birth were particularly striking, suggesting two major steps to beta cell maturation. [27]

The dynamic neonatal environment that is very different from that of the adult provides the landscape of these changes: at day 1 glucose levels are low, as are levels of thyroid hormone (T_3 and T_4), of corticosterone (the main glucocorticoid of the rat) and of prolactin and the diet is only milk. Serum T_4 levels start changing around day 7 and peak at day 13 while leptin surges also about day 8, these overlap with the first inflection point of the gene expression patterns and so may regulate the changes seen. There are surges of corticosterone and prolactin around day 15, the second inflection point. The timing of these hormonal surges suggests that they are the physiological regulators of the genes that need to be regulated for neonatal beta cells to become functional. Understanding the mechanisms of functional maturation of insulin-positive beta cells should have a potential in vitro application in the development of replacement therapies for type 1 diabetes. [28]

As previously mentioned several beta cell genes could be previously identified. Those genes are expressed neonatally at very low levels, many of which are regulated by MafA, a glucose responsive transcription factor specific to beta cells expressed neonatally at about 10% of adult mRNA levels.Pdx1;NeuroD1 and Nkx6.1 also are expressed at less than 20% of adult levels. These low values for beta cell genes are not due to differences in proportion of beta cells per islet. By day 7 Pdx1 and NeuroD1 no longer differ from adult in contrast MafA expression which remains significantly lower than adult through day 21. After infection with adenovirus expressing MafA, Pdx1 and Gfp for 4-5 days, day 2 rat islets were evaluated by q PCR and for insulin secretion with static incubation and reverse haemolytic plaque assay. Over-expression of Pdx1 increased MafA, NeuroD1 and glucokinase mRNA and insulin contend but failed to enhance glucose responsiveness. Similar over-expression of MafA resulted in increased NeuroD1, Nkx6.1, glucokinase and GLP1R mRNAs and no change in insulin contend but, importantly, acquisition of glucose- responsive insulin secretion. [29]

Since the endogenous levels of MafA are so low at this age, these experiments are essentially reconstruction or rescue experiments. After Adenoviral MafA infection both the percentage of secreting beta cells and the amount of insulin secreted per beta cell increased, approaching that adult beta cells which again identified, MafA as a key transcription factor driving the functional maturation of beta cells, but the question becomes what regulates its expression in vivo during this neonatal time. In search for physiological factors regulating the functional maturation of beta cells, thyroid hormone (T_3, T_4) is a strong candidate. The circulating thyroid hormone concentration rises significantly during the second postnatal week and promotes the development of the central nervous system and the digestive track.

Hypothyroidism prevents or delays maturation of these systems, and conversely thyroid hormone administration results in accelerated development. During the first month of postnatal life, serum levels of thyroxine (T_4) peaked at day 15, being 6.5 fold higher than at day 1 and 2 fold higher than adult levels.

In addition to the increasing thyroid hormone in the first two postnatal weeks, there are other systemic hormonal changes during this first postnatal month that could be physiological triggers for the beta cell maturation. Overlaying these hormonal changes are dietary gene expression changes: rats start eating chow at about day 14 after birth but are usually not weaned until day 24. It is entirely possible that there is an internal self-regulating "clock" for maturation, but preliminary data of

Thyroid hormone receptors increases sharply between P7 and P9

*p<0.05 respect to previous age
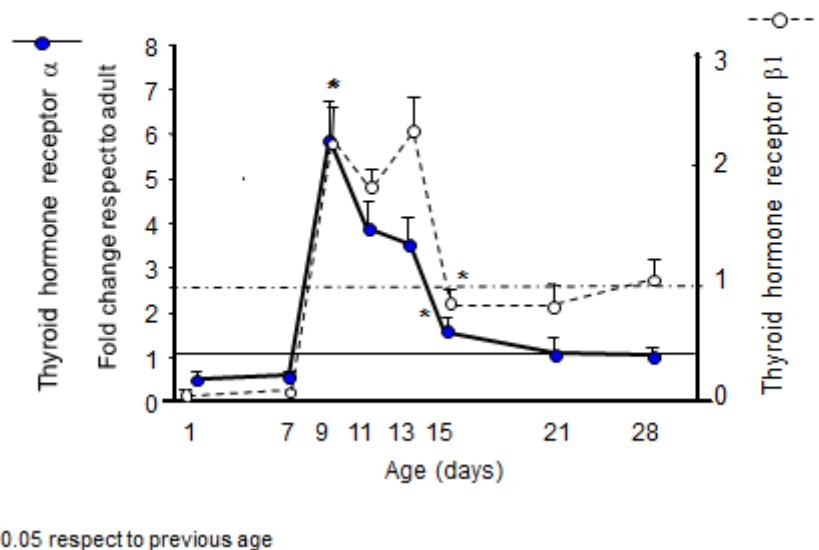
Fig.11 Change in Thyroid hormone receptor expression as an example of the rapid changes occurring during the first inflection point between day 7 and day 10. These changes might influence MafA activation and thereby direct beta cell maturation.

thyroid hormone (T_3) shows that beta cell maturation can be manipulated by external signals. T_3 regulates MafA mRNA levels in vitro, and plasma T_4 and thyroid receptors numbers in islets increase around the day 7 to day 10 inflection point when MafA expression goes from about 10% of adult level to 43%. About the same time is a leptin surge that is key to development of the hypothalamic wiring that regulates adult feeding behaviour. [30]

This surge has been confirmed in rats to be between day 7 and day 10. Both prolactin and corticosterone start to increase in parallel from extremely low levels before day 14, a time when genes Pdx1, NeuroD1, MafB and glucagon are starting to drop to adult expression levels. From day 15 to day 28 circulating levels of both these hormones increase impressively. This is of particular interest since these two hormones had opposing effects on transcriptional regulators of energy metabolism in islets in studies done to mimic the changes during pregnancy when both hormones increase with prolactin being known to be trophic for the neonatal and adult beta cells, stimulating their proliferation, inhibiting their apoptosis, decreasing the threshold for glucose stimulation for insulin secretion and promoting insulin gene transcription while corticosterone has been shown to counter the beneficial effects of prolactin on insulin secretion and proliferation of neonatal beta cells . These data suggest that these two hormones may be key for the second inflection point and the subsequent increases of some of the metabolic genes. [31]

Based on the temporal overlap of inflection points, expression of numerous genes and increase in circulating hormones, it is very likely that various physiological factors regulate the maturation of beta cells. To determine if the physiological factors that are effective in maturing the neonatal rat beta cell will similarly direct in vitro functional maturation of the immature human pancreatic progenitors, the hormones (T_3,leptin,corticoid and prolactin) that led to functional maturation in the neonatal rat will be applied to human pancreatic tissue. While this may seem like a reach it is a very important translational step. While the ultimate goal is to have an in vitro protocol for human stem cell- derived progenitors, the developed treatment will first be tested and explored using islet cell clusters (ICC) from 19- 22 week old human fetal pancreases. These pancreases are available consistently and are reproducible at the same stage. Due to how little is known about the

human fetal pancreas as to temporal expression of key genes and concentrations of circulating hormones, it is impossible to give precise protocols as of now. Based on previous research on neonatal rat islets, experiments on human pancreatic tissue will be carried out. These experiments will utilize dose response curves determined in experiments with rat pancreatic tissue, first testing one hormone at a time, then a combination of different hormones. If a protocol could be developed that accelerates maturation of these immature human cells, collaborations with other research groups could then be pursued that will focus on the differentiation of human embryonic stem cells (hESC) or induced pluripotent stem cells (iPS) to pancreatic progenitors

Fig.12 The specialized pathways that normally define the unique metabolism of mature beta cells are expressed poorly in the neonatal islet but increase gradually with age. Genes with lower expression in the neonates are shown in blue

to extend this study to clinically relevant cells. [32]

Glucose-stimulated insulin secretion only develops postnatally in rodents. In previous studies microarray analysis was used to determining differentially expressed genes of 1 day old neonatal and 4 week adult rat beta cells. Major phenotypic differences in LCM excised beta cells from neonatal and adult rat islets could be identified using microarrays of LCM samples.

The results showed a set of "disallowed genes" in the neonatal rat beta cells which are expressed normally low in adult beta cells (e.g. Monocarboxylate transporter 1 etc.) and a set of Important genes that normally define the unique metabolism of adult beta cells which were expressed low in neonatal beta cells (e.g. Malate dehydrogenase 1). [32]

The overall low activation of important metabolic genes and enzymes is ultimately responsible for the poorly developed glucose responsiveness of neonatal beta cells. The work done for this thesis intended to close the gap of previous research by focusing on the established infliction points of hormones, genes and transcription factors at day 7 and day 10 after birth to pinpoint the factors necessary for rat beta cells to mature.

1.9. Laser Capture Microdissection for Microarray analysis

LCM will be used to dissect the beta cells out of the pancreas in 7 day and 10 day old rats. Their gene expression will be compared using microarray analysis

Laser capture microdissection holds many promises to study the unaltered expression of specific cell types from complex tissue specimen and is often considered a substantial advance compared to existing dissection, purification and separation methods. [34]

However LCM has many requirements and challenges that make a quick optimisation of this new technique for microarray expression analysis difficult. The suitability of tissue samples collected with LCM for microarray analysis depends greatly on the right quality and quantity of starting material and the quality of extracted RNA and the necessary RNA integrity. [35]

The LCM in connection with Microarray analysis involves many methodological obstacles which are normally not encountered when using whole tissue analysis or gene analysis techniques such as PCR. These obstacles include tissue handling (e.g fixation, storage, and staining), consumables (e.g. slide choice), staining reagents (conventional H&E vs. fluorescence) and extraction methods which at the end have to yield RNA samples that have suitable concentrations and quality for microarray analysis . [36]

Despite previous publications that claim suitability of LCM in connection with Microarray analysis the actual implementation of this experimental set up as protocol is a balancing act. [26] [34]

1.10. Aim

This thesis will present the effects of laser capture microdissection on RNA quantity and quality by attempting to analyse the gene expression of previously mentioned key hormones and genes of isolated beta cells at the inflection points during the first two neonatal weeks at day 7 and day 10 by microarray analysis of laser capture microdissected (LCM) rodent beta cells. Simultaneously the suitability of LCM for convincing results with Microarray analysis will be evaluated

2. Methods

7 and 10 day old Sprague-Dawley rats are purchased from Taconic Farms, Germantown, New York, USA. Animals are checked until delivery in order to have healthy day 7 or day 10 animals. Rats were killed by anaesthesia with Nembutal injections for pancreas exision and islet extraction. The dissected pancreas was embedded in Tissue Tek OCT medium and snap frozen in chilled isobutane and immediately stored at −80°C. Frozen OCT blocks are sliced into sections of 8 µm and placed onto uncoated glass slides which are then later used for LCM. Beta cell riched cores of islets were dissected using the PixCell II LCM system For each RNA sample, at least 5,000 'hits' (laser pulses) from 5 to 8 islets per section were excised from 20 to 25 tissue sections. Total RNA from each sample was isolated using the Arcturus Pico Pure RNA isolation protocol. RNA amplification based on T7 polymerase is carried out to obtain enough RNA for microarray analysis. RNA is transcribed to cDNA, labelled with biotin and hybridized to the Affymetrix GeneChip Rat Genome 230 2.0 Array. [26]

Experimental Design
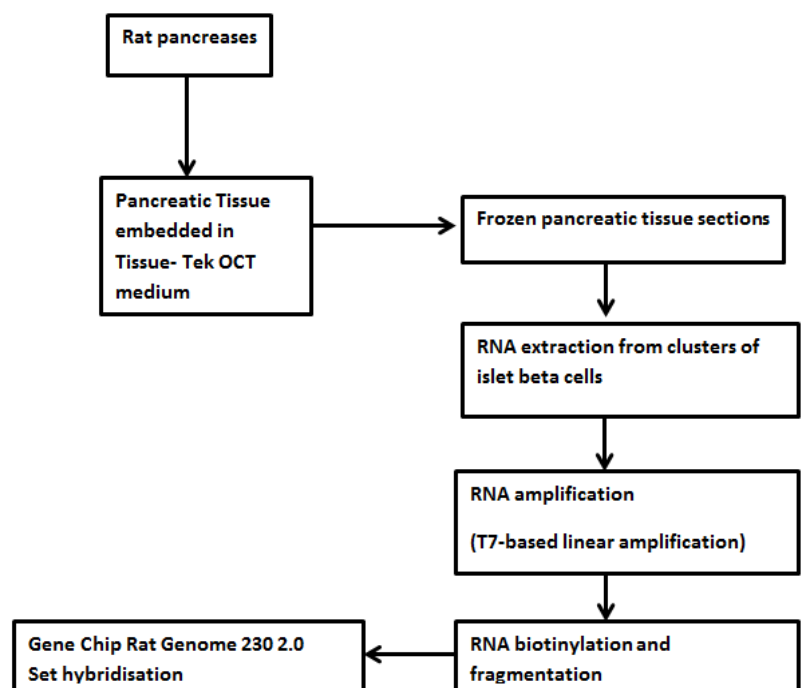
Fig.13 Flow chart of the experimental design and the procedures used. The experimental design is a multistep procedure where the success of one step depends on the success of the previous step

2.1. Animals

The Sprague-Dawley rat is one of the most widely used animal models in medical research with outstanding economic reproductive performance which makes it the rat of choice when tissues of specific age are needed. To be able to monitor the development and

determine the age of the rats accurately is vital for the success of this study. Sprague- Dawley rats are

Fig.14 Sprague-Dawley rats 7 days after birth. Neonatal pups were nursed until they were killed at day 7 or day 10 respectively.

raised specifically so that on the day of pancreas dissection they are 7 or 10 days old exactly. This is what facilitates research of neonatal development, and requires cell harvesting and killing of the neonates on the targeted neonatal ages. [36]

All animals are kept under appropriate conditions in climatised rooms, with free access to standard pelleted food and tap water. All procedures were in compliances with the Joslin Institutional Animal Care and Use Committee Guidelines. Neonatal pups were nursed until they were killed at day 7 or day 10 after birth respectively. For each age there were 6 to 7 animals. The pancreases were excised and embedded in optimal cutting tissue compound (OCT) and processed for frozen block sectioning.

2.2. Dissection of the Pancreas

Animals were beheaded or overdosed with anaesthesia (0.05-0.07 ml per rodent). Pancreatic tissue was obtained from heart beating cadaveric donors within 20 minutes of surgical procedure. The specimens are snap frozen immediately after the procedure. The abdomen is opened up and the pancreas is exposed as much as possible by making a "V" cut from the lower abdomen. After the spleen and the first loop of the duodenum are evident the colon is pulled away to display the "head of the pancreas" which is behind the colon. Once the whole pancreas is in view the splenic end is disengaged from the stomach and from strands
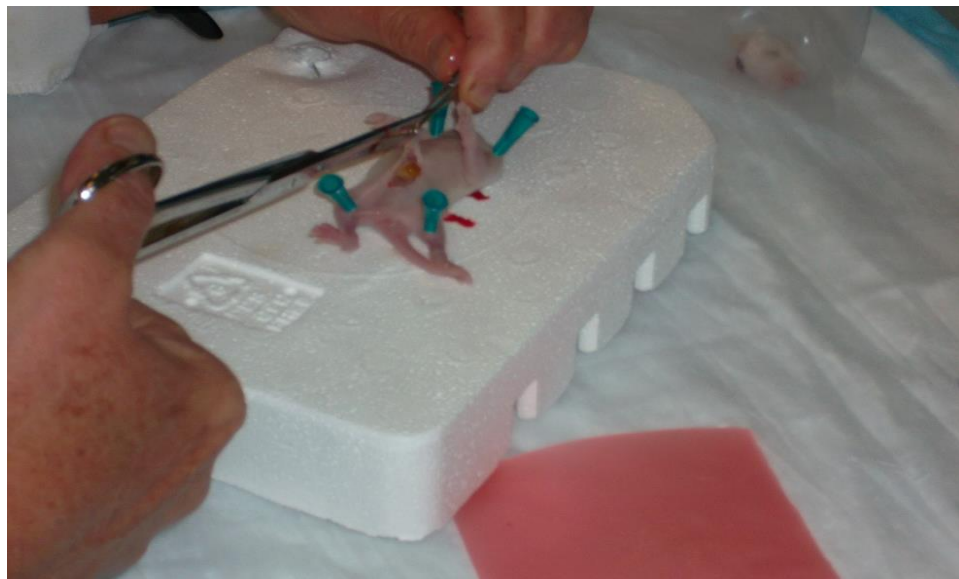
Fig.15 Excision of the pancreas .Animals are overdosed and while under deep anaesthesia the whole pancreas is dissected.

of fat around the stomach and spleen. The spleen is left attached to act as a handle. The spleen now is being held and pulled forward so that remaining small attachments can be snipped off. A central cut across the pylorus is carried out. It is very important not to cut off the small flap of pancreatic tissue that overlays the pylorus while performing the cut. Now the lower part of the duodenum is cut at exactly that position where the pancreas ends. This part is normally "hidden" behind the colon.

While holding on to the duodenum and spleen the pancreas and attached organs are lifted out to enable snipping across the blood vessels and lymphatics that cross the duodenum as well as any remaining attachments behind the pancreas such as ties for perfusion. The pancreas is spread out on wax board and the spleen, duodenum as well as large lymph nodes are separated and discarded. [37]

2.3. Tissue freezing

Methyl butane is put into a glass beaker and covered with parafilm. Dry Ice is filled into a plastic beaker so that its base is entirely covered and the glass beaker containing the methyl butane is put inside. More dry ice is added until the space between the two beakers is filled until the upper edge of the glass beaker is reached. Ethyl alcohol is put on the dry ice sited in the space between the two beakers. In this way a freezing system is obtained. Subsequently the etyl alcohol on the dry ice between the beakers is incubated for 20 minutes so that the methylbutane inside the glass beaker can cool down. OCT is filled into the mold bases to form a lower layer (about 1/3 the depth of the mold), while avoiding the
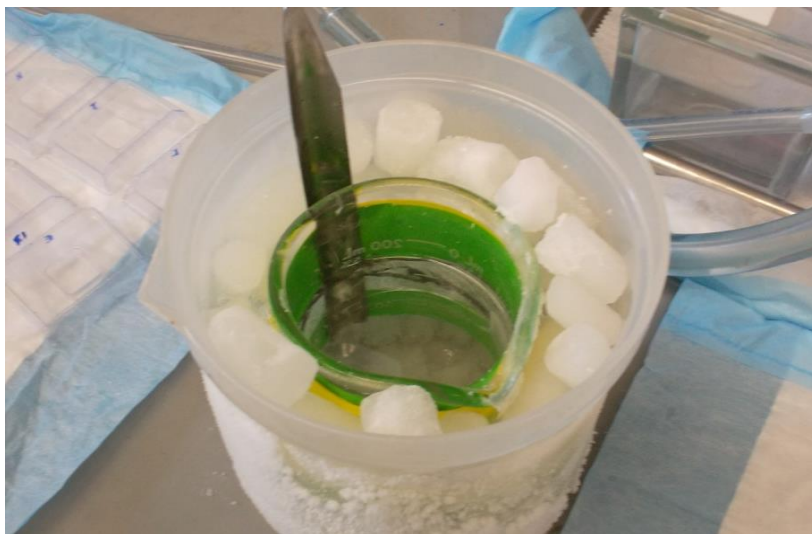
Fig.16 For best RNA preservation, pancreatic tissue specimens are frozen immediately after dissection.

formation of bubbles. In case bubbles do form they can be removed using a tip or a needle. Now the pancreatic tissue is placed into the mold and covered with OCT. It is especially important that the desired cutting surface of the tissue lies flat on the bottom. More OCT is added until the specimen is completely covered and the cryomold is filled to the top with OCT. The tissue should be centred in the mold having a border of embedding compound surrounding the tissue.

This orientation will later ease the cutting of frozen sections in the cryostat. The base mold is then plunged into the icy methylbutane and left there for 15-30 seconds and moved onto dry ice at an angle that allows the solvent to drain and evaporate.As soon as the OCT losses its shiny appearance, after about 5 minutes, it can be wrapped in aluminium foil and is stored at -80 °C. [38]

2.4. Tissue sectioning & Slide Preparation

Sectioning must be performed in a cryostat at −20°C. Before sectioning the internal surfaces of the cryostat, blade, object holder, and paintbrush have to be precleaned with 100% ethanol and equilibrated to the cryostat temperature which usually takes about 15-20 minutes. The frozen tissue block is fixed to the object holder by spreading a small amount of embedding medium onto the surface and placing the block on top. When the embedding medium around the block is completely frozen and the tissue is firm, the object holder can be mounted on the microtome head. Now the tissue is brought closer to the blade edge. The block is trimmed until an even cutting plane is reached. 8 µm thick sections are cut with a slow, smooth, and steady motion of the turning wheel on the right side (see Fig.7) of the cryo chamber. As the sections begin to form on the blade

Fig.17 A cryostat can help to overcome conventional obstacles of disinfection since the cold disinfection facilitates complete none-toxic disinfection of the entire cryo-chamber

edge, a paintbrush is used to gently guide the section down the face of the blade; this helps to keep the section flat.

The section is mounted in the middle of a labeled, plain, uncharged glass slide by bringing the surface of a room-temperature slide very close to the section. The section will seem to jump onto the slide and the mounting medium will melt. It is important that the motion of the wheel is slow, smooth, and steady for this helps to avoid breakage of the tissue and makes it possible to obtain sections without wrinkles, which, when present, complicate the LCM procedure later. The blade edge is cleaned with 100 % ethanol and clean dry paper towels. The section slides are stored on dry ice for short term (1-2 h) and at −80°C for long term storage (for up to 6 months). During the sectioning OCT may stick to the blade, making its surface uneven, and, as a consequence, lead to breakage of sections. To avoid this problem, OCT is removed by wiping down the blade using tissue paper soaked in 100% ethanol. The blade is cleaned in an upward direction to avoid injury of hands and fingers. During sectioning, one slide of every fifth section performed is marked; kept apart and stained with hematoxylin/eosin (H/E). The H/E staining helps to locate islets quickly during the LCM procedure. [39]

2.5. Hematoxylin-Eosin Staining for LCM

In order to microscopically locate the islets in the pancreatic tissue, the specimen must be stained in order to make the islets visible. The hematoxylin and eosin stain uses two separate dyes, one staining the nucleus and the other staining the cytoplasm and connective tissue. Hematoxylin is a dark purplish dye that will stain the chromatin within the nucleus, leaving it with a deep purplish-blue color. Eosin is a pink to red dye that stains the cytoplasml including collagen and connective tissue, and leaves an pink counterstain. This counterstain acts as a sharp contrast to the blue – purplish nuclear stain of the nucleus and helps to discriminate between different tissues within the pancreas such as acinar cells and islets. The process of performing the H&E stain
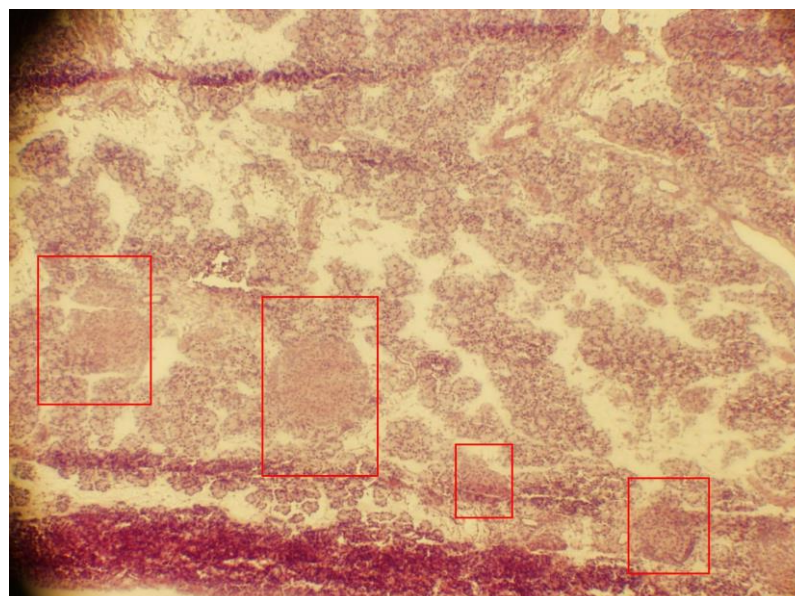
Fig.18 Hematoxylin /Eosin stain of pancreatic section slide. Islets are photographed to pin down their location for a speedy LCM procedure.

is straight forward. After the tissue has been embedded in OCT, sectioned and placed on a slide, the slide is taken and put through brief changes of xylene, alcohol and water to 'hydrate' the tissue.

This process is called 'running the slides down to water' and must be done to give the cells an affinity for the dyes. The slides are then stained with the nuclear dye (hematoxylin), rinsed, and stained with the counterstain (eosin). Now the slides are rinsed and run in the reverse manner from the run down (dehydration through, alcohol, and xylene), then coverslipped [40]

2.6. Tissue Dehydration and LCM

In order to reveal cell specific expression profiles which would be otherwise obscured in mixed cell samples microdissection was used to collect pure populations of beta cells. After locating the islets, a LCM Cap is placed over the target area. Pulsing a laser through the cap causes the thermoplastic film to form a thin protrusion that bridges the gap between the cap and tissue and adheres to the target cells. Lifting of the cap removes the target cells now attached to the cap. The isolated beta cells are now available for extraction of RNA.
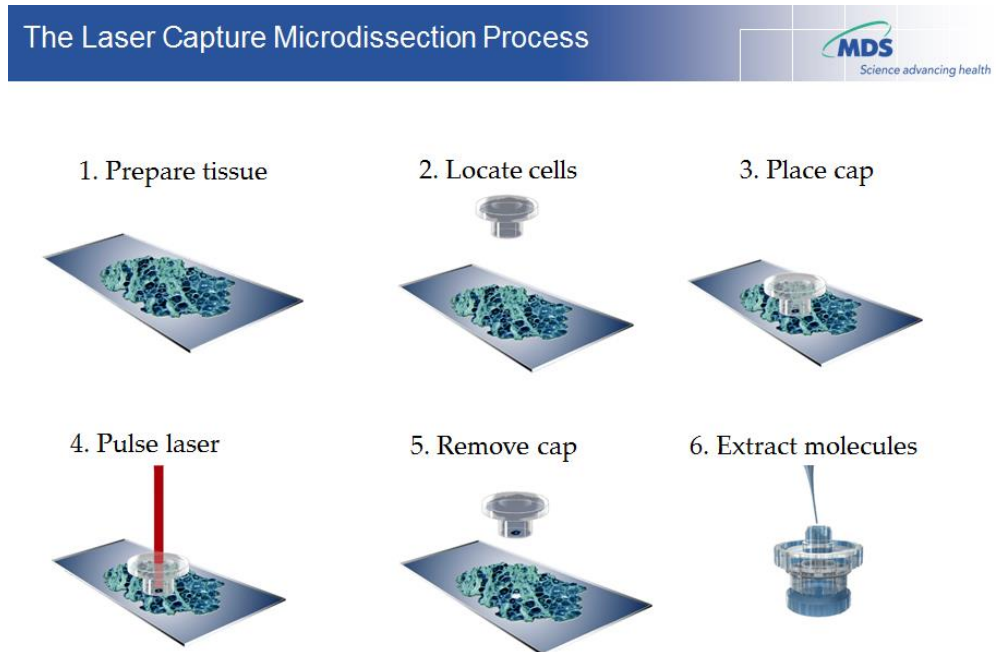
The Laser Capture Microdissection Process

1. Prepare tissue
2. Locate cells
3. Place cap
4. Pulse laser
5. Remove cap
6. Extract molecules

Fig.19 LCM under direct microscopic visualization permits rapid procurement of selected beta cell populations from a section of complex heterogeneous tissue.

Homogeneous cell populations reveal hidden molecular signatures and are therefore a powerful tool in cell specific molecular analysis. Pancreatic beta cells have a specialized phenotype maintained by the expression of a unique set of genes and the suppression of others, but this pattern is altered in diabetes. Gene profiling studies of a pure beta cell population not exposed to the trauma of isolation procedures and ischemia that causes changes in gene expression by dissecting beta cells from the pancreatic tissue directly using the laser capture microdissection allows the sampling of single cells or groups of cells located in histologically complex tissues that can be analysed for unique gene expression.

The technique enables identification of islets prior to dissection. Dissection of beta cells from rodent islets is straight forward since non- beta -cells are restricted to the islet mantle and rarely found in the islet cores. This is fortunate because the autofluorescence of rodent beta cells is not strong enough to be useful for LCM.

The successful application of this technique requires careful procurement of pancreatic tissue. Prior to dissection of islets the tissue on the slides must be completely dehydrated. The sections are fixed in 70% ethanol washed in DEPC-treated water, and dehydrated in 100% ethanol and xylene.The dehydration reagents must be prepared fresh every time and cannot be reused since once open ethanol absorbs water from the air, which causes reduction of the ethanol dehydration efficiency and affects the ability to perform LCM. It can be helpful to switch to a new set of dehydration reagents and DEPC- treated water. The use of fresh reagents also helps avoid possible contamination by tissue RNases. The use of polypropylene conical tubes for dehydration solutions is mandatory since polypropylene is resistant to the corrosive activity of xylene. The laser power and duration must be

tested with each new pancreas sample, and, if adjustments are needed to obtain an effective laser spot, it is recommended to increase the power instead of the duration; since increasing the duration will increase spot diameter, the duration must be kept as low as possible. The microdissection session must not be longer than 15 min in order to preserve the RNA from degradation. After the LCM procedure the RNA is extracted using guanidine isothiocyanate buffer and stored at -80°C. [34]

2.7. RNA Isolation

RNA is isolated from the LCM extract solution using the RNA Pico Pure spin column based method. This method does not make use of compounds such as such as phenol, chloroform or CsCl gradient centrifugation. Instead it uses a lysis buffer that contains chaotrophoc salts like guanidine HCL which destabilizes van der Waals forces, hydrophobic interactions and hydrogen bonds. The denaturing properties of guanidine salt inhibit RNA degradation by destabilising RNases and disrupting the association of RNA with water while at the same time facilitating binding of RNA to the silica membrane.

The flow chart (Fig.20) illustrates the RNA isolation procedure. First the RNA is extracted from an LCM slide. Next the cell extract is loaded onto a pre- conditioned purification column. The extract is spun through the column to capture RNA on the purification column membrane. The column is washed twice with wash buffer, and the RNA is eluted in low ionic strength buffer. The entire process can be completed in less than one hour. [41]
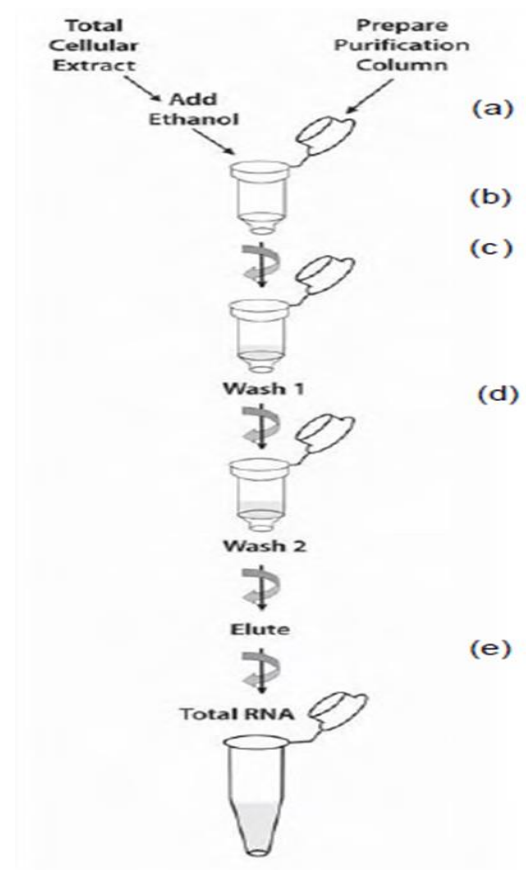
Fig.20 Summary of RNA isolation procedure. The isolation is designed to recover high-quality total RNA consistently from fewer than ten cells.

RNA can be easily degraded during the isolation procedure therefore precautions must be taken to avoid the activation of tissue RNases and the sample contamination by environmental RNases. For this the operator must avoid any biological contact with the sample, wear disposable gloves, and change them frequently.

The laboratory bench surface, pipettes and any other surfaces that may come in contact with the sample must be cleaned using RnaseZap (Ambion). All the solutions and plastic ware must be RNase free. Column based nucleic acid purification is a solid phase extraction method to quickly purify nucleic acids. This method relies on the fact that the nucleic acid binds by adsorption to the solid phase of the silica membrane inside the column depending on the pH and the salt content of the buffer. [42]
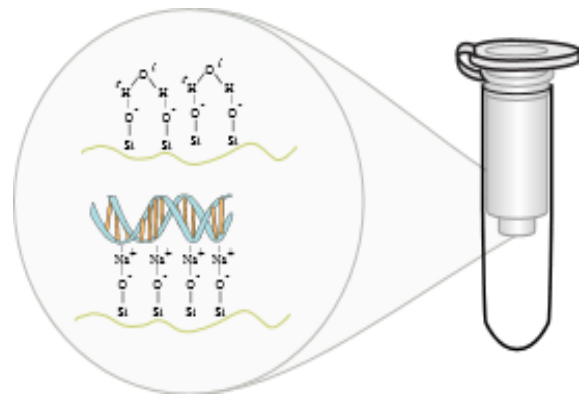
Fig.21 With the advanced techniques of silica membrane in purification spin columns, RNA can be selected and reversibly bound.

2.8. Purity Assesment of RNA

For quantitating the amount of RNA obtained after isolation, readings are taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of the nucleic acid in the sample. An OD of 1 corresponds to a concentration of 40 ng/µl of single stranded RNA. The ratio of absorbance at 260 nm and 280 nm is taken to estimate the purity of the RNA. A ratio of around 2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower it may indicate the presence of protein, or other contaminants that absorb strongly at or near 280 nm. [43]

A 1 ul sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends.

Fig.22 Spectrophotometric analysis is commonly performed to determine the average concentrations of RNA present in a mixture, as well as it's purity

The gap is controlled to both 1mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear charged coupled device array (CCD) is used to analyse the light that passes through the sample. The instrument is controlled by a PC based software, and the data is saved in an archive file on the PC. [44]

One of the disadvantages of using conventional spectrophotometers is that the cuvettes are large, making it difficult to measure low concentrations of RNA without losing an unacceptable fraction of the sometimes precious and valuable RNA sample. The NanoDrop 1000 UV-Vis Spectrophotometer enables highly accurate analyses of extremely small samples with remarkable reproducibility. The sample retention system eliminates the need for cuvettes and capillaries, which decreases the amount of sample required for the measurement. Surface tension is used to hold a column of liquid sample in place while a measurement is made. The measurement typically takes less than 10 seconds, and the spectrum and its analysis is shown on the screen of the attached PC and archived. Once the measurement is complete, the sample is simply wiped from the measurement pedestals. The archived data can be manipulated by a spreadsheet program such as MS Excel. [45]

2.9. RNA Amplification

The isolated and RNA of the laser captured cells is now amplified by T7- polymerase based amplification using T7-oligo-dT-primers.Two rounds of amplification are performed using the RiboAmp HS RNA Amplification Kits from Arcturus. The RNA Amplification enables the researcher to perform molecular analysis on RNA samples that have a too low concentration for microarray analysis. The Amplification process offers a unique linear amplification from as little as 100 pg of total RNA. RNA is amplified up to 1,000,000-fold in two rounds. The Amplification produces amplified antisense RNA (aRNA), ready for labelling and microarray hybridization. The RNA Amplification Kit is optimized to amplify pico or low nanogram amounts of starting RNA to produce micrograms of total cellular RNA in a five-stage process. These five stages are listed and shown in Figure 23. At the beginning the first strand is synthesized in a reaction that produces cDNA and makes use of a T7 promoter sequence.
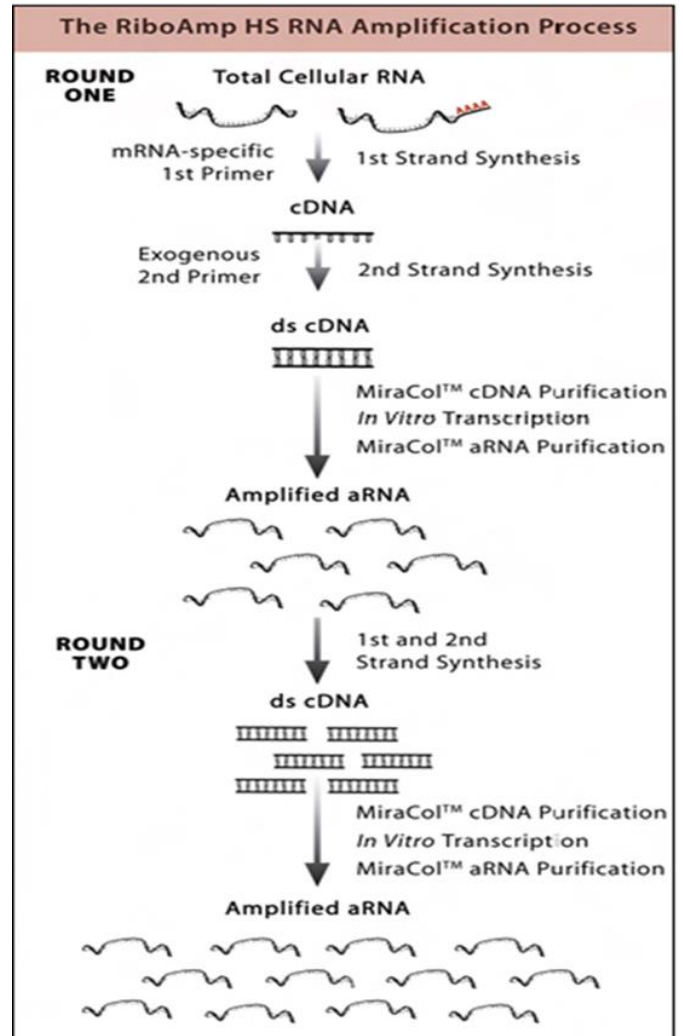
Fig.23 RNA amplification procedure. The amplification of RNA is necessary to obtain enough RNA for Biotin labelling and microarray analysis

Now the 2nd-strand is synthesized in a reaction utilizing exogenous primers that yields double- stranded cDNA. Now the cDNA is purified using purification spin columns. In vitro transcription that uses utilizing T7 RNA polymerase produces then antisense RNA (aRNA) which is again isolated using purification spin columns. The RNA amplification is quite a long procedure; it requires three full days during which the protocol can be stopped overnight. The process can be completed by the morning of the third day, and the product is now ready for labelling and microarray hybridization. The success of amplification using the RNA Amplification depends on the quality of the source RNA. Integrity is affected by exposure to internal and external sources of RNases. RNase contamination will cause experimental failure. Due to the ultra-sensitivity of the amplification, it is very important to prevent RNA, DNA, and nuclease contamination and to clean work surfaces before and after each use. To minimize RNase contamination it is recommended to wear disposable gloves and change them frequently.   Work surfaces must be cleaned with commercially available RNase decontamination solutions prior to performing reactions to avoid RNAse contamination. [46]

2.10. Quality Assesment of RNA

To obtainin meaningful gene expression results in the microarray analysis the RNA extracted from the LCM samples has to be in one piece which means it needs to have a good quality. The quality of RNA is in this experiment measured using the Agilent 2100 Bioanalyzer and assigns what is known as the RNA integrity number to the individual RNA samples. The RNA integrity number is a measure for the degradation of the RNA sample under investigation. The Agilent system gives a number to each RNA sample submitted for analysis in the range from 1 to 10 whereby 1 is the most degraded and 10 is the most intact. Degradation in this case refers to the state the RNA strand is in at the time. The RNA could be fragmented from purification procedures and or degraded by contamination sources such as RNAses. Depending on the previously measured RNA concentration and purity determined with the Nanodrop spectrometry either the Nano or the Pico Agilent LabChip is employed. The Nano LabChip can analyse RNA concentrations in the nanogram range and the Pico LabChip can analyse RNA sample concentrations in picogram range. Once applied to the chip the RNA samples are separated by electrophoresis and afterwards detected via laser induced fluorescence detection. The bioanalyzer software generates an electropherogram and gel-like image and displays the sample concentration and the so called ribosomal ratio. [47]

By assessing the appearance of the obtained electropherogram the condition of the RNA can be evaluated. If the RNA has a low RNA integrity number (RIN) it can be deemed unsuitable for gene expression studies as degraded RNA would yield an incomplete picture of the genes present on the RNA of the beta cells at day 7 and day 10. Therefore the RNA integrity number should ideally be above 5 in order to get good results in the differential gene expression study. The RIN is intended to act as a standard for evaluation of
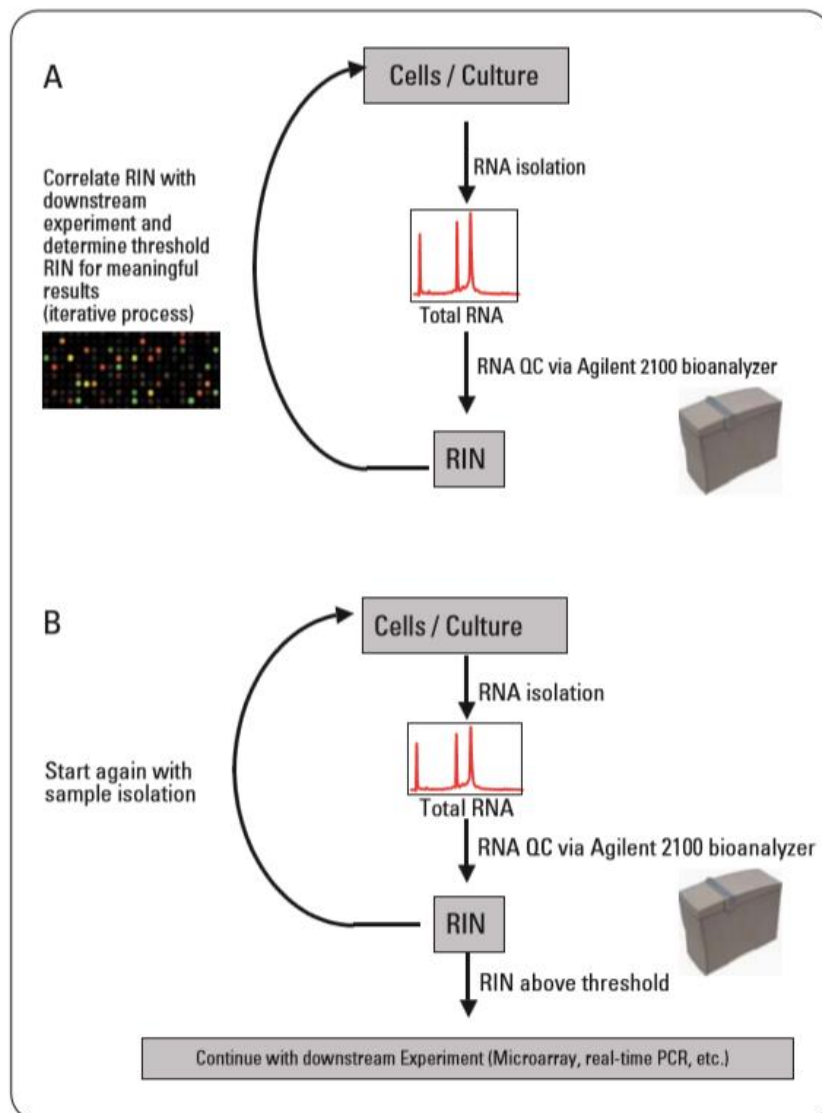
Fig.24 A. Correlate RIN values with downstream experiments (e.g. microarray or RT-PCR) and determine threshold value for obtaining meaningful gene expression results.

B. Once initial correlation experiment has been performed, and data thresholds have been set, RIN values can be used to discard samples that do not pass the sample QC on the Agilent 2100 Bioanalyzer

RNA quality. If two samples with the same RNA integrity are compared the

results are meaningful because it indicates that

both samples have the same degree of degradation and differences in their gene expression profiles later on are not due to different degrees of degradation but to actual differences in gene expression. The RIN itself is determined using an algorithm that uses information originating from bioinformatic databases which track features of the electropherogram (e.g. 18S, 28S rato, peaks) of RNA samples of the same or similar tissues types that have been previously analysed elsewhere to establish a standard and eliminate individual interpretation of RNA integrity.

Once all RNA samples have been assigned a RNA integrity number, they can be correlated. Ideally two samples that have the same RIN should be compared on the microarray. By establishing a threshold for minimum RNA integrity below which RNA samples are no longer suitable for the expression analysis is sensible since it introduces an element of quality control into the experiment. This ensures that the differences in expression are due to differences in gene expression not RNA degradation. [47]

2.11. Biotin labelling

After the final qualification using the Agilent 2100 bioanalyzer RNA was labelled using biotin for subsequent microarray analysis. In Figure 25 a summary of the labelling process is shown. The labelling process is a non-enzymatic technique for the labelling of unmodified, amplified RNA (aRNA). The unmodified RNA is labeled after the amplification. This allows incorporation of modified nucleotides during RNA amplification. By making use of nucleotides that are unmodified in the amplification process produces higher concentrations and longer aRNA fragments of RNA thus providing better representation of the RNA transcript for downstream analysis.

The nucleotides of the RNA are labelled

with biotin in a reaction that takes about 15 to 30 minutes and is carried out at a temperature of 85 °C. The now labelled RNA is isolated using purification spin colums in order to clear the samples of any impurities left, mostly leftover biotin and

Fig.25 Schematic Representation of the biotin labelling process for microarray hybridization. The reagents facilitate effective labelling of RNA samples with biotin for hybridization to cDNA or micro arrays.

any degraded RNA. Finally the RNA is hybridized with a blocking reagent and mixed with a hybridisation mix to prepare the RNA samples for microarray analysis. [48]

2.12. Microarray Analysis

The labeled RNA samples are hybridized with the the GeneChip Rat Genome 230 2.0 microarray with the intend to compare the two different gene expression profiles (of day 7 and day 10) for differential gene expression analysis. The data is analysed using various bioinfomatic tools such as the Database for Annotation, Visualization and Integrated Discovery (DAVID) software and GenePattern.

Microarrays are small, solid surfaces onto which the DNA sequences from many of different genes are immobilized and

Fig.26 A microarray is a tool for analyzing gene expression that consists of a small membrane or glass slide containing samples of many genes arranged in a regular pattern

attached, at certain locations. The surfaces themselves are usually glass, silicon or nylon slides that have the size of two side by side thumbs. DNA is synthesized and printed directly onto the surface by using hybridization probing, a technique in which nucleic acid molecules are immobilized as probes onto the surface and now complementary fluorescently labeled nucleotides coming from the sample under investigation can be identified

through base pairing of

DNA sequences that are able to bind to one another (e.g. Adenine and Thymine).This way the presence of specific genes can be confirmed. Upon binding of complementary DNA strands a fluorescence signal will indicating the presence or absence of a certain gene sequence in the sample RNA. [49]
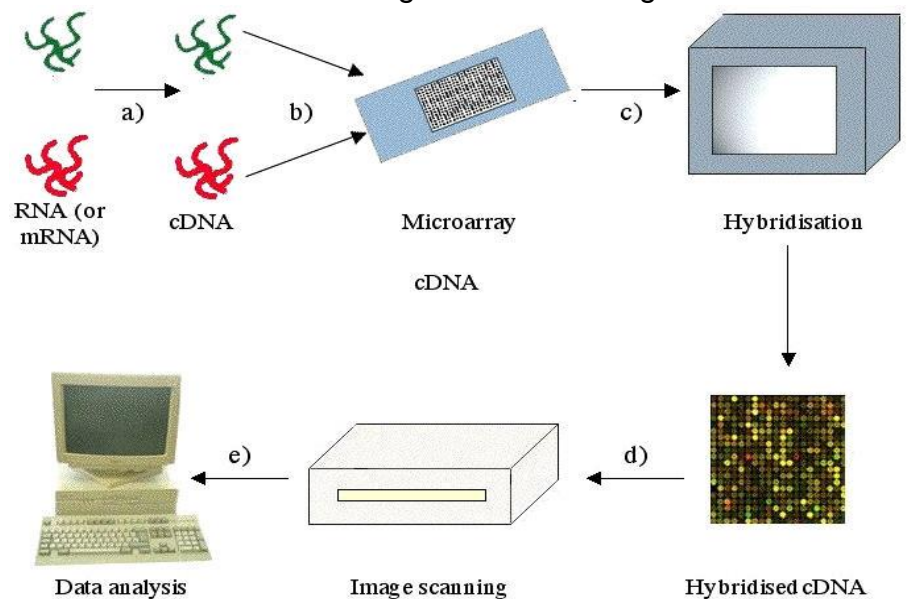
After this hybridization step is complete, the microarray is placed in a "reader" or "scanner" that consist of multiple lasers, a special microscope, and a camera. The fluorescent tags are excited by the laser, and the microscope and camera work together to create a digital image of the array.

Fig.27 a) Reverse transcription b) Prepare DNA chip using chosen target DNAs c) Incubate your hybridization mixture containing fluorescently labeled cDNAs with your DNA chip. d) Detect bound cDNA using laser technology and store data in a computer e) Analyze data using computational methods.

The data is then stored in a computer, and a special program is used to calculate the red-to-green fluorescence ratio and to subtract out background data for each microarray spot by analyzing the digital image of the array.

The difference in expression is presented using the Database for Annotation, Visualization and Integrated Discovery (DAVID) which enables to create a heatmap that shows which genes are expressed stronger at day 7 or day 10 respectively using a specific colour-code.

Using traditional methods to assay gene expression, researchers were able to analyse only small number of genes at a time. The advent of new tools like microarrays enables researchers to analyze expression of many genes in a single experiment and address previously intractable problems and to uncover novel potential targets for therapies.

In order to compare the gene-expression profiles of the day 7 and day 10 neonatal rat beta cells the "Rat Genome 230 2.0 Array" was chosen since it provides an overview over a vast selection of rat genes. The Gene chip allows to interrogate over 30,000 transcripts and variants from the rat genome, including over 28,000 well- substantiated rat genes. The main advantage of the Rat Genome 230 2.0 Array is that each high-density array contains multiple probe pairs per probe set which provides several independent measurements from every transcript.

The sequences used in the design of the Gene chip Rat Genome 230 2.0 Array are selected from the bioinformatics databases GenBank, dbEST, and RefSeq. The sequences are synthesized as complementary Oligonucleotide probes onto the arrays [50]

45

Since previous reports elucidated sharp increases of transcription factors that lead to beta cell maturation occurring between day 7 and day 10 after birth of the neonatal rat, the gene expression profiles that are the result of the microarray analysis trials performed for this thesis could provide a map for maturation of beta cells according to which maturation of beta cells such as beta cells derived from induced pluripotent stem cells could be validated in a way that the microarray outputs would serve as a guideline as to which genes have to be activated for beta cells to mature. This map would be an invaluable contribution to the development of an overall maturation protocol for human beta cell transplantations. [50]

3. Results

3.1. Purity Assessment of none amplified RNA

After dissecting the beta cells from the islet core using laser capture microdissection and isolating the beta cell RNA, all samples where checked for their overall quality and amount of RNA. At the ages examined (P7;P10) the average islet has only approximately 250 vs. 1500 islets of an adult islet equivalent. The number of cells captured with laser capture microdissection cannot be easily estimated since the dehydration of the tissue alters the morphology of the cells. The number of captured cells is also strongly influenced by the efficiency of the microdissection. However, a rough estimation of the quantity of captured tissue can be obtained by counting the laser pulses performed during the microdissection

D7	ng/µl	260/230	260/280	D10	ng/µl	260/230	260/280
A	3.9	2.81	2.76	F	4.7	2.1	1.33
F	9.1	0.74	1.43	E	3.3	1.8	1.7
I	5.1	1.58	1.71	B	6.2	1.74	1.43

Fig.28 RNA concentrations measured with nanodrop spectroscopy. The concentration of RNA suggests an amount of RNA that is higher than the actual concentration present in the sample. The 260/280 ratios suggest a decent purity of the sample

session. In my experience samples obtained from 5,000 laser pulses generate enough RNA for microarray analysis. As can be seen in Fig.28 the amount of RNA after isolation is rather low being less than 10 ng/µl. Quantitation of isolated RNA through UV spectrophotometry is usually not possible for samples containing less than 1µg of total RNA. Therefore measurements will be affected by components in the eluted sample, which cause overestimation of the total RNA content. Nanodrop spectrophotometry measurements frequently give too high readings for RNA concentration in samples that have only small RNA contents and can therefore be misguiding.

The measured RNA readings normally turn out to be much lower when analysed with the Agilent gene chip as it is much more precise and effective for determining RNA concentrations that are lower than 100 ng/ µl. The analysis of the samples on a microarray requires an amount of RNA that amounts to at least 250 ng in 3 µL solution or less which necessitates the amplification of RNA samples obtained from laser capture microdissection. The RNA amplification procedure applied has the potency to produce microgram quantities of amplified antisense RNA (aRNA) from picogram quantities of total cellular RNA. The minimum total RNA input requirement for the RNA amplification is 100 pg of total RNA and will yield ideally more than 15 µg. The 260/280 ratios in Fig.28 reveal that most samples have a 260/280 absorbance ratio close to 2.0. A ratio around 2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm

Abnormal 260/280 ratios may also indicate that there was an issue with the measurement. Although purity ratios and spectral profiles are important indicators of sample quality, the best indicator of RNA quality is functionality in the downstream application of interest. It is important to note that there are occasions when the purity ratios are within expected limits, yet there is a problem with the sample.The spectrophotometric analysis of the isolated RNA samples is ultimately a first checkpoint if the RNA has the appropriate quality and quantity to be used in later stages of the gene expression profiling experiment.

The main disadvantage to use absorbance is the lack of specificity as well as the lack of sensitivity to quantitate low-level samples. All nucleic acids (dsDNA, RNA and ssDNA) absorb at 260 nm, and the method is not capable of distinguishing between the various forms of nucleic acid. While the A260/A230 ratio can be used to estimate nucleic acid purity, the amount of genomic DNA present in an RNA preparation cannot be

determined by absorbance. Also, if significant amounts of contaminants that absorb around 260 nm are present in a sample, the contaminants themselves can contribute to the absorbance value, resulting in an overestimation of nucleic acid concentration. If RNA samples are degraded due to the nature of the sample or sample handling and preparation, changes in RNA integrity are not reflected in the measurement because single nucleotides also will contribute to the 260nm reading.

3.2. Quality Assessment of none amplified RNA

Analysis of the RNA samples using the Agilent 2100 Bioanalyzer technology revealed that the RNA concentration was far lower than the analysis with Nanodrop spectrometry promised. While the RNA concentration in the Nanodrop measurement was on average 5 ng/µl the Agilent technology showed that the actual concentration is about 10 times less that value. Since the minimum RNA input requirement for microarray analysis requires an amount of RNA that amounts to at least 83 ng in 1 µl or 250 ng in 3 µl solution, the Agilent measurement underlines the fact that the samples have to be amplified first before they can be used for gene expression studies. It can be taken from table 29 that all RNA samples possess a low RNA integrity number. The RNA Integrity Number (RIN) software algorithm allows the classification of total RNA, based on a

D7	pg/µl	RIN	D10	pg/µl	RIN
I	469	1.9	F	283	1
F	709	1	E	405	1
A	524	1	B	1073	1.3

Fig.29 Agilent measurement of unamplified RNA samples. The RNA concentration is lower compared to the Nanodrop measurement. The RNA integrity number suggests degradation of the RNA samples. Similar RNA integrity numbers facilitate correlation of RNA samples in expression

numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact.

It is important to note that RIN values are often not applicable to low concentration RNA samples, such as RNA samples originating fromLCM. When using lower concentrations, higher sample to sample variances of the RIN may be observed. So although the RNA integrity number in table 29 suggests a strong degradation of the RNA, the assigned RNA integrity might not be accurate for the RNA samples. Of course it is recommendable to work with samples that have the highest possible RNA integrity numbers for gene expression studies. A widely accepted cut-off value for the RNA integrity number is ≥5. [47]

Fig.30 Electropherograms of unamplified RNA. The peak in the small molecular range and the RNA integrity number would suggest degradation but might just be due to low RNA starting concentration.
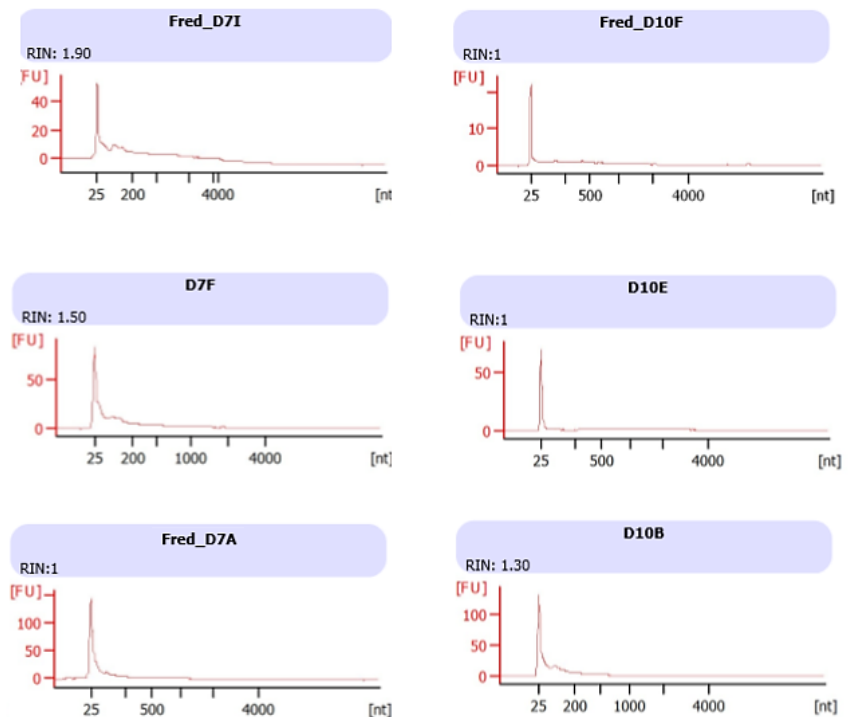
Unfortunately, when working with neonatal islet tissue, it is not easy to collect a sufficient amount of RNA that meets the threshold value. Thus, at least biological replicates showing similar RNA integrity numbers should be used. It can be seen in the table 29 that the RNA samples possess a fairly similar RNA integrity number and therefore the comparison of gene expression between the samples is enabled.

The RNA quantification results of the Nanodrop and the Bioanalyzer should agree to a large extend. This is particularly true if the samples have a high concentration of RNA that is highly pure. If the results of the Nanodrop and the Bioanalyzer do not agree, there is most likely a low concentration level of RNA and/or the material is not pure. The Bioanalyzer measures the amount of fluorescence as the RNA sample is pulsed through a microchannel over time. The Agilent Bioanalyzer software creates a graph called an electropherogram which can be seen in Figure 30.

The electropherogram of the unamplified samples shows extreme degradation. The example lacks 28S and 18S peaks altogether and consists solely of low molecular weight species. Usually if samples perform in a similar manner on the Bioanalyzer it would be advisable to examine the RNA extraction technique and try submitting new samples. Since previous research data gathered in the lab showed similar RNA degradation the decision was made to continue the experiment despite the obtained RNA integrity number values. To determine absolute or relative purity of the final RNA sample can be rather difficult. The RNA or the solution it is dissolve in may include variants of unknown or unexpected impurities. The Purity should therefore be assessed by Nanodrop Spectrometry and Agilent analysis to be sure to avoid any kind of contamination occurring.

3.3. Purity Assessment of amplified RNA

The RNA amplification is quite a long and demanding procedure. It requires three full days during which the protocol can be stopped overnight. Appropriate stopping points are suggested in the RNA amplification protocol. It can be gleaned from table 31 that the amplification of RNA was a success.

D7	ng/µl	260/230	260/280	D10	ng/µl	260/230	260/280	Control	ng/µl	260/230	260/280
I	263.86	2.76	2.46	F	130.68	2.72	2.33	Control Amp 1	600.22	3.14	2.56
F	419.52	3.09	2.48	E	821.11	3.26	2.63	Control Amp 2	128.23	2.94	2.44
A	119.68	2.64	2.3	B	370	3.45	2.6				

Fig.31 Nanodrop measurement of amplified RNA samples. The use of control RNA's during the amplification is recommended to proof that the amplification was successful

All RNA samples could be amplified to a concentration higher than100 ng/µl which had been previously specified by the microarray core as necessary RNA concentration for attaining meaningful results in differential gene expression studies. It is recommended in the RNA amplification protocol to use the in the kit provided control RNA to make sure the amplification has worked out for the best. The 280/260 ratios in figure 31 show that all RNA samples possess a sufficient degree of purity as the 260/280 ratios are around 2. The 260/230 ratios are used as a secondary measure of nucleic acid purity and these values are for "pure" nucleic acid often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2.

If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm. When looking at figure 31. it is plain to see that all the amplified RNA samples show a good 260/230 ratio that suggests that they are not contaminated by protein or other contaminants such as degraded RNA. Although the Nanodrop measurement of the amplified RNA samples suggests a high concentration of RNA after the amplification it is not possible to predict the quality and integrity of the RNA with the Nanodrop readout alone. The purity is a good indicator if further proceeding with the amplified RNA samples is at all reasonable but it cannot guarantee that RNA is intact and suitable for gene expression analysis using high throughput gene expression tools such as microarrays.

Therefore it is imperative that at the end of the RNA amplification, before proceeding to the RNA biotinylation and microarray analysis, the RNA integrity is assesd by running amplified RNA on the Nano LabChip of the Agilent 2100 Bioanalyzer. As previously mentioned the concentration of RNA is usually higher when measuring it with the Nanodrop spectroscopy method compared to the readouts the Agilent technology gives for the same RNA samples. Due to a more automated design and many different attributes that are considered in the Agilent analysis it can be considered a more accurate and reliable technique for determining the RNA concentration.

3.4. Quality Assessment of amplified RNA

The Agilent analysis results of the amplified RNA samples confirmed that each sample was positively amplified to have a RNA concentration higher than 80 ng/µl as can be seen in table 32 which was previously specified as the minimum concentration for biotin labelling. The RNA integrity numbers are considerably low ranging from 2.0-2.3 The low RNA integrity number combined with the fact that the RNA samples barely exeed the minimum concentration for labelling and hybridisation of 80 ng/µl clearly points towards a strong degradation of the RNA. This can also be observed when looking at the overall appearance of the electropherograms coming from the Agilent 2100 Bioanalyzer (see Fig.33). In most of the samples the 28S and 18S peaks are absent and almost all RNA has been degraded into 100bp or less which is marked by an overall shift of the peaks to the right in the

D7	ng/µl	RIN	D10	ng/µl	RIN
I	123	2.3	F	88	2.3
F	331	2	E	682	2.1
A	210	2.3	B	226	2.2

Fig. 32 Agilent measurements of amplified RNA samples. All samples are equally degraded as can be deduced by the RNA integrity numbers ranging from 2.0-2.3

electropherogram. The peak heights in the electropherograms are related to quantity. The lower the peak, the less fragmented cRNA is present. Since the peaks are fairly high in the electropherograms obtained as can be observed in figure 33 it is likely that the RNA is fragmented to a large extend. It is recommended to use RNA samples with high RIN scores (7-10) [47] which also have a reasonably narrow distribution of RIN scores which should be typically a variation of 1-1.5 difference of RIN between the samples. As can be seen in figure 32 all six RNA samples possess a similar RNA integrity ranging from 2.0 -2.3 which could imply that despite their obvious fragmentation and degradation they could be potentially compared on the ground that they have a narrow RNA integrity number distribution.

Despite the values of total RNA obtained with the Agilent measurement the total RNA of each sample just before labelling amounted to only 1 to µg of total RNA per sample.

The amount of RNA necessary to prepare for the Affymetrix Rat Genome 230 2.0 array is 5-10 µg of total RNA in 10 µl RNase-free water or elution buffer and alternatively 15 µg of fragmented RNA in 40 µl.

The amount of 1 µg of RNA obtained after amplification is the bare minimum concentration necessary for labelling and microarray analysis. Usually the amplified RNA is greater than 1 µg after amplification. The RNA amplification kit has been validated down to as little as 100 pg of input RNA to yield a concentration of amplified RNA that is larger than 30 µg of amplified RNA. The obtained yield of amplified

Fig.33 Different stages of RNA degradation with their corresponding Agilent electopherogram. The electropherogram on top shows an example of completely intact RNA with clear peaks representing the 18s and 28s ribosomal RNA fragments

RNA in this experiment is therefore significantly below the target RNA concentration which should be anywhere between 20-30 µg after amplification. It is essential to determine the quality of RNA samples prior to microarray
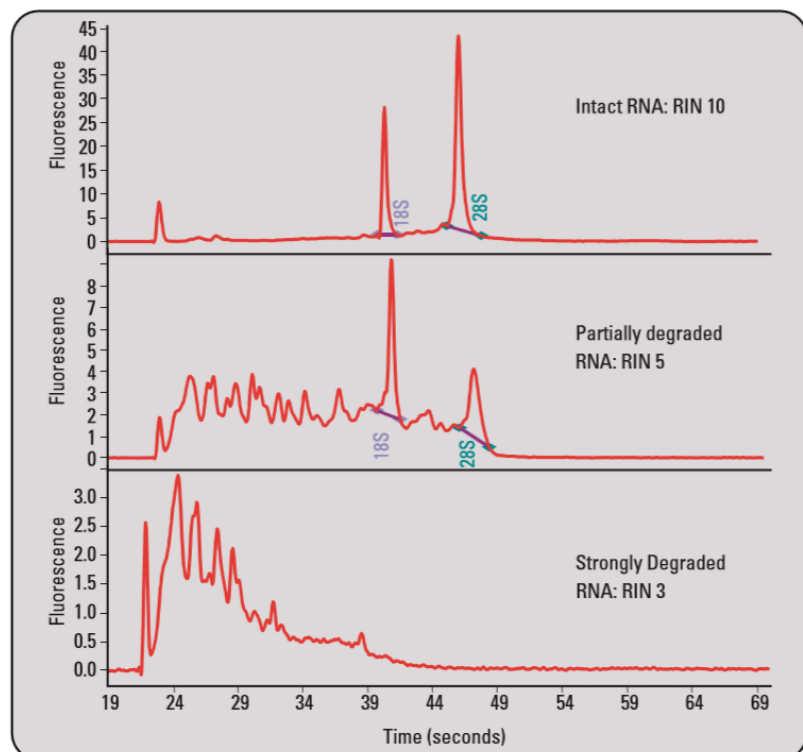
analysis to ensure that differential degradation of samples is not later mistaken for differential expression. The quality of the samples is by far the single most important factor in the whole process. The fact that the RNA loses its peaks corresponding to the 18S and 28S ribosomal RNA among other signs of degradation mentioned above has been taken into account in previous pilot projects to determine the best cell isolation technique for RNA purification of neonatal pancreatic islet tissue.
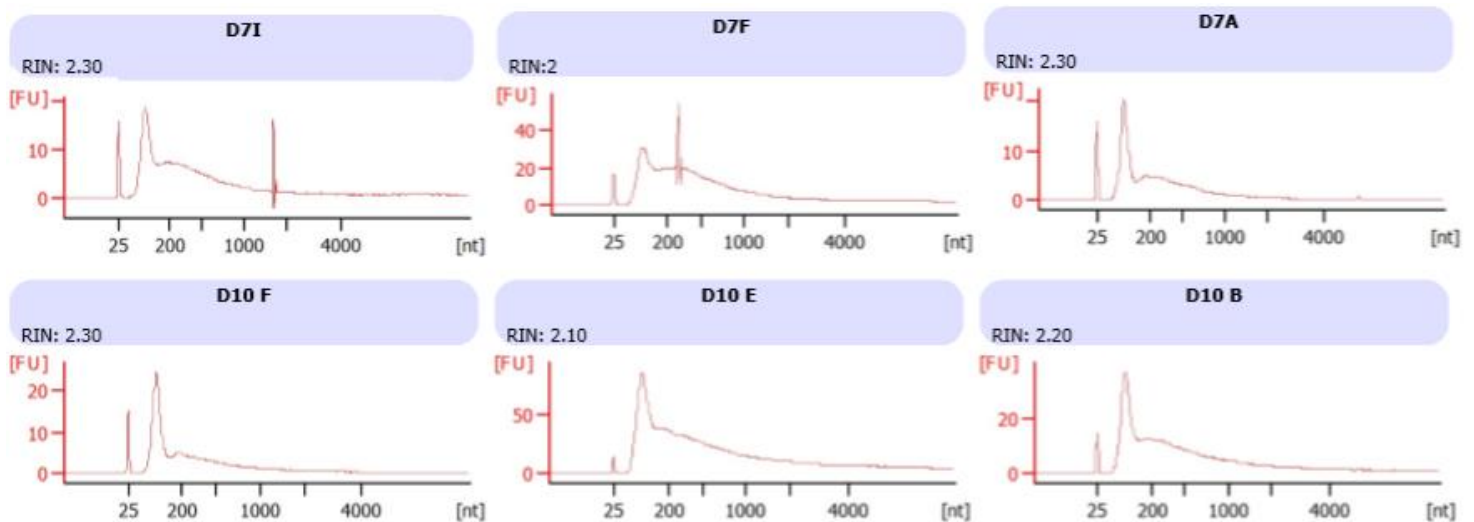

Fig.34 Agilent Analysis of amplified RNA samples. The electropherograms lose the peaks corresponding to the 18S and 28S ribosomal RNA.

It was assumed that laser capture microdissection significantly lowers the RNA integrity compared to other tissue extraction methods and it was anticipated that the RNA is to be at least partially degraded. Therefore it was assumed that the detection of large differences in gene expression might still be possible, whereas small expression differences would not be detectable due to degradation. Furthermore did the data gathered in the pilot project suggests that the influence of RNA integrity on microarray expression might not be immediately visible as you can compare whatever is left of the genes in the microarray analysis. But the influence of the RNA integrity number has to be considered in gene expression studies to avoid the risk of data extenuation.

3.5. Biotin labelling and Hybridisation of RNA samples

In order to label the RNA samples with biotin the Turbo Labelling Kit from Life Technologies was used according to the recommendations of both Life Technologies and Affymetrix. 1ug of the previously obtained amplified RNA was used for labelling and hybridisation.

RNA Sample	ng/uL	260/280	260/230
D10F	10.03	2.36	0.33
D10E	10.7	2.11	0.44
D10B	8.2	2.13	0.36
D7I	14.13	1.89	0.31
D7F	9.92	1.65	0.35
D7A	12.55	1.54	0.28

Fig.35 Nanodrop measurement of labeled RNA samples.

The RNA samples were amplified once more in another round of amplification and labeled using the biotin labelling kit. During the amplification the RNA is first reverse transcribed into complementary DNA and afterwards in vitro transcription yields biotinylated cRNA. The RNA products are purified using specific purification spin columns . After running the samples through the purification column, 150 ng of labeled RNA per sample, or about 7ug/ul of RNA in 14 ul where obtained which is significantly below the yield required for hybridization. The minimum concentration of RNA in order to hybridize the RNA to the microarray probes is usually 10 µg.

The columns used for purification are designed to clear the samples of any impurities mostly leftover biotin and any degraded RNA. It should leave only the labeled amplified RNA. When using low quantity and low quality samples it is common to lose a significant portion of the RNA through the spin column. Post-labelling purification is recommended for improved signal-to-noise ratio and lower background levels during hybridization and provides consistently superior hybridization performance after labelling of total RNA. As can be seen in figure 24 the 260/280 ratios of the labelled RNA samples are fairly regular ranging from 1.5 to 2.36 but the 260/230 ratio is appreciably lower than expected and probably indicates the presence of contaminants which absorb at 230 nm such as protein or chaotropic salts like guanidinium. If RNA yields fall below a certain RNA concentration threshold of 20 ng/µl, the 260/280 ratios are not accurate anymore. It is possible to find that the RNA peak at 260 nm is not significantly high enough to balance the 280 nm or 230 nm readings, resulting in ratios that may look right but lead in fact to false high 260 nm readings which means the concentration of the labeled RNA samples after purification might even be lower than indicated in figure 34. Since the concentrations of the labeled RNA appear to be far below the recommended minimum concentration of RNA required for hybridization with the array there is a chance that the signals received from the array of the gene expression of day 7 and day 10 would be indistinguishable from one another. Therefore the decision was reached to stop the experiment at this point in time and not to proceed to microarray analysis.

4. Discussion

Due to the inadequacy of the RNA samples at the end of the labelling step it was not possible to analyse the gene expression of the RNA samples with microarray analysis. The suitability of laser capture microdissection for gene expression studies of purified beta cell tissue suggested by Marselli et al. and other scientists could not be confirmed.

Of course there is a multitude of reasons that may account for the low amount of RNA obtained after the biotin labelling process. Here different factors will be taken into considerations that may have contributed to the ultimate outcome of this study and could be improved should the experiment be repeated again.

Laser capture microdissection presents many challenges, particularly with regard to isolating intact RNA for downstream applications such as gene expression arrays where the recommended minimum RNA integrity number is 7.0. Although frozen tissue yields more intact RNA than its formalin-fixed counterpart issues of collection (time to freezing) and storage markedly affect RNA integrity.

High RNA sample quality is essential for successful microarray experiments. RNase contamination is the number one cause for experimental failure when studying RNA for gene expression analysis. Therefore it is most important to minimize the threat of RNase contamination by protecting the RNA at all stages and in particular precautions and quality assurance steps have to be taken into consideration when proceeding through the different stages of the experiment.

Stray amplified RNA in the work area can contaminate precious RNA samples if the work area is routinely used for performing amplifications reactions.

To ensure a work area is free of amplified RNA irradiation of the work bench in the lamina hood with UV overnight every three to four days is a good way to decrease the amount of RNases present in the working area. All surfaces on which RNA purification or amplifications are performed must be cleaned with commercially available decontamination solutions first. Since Autoclaving will not kill all RNases, as they are quite stable, it is strongly recommended to use only RNase and DNase free tubes and aerosol filtered pipette tips. The safest bet is to simply use freshly opened pipette tips and tubes as the threat of contamination is small compared to tips and tubes which have been laying open in the laboratory for long periods of time. Another way to to deal with RNase contamination could be the constant and frequent use of RNaseZap (by Ambion) to clean not just the workbench but also all the pipetters needed for carrying out any of the isolation or amplification reactions. Powder free disposable latex gloves should be worn at all times when handling the RNA samples at they should be changed frequently. After putting on the gloves touching of any surfaces that may introduce RNases onto the glove surface must be avoided.

Moreover the LCM tube with captured cells should never be stored at -80°C overnight because the recovered RNA will be significantly reduced. Storage for several hours at room temperature is acceptable. On- column DNase treatment reduces the amount of recovered RNA..[51]

The cryosectioning unit should have a new blade installed and be thoroughly decontaminated of RNases before cutting and only RNase-free LCM slides, slide boxes, and reagents should be used for each sectioning run.

Despite the increasing acceptance and application of LCM, there seems to be no consensus regarding tissue preparation prior to LCM. Many sources indicated that variations in tissue preparation methods can compromise the quality of RNA by up to 75%. [52]

Preservation of RNA integrity by inclusion of RNase inhibitor into dehydration solutions for frozen tissue sections just before LCM could therefore represent a necessary step to reduce RNA degradation further.

When preparing the dehydration solutions for the LCM and the 70% ethanol solution for the RNA isolation procedure one important safety measure is to avoid RNase contamination by using exlusively freshly opened bottles of diethylpyrocarbonate (DEPC) treated water as this type of water is certain to be free of RNases.

Ultimately the integrity and the quantity of the RNA right after laser capture Microdissection will determine all subsequent steps in the presented expression protocol therefore evaluating the RNA quality by an Agilent Picochip right after the LCM preparation, after LCM itself and after RNA isolation and after RNA amplification before proceeding to the biotin labelling procedure is the best way to ensure that the RNA yield is not diminished during column purification.

5. Conclusion and Outlook

RNA is a particularly labile bio-molecule and is highly susceptible to degradation by endogenous and exogenous nucleases and to non-specific degradation by divalent cations, heat, elevations in pH, and storage of tissue or cells over extended periods prior to RNA extractions.

The proposed setup of the experiment did not reach its goal due to insufficient concentrations and integrities of the RNA samples just before hybridisation.

The establishment of an alternative protocol employing a degradation profile that defines a detection limit below which differences in gene expression are caused by RNA degradation not by gene expression could improve the applied protocol and bring about expression results despite existing minimums for RNA integrity and quantity for Microarray analysis.

An observed failure in a multi-step procedure can result from a variety of causes. Each of the steps in the procedure is associated with a possible risk to cause a failure. Therefore in order to reduce the risk it might be best to design quality control checkpoints and alternative routes for every single step in the experiment to get rid of the risk and achieve the desired outcome. The suitability of the RNA samples for microarray analysis is strongly influenced by the fact that they are from frozen neonatal tissue that was dissected using laser capture microdissection. All these factors are likely to contribute to the overall degradation of the RNA.

This is critical because in an application such as microarray analysis it is important to be sure to analyse the RNA in one piece in order not to lose specific fractions of the RNA strand.

If the setup of the experiment to study the gene expression of beta cells would be changed as to determine the presence of one particular exon within an RNA sample, it would not matter if the total RNA would have a RNA integrity number of 1. As long as the splice variant could be detected either by size or by sequencing the PCR the RIN could be between 1-5 and it still would be legitimate to conclude that the transcript is expressed in that particular tissue.

To look for individual genes would be especially sensible since certain genes like Pdx1 and NeuroD are known to be a hallmark of beta cell maturation.

Taking these steps into consideration there is little doubt that the continuation of this study will successfully characterize the gene expression of beta cells at day 7 and day 10 after birth and advance the development of functional beta cell transplants.

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