Gene expression and cell cycle regulation in human pancreas development and congenital hyperinsulinism

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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Abstract

The dynamics of β-cell mass are at the focus of an extensive international effort to develop β-cell replacement therapies for type 1 diabetes. During normal fetal development endocrine cells emerge from a pool of PDX1+/SOX9+ multipotent progenitors that transiently express the proendocrine gene NGN3. These cells become hormone-positive and are seen to bud from the ductal structures and aggregate into islet clusters.

Congenital hyperinsulinism in its diffuse form (CHI-D) is characterised by an increase in hormone-positive cells associated with ducts and diffuse patterns of insulin expression. CHI-D arises from mutations inactivating the KATP channel and is diagnosed following persistent episodes of hypoglycaemia caused by an inappropriate secretion of insulin. Whilst existing knowledge has focused on the β-cell, we have explored the histology of CHI-D across multiple pancreatic cell lineages. The starting hypothesis considered CHI-D as an over-exuberance of endocrine differentiation with a progenitor population underlying this process. We suggest CHI-D is not simply an excessive proliferation of pre-existing β-cells.

Expression of many transcription factors involved in endocrine differentiation were unchanged in CHI-D, NKX2.2 was increased and persisted in δ-cells. The incidence of nucleomegaly was also confirmed in CHI-D samples, predominantly in the β- and δ-cell lineages. Whilst increases in endocrine cell proliferation were subtle, the ductal and acinar cell lineages had significantly elevated proliferation correlating with changes in cell cycle regulation.

The expression of NGN3 was profiled in a range of human fetal samples to determine whether a competence window for endocrine differentiation exists during development. Peak expression was observed between 10-17 wpc whilst protein and transcript expression were both reduced by birth and postnatally. Combined with the data in CHI-D and postnatal controls, it is likely that endocrine commitment ceases in human towards the end of gestation and that further increases in β-cell mass rely on proliferation or NGN3-independent pathways.

These data provide new clues for the pathological mechanisms of CHI-D and the establishment and maintenance of the β-cell mass in the human pancreas. We have shown an altered potential for cell proliferation in CHI-D in previously unappreciated ways and provide a rationale for studying molecular components of the β-cell to help unlock β-cell proliferation as a therapeutic option in diabetes.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td><strong>ABCC8</strong></td>
<td>ATP-binding cassette transporter sub family C member 8</td>
</tr>
<tr>
<td><strong>ANOVA</strong></td>
<td>Analysis of variance</td>
</tr>
<tr>
<td><strong>ARX</strong></td>
<td>Aristaless related homeobox</td>
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<tr>
<td><strong>ATP</strong></td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td><strong>Ca2+</strong></td>
<td>Calcium</td>
</tr>
<tr>
<td><strong>CDKN1B</strong></td>
<td>Cyclin-dependent kinase inhibitor 1B</td>
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<tr>
<td><strong>CDK6</strong></td>
<td>Cyclin-dependent kinase 6</td>
</tr>
<tr>
<td><strong>CHI</strong></td>
<td>Congenital hyperinsulinism of infancy</td>
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<tr>
<td><strong>CHI-D</strong></td>
<td>Diffuse form</td>
</tr>
<tr>
<td><strong>CHI-F</strong></td>
<td>Focal form</td>
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<td><strong>CK19</strong></td>
<td>Cytokeratin 19</td>
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<td><strong>DAB</strong></td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td><strong>DAPI</strong></td>
<td>Diamidine-2-phenylindole</td>
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<td><strong>Dpc</strong></td>
<td>Days post conception</td>
</tr>
<tr>
<td><strong>DM</strong></td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>Embryonic day (mouse)</td>
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<td><strong>18F-DOPA PET</strong></td>
<td>18F-fluoro-L-3,4-dihydroxyphenylalanine positron emission tomography</td>
</tr>
<tr>
<td><strong>FOXA2</strong></td>
<td>Forkhead box A2</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td><strong>GATA4</strong></td>
<td>GATA-binding protein 4</td>
</tr>
<tr>
<td><strong>GCK</strong></td>
<td>Glucokinase</td>
</tr>
<tr>
<td><strong>GLUT2</strong></td>
<td>Glucose transporter 2</td>
</tr>
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<td><strong>GSIS</strong></td>
<td>Glucose stimulated insulin secretion</td>
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<tr>
<td><strong>H&amp;E</strong></td>
<td>Haematoxylin &amp; Eosin</td>
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<td><strong>HPRT</strong></td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
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<td>Immunohistochemistry</td>
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<td><strong>IF</strong></td>
<td>Immunofluorescence</td>
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<td>Insulin like growth factor 2</td>
</tr>
<tr>
<td><strong>ISL-1</strong></td>
<td>Isl lim homeobox 1</td>
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<tr>
<td><strong>K&lt;sub&gt;ATP&lt;/sub&gt;</strong></td>
<td>ATP-sensitive potassium channel</td>
</tr>
<tr>
<td><strong>KCNJ11</strong></td>
<td>Potassium inwardly rectifying channel subfamily J member 11</td>
</tr>
<tr>
<td><strong>K/O</strong></td>
<td>Knock out</td>
</tr>
<tr>
<td><strong>LCM</strong></td>
<td>Laser capture microdissection</td>
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<tr>
<td><strong>MAFA</strong></td>
<td>Musculoaponeurotic fibrosarcoma oncogene homolog A</td>
</tr>
<tr>
<td><strong>MAFB</strong></td>
<td>Musculoaponeurotic fibrosarcoma oncogene homolog B</td>
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<tr>
<td><strong>Mo</strong></td>
<td>Months</td>
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<tr>
<td><strong>MODY</strong></td>
<td>Maturity onset diabetes of the young</td>
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<tr>
<td><strong>NEUROD1</strong></td>
<td>Neurogenic differentiation 1</td>
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<td><strong>NKX6.1</strong></td>
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<tr>
<td><strong>NLS</strong></td>
<td>Nuclear location signal</td>
</tr>
<tr>
<td><strong>NS</strong></td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Term</td>
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<td>PDX1</td>
<td>Pancreatic and duodenal homeobox 1</td>
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<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
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<td>Re-Op</td>
<td>Re-operation</td>
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<td>Sry (sex determining region Y) homeobox gene 9</td>
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<tr>
<td>SS</td>
<td>Somatostatin</td>
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<tr>
<td>SUR1</td>
<td>Sulphonylurea receptor 1</td>
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<tr>
<td>TF</td>
<td>Transcription Factor</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling</td>
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<td>UCSC</td>
<td>University of California Santa Cruz, USA</td>
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<td>Wpc</td>
<td>Weeks post conception</td>
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Publications arising from this thesis


Chapter 1 - Introduction

1.1. Introduction

The pancreatic islet has been at the centre of considerable interest in recent years because of the impact knowledge of these cell types could have on regenerative therapy. By understanding the development, growth and maintenance of these cells in embryogenesis and the neonate through to adulthood it is hoped that the science will unlock pathways and targets to reinstate such processes in conditions of disease and dysfunction.

Diabetes mellitus is estimated to affect approximately 3.2 million people in the UK (QOF 2013) and is rapidly becoming a worldwide epidemic contributing to significant impacts on health, society and the economy. Diabetes results when the β-cells of the pancreatic islets are unable to produce sufficient insulin to meet the body's metabolic demands. This can either be as a result of an autoimmune disorder where the β-cells are severely reduced in number [Type 1 Diabetes Mellitus (T1DM) or Type 2 (T2DM)], or due to an insufficiency of insulin production or tissue responsiveness (T2DM).

To date, T1DM has been mostly treated by insulin injection which, whilst allowing the continuation of a relatively normal lifestyle, is not a cure. Interest has therefore been directed into regenerative medicine, and the potential to stimulate the regeneration of insulin-producing β-cells in deficient diabetic patients by reinstating the processes that occurred during in utero development.

Whilst diabetes results from an insufficiency of insulin production and a lack of β-cells, hyperinsulinism of infancy (HI) can be considered the pathological opposite. HI is defined by the inappropriate release of insulin in response to the levels of circulating glucose, resulting in a state of prolonged hypoglycaemia (Dunne, et al. 2004). Genetic mutations have been identified in approximately 50% of patients diagnosed with HI causing defects in the secretion of insulin from the β-cells and the transportation of glucose across their membrane (Dillon 2013).

The subject area for β-cell regeneration is highly contentious and as a result the surrounding literature can be conflicting with regards to the existence of a cell source capable of giving rise to β-cells postnatally. If such a population of progenitor cells were to exist we seek to discover if there is evidence for their involvement in the maintenance of β-cell mass after birth and also if there is a role for these progenitors as a cause for new β-cell growth. It is hoped that by understanding the pathways involved in embryogenesis it will be possible to activate progenitors therapeutically offering potential towards treatments for currently incurable diseases.
1.2. Role of the pancreas

1.2.1. Anatomy of the pancreas

The mammalian pancreas is a complex organ situated transversely across the posterior wall of the abdomen, behind the stomach and the peritoneum. It forms a long and irregularly prismatic shape which can vary in size between individuals from approximately 12.5 - 15cm long in the adult.

The embryonic pancreas can be first identified at 26 days post-conception (dpc) as two thickenings around the dorsal and ventral regions of the distal foregut (Piper, et al. 2004). For the next few days these dorsal and ventral buds elongate either side of the duodenum and fuse to form the single pancreatic organ (Gittes 2009; Piper et al. 2004).

![Figure 1-1. Anatomy of the pancreas](Image)

The pancreas is an unusual organ as it functions not only as an exocrine organ of the digestive system where it is considered an accessory gland along with the liver and the salivary glands, but also as an endocrine organ that secretes hormones important for the regulation of metabolism (Germann and Stanfield 2005).

The early progenitor cells that make up the primitive pancreas give rise to three differentiated components each serving their own function within the organ. The acinar
cells make up the majority of the pancreatic tissue and constitute the exocrine portion of the pancreas, secreting various enzymes into the digestive system via the duodenum. Epithelial cells form the ducts which convey the exocrine secretions. The endocrine cells of the pancreas form clusters amongst the exocrine tissue organised in micro-organs named the islets of Langerhans. The islets play a crucial role in maintaining normoglycaemia defined by the maintenance of blood glucose levels within narrow limits (4.4-6.1 mmol/L).

**Figure 1-2. Histology of the pancreas**
A magnified image showing the architecture of the mature pancreas consisting of the exocrine, endocrine and ductal compartments (Taken from Gittes, 2009).

1.2.2. Role of the exocrine cells

The majority of the pancreas is composed of pancreatic exocrine cells and their associated ducts. The exocrine tissue is made up of acinar cells that are arranged in grape-like clusters. These acini secrete a mixture of digestive enzymes into the pancreatic duct draining into the gastrointestinal tract. The exocrine cells contain membrane-bound secretory granules that hold the digestive enzymes that are released into the lumen of the acinus by exocytosis. This pancreatic juice is rich in digestive enzymes including pancreatic amylase, to break down starch and glycogen, pancreatic lipases that break down fats, a number of proteases to digest proteins, and nucleases which break down nucleic acids.

1.2.3. Role of the ductal cells

During development, fusion of the ventral and dorsal pancreatic buds is normally accompanied by the anastomosis of their respective ducts. The prominent pancreatic duct
of the dorsal pancreas fuses with that of the ventral pancreas forming a single, main pancreatic duct spanning the transverse length of the pancreas.

The pancreatic ductal system is a network of ducts converging from small intercalated ducts through a system of progressively larger ducts that eventually drain into the main pancreatic duct towards the duodenum.

The cells lining the ductal system form a simple epithelial layer of mostly columnar shaped cells. The epithelial cells of the ducts also secrete bicarbonate and water that helps to convey the enzyme-rich fluid produced by the acini towards the gastrointestinal tract and neutralise the acid secreted by the stomach as they enter the duodenum.

1.2.4. Role of the endocrine cells

The role of the endocrine pancreas is to produce and secrete a mixture of hormones involved in glucose homeostasis. Endocrine cells within the islets of Langerhans secrete their hormones directly into the blood stream. The role of the pancreatic islets therefore relies crucially on the development of an intimate blood vessel network both for monitoring the circulating glucose levels and for maintaining normoglycaemic concentrations.

1.2.4.1. Islets of Langerhans

The islets of Langerhans were first described by a German pathological anatomist, Paul Langerhans, in 1869. The pancreatic islets are responsible for maintaining appropriate levels of circulating glucose and constitute approximately 1-2% of the total combined mass of the pancreas (Naya, et al. 1997). Islets typically consist of four different secretory cell types; glucagon-producing alpha (α) cells, insulin-producing beta (β) cells, somatostatin-producing delta (δ) cells and pancreatic polypeptide (PP) cells. In recent years a fifth mammalian islet cell type, the ghrelin-producing epsilon (ε) cell has also been described (Wierup, et al. 2002). Although little is understood about the role of ghrelin in the human adult pancreas, a report by Andralojc and colleagues discovered a relative abundance of ε-cells during pancreatic development which are greatly reduced upon maturity. It is speculated that the ε-cells may play a role in the differentiation pathway for β-cells and for regulating β-cell survival and function after birth (Andralojc, et al. 2009; Granata, et al. 2007; Irako, et al. 2006).

Whilst the rodent islets appear to conform to a regular and ordered structure, with a β-cell core surrounded by a mantle of α-, δ- and PP- cells, the cells in the mammalian islets appear more disordered (Cabrera, et al. 2006). There are no obvious subdivisions within the human pancreatic islets and β-cells do not tend to form clusters, but are rather
intermingled freely with other endocrine cell types throughout the islet. Irrespective of cell type the islet cells appear located close to, and with equivalent and random access to blood vessels (Cabrera et al. 2006).

Cell composition varies strongly from islet to islet and also between pancreatic regions, as the neck of the pancreas, for example, show higher proportions of glucagon immunoreactive cells in the islets (Brissova, et al. 2005; Cabrera et al. 2006; Stefan, et al. 1982) but there is general consensus across the literature as to the relative proportions of the different cell types constituting the adult human pancreatic islet. Beta cells prevail as the most prominent, constituting, on average, approximately 70% of the islet cell population, α-cells approximately 20%, δ-cells <10%, PP-cells < 5% (Cabrera et al. 2006; Clark, et al. 1988; Rahier, et al. 1983; Stefan et al. 1982) and ε-cells approximately 1% (Wierup et al. 2002). The unique cellular organisation of human islets has implications on islet cell function and the intermingled arrangement in primates has been suggested to allow β-cells to respond to low concentrations of glucose (1mM) to which normal mouse islets cannot (Kim, et al. 2009). This could be due to the relatively large contribution of α-cells within human islets compared to those of the mouse (Cabrera et al. 2006). The differences observed between species (and in particularly between rodent and human) may reflect physiological and evolutionary adjustments in order to accommodate changes in demands for insulin (Kim et al. 2009). Humans have adapted a lifestyle where meals are eaten at intervals throughout the day whilst the mouse will graze more constantly therefore requiring a more sustained release of insulin and are less likely to experience periods of high or low blood sugar levels.

The pancreatic islets secrete hormones with opposing effects in response to changes in glycaemia. After a meal, when the blood glucose concentration increases, the β-cells secrete insulin to reduce glycaemia. Insulin stimulates the uptake of glucose by cells of the body and stimulates its conversion into glycogen by the liver. If the glucose levels fall too low, the α-cells of the islet secrete glucagon which stimulates the breakdown of glycogen back into glucose in order to raise the blood glucose concentration (Bouwens and Rooman 2005).

1.2.4.2. Beta cells

The β-cell is the most abundant islet cell type. In order to successfully perform their role in glucose homeostasis, not only do β-cells synthesise, package and secrete insulin, they also monitor the circulating glucose concentrations and ensure that this release is appropriate to the level of glycaemia.
Insulin is a dominant hormone involved in fetal growth and, as insulin does not cross the placental barrier, it is crucial that the fetal β-cells are functional from early on. Insulin deficiency during the fetal period often leads to growth retardation, whilst excessive insulin secretion can cause fetuses to be born with excessive body weight, a term known as macrosomia (Fowden 1989).

Insulin is the first hormone to be detected within the pancreas in human fetal development at 8 weeks post-conception (wpc), and remains the most prevalent of the islet hormones during early development (Clark and Grant 1983; Piper et al. 2004; Stefan et al. 1982). For these first few months fetal insulin secretion is influenced by the surrounding concentrations of amino acids as glucose levels are low (Eriksson, et al. 1980). It is only later in gestation, during the last trimester of pregnancy, that the fetal β-cells become progressively more responsive to glucose (Dunne, et al. 2004). Despite this relatively late maturity for glucose responsiveness evidence for mRNA of the key components of glucose sensing apparatus within the β-cells has been identified from 7-10 wpc (Dunne et al. 2004).

At birth the newborn must become independent in sensing and producing glucose and maintaining a glycaemic homeostasis. The glucose sensing and releasing apparatus of the mature β-cell include the glucose transporters GLUT1 and GLUT2 (although GLUT1 appears to be the major isoform in the mammalian pancreas (Ferrer, et al. 1995)), glucokinase (GCK), which is involved in metabolising the glucose, ATP-sensitive potassium channels (K<sub>ATP</sub>) and voltage-gated calcium channels (Best, et al. 2008; Dunne et al. 2004; Richardson, et al. 2007). Within the granules secreted by the β-cells are a variety of factors that are necessary for processing the insulin. Insulin is initially synthesised as proinsulin which is cleaved into proinsulin in the rough endoplasmic reticulum of the β-cell. Proinsulin is packaged into immature granules within the golgi network and is consequently matured by the action of the enzyme prohormone convertase 1/3 (PC1/3) into active insulin. C-peptide is co-secreted in equimolar amounts as a by-product (Best et al. 2008; Goodge and Hutton 2000; Steiner 1998). Also secreted in the granule is islet amyloid polypeptide (IAPP) however there is a lack of compelling data to suggest a specific physiological role for this protein (Brass, et al. 2010). These additional factors provide a selection of markers that can be targeted in order to judge the phenotype and maturity of differentiated β-cells.

1.2.4.2.1. Glucose monitoring and insulin release

A unique feature of the pancreatic β-cell is its ability to monitor and respond to changes in the circulating glucose concentrations in order to maintain normoglycaemia.
Glucose is the major physiological stimulus for initiating insulin secretion which is triggered by circulating blood sugar levels exceeding 5.5mM (Dunne et al. 2004). The β-cell secretes insulin in a “first-phase” and “second-phase” manner (Dunne et al. 2004). The first phase of insulin release is glucose-stimulated and follows the transport and subsequent metabolism of glucose within the β-cell.

The glucose sensing phenotype of the β-cell is derived, in part, by the transport of glucose across the β-cell membrane, facilitated by the glucose transporter GLUT1 and/or GLUT2 and, particularly in humans, the subsequent phosphorylation of glucose by GCK producing glucose 6-phosphate which is incapable of leaving the β-cell (Best et al. 2008).

The successive rise in intracellular glucose generates an increase in ATP production which in turn closes the $K_{\text{ATP}}$ channels causing depolarisation of the cell membrane. Voltage-gated calcium channels determine the metabolic sensing of glucose by the β-cell. When activated these channels allow an influx of calcium that promotes release of insulin by the fusion of the secretory granule with the plasma membrane (Best et al. 2008; Dunne 2000). The first insulin released is likely to be pre-formed insulin-containing granules of which there are several hundred present in each β-cell (Dunne et al. 2004).

![Figure 1-3: Glucose-stimulated insulin secretion of pancreatic β-cell](image)

A schematic diagram showing the process of glucose-stimulated insulin release from the β-cell. (Adapted from Dunne et al. 2004).

The second phase of insulin secretion remains poorly understood but is believed to result from a gradual augmentation and potentiation of calcium-triggered insulin release, again involving the $K_{\text{ATP}}$ channel. However, following the initial secretion of the pre-formed insulin granules in the first phase, the continued stimulus on the β-cell triggers a process involved with preparing the previously non-releasable granules for exocytosis known as “priming” (Dunne et al. 2004).
When the mechanisms that control insulin release are compromised, potentially lethal disorders such as diabetes and hyperinsulinism can manifest (Dunne et al. 2000).

1.2.5. Dysfunction of the beta cell

1.2.5.1 Diabetes

The term “diabetes” encompasses a common group of heterogenous disorders characterised by hyperglycaemia. Diagnosis usually follows a glucose tolerance blood test, either in a fasted state (fasted plasma glucose) greater than 7.0 mmol/L, or following an oral glucose challenge with a two hour plasma level greater than 11.1 mmol/L. An HbA1c test can also be used with diagnosis following a result exceeding 6.5% saturation (Kirkman and Kendall 2010). Diabetes can occur as a consequence of absolute β-cell loss usually a result of autoimmune destruction (T1DM), or simply by insufficient β-cell function and inadequate insulin secretion and/or diminished tissue response to insulin (T2DM) (Kaneto, et al. 2005).

T1DM is the most common presentation among children and adolescents and can be characterised generally by its rapid onset and dependence on exogenous insulin. In most cases T1DM is a complex disorder involving multiple genes and environmental factors. Classic symptoms include polyuria, caused as a result of dehydration due to osmotic diuresis. This in turn stimulates thirst and polydipsia. Other symptoms include polyphagia and weight loss with elevated blood glucose levels on testing (Roche, et al. 2005). Whilst current insulin treatment permits the patient to live a relatively normal lifestyle associated problems with diabetes include cardiovascular disease, retinopathy, neuropathy and other end-organ damage which are accelerated by poor glycaemic control (Ali 2010).

The typical T2DM patient is overweight and often without symptoms. Some can present with hyperglycaemic conditions similar to those seen in T1DM but more often it is diagnosed incidentally by screening or medical examination for other conditions. T2DM can typically be improved with exercise and diet control although therapeutics can also be initiated to help control glycaemia. Similar cardiovascular risks to those experienced in T1DM exist, often potentiated by the period of elevated glucose levels that often precede diagnosis (Fonseca and John-Kalarichal 2010).

As an epidemic the impact of diabetes is substantial, not only on health but also the socio-economic costs associated with the care and treatment of patients. As a result there has been significant focus in the field of diabetic research and finding more sustainable and effective therapy for the disease. Transplantation of whole pancreata has been explored with some success (Farney, et al. 2000), but is restricted by the high invasiveness or surgery and post-surgical requirement for immunosuppression. Likewise, isolated islet
transplant has also been explored (the Edmonton protocol). Shapiro et al. were able to provide the most promise for the disease so far by successfully transplanting β-cells into a group of seven patients (Shapiro, et al. 2000). Whilst promising, the protocol again requires a level of immunosuppression following transplant and both procedures suffer a substantial deficit in the large and continued supply of cadaveric material that is necessary to treat DM patients. The demand for DM therapies is simply too great for the supply to sustain and is therefore not a reliable therapy option for the majority of patients.

1.2.5.2 Hyperinsulinism of Infancy

Congenital hyperinsulinism of infancy (CHI) is a rare condition where inappropriate insulin release from the pancreatic β-cells results in persistent or recurrent episodes of hypoglycaemia.

Most cases of CHI have been attributed to a mutation in the genes encoding subunits of the \( \text{K}_{\text{ATP}} \) channel within the pancreatic β-cell which ultimately alters the ability to monitor and regulate insulin release (Dunne, et al. 1997). However, in 40-50% of CHI cases, the genetic cause is unknown (Dillon 2013; Flanagan, et al. 2011).

The histological features of CHI can be subdivided into two major forms; Diffuse (CHI-D) and Focal (CHI-F) (Jaffe, et al. 1980; Rahier, et al. 1984). CHI-D is the most common form, where all the β-cells throughout the pancreas are affected by an abnormality in function. CHI-F is generally most easily recognized as it consists of an adenomatous hyperplasia of islet cells in a localised region of the pancreas (Rahier et al. 1984) whereas the rest of the pancreas appears normal for its age (Kassem, et al. 2000).

For a high proportion of CHI patients, medical therapy is of limited use (Dunne, et al. 1999; Glaser, et al. 1999). As a result, the only potential for curing the disease is near-total or total pancreatectomy.

The condition of CHI is explained in more detail later.
1.3. Lessons learned from development

1.3.1. Morphological development

The embryonic pancreas develops from a pool of apparently identical progenitor cells and transforms into the adult pancreas composed of three distinct cell types; exocrine, endocrine and ductal (Gu, et al. 2002). The architecture of the mature pancreas is formed from a series of morphological events and complex tissue interactions that are carefully orchestrated throughout embryonic organogenesis.

Cells fated to form the pancreas arise from the endoderm of the primitive gut tube (Wells and Melton 1999). The region of the endoderm at the foregut-midgut junction contains cells already committed to a pancreatic fate marked by the expression of the pancreatic and duodenal homeobox factor-1 (PDX1) prior to any observation of morphological changes (Bouwens and Rooman 2005; Puri and Hebrok 2010).

In humans the first visible evidence of the pancreas presents as a dorsal outgrowth from the gut endoderm apparent at 26 dpc, a structure known as the dorsal bud (Gittes 2009; Piper et al. 2004). Six days later an outgrowth on the opposite aspect of the endoderm, the ventral bud, begins to evaginiate into the mesenchyme (Gittes, 2009). During embryogenesis these buds undergo elongation and rotation behind the duodenal loop before fusing with each other by 56 dpc, which marks the end of the embryonic period and the formation of the single pancreatic organ (Gittes, 2009; Piper et al. 2004).

Little cellular ultrastructural differentiation is seen in these early periods of development (Gittes, 2009). During the initial stages of bud elongation the epithelial cells of the pancreas are arranged as simple tubular structures within a loose mesenchymal stroma. It is only from the commencement of the fetal period that they become apparent as more branched epithelial clusters (Piper et al. 2004).

Each bud develops independently of the other, developing their own exocrine and endocrine compartments and individual branching networks of the ductal system that will conjoin upon organ fusion at 56 dpc (Jensen 2004).

Cells fated down the endocrine lineage delaminate from the developing epithelium, whilst the early exocrine cells remain within the epithelium (Puri and Hebrok, 2010), although it is ultimately the regulation of gene transcription that will establish the mature pancreatic cells and their unique phenotypes (Wilson, et al. 2003).

Insulin is the first hormone to be expressed in the developing human pancreas, evident as positive immunoreactive staining in rare individual epithelial cells at 52 dpc (Piper et al.
By 8.5 wpc both glucagon and somatostatin are also identified by separate expression within isolated epithelial cells (Piper et al. 2004).

Islets form relatively early in human gestation compared to that of the rodent (Piper et al. 2004). At commencement of the fetal period insulin cells form clusters up to 5 cells in diameter and are the more distinct of the pancreatic cells. As a result, insulin remains the most prevalent of the hormones, with its expression approximately 13 fold greater than that of glucagon which is subsequently greater than somatostatin expression (Piper et al. 2004). By 10 wpc the functional endocrine pancreas is completed by the expression of PP and with continued development, the number of hormone positive cells increases (Piper et al. 2004).

Whilst the expression of insulin within clusters of hormone positive cells suggests relatively mature β-cells by the end of the first trimester, their function as endocrine cells depends on the coordinated development of an intricate blood vessel network (Piper et al. 2004). In the early stages of development scattered insulin positive cells are not in particularly close association with vascular endothelial cells. The primitive islet structures of the pancreas become evident between 12 and 13 wpc when aggregations contain cells independently expressing all 4 of the islet hormones (Bouwens and Rooman 2005; Falin 1967; Piper et al. 2004). Developing vessels finally penetrate the fetal islets at 14 wpc (Piper et al. 2004).

In the rat the fastest expansion of β-cell mass occurs from E16 where doubling of the β-cell population can be observed each day (Bouwens and Rooman 2005). In human fetal development the β-cell population is seen to expand from 8 wpc as cell clusters of the primitive islet increase from five cells to a maximum of 20 cells in diameter by 12 wpc. The islet size continues to increase during the 2nd and 3rd trimester of pregnancy (Piper et al. 2004). As only a limited number of β-cells (<10%) show mitotic activity, it is believed that this increase is due to neogenesis and rapidly proliferating undifferentiated precursors rather than cell division alone (Bouwens and Rooman 2005).

1.3.2. Endocrine differentiation

It is ultimately the regulation and timely expression of gene transcription that provides the mature pancreatic cells with their unique phenotypes (Wilson et al. 2003). Simply cataloguing the different transcription factors involved does not delineate the differentiation process. It is through intense research that a hierarchy of transcriptional factors has emerged for regulating the transition from pancreatic epithelial cells to adult cell lineages (Wilson et al. 2003).
All pancreatic cell types arise from a common pool of cells that contain the homeodomain transcription factor PDX1, the basic helix-loop-helix (bHLH) pancreas-specific transcription factor 1A (PTF1A) and Sry Box 9 (SOX9) positive cells of the early pancreatic buds, including the acinar and ductal cells of the exocrine pancreas as well as the endocrine lineages constituting the islets of Langerhans (Akiyama, et al. 2005; Gu et al. 2002; Kawaguchi, et al. 2002; Seymour, et al. 2008).

Whilst PDX1+ progenitors have been shown to contribute to all pancreatic lineages, PDX1+ cells are more likely to become duct cells before E12.5, with acinar or islet cells predominantly arising before E8.5 or after E12.5 (Gu et al., 2002). SOX9 is excluded from endocrine lineage-committed progenitors and differentiated cells, and becomes restricted to cells of the ductal system in humans after birth (Seymour et al. 2007, 2008) where weak PDX1 staining has also been observed (Piper et al. 2004). This ductal compartment has been suggested as a source of endocrine differentiation-competent progenitors in the mature pancreas (Bonner-Weir, et al. 2000; Seymour et al. 2008; Xu, et al. 2008) but is still subject to much controversy and continued investigation.

The endocrine portion of the pancreas diverges early before the onset of hormone expression by the presence of transcription factors (Herrera 2002). During fetal development a subset of epithelial progenitors positive for PDX1 and SOX9 give rise to cells that transiently express the bHLH transcription factor neurogenin3 (NGN3) committing cells to an endocrine fate (Gradwohl, et al. 2000). Notch signalling determines which cells from the multi-potential progenitor pool will differentiate into an endocrine lineage, and normally inhibits NGN3 expression (Wilson et al. 2003).

Without Ngn3 expression islet differentiation fails in the mouse (Gradwohl et al. 2000) and the cells are fated to an exocrine or ductal lineage with further factors required to drive the differentiation, including the former multi-potential progenitor marker PTF1A (Murtaugh and Kopinke 2008). Unlike islet cells, which are generally considered post-mitotic and do not have a tendency to divide in utero, newly-formed acinar cells are highly proliferative and rapidly expand to become the most abundant pancreatic cell-type by birth (Jensen 2004; Murtaugh, et al. 2008; Pictet, et al. 1972).

Whilst NGN3 expression is both necessary and sufficient to initiate pancreatic endocrine differentiation, further factors are required downstream of the NGN3 positive precursors in order to control the decision of islet cell specification towards one of the five different endocrine cell types (Wilson et al. 2003). A number of transcription factors that are selectively expressed in the developing endocrine lineage could play a role in fate determination including a large number of homeodomain proteins. These include: PAX4, NKX2.2, and NKX6.1 which are co-expressed with NGN3 and therefore considered early factors, and NEUROD1 which is expressed slightly later than NGN3 and persists in
mature islet cells. PAX6, ISL1 and PDX1 are considered late factors and appear to have involvement in the maturation of the endocrine cells (Wilson et al. 2003).

There is controversy about the subsequent pattern of transcription factor expression or their interactions during endocrine differentiation. The roles of many of these factors have been attributed through various knock-out experiments in mice and other animals. A generally well conceived version of the proposed cascade of genetic expression has been devised by Wilson et al. (2003) and is shown in figure 1-4, followed by a brief description of each of the key transcription factors involved in β-cell differentiation.

Figure 1-4: Endocrine pancreas differentiation during fetal development
An illustration showing the simplified hierarchy of transcription factor expression in developing pancreas. The model does not strictly represent the signalling pathways but rather a consensus of gene expression toward the terminal differentiated state (Habener, et al. 2005; Wilson et al. 2003).

NEUROD1 is believed to hold a crucial role in regulating pancreatic islet development as well as having a role in insulin gene transactivation (Itkin-Ansari, et al. 2005; Wilson et al. 2003). Overexpression and targeted disruption studies have helped to establish this developmental role with gene knock-out mice showing mild and moderate reductions in δ- and α-cells respectively and a severe loss of β-cells (Naya, et al. 1997). NEUROD1 is predominantly restricted to β-cells in the adult pancreas (Itkin-Ansari et al. 2005).

PAX4 is an early factor important for the full differentiation of β-cells (Ketola, et al. 2004). It is selectively expressed in the developing islets and later restricted to endocrine cells (Dohrmann, et al. 2000; Smith, et al. 1999). In knock-out models mice develop severe diabetes as a result of alteration in islet development characterised by a deficiency in β- and δ-cells and an increase in α-cells (Sosa-Pineda 2004).
MAFA plays a role in islet differentiation, probably by direct interaction with PDX1, but null mice showed no defects in β-cell development (Artner, et al. 2006). MAFA is, however, present in the first insulin positive cells of the fetal pancreas and is maintained through to adulthood (Matsuoka, et al. 2004; Oliver-Krasinski and Stoffers 2008).

MAFB is expressed in both β- and α-cells early in differentiation, but becomes restricted to α-cells postnatally (Artner et al. 2006; Oliver-Krasinski and Stoffers 2008).

NKX2.2 can be identified early in the epithelium of the pancreatic bud (Oliver-Krasinski and Stoffers 2008; Sussel, et al. 1998) then becomes localized to NGN3 progenitor cells. It persists in mature endocrine cells including β-cells, but also the α- and PP-cells. Nkx2.2-null mice lack β-cells and no NKX6.1 is apparent either, suggesting a regulatory role for Nkx2.2 upstream of Nkx6.1 (Sussel et al. 1998).

NKX6.1 is expressed in NGN3 positive endocrine precursors before becoming restricted to pancreatic β-cells where it is maintained postnatally (Oliver-Krasinski and Stoffers, 2008).

PDX1 not only has an early role in pancreatic bud formation, but also in the final differentiation of mature islets. After early expression PDX1 is down-regulated and becomes undetectable in NGN3+ progenitors (Schwitzgebel, et al. 2000). It is reactivated in most differentiated β-cells where it plays a role in maintaining the differentiated phenotype (Wilson et al. 2003). PDX1 also functions in mechanisms of insulin gene transcription as well as having a role in modulating transcription of other genes preferentially expressed in pancreatic β-cells, including the GLUT2 transporter, GCK, and the SUR1 and Kir6.2 proteins (Dunne et al. 2004). Postnatally in humans PDX1 is most strongly detected in the pancreatic β-cells but has also been reported within ductal cells (Piper et al. 2004).

GATA factors are from a family of zinc finger proteins that regulate gene expression, development and cell proliferation in a variety of tissues (Ketola et al. 2004). In the mouse GATA4 is required for pancreatic development whilst its role in humans is not as clear. GATA4 does not appear to have an essential role in pancreatic organogenesis but may play a role in gene expression and function of the exocrine pancreas (Ketola et al. 2004). GATA4 has early and widespread expression in the developing human pancreas but is later restricted to the exocrine pancreas (Shaw-Smith, et al. 2014). GATA6 is preferentially expressed in the endocrine pancreas and may have a distinct role in the differentiation and function of the endocrine pancreas, especially during postnatal development but this is not well understood. More recently data has emerged indicating a role for GATA4 in endocrine neogenesis and normal pancreas formation following the
identification of mutations in the GATA4 gene causing neonatal-onset and childhood diabetes with or without exocrine insufficiency (Shaw-Smith et al. 2014).

1.3.2.1. Neurogenin factors

Neurogenin3 (NGN3) is a member of the neurogenin family of basic helix-loop-helix (bHLH) genes which have been shown to play critical regulatory roles in neural stem cell differentiation. Proteins of the bHLH family are important transcriptional regulators in determining cell fate and differentiation in a wide variety of tissues and developmental stages in multicellular organisms (Hassan and Bellen 2000; Massari and Murre 2000). Three NGN proteins have been described in the mouse; NGN1, NGN2 and NGN3 (Ma, et al. 1996; Sommer, et al. 1996). Whilst NGN1 and NGN2 show involvement in the development of several neuronal lineages (Fode, et al. 1998), NGN3 shows a similar precursor selection role in the mammalian pancreas. NGN3 is transiently expressed during pancreas development controlling endocrine fate decisions from multipotent progenitors (Gradwohl et al. 2000).

It is well established that NGN3 plays an essential role in determining which PDX1+/SOX9+ multipotent progenitor cells will eventually become islets. When expressed, NGN3 marks a population of cells that are in transit from undifferentiated progenitors to mature endocrine cells. Lineage tracing experiments in mice reveal that all endocrine cells are derived from NGN3+ precursors (Gu et al. 2002) and that when Ngn3 is deficient islet differentiation is virtually abolished and islets of Langerhans fail to form (Gradwohl et al. 2000; Wang, et al. 2009a). Further evidence for the importance of NGN3 in endocrine differentiation emerges when ectopic expression of Ngn3 is able to convert early endodermal progenitors into hormone-positive islet cells at the expense of exocrine differentiation (Apelqvist, et al. 1999; Johansson, et al. 2007; Schwitzgebel et al. 2000). These experiments show that Ngn3 is both necessary and sufficient to drive the formation of islet differentiation during pancreatic development. These transient NGN3+ cells can be found to coexpress some markers of differentiating endocrine cells including the transcription factors PAX6, NEUROD1, NKX2.2 and NKX6.1 but do not express islet hormones or markers of mature endocrine cells (Apelqvist et al. 1999; Gradwohl et al. 2000; Jensen, et al. 2000; Schwitzgebel et al. 2000).

Despite this, knowledge of the NGN3-regulated programme is still somewhat incomplete. NGN3 expression appears to depend on gross positional cues and local signals that limit its expression to only specific cells. Active Notch signalling pathways usually maintains the multipotency of pancreatic progenitor cells and prevents NGN3 expression in most but a few scattered cells within the developing pancreas which become NGN3+ as a result of
inhibition of this Notch signalling (Apelqvist et al. 1999; Jensen et al. 2000). When the Notch signalling pathway is disrupted within the pancreas premature and expanded expression of NGN3 can be observed resulting in accelerated endocrine differentiation (Apelqvist et al. 1999; Jensen et al. 2000). However the positive signals driving NGN3 expression in the pancreas are less well understood.

The proendocrine Ngn3 gene is found on chromosome 10 and is thought to control a complex network of transcription factors leading to the mature islet cell, including Arx, Pax4, Pax6, NeuroD1, Nkx6.1 and Nkx2.2 (Gasa, et al. 2004). These factors are downregulated when Ngn3 is knocked-down (Gradwohl et al. 2000) but when Pax6, NeuroD1, Nkx6.1 or Nkx2.2 are knocked-out Ngn3 expression is still present (Schwitzgebel et al. 2000). This suggests a downstream position of these factors as well as a necessity for Ngn3 in initiating endocrine differentiation. Whilst it has not yet been shown that NGN3 binds to the promoter region of these downstream gene targets in vivo, there is the possibility that the expression of Ngn3 permits the opening of the chromatin where these genes are found, permitting transcription during the transient NGN3 expression window (Soyer, et al. 2009). Whilst NGN3 initiates the process of endocrine differentiation commitment to the specification of one of the five islet cell types appears tightly controlled by both temporal regulation and the ensuing expression of downstream genes (Johansson et al. 2007).

NGN3 is first detected in the mouse epithelium at E9 (Apelqvist et al. 1999; Jensen et al. 2000; Schwitzgebel et al. 2000; Sommer et al. 1996). This NGN3 expression is seen to peak around E15.5 after which it is downregulated and other factors takeover such that by E17.5 and in the neonatal pancreas only a few NGN3+ cells can be observed (Apelqvist et al. 1999).

There are contradictory findings regarding the persistence of NGN3 expression in the adult pancreas. IHC observations have concluded that NGN3 expression is turned off in the postnatal mouse pancreas (Jensen et al. 2000; Schwitzgebel et al. 2000) however, transient and/or low expressing cells may have escaped detection by this method. Several reports have since identified Ngn3 transcripts in wild-type (WT) isolated adult pancreatic islets (Dror, et al. 2007; Gu et al. 2002; Wang et al. 2009a) and Xu et al have demonstrated that regenerative conditions can cause a modest but measurable induction in Ngn3 transcript and protein expression (Xu et al. 2008). However, these analyses have not yet established the profile for this Ngn3 expression; whether it is restricted to only a few putative endocrine progenitor cells or whether Ngn3 is maintained in differentiated islet cells and to what purpose this may have on endocrine function. In a report by Wang and colleagues a measurable presence of Ngn3 transcripts was observed in the postnatal pancreas that was associated with the expression of several genes, including Insulin,
MafA and, to a lesser extent, Nkx6.1 and NeuroD1. When Ngn3 was inactivated in these mature cell populations a downregulation of these genes was observed. The exact function of Ngn3 after birth is not known, however emerging evidence suggests that these transcripts may maintain chromatin structure within cells, permitting the expression of key genes that are needed for islet cell function (Wang et al. 2009a).

1.4. Maintenance of islets postnatally

1.4.1 Postnatal islet cell “replication” vs “differentiation” vs “trans-differentiation”

There is considerable interest to be had in understanding how β-cells maintain their mass postnatally. It is important that there is a balance in the total population of the β-cell; too many can be just as serious as too few, resulting in hypo or hyper-glycaemia respectively. It has been proposed that the β-cell mass is dynamic from birth and into adulthood in order to maintain glucose metabolism and is now generally well accepted (Bonner-Weir, et al. 2004). Under normal and pathophysiological conditions a variety of mechanisms could be involved in mass dynamics, including changes in cell replication, neogenesis, changes in individual cell volume and changes in the cell loss or death rates within the tissue (Bonner-Weir, et al. 2010). By understanding the underlying processes it is conceivable that a best strategy could be devised to minimize β-cell loss and stimulate β-cell regeneration as a potentially curative treatment for diabetes (Hanley, et al. 2008).

As with many other tissues and organs, after birth there is evidence in support of the β-cell population growing postnatally, at least until adolescence (Bonner-Weir et al. 2010). In the initial month of life in the rat the β-cell mass increases with body weight, the result of increased β-cell number and size (Bonner-Weir et al. 2010; Montanya, et al. 2000). There is also evidence to suggest an adaptive mechanism of the β-cells to change their size, number and function in response to a variety of conditions including pregnancy, obesity and conditions of insulin resistance (Genevay, et al. 2010; Khalaileh, et al. 2008). It has long been known that rodents undergo rapid and substantial β-cell growth due to hypertrophy and hyperplasia in pregnancy (Parsons, et al. 1992). However, a more recent investigation in humans by Butler and colleagues, whilst observing only a slight increase in β-cell density, accounted this growth to neogenesis rather than replication (Butler, et al. 2010). This neogenesis was concluded by the observation of an increased number of scattered and duct-associated β-cells in the absence of detectable changes in β-cell replication or apoptosis (Genevay et al. 2010).
Assuming a dynamic capacity of the adult pancreas, the mechanisms by which this maintenance occurs and the identity of the cell type from which new β-cells derive has been the subject of much controversy in recent years. There are generally two viewpoints that divide this area of research, the concept of replication from existing β-cells and that of neogenesis.

It is well established that all cell types of the mature pancreas, including the islets and their components, are generated by differentiation from PDX1- and PTF1A- and SOX9-positive progenitors during embryogenesis. However, throughout the course of development, neither PDX1 nor PTF1A are exclusive indicators of progenitor cells as each becomes a marker of mature pancreatic cell types (Seymour et al. 2008). It is therefore still unclear as to whether multipotential progenitor cells persist in the mature pancreas, and how to define them. The ductal compartment has been the focus of significant interest and suggested to harbour endocrine-differentiation competent progenitors (Bonner-Weir et al. 2000; Xu et al. 2008).

SOX9 is coexpressed with PDX1 in undifferentiated multipotent progenitors during pancreatic development. The Sox genes have been implicated as maintaining multipotent states in many tissues, and Sox9 fulfils this role in the embryonic pancreas (Seymour, et al. 2007). The persistent expression of SOX9 and the reports of PDX1 observation in adult pancreatic ductal cells (Piper et al. 2004) raises the possibility that these factors continue to define a progenitor population in the adult pancreas (Seymour et al. 2008). It has been hypothesized that ductal epithelial cells can transiently dedifferentiate and serve as multipotential progenitor cells (Seymour et al. 2007), and that β-cells can be generated in the adult pancreas from transient endocrine progenitors positive for NGN3 (Gradwohl et al. 2000; Gu et al. 2002). Formation of β-cells is absolutely dependent on NGN3 expression (Gradwohl et al. 2000), and is therefore a crucial indicator of neogenic activity. There have been contradictory reports on the presence of NGN3 in the postnatal pancreas. Many have determined it to be undetectable after birth in humans (Jensen et al. 2000; Lee, et al. 2006; Schwitzgebel et al. 2000) whilst others have argued that it may be expressed albeit transiently and/or in small amounts, seemingly undetectable by IHC study. Publications have since reported detection of Ngn3 by RT-PCR being isolated from whole islets of 8 week old mice (Gu et al. 2002). Even in light of this evidence the migration of NGN3 positive cells from the ductal compartment to existing islets cannot be ruled out, nor is the possibility that transcripts remain without protein accumulation postnatally. On top of this there is still no further understanding of the cell of origin that could serve as a progenitor in the postnatal pancreas. As a result there is still a lot to be learned from the expression profile of Ngn3 in the mature pancreas, and whether it continues to define a marker of endocrine differentiation or has some alternate role postnatally.
The first solid demonstration of \( \beta \)-cells being generated from NGN3 positive embryonic-type endocrine progenitor cells in the adult pancreas came from Xu et al. (2008). A pancreatic duct ligation model has previously been shown to cause \( \beta \)-cell hyperplasia (Rosenberg 1998) and increases in islet cell mass and duct proliferation (Wang, et al. 1995). Following the same model with genetic tracing, Xu et al. (2008) demonstrated that NGN3 was strongly induced in the ductal cells.

Further evidence for assuming a process of neogenesis to be occurring postnatally is largely based on histological observations of new islets described to be “budding” from ducts which has been seen after birth and express similarities to the embryological events of islet generation (Bonner-Weir et al. 2004; Gu et al. 2002).

Whilst the proposal of progenitor recruitment and embryogenesis being reinstated postnatally is appealing, mechanisms of growth and mass maintenance by other processes such as proliferation of existing cells cannot be ruled out.

Those who counteract the neogenesis argument favour the view that increases seen in \( \beta \)-cell mass are due to replication of existing \( \beta \)-cells. The groups of both Dor and Teta have offered significant contribution to this concept with lineage tracing experiments and measurements of proliferative capacity in ageing rodents. Neither group found any conclusive evidence in favour of neogenesis and therefore concluded that growth seen in the first year of life is due to the proliferation of existing \( \beta \)-cells only (Dor, et al. 2004; Teta, et al. 2005).

Following a 90% pancreatectomy in adult rats there is extensive regeneration of the remnant organ with eightfold and six-fold increases in endocrine and exocrine pancreas respectively after 4 weeks (Bonner-Weir, et al. 1993). This is a less robust process than the liver is able to demonstrate, as the regrowth is proportionate to the level of excision (and a near total pancreatectomy is required to see a marked growth response) but does offer promise that the pancreas has capacity at least for modest regeneration (Bouwens and Rooman, 2005).

The experiments by Dor and Teta only used 50-70% pancreatectomy and failed to show any occurrence of neogenesis in their regeneration process. It may be that the extent of excision used in these experiments may not have been sufficient to stimulate a differentiation pathway, assuming replication may therefore have been a more favourable mechanism for regeneration in this instance (Dor, et al. 2004; Teta, et al. 2005).

Further problems in extrapolating regenerative data come from the investigative models used.
Evidence from these studies arise close to entirely from models of rodent regeneration that often require pancreatic injury to yield a quantifiable response. The evidence is persuasive that, under normal circumstances and some pathological scenarios, β-cell mass can be maintained by regeneration from duct-like cells as well as by β-cell proliferation (Bonner-Weir et al. 2000; Xu et al. 2008).

However, the reliability of extrapolating such data towards human capacity is restricted by the heightened proliferative capacity of the rodents compared to that of humans (Forsyth, et al. 2002). Mice often die as the result of tumour formation (Lipman, et al. 2004). With evolution humans have developed an anti-tumour defense as a consequence of their much larger bodies and extended life span, resulting in cells with lower rates of proliferation. In the rodent, however, telomerase expression persists and their cells continue to have long telomeres (Wright and Shay 2002). In the context of β-cell mass maintenance it may be feasible that mouse models tend to respond to injury by proliferative restoration whilst humans might have more tendency towards differentiation pathways from stem cell or precursor cell-types under the same conditions (Hanley et al. 2008).

It could be viable that replication and neogenesis are not mutually exclusive. It may be possible that both processes can occur simultaneously but that different stimuli are required to initiate each (Bonner-Weir et al. 2010).

### 1.4.2. Cells that have potential as progenitors

There are several tissues throughout the human body that are well documented for their continuous and rapid turn-over of cells such as the skin and the intestine. Tissue-specific stem cells often account for such features and are a key area of interest for regenerative medicine.

The pancreas shows intriguing similarities with the liver, which is not entirely surprising given the two organs both develop from the same endodermal origin. Following partial hepatectomy the liver is able to rapidly regenerate by proliferation of surviving hepatocytes. If this process is restricted, the liver will adopt a regeneration programme based on stem-cells which are able to proliferate and differentiate into functional hepatocytes to restore the lost mass (Fausto and Campbell 2003; Khalaileh et al. 2008; Li, et al. 2010).

It is now evident that terminal differentiation, long thought to be an irreversible process can be reversed in cells from an adult animal. The process described as trans-differentiation means that fully differentiated cells can change fates from one cell type to
another, for example exocrine to endocrine, α- to β-cell, hepatocyte to pancreatic cell (Li et al. 2010; Puri and Hebrok 2010).

Ferber and coworkers demonstrated that ectopic expression of a single transcription factor, Pdx1, delivered by adenovirus to mice is sufficient to induce mature and biologically active insulin production in the liver. Whilst it is not fully understood which cells are undergoing this transformation, there is possibility that it may have occurred in a multipotent population that are able to change their fate by expression of a single gene (Ferber, et al. 2000; Puri and Hebrok 2010).

The pancreatic α-cell is functionally very similar to the β-cell and shares many of the same components in glucose metabolism, making it an appropriate candidate for reprogramming to a β-cell phenotype.

Thorel and colleagues were able to demonstrate a trans-differentiation from adult α- to β-cell in a series of experiments generating near-total β-cell loss by administration of diptheria toxin to mice. Rapid increases seen in β-cell mass within the first month of ablation are attributed to non β-cell origins due to the almost negligible remaining population of β-cells and their low rates of proliferation. Following lineage tracing experiments Thorel’s group showed that 65% of insulin positive cells one month after ablation contained a marker for the adult α-cell (Thorel, et al. 2010).

Similarly, Zhou and co workers recently published data unveiling an ability of the pancreatic acinar cells to be remodelled into functional β-cells in vivo. Adenoviral infection with three transcription factors, Pdx1, Ngn3 and MafA appeared sufficient to induce β-cell formation in mice. The generated insulin-positive cells closely resembled β-cells, with most expressing genes essential for endocrine function, including the apparatus for glucose sensing and metabolising. However, the fate change was variable and the cells generated may not be fully functional, but this does propose exciting possibility (Zhou, et al. 2008).

Despite dramatic advances in understanding the molecular basis for β-cell dynamics and generation there is still a lot to be learned. Eventually it is hoped that one or more of the pathways involved in pancreatic regeneration and growth may be manipulated for the therapeutic treatment of diabetes.
1.5. Lessons learned from hyperinsulinism

1.5.1. Overview of hyperinsulinism of infancy

It is not uncommon to witness abnormalities in glucose homeostasis, such as hypoglycaemia and hyperglycaemia, during the neonatal period. These symptoms can occur as a result of varying causes including prematurity, hormonal deficiencies and metabolic conditions (Hussain 2010). Hypoglycaemia can occur, however, in a more severe form as the result of a rare genetic disorder.

Hyperinsulinism of Infancy (HI) appears to have first been described more than 50 years ago by McQuarrie and for decades it was ascribed to a process of nesidioblastosis (McQUARRIE 1954). Laidlaw first coined the term nesidioblastosis in 1938 to describe the persistent endocrine cell proliferation and formation of new islets that appear to be budding from the pancreatic duct epithelium seen in cases of hypoglycaemia and also adult insulinomas (Laidlaw 1938). Nesidioblastosis is defined by observations of poorly defined islets, small clusters of endocrine cells scattered throughout exocrine tissue, and a high frequency of hormone-positive cells interposed amongst ductal cells (Kassem, et al. 2001; Rahier, et al. 1981). The observation of nesidioblastosis however has since been discovered in the pancreata of normoglycaemic neonates and is therefore not pathognomonic of hyperinsulinism per se (Rahier 1989; Sempoux, et al. 1995), and is no longer used as a defining term. However in the healthy neonate the islets normally evolve to a more conserved, mature architecture during the first year of life whilst in HI the neonatal form resembling nesidioblastosis is believed to persist (Kassem et al. 2000).

Over the years the syndrome, described by the recurrent and severe hypoglycaemia, has been referred to by a number of different names including islet cell adenomatosis, β-cell dysregulation syndrome and more recently persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) or simply hyperinsulinsim of infancy (HI).

The adjective “congenital” is often added to hyperinsulinism of infancy in order to differentiate this entity from discrete insulinomas and other neoplastic diseases which are rarely, if ever, diagnosed in the new born period (Glaser et al. 1999). Congenital hyperinsulinism of infancy (CHI) is generally more accepted now as the term of choice to describe the hypoglycaemic disorder.

Until relatively recently CHI was considered an enigmatic condition but advances in knowledge and information concerning the genetics, cell biology and physiology of CHI have provided new insights into the disease (Dunne et al. 2004).

Whilst it is still a very rare disease, occurring in approximately 1/27000 - 1/50000 live births in out-bred populations (Arnoux, et al. 2011; Rahier, et al. 2011), the implications
can be critical. CHI causes persistent or recurrent hypoglycaemia which, in the growing infant, is a major cause of irreversible neurological damage, mental retardation, epilepsy and iatrogenic diabetes (Aynsley-Green 1981).

Clinical manifestations are mainly experienced shortly after birth with most presenting during the first few postnatal days and others within the first year (Dunne et al. 2004). Diagnosis usually follows the observation of a positive glycaemic response to glucagon at times of hypoglycaemia along with symptoms evident of excessive insulin secretion, such as inappropriate suppression of lipolysis and ketogenesis; it is rarely as a result of hypersecretion of insulin (Cosgrove, et al. 2004; Finegold, et al. 1980).

CHI itself is a heterogeneous condition with varying descriptions characterizing it over the years. Whilst the aetiology of the condition is still unclear, it is generally well accepted there are two main clinical subtypes; a diffuse form (CHI-D) defined by functional abnormality of the islets throughout the pancreas, and a focal form (CHI-F) in which localised islet cell adenomatous hyperplasia is confined to a single region (or regions) of the pancreas (Rahier, et al. 2000).

CHI-D is generally considered the more common of the two and bears some histological characteristics of the nesidioblastosis that originally defined it (Kassem et al. 2001). However, the CHI-F has also been reported to have a variable prevalence from 40-65% of CHI cases (Dunne et al. 2004; Stanley 2002).

CHI-D appears to involve the whole pancreas, with discrete changes in function affecting all the β-cells throughout. In line with the nesidioblastosis comparisons, insulin-producing cells have been observed within acini and outside of well-defined islets, but it is difficult to distinguish this from normal neonatal development. It is generally well believed now that CHI-D is predominantly inherited as an autosomal recessive disorder causing mutations in the $K_{ATP}$ channel genes and consequently a non-functional $K_{ATP}$ channel (Dunne et al. 1997).

Until 1997 the existence of a focal form of CHI was controversial, it was de Lonlay et al. (1997) that provided the first molecular proof that two genetically distinct forms existed where CHI-F is due to loss of the maternal chromosome (de Lonlay, et al. 1997). CHI-F is limited to one or several regions throughout the pancreas where there appears to be increased proliferation and islet adenomatosis (Rahier, et al. 1998). These regions are between 2-5mm in size, thought to develop as a result of an imbalance in the expression of tumour suppressor genes which are imprinted in similar regions to those implicated in mutations or loss of heterozygosity (Dunne et al. 2004). Away from the lesion(s) the remaining pancreas has a normal histological appearance (Dunne et al. 2004) and often patients can recover to a normoglycaemia following partial pancreatectomy of the
dysfunctional region (Glaser et al. 1999). However, these regions are difficult to identify with the naked eye, as they are small and do not appear tumourous and are therefore difficult to identify at surgery resulting in what could be a more than necessary excision. The focal lesion maintains a usual lobular structure due to aggregation of otherwise normal islets sometimes also containing exocrine elements (Rahier et al. 2000). CHI-F does not appear to follow a Mendelian inheritance but is rather the result of gene silencing and loss of function. It is thought to be the consequence of two events, one being inherited (heterozygous mutation of a paternal gene), and the other acquired (by a somatic event involving loss of maternally inherited chromosome) occurring only in the affected region(s) of the pancreas (Kassem et al. 2000).

CHI represents a group of clinically, genetically, morphologically and functionally heterogenous disorders (Dunne et al. 2004). Rarely are elevated levels of insulin actually recorded, but rather that these levels are inappropriate to the levels of glycaemia (Dunne et al. 2004). It is known that CHI can be caused by mutations in at least seven different genes, each affecting the β-cell or its function differently resulting in divergent phenotypes associated with the syndrome (Flanagan et al. 2011; Glaser 2011). The most common cause of CHI, occurring in 40-45% cases, results from mutations affecting genes encoding the SUR1 or Kir6.2 proteins, two subunits of the β-cell K\textsubscript{ATP} channel (Dillon 2013). A minority of patients have glucokinase (GCK) or glutamate dehydrogenase mutations although these patients usually present with milder forms of the disease, whilst 40-50% of patients still have an unknown genetic cause (Dillon 2013; Flanagan et al. 2011).

1.5.2. Mutations in the K\textsubscript{ATP} channel

There has been great progress over recent years in uncovering the underlying causes of CHI although much about the disease remains unknown. For most cases of CHI, where a cause has been identified, it is the consequence of mutations leading to a non-functional K\textsubscript{ATP} channel in the pancreatic β-cell which results in the inappropriate release of insulin (Sempoux, et al. 2003). These comprise a mixture of missense, nonsense, frameshift, insertions/deletions and splice-site mutations (Bennett, et al. 2010).

1994 saw the first evidence of a mutation on a locus located in the human genome on chromosome 11 (11p15.1) being linked to both CHI-D and CHI-F (Glaser, et al. 1994). Although some dominant mutations have been reported the majority are inherited recessively with heterozygous parents rarely being affected (Flanagan et al. 2011; Glaser 2000).
The human \(K_{\text{ATP}}\) channel is a hetero-octameric protein organised as a complex of four Kir6.2 subunits arranged around a central pore coupled to four SUR1 subunits (Figure 1-5). The Kir6.2 subunit forms the potassium channel whilst the SUR1 protein is part of the ABC binding cassette family. The \(K_{\text{ATP}}\) channel couples metabolic activity (glucose) to a cellular response (insulin release), and the sensitivity of this channel involves both subunits (Bennett et al. 2010; Dunne et al. 2004). The \(K_{\text{ATP}}\) channel can only function if all the subunits are correctly assembled and transported to the cellular membrane (Bennet et al. 2010). Hyperinsulinism results when the \(K_{\text{ATP}}\) channel is either absent or dysfunctional and remains closed even at low blood glucose levels causing the \(\beta\)-cell membrane to remain depolarized (Bennett et al. 2010; Cosgrove et al. 2004).

**Figure 1-5: Schematic structure of the \(\beta\)-cell \(K_{\text{ATP}}\) channel**

An illustration showing the structure and composition of the \(\beta\)-cell \(K_{\text{ATP}}\) channel. In all images, SUR1 subunits are blue and Kir6.2 are purple. (A) Cross-section of the octameric \(K_{\text{ATP}}\) channel in the plasma cell membrane. Four Kir6.2 subunits create the central pore of the \(K_{\text{ATP}}\) channel, surrounded by four regulatory SUR1 subunits. (B) Illustration of the transmembrane composition of a single Kir6.2 subunit. Binding of ATP to the channel closes the channel. (C) Transmembrane topology of a single SUR1 subunit. Binding of sulphonylureas to intracellular loops of the SUR1 subunit inhibits activity of the \(K_{\text{ATP}}\) channel. (Adapted from Saint-Martin et al. 2011).

The SUR1 subunit is encoded for by the \(ABCC8\) gene, whilst Kir6.2 is coded by the \(KCNJ11\) gene, both found in the \(\text{Ch}11\text{p}15.1-\text{Ch}11\text{p}15.5\) region on the short arm of chromosome 11. Mutations in the \(ABCC8\) gene were the first to be discovered and represent the most common cause of medically unresponsive CHI with more than 150 mutations being described to date (Flanagan, et al. 2009; Thomas, et al. 1995). Only 24 \(KCNJ11\) mutations have been discovered (Flanagan et al. 2009; Marthinet, et al. 2005;
Nestorowicz, et al. 1997). Despite the advances that have been made more than 50% of CHI-D cases and approximately 30% of CHI-F have failed to define any genetic basis (Dunne et al. 2004; Flanagan et al. 2011).

CHI-D predominantly arises from the autosomal recessive inheritance of $K_{ATP}$ gene mutations, where a mutant allele is inherited from each parent affecting all of the islets of Langerhans throughout the affected pancreas (Glaser, 2000).

CHI-F is defined by a non-Mendelian mode of inheritance of $K_{ATP}$ dysfunction along with β-cell hyperplasia (Cosgrove et al. 2004). It is the consequence of two events, one being inherited and the other acquired. The first involves a constitutional heterozygous mutation, usually in the $ABCC8$ gene but also less commonly the $KCNJ11$ gene, each of paternal origin. The second event is somatic and involves the loss of part of the maternal chromosome 11 in the 11p15.1-11p15.5 region (Bennet et al. 2010; Dunne et al. 2004; Kassem et al. 2001). A compensatory duplication of the paternal 11p15.1-11p15.5 region causes all the β-cells in the affected lesion to be homozygous for the $ABCC8/KCNJ11$ mutations (Bennett et al. 2010). The probability of this somatic chromosomal loss occurring in neonates carrying a paternally derived mutation is <1% (Fournet, et al. 2001).

The 11p15.1-11p15.5 region of maternal chromosome 11 also contains several imprinted genes involved in cell proliferation, including the tumour suppressor genes H19 and P57$^{kip2}$ which negatively regulate cell proliferation (Kassem et al. 2001). Thus, when the maternal chromosome is lost, affected cells proliferate abnormally and express the mutant $K_{ATP}$ channel.

It is not fully understood whether the defected $K_{ATP}$ channel alone can account for inappropriate insulin release from β-cells as the system is still reliant on calcium-dependent exocytosis and insulin production. It may be possible that other factors resulting as a consequence of mutations and/or abnormal development of the islets may play a role in the pathogenicity of the disease and the histological features that manifest.

1.5.3. Evidence for a progenitor population

Early descriptions of CHI pathology are based on observations of islet cell hyperplasia and nesidioblastosis. Whilst this has since been shown as a common physiological feature of the normoglycaemic neonate (Jaffe et al. 1980) it is still a recurring presentation in the literature and a highly reported characteristic of CHI.

During fetal development it is well documented that the islets are generated from progenitors that appear to emerge from within the ductal epithelium. Whether such a progenitor population exists after birth that can be stimulated in cases of CHI remains unknown. However, the observations of islet hyperplasia and budding of hormone positive
cells, as seen in many CHI subjects, is an encouraging assumption of neogenesis and a recapitulation of this embryonic programme.

Despite the resemblance of these CHI characteristics to the process of embryonic pancreas development there seems to be confusion in the literature about nesidioblastosis and the mechanisms involved. Many authors, including Rahier et al. (2000), have ruled out nesidioblastosis as being pathogenic of CHI, not only because of its occurrence in strictly normoglycaemic subjects, but also because of the deficiency in conclusive evidence for an increase in proliferation rates within diseased tissue, marked by the presence of the Ki67 protein (Rahier et al. 1984; Rahier et al. 2000; Sempoux, et al. 1998a). Nesidioblastosis has often been attributed to the proliferation of islets (Dahms, et al. 1980; Laidlaw 1938; Rahier et al. 1984) however this process would not account for the changes in ductal structure and budding observations that have been described. Consequently, the appearance of new islet formation and the budding of hormone positive cells from duct-like cells within the ductal epithelium is a striking characteristic of neogenesis, rather than a feature of proliferative activity.

It is possible that CHI-F could result from increased proliferation and a tumour-like growth, accountable by the reduced expression of the tumour suppressor genes P57KIP2 and H19, both lost on the maternal chromosome. However, in CHI-D proliferation rates appear no higher than those of control subjects despite insulin-positive cells, distinct from defined islets, still being recognised (Sempoux et al., 1998). If proliferation was accountable for these characteristics, it would be expected that only certain cell populations would be affected, as the new growth is generated from individual cell sources. However, a report by Heitz et al. (1977) showed that whilst β-cells were the predominant cell type to increase in number (62%), the other islet cell types also increased proportionately (Dahms et al. 1980; Heitz, et al. 1977). This is suggestive of a whole islet generation rather than simply an expansion of its individual components.

Alexandrescu et al. (2010) performed a histological study on CHI-D and also observed islet budding from mature epithelium. Despite an increase in islet cell number no mass effect was noted, indicative that this neogenesis may be a mechanism of replacement rather than new growth (Alexandrescu, et al. 2010).

Kassem et al. (2000) also found that, whilst normoglycaemic neonatal pancreata are characterised by small, poorly formed islets, this distribution rarely persists beyond 6 months of age. At this time the islets conform to a more mature-type architecture with well-defined islets and fewer extra-islet insulin positive cells (Kassem et al. 2000). In the case of CHI-D the neonatal form persists. Kassem et al. hypothesized that a continued
rate of apoptosis and proliferation was maintained in CHI-D and is responsible for the process of remodelling seen in the pancreata of these patients. This immaturity could result as a consequence of mutations occurring during development and resulting in a state of injury to which the pancreas is trying to respond.

The trigger for neogenesis in CHI tissue has been attributed by some authors to an increase in endocrine cell apoptosis (Jack, et al. 2000; Kassem et al. 2000; Rahier et al. 2000). It has been found that SUR1 mutations in β-cells can induce β-cell apoptosis in the rat and islet cell hyperplasia and nesidioblastosis in humans (Efanova, et al. 1998; Rayman, et al. 1984). It is theorized that functional abnormalities in the SUR1 and Kir6.2 subunits may be apoptotic triggers (Jack et al. 2000; Kassem et al. 2000) and that the ductal proliferation seen is a response to an increase in apoptotic activity in an attempt to maintain normal endocrine mass. This would also explain why no specific increases in endocrine mass have been observed in cases of CHI (Goudswaard, et al. 1986; Rahier et al. 1984).

In either situation there is still considerable information to be gained from CHI patients and the underlying pathology that may cause the inappropriate insulin secretion and persistent prematurity of β-cells seen in this disease.
1.6. Aims of the project

Our group has primarily focused research into understanding the development of the human pancreas. We are in a unique position to study such an elusive area of research having collected almost 3000 specimens of human embryonic and fetal material from voluntary terminations of pregnancy over the last decade. As such the group has published a number of high impact articles detailing events involved in the differentiation of the endocrine lineage and continue to identify differences between human and mouse development, reinforcing our research as a valuable resource to understanding our own species in health and disease.

In collaboration with Mark Dunne’s lab this project will profile for the first time samples of CHI-D pancreas to investigate whether aspects of fetal development are being reinstated in pathological conditions. It is hoped that knowledge gained from studying the pancreas in an altered physiological condition will underpin interpretations and exploitation of human pluripotent stem cell differentiation towards a β-cell fate. The overall aim of the project concerns the potential for pancreatic β-cell regeneration which is desirable as an ambitious form of cell therapy in diabetes. There has been a lot of interest in recent years in the processes governing the maintenance and generation of β-cells postnatally and also the development of models to investigate them. To date there has been little consideration of CHI as a prerequisite to islet generation or to the knowledge that can be gained by understanding the disease.

The specific aims of the project therefore include:

1. Carry out histological comparisons of CHI-D tissue with age-matched controls and fetal samples as a reference to development.

2. Establish whether neogenesis is being induced in CHI-D samples.

3. Explore cell mass dynamics including proliferation and its regulation as well as apoptosis in CHI-D and age-matched controls.

4. Investigate the phenotype of NGN3-positive cells in human fetal samples to better understand the fetal endocrine differentiation programme.
Chapter 2- Materials and methods

2.1. Preparation of tissue sections

2.1.1. Tissue collection

2.1.1.1. Human fetal samples

All human embryonic and fetal samples were collected, used and stored with full ethical approval from the North West 5 Research Ethics Committee (of REC reference: 08/H1010/28)(Appendix 1-1), and under the codes of practice issued by the Human Tissue Authority. Informed, written consent was obtained from women undergoing voluntary terminations of pregnancy, inline with guidelines issued by the Polkinghorne Committee (Polkinghorne 1989). Human embryos and fetuses from the first and second trimester were obtained following medical (Mifepristone) or surgical terminations and staged immediately by stereomicroscopy according to the Carnegie classification (O’Rahilly and Müller 2010). The Carnegie staging system uses morphology to ascribe the age of the embryo or fetus, given in days post conception (dpc). Fetal tissue was staged using a combination of ultrasonographic-guided gestational aging as well as hand and foot length measurements (in millimeters), and given as weeks post conception (wpc). Once staged samples were prepared for experimentation using techniques described below.

2.1.1.2. CHI-D patient samples

Pancreatic tissue was collected for research from five CHI-D patients aged between 2-13 months following clinical pancreatectomy at the Royal Manchester Children's Hospital, UK. Full ethical approval and informed consent was obtained for each patient (NRES 07/G1010/88, Trust R&D PIN 5276) (Appendix 1-2). In all, the diagnosis of CHI-D was made at the hospital according to accepted clinical criteria, identification of mutations in either the ABCC8 or KCNJ11 genes and upon established histopathological criteria (Dunne et al. 2004; Glaser 2011). All patients presented with sustained hypoglycaemia that was unresponsive to Diazoxide or Somatostatin therapy. Details of each patient are summarized in Table 2-1. After surgery pancreatic tissue was fixed in formaldehyde within 5 minutes of retrieval to ensure immobilization of antigens whilst retaining cellular and subcellular structures. After fixing samples were embedded in paraffin wax before receipt by our lab group.
Table 2-1: Summary of CHI-D patient information.

All samples were previously diagnosed with CHI-D and have a genetic mutation identified on either the ABCC8 or KCNJ11 gene. Samples were available as paraffin-embedded specimens only. Patients CHI-D 3 and CHI-D 5 underwent a second operation (R) to treat recurring hypoglycaemic symptoms at 31 and 34 months respectively.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Birth Weight (kg)</th>
<th>Age of Onset (months)</th>
<th>Age at Surgery (months)</th>
<th>Histology</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHI-D 1</td>
<td>M</td>
<td>6.7</td>
<td>1</td>
<td>2</td>
<td>Diffuse</td>
<td>ABCC8</td>
</tr>
<tr>
<td>CHI-D 2</td>
<td>M</td>
<td>4.3</td>
<td>1</td>
<td>2</td>
<td>Diffuse</td>
<td>KCNJ11</td>
</tr>
<tr>
<td>CHI-D 3</td>
<td>M</td>
<td>4.5</td>
<td>1</td>
<td>3 31 (R)</td>
<td>Diffuse</td>
<td>ABCC8</td>
</tr>
<tr>
<td>CHI-D 4</td>
<td>M</td>
<td>2.9</td>
<td>1</td>
<td>6</td>
<td>Diffuse</td>
<td>ABCC8</td>
</tr>
<tr>
<td>CHI-D 5</td>
<td>F</td>
<td>4.6</td>
<td>1</td>
<td>13 34 (R)</td>
<td>Diffuse</td>
<td>ABCC8</td>
</tr>
</tbody>
</table>

2.1.1.3. Neonatal control samples

Archival pancreatic tissues were obtained from post-mortem donations of individuals who died from non-pancreatic diagnoses. Samples ranged in age from full term fetuses (35-41 wpc) and neonatal (6 weeks – 12 months), as well as five samples from individuals aged 3, 4, 11, 12 and 13 years. All samples were generously donated by Dr. Ronald de Krijger, Erasmus MC, Netherlands and showed unremarkable pancreatic histology. All samples were formaldehyde fixed and paraffin embedded before receipt.

2.1.2. Tissue fixing and embedding

All embryos and dissected fetal tissue were promptly and identically fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St Louis, MO, USA) for approximately 8-16 hours with gentle agitation, depending on the size of the specimen. Samples were subsequently dehydrated following incubations in increasing concentrations of ribonuclease- (RNAse-) free ethanol solutions (70, 80, 90 and 100% ethanol diluted with diethylpyrocarbonate-
[DEPC-] treated water [Sigma Aldrich]), each time rocking at room temperature for two hours. Samples were incubated in chloroform (Fisher Scientific, Loughborough, UK) for 16 hours at room temperature to remove residual ethanol from the tissues before proceeding with the embedding procedures.

Samples were infiltrated with molten paraffin wax (Fisher Scientific) following three incubation periods of 2 hours each at 72°C. The final incubation was performed under vacuum pressure to facilitate removal of chloroform and thorough infiltration of paraffin into the tissues. The vacuum was released slowly and the specimens embedded into paraffin (Fisher Scientific) with plastic cassettes and allowed to solidify before storing at room temperature until use.

2.1.3. Microtome sectioning and mounting

Embedded tissue was sectioned to 5 µm thickness using a Leica RM2235 Microtome (Leica Microsystems, Wetzlar, Germany). Sections were mounted onto Leica X-tra Adhesive pre-cleaned microscope slides in a sequential manner (2 - 4 sections per slide depending on the size of the tissue). To ensure section adherence to the slides DEPC-treated water was pipetted underneath each section and the slide (with floating section) was placed on a hot block set at 42.3°C to allow expansion and prevent creasing of the tissue. The water was removed by suction and the slides, now mounted with tissue sections, were allowed to dry overnight at 37°C before storing in RNAse-free boxes at 4°C.

2.1.4. H&E staining

For accurate orientation and analysis of embryos and fetal tissues, as well as assessment of tissue quality for CHI-D and control samples, sections were stained with haematoxylin and eosin (H&E). For embryos, every ninth section was mounted onto a separate slide (ie sections every 40 µm) for H&E analysis. For fetuses, sections after every 8th slide were stained.

Slides were placed in a xylene wash twice, for three minutes each, to remove the paraffin. Sections were then rehydrated in 100% and 90% ethanols for 2 minutes and then rinsed in de-ionized water for a further 2 minutes. Slides were submerged in Shandon TM Harris Haematoxylin (Thermo Fisher Scientific Inc, Waltham, MA) for 3-5 minutes then rinsed in de-ionized water until the water ran clear. Slides were then counter-stained with Eosin (Thermo Fisher Scientific) for 3 minutes before rinsing again in de-ionized water.

Sections were dehydrated following washes in 70, 90 and 100% ethanol solutions for 2 minutes each, then placed in xylene for a further 5 minutes. Slides were mounted with a
coverslip using Entellan® Mounting Medium (Merck Millipore, Darmstadt, Germany) and left to dry at room temperature.

2.2. Immunostaining

Immunostaining was performed on prepared slides to enable detection of proteins of interest. This process utilizes antibodies to detect and visualize antigens (proteins of interest) in tissues, by virtue of a colour reaction or conjugation to a fluorophore (Figure 2-1).

![Image](image.png)

**Figure 2-1: Principles of immunostaining on fixed tissue.**
A; Immunohistochemistry of a single protein. The primary antibody (gold) binds to a specific epitope on the antigen of interest. A biotinylated-secondary antibody (blue) recognizes the primary antibody and binds. A streptavidin-conjugated horse-radish peroxidase (S-HRP) is then added to the reaction. Streptavidin binds to the biotin molecules on the secondary antibody thereby amplifying the signal which is generated by a colour-change reaction where HRP oxidizes 3,3’Diaminobenzidine (DAB) in the presence of hydrogen peroxide. An insoluble brown-coloured substrate is produced allowing visualisation of the protein of interest by bright-field microscopy.

B; Dual immunofluorescence of two distinct proteins. The initial primary antibody, specific to the first protein of interest, is visualized by the addition of a biotinylated-secondary antibody followed by streptavidin that is directly conjugated to a fluorophore (red). The second primary antibody, specific to a second epitope of interest is detected by the addition of a secondary antibody directly conjugated to a fluorophore (green). Each fluorophore is excited at a different wavelength and can therefore be visualized independently in the same sample on a light microscope using different filters.

2.2.1. Immunohistochemistry

Previously prepared slides were first de-waxed following washes in xylene, twice, for three minutes each time, then rehydrated in 100 and 90% ethanol solutions for 2 minutes each. Slides were then submerged in de-ionized water before incubating in 0.1% hydrogen peroxide (Sigma-Aldrich) prepared with 0.01M phosphate-buffered saline (PBS) for 20 minutes at room temperature to quench endogenous peroxidase activity. Following this slides were washed three times in 0.01M PBS at room temperature for five minutes each time.
Tissue was permeabilised by boiling in 10 mM sodium citrate solution (pH 6.0) for between ten and thirty minutes (optimized for each antibody and tissue) to maximize antigen retrieval. Slides were allowed to cool in the buffer for 20 minutes before a further three, five-minute washes in PBS. Individual sections were bordered with an ImmEdge™ hydrophobic barrier pen (Vector Laboratories, Peterborough, UK) to prevent antibody solutions on different sections from mixing. The primary antibody of interest was prepared according to the optimized dilution (Table 2-2) using a solution of 0.1 % PBS/Triton™ X-100 (non-ionic surfactant) (Sigma-Aldrich) and containing 3 % host serum from the species in which the secondary antibody was raised in, to minimize background and non-specific binding of the secondary antibody. Enough antibody solution was applied to cover each section before placing the slides in a humidified container to prevent drying, and incubating at 4°C overnight.

The following day slides were washed in PBS, three times for five minutes each, before incubating with a biotin-conjugated secondary antibody solution (species specific), diluted in 0.1 % Triton™ X-100/PBS, for two hours at 4°C.

Slides were washed in PBS three times for five minutes each and then incubated at 4°C for one hour with streptavidin horseradish-peroxidase (SA-HRP; Vector Laboratories) diluted to 1:200 with 0.1 % Triton™ X-100/PBS. Colour staining was achieved with the addition of 3,3’-diaminobenzidine (DAB; Merck Millipore) for 2 minutes, followed by DAB containing 0.03 % hydrogen peroxide for 3 minutes. In the presence of hydrogen peroxide and horseradish peroxidase DAB is converted into an insoluble brown product. Slides were then washed in PBS, once, for five minutes. Sections were counter-stained with Toluidine Blue (Fluka Analytical) for one-two minutes before rinsing in de-ionized water. Sections were dehydrated by washes in 70, 90 and 100% ethanol for 10 seconds x2 and 3 minutes respectively, before washing twice in xylene for 2 minutes each to remove any traces of the barrier pen. Slides were mounted with a coverslip using Entellan™ Mounting Medium.

2.2.2. Dual immunofluorescence

Dual immunofluorescence was performed to enable the detection of multiple proteins in each tissue section. The process is similar to that of IHC (section 2.2.1). Following ethanol rehydration and rinsing in de-ionized water slides were immediately incubated in 10 mM boiling sodium citrate solution (pH 6.0) for between 10-30 minutes (depending on antibody and tissue optimisation), to ensure tissue permeabilisation. Following 20 minutes cooling down in the citrate buffer and three five-minute washes in PBS, slides were incubated with primary antibody as detailed in section 2.2.1.
The following day slides were washed three times in PBS before administering the biotinylated secondary antibody and incubating for two hours at 4°C. Thereafter slides were washed three times in PBS and incubated with a streptavidin-conjugated fluorochrome at 4°C for one hour. Slides were washed three times in PBS, five minutes each time, before a second primary antibody (against a different protein of interest) was applied.

After over-night incubation slides were washed in PBS and a secondary antibody directly-conjugated to a fluorophore was applied before incubating, in the humidified container, at room temperature for two hours.

Slides were washed in PBS three times, for five minutes each, and then dehydrated following immersion in 70, 90 and 100% ethanol solutions for 10 seconds x2, and 3 minutes respectively, followed by two three minute washes in xylene, before incubating in 100% ethanol for a further 2 minutes. Nuclear counter-staining was achieved with the addition of Vectashield® Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) which was used to mount a coverslip, with the edges sealed with nail varnish.
<table>
<thead>
<tr>
<th><strong>Primary Antibody</strong></th>
<th><strong>Raised In</strong></th>
<th><strong>Dilution</strong></th>
<th><strong>Supplier</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal anti-Insulin</td>
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<td>Abcam</td>
</tr>
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<td>Guinea Pig</td>
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<td>Rabbit</td>
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<td>Zymed</td>
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<td>Abcam</td>
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<td>Rabbit</td>
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<td>Abcam</td>
</tr>
<tr>
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<td>DSHB</td>
</tr>
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<td>Novocastra</td>
</tr>
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<td>1:200</td>
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</tr>
<tr>
<td>Polyclonal anti-NGN3</td>
<td>Mouse</td>
<td>1:1000</td>
<td>DSHB</td>
</tr>
</tbody>
</table>

**Table 2-2: Antibodies used in immunostaining of fixed tissue.**

Details of antibodies used in immunostaining experiments along with the species each was produced in, optimized dilution and the supplier.
2.2.3. TUNEL

The terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay is an established method for detecting apoptotic cells based on the extensive DNA fragmentation that results following apoptotic signaling cascades in the late stages of programmed cell death. The method utilizes the ability of TdT—an enzyme that catalyzes the addition of dUTPs to the blunt ends of double-stranded DNA breaks. TUNEL assay kits contain highly purified forms of TdT enzyme along with biotinylated nucleotides, which are incorporated into the DNA breaks and detected by the addition of a streptavidin-conjugated fluorescein (See Figure 2-2).

Figure 2-2: Principle of TUNEL assay on fixed tissue
DNA fragmentation is a characteristic hallmark of cells undergoing apoptosis. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) is an established method for detecting DNA fragments. The TACS® 2 TdT-Fluor In Situ Apoptosis Detection Kit contains highly purified TdT enzyme which functions to repair DNA fragments by the incorporation of biotinylated nucleotides. The addition of a streptavidin-conjugated fluorophore binds to the biotin molecules on the nucleotides, identifying damaged DNA within cells that can be visualized in fixed tissue samples using fluorescent-light microscopy.

2.2.3.1. In Situ TUNEL detection

Cells undergoing apoptosis were identified using prepared slides as described previously (Section 2.1.2.) by virtue of fluorescence detection using the TACS® 2 TdT-Fluor In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA), according to manufacturers instructions. Briefly, slides were first deparaffinised following two washes in xylene, for 3 minutes each, and then rehydrated in washes with 100 and 90% ethanols for 2 minutes each before rinsing in deionized water. Slides were immersed in 1xPBS solution for 10
minutes before incubating with Proteinase K (supplied) for 20 minutes (optimized to the tissue), at room temperature in a humidified container. Slides were washed twice in deionized water for 2 minutes each and then immersed in 1x TdT Labelling Buffer (supplied; diluted from 10x stock with deionized water). Following this slides were covered with Labelling Reaction Mix containing TdT dNTPs, 50X cobalt cations and TdT enzyme, and prepared in 1X TdT Labelling Buffer (all supplied). Slides were incubated at 37°C for one hour in a humidified container before washing in a 1x TdT Stop Buffer (supplied) for five minutes, to stop the labeling reaction. Slides were then washed in 1X PBS twice, for five minutes each to remove any unbound conjugate. A streptavidin-conjugated fluoroscein (supplied) was prepared at 1:200 dilution with 1X PBS and applied to the samples by pipette. Slides were incubated at room temperature, in the dark, for 20 minutes and then washed, three times, in 1X PBS for 2 minutes each. Samples were dehydrated following washes in 70, 90 and 100% ethanols for ten seconds (x2) and three minutes respectively before mounting with DAPI enriched mounting medium (Vector Laboratories) and the coverslip sealed with nail varnish.

2.2.3.2. Double labelling TUNEL and insulin

When samples were dual labeled with TACS® 2 TdT-Fluor and Insulin, slides were first processed for apoptosis detection using the method described above (section 2.2.3.1.). Proteinase K treatment provided sufficient antigen retrieval for protein detection with an insulin-specific primary antibody (See Table 2-2). As such, after the addition of Strep-Fluor and washes in 1X PBS (see protocol description section 2.2.3.1.), samples were incubated with primary insulin antibody overnight at 4°C in a humidified container, in the dark. The following day slides were washed three times in 1X PBS before incubating with a secondary antibody directly conjugated to a fluorophore (red), at room temperature for two hours. Slides were again washed, three times in xPBS, then dehydrated following immersion in 70, 90 and 100% ethanols. Slides were mounted using DAPI-enriched mounting medium (Vector Laboratories) and the coverslip sealed with nail varnish.

2.2.3.3. TUNEL assay controls

Positive and negative controls were included with each sample, in each experiment, to validate staining.

For the positive control, one sample was treated with TACS-Nuclease (supplied) to generate breaks in the DNA of all the cells in the treated section. TACS-Nuclease was added to the 1X TdT Labelling Mix (see method described previously section 2.2.3.1.). All
further processes and incubations were carried out according to normal protocol. The TACS-Nuclease treated control confirms a positive permeabilisation method with Proteinase K as well as a positive labeling reaction for TdT Labelling Reaction Mix and Streptavidin-Fluoroscein.

An experimental negative control sample was also included in each experiment. This involved following the procedures as previously described (Section 2.2.3.1.) with the omission of the TdT enzyme in the Labelling Reaction Mix. Omitting the enzyme will identify any unspecific binding of Strep-Fluor or tissue autofluorescence.

2.2.4. Image analysis

Slides were viewed using the Axiovert Imaging System (Carl Zeiss Ltd., Cambridge, UK) and images captured using Axiovision 4.7 software (Zeiss). Further image analysis and editing was achieved using Adobe Photoshop CS4 (Adobe Systems, Uxbridge, UK).

2.2.5. Cell counting

Immunohistochemistry or immunofluorescence was performed as described previously (sections 2.2.1 and 2.2.2). Protein of interest-positive cells were counted in 10 randomly selected fields of view at x200 magnification in at least two different positions within each CHI-D, fetal and control pancreas. If 10 fields of view were not possible (ie in smaller fetal samples) all the protein-positive cells were counted.

Proliferation was characterized by nuclear Ki67 (Novocastra) stain with population analysis achieved with co-expression for insulin, glucagon, SOX9 and GATA4.

Apoptosis was characterised by a positive nuclear TUNEL stain (see section 2.2.3) with TUNEL+/insulin+ -cells counted as a percentage of total TUNEL-positive cells in each field of view at x200 magnification or as a proportion of insulin-positive cells in the same area.

The proportion of NKX2.2-positive cells that co-stained with each hormone was calculated as a percentage of the total population of cells positive for that hormone.

Large cell nuclei were identified visually either by DAPI staining on the microscope or on digital images of sections following H&E or IHC staining (See sections 2.1.4 and 2.2.1 respectively). The slide scanning procedure, in brief, involved slides, previously stained for H&E or IHC and mounted with a coverslip, being digitally scanned at a 45X resolution on the Pannoramic 250 Flash II slide scanner (3DHISTECH, Hungary) and visualized with the Pannoramic Viewer software (3DHISTECH).
Large nuclei (defined as being >1.5 fold larger than the average diameter of 10 surrounding cell nuclei within each islet) were confirmed by measuring the cell diameter in the Pannoramic Viewer software.

2.3. Laser capture microdissection

Laser capture microdissection (LCM) is a method used to isolate specific cells or regions of interest from microscopic sections of tissue. The PALM® MicroBeam system comprises an infra-red laser fitted to an inverted microscope which allows you to accurately visualise specific cells or regions and dissect them with pin point accuracy. Since there is no contact of the tissue or equipment there is reduced risk of contamination whilst the precision of the laser also prevents contamination from surrounding cells or tissues in the retrieved product.

After “cutting” around the desired area with the laser a change in focus of the beam causes a pressure change which in turn catapults the dissected material into an adhesive cap that is positioned over the tissue section. Multiple cells or tissue regions can be collected into one cap and harvested for use in downstream applications such as gene arrays and RNAseq (see Figure 2-3).
Tissue sections, mounted onto a microscope slide and immunostained to allow for the identification of target cells, are placed onto the PALM® MicroBeam system and an adhesive cap placed over the sample. A UV laser beam is focused and dissects around the target tissue, visualised via an inverted microscope. The dissected tissue is collected by photonic pressure which catapults the sample into the collection cap where it can be further processed for RNA extraction and downstream applications.

2.3.1. Preparation of slides for LCM

LCM was performed on PFA-fixed, paraffin embedded tissues. Slides were prepared as previously described (section 2.1) and IHC towards the protein of interest performed according to normal protocol (Section 2.2.1). All steps were carried out with RNAse-free precautions (ie treatment of all equipment with DEPC-treated ethanol or RNAseZap® [Ambion, Life Technologies, Paisley, UK]) and all dilutions were made with RNAse-free water (Sigma). After counter-staining with Toluidine Blue, sections were dehydrated by submergence in RNAse-free 70, 90 and 100% ethanols followed by two washes in xylene. The application of a coverslip was omitted. Prepared slides were stored in an RNAse-free 50 ml Falcon tube at -80°C until use.
2.3.2. Laser capture microdissection

LCM was performed using a PALM® MicroBeam 3 system (Zeiss). Prior to use the microscope, stage and surrounding areas were treated with RNaseZap®. Sections or cells of interest were dissected using a focused laser beam at 40x/1.30 Plan Apo objective and laser power of 35%. For the visualisation of NGN3-positive cells a liquid cover glass (Zeiss) was applied to help focus on the sections, whilst this was omitted for islet-capture.

Catapulted material was collected in a 200 µl AdhesiveCap® collection tubes (Zeiss) which permits buffer-free adherence of the sample to the collection lid. Following LCM, collected samples were stored at -80°C before being processed for RNA extraction (section 2.4.1).

2.4. cDNA synthesis from RNA

All steps involved in RNA extraction were carried out with RNase-free precautions including baked glassware at 180°C, pre-treatment of equipment and bench area with RNaseZap® and the use of RNase-free filter tips.

2.4.1. RNA extraction (Qiagen RNeasy Kit)

Total RNA was isolated from previously prepared slides of PFA-fixed, paraffin-embedded samples (section 2.1), using the RNeasy FFPE Mini Kit (Qiagen, Manchester, UK) according to manufacturer’s guidelines.

Briefly, slides were deparaffinised with washes in xylene followed by sequential washes in 100 and 90% ethanol to rehydrate the sections. Tissue sections were scraped from the slide into an eppendorf using 150 µl Buffer PKD (supplied in kit). To the buffer 10 µl of Proteinase K (supplied) was added, the sample vortexed and covered in para-film then incubated at 56°C (in an RNAse-free hybridisation oven) overnight. The following day samples were incubated at 80°C for 15 minutes to reverse PFA cross-linking of nucleic acids, centrifuged and then put on ice.

Any contaminating DNA was removed by the addition of 10 µl DNAse I and 16 µl of DNAsse Booster Buffer (supplied) and incubated at room temperature for 15 minutes. 320 µl Buffer RBC (supplied) was then added to each sample to adjust the binding conditions, followed by 720 µl 100% ethanol, with samples mixed by pipetting.
Sample solutions were then transferred to an RNeasy MinElute Column (supplied) and centrifuged for 15 seconds at 10,000 rpm. The column was washed twice with Buffer RPE (supplied) to reduce contamination. A further centrifugation step at full-speed for five minutes ensured complete removal of Buffer RPE before eluting the RNA (bound to the membrane in the MinElute column) with 25 µl RNase-free water (supplied). The RNA solution was quantified (see section 2.4.2) before storing at -80°C until use.

2.4.2. RNA quantification

RNA purity and quantity was assessed using a spectrophotometer (NanoDrop-2000, Thermo Fisher Scientific, Wilmington, DE, USA), which can measure RNA nucleic acid concentrations from 2 - 3000 ng/µl and determines the quality by measuring the ratio of absorbance at 260 nm and 280 nm. A ratio of ~2.0 is generally accepted as pure for RNA, indicating the sample to be free from DNA, protein or other contaminants (such as ethanol).

2 µl of the sample was pipetted directly onto the measuring surface after recording a blank measurement with RNase-free water. Concentration was determined using the absorbance reading at 260 nm, where a reading of 1 is equivalent to ~40 µg RNA/ml.

2.4.3. First strand cDNA synthesis (ABI Method)

cDNA was generated from total RNA using a reverse transcriptase High Capacity RNA-cDNA kit (Applied Biosystems, Life Technologies), according to the manufacturer’s guidelines. The kit converts up to 2 µg RNA per 20 µl reaction, into single-stranded cDNA. This process utilises Oligo(dT) primers which bind to the 3’ polyadenylated (PolyA) tail of the mRNA transcript, and then extended by reverse transcriptase (RT). Briefly, the required volume of RNA (<9 µl) was added to 10 µl 2x RT Buffer (containing MgCl₂, RNase inhibitors, Oligo(dT) primers, dNTPs), and 1 µl 20x RT Enzyme, with the total volume made up to 20 µl with the addition of RNase-free water. The same mixes with the omission of the enzyme were prepared concomitantly for use as a negative control (minus-RT) to identify DNA contamination in downstream applications.

The reaction samples were briefly centrifuged to mix the contents and disperse any air bubbles before loading into a thermal cycler (Bio-rad Tetrad thermal cycler; Bio-rad, Hemel Hempstead, UK). Following the process of reverse transcription, samples were stored at -20°C until use.
2.5. RNA amplification

Total RNA isolated from LCM tissues was amplified using the Ovation® RNA-Seq System V2 (NuGEN Technologies, San Carlos, CA, USA). This kit utilises Ribonuclease (single primer isothermal amplification) technology which provides a rapid and sensitive RNA amplification process (outlined in Figure 2-4). Ribonuclease technology allows for the amplification of small amounts of RNA (~500pg) into microgram quantities of cDNA suitable for sequencing library construction, next-generation sequencing systems, as well as qPCR and microarray analysis.

All procedures were carried out on ice and all reagents were included in the kit, unless otherwise stated.

2.5.1. Ovation® first strand cDNA synthesis

This initial step uses a DNA/RNA chimeric primer that hybridizes either to the 5’ portion of the Poly(A) tail of mRNA molecules, or randomly across the transcript, and is then extended to the 3’ DNA end of each primer by RT enzyme. This process generates a cDNA/mRNA hybrid molecule.

5 µl total RNA sample was added to a mixture containing 2 µl First Strand Primer Mix and heated to 65°C for five minutes to anneal the primers to the RNA. 3 µl First Strand Master Mix, containing a buffer and RT enzyme, was then added to each samples and heated to 25°C for ten minutes, 42°C for ten minutes to synthesize the cDNA, and then 70°C to stop the reaction.

2.5.2. Ovation® generation of DNA/RNA heteroduplex double-stranded cDNA

During this step the mRNA within the cDNA/mRNA complex is fragmented, creating priming sites for DNA polymerase to bind and begin synthesizing a second strand. The result is a double-stranded cDNA molecule with a DNA/RNA heteroduplex at one end.

To the sample 10 µl of Second Strand Master Mix (containing buffer and enzyme) was added and the mixture heated to 25°C for ten minutes, followed by 50°C for 30 minutes and 80°C for 20 minutes.

2.5.3. Ovation® purification of cDNA

Synthesized cDNA was purified using Agencourt RNAClean XP beads (supplied). Briefly, the cDNA sample was combined with 32 µl (1.6 volumes) bead suspension and incubated
at room temperature for ten minutes to allow the cDNA to bind to the beads. The sample was then transferred to a magnet and allowed to stand for five minutes until the beads had completely cleared the solution. With the samples still on the magnet, the buffer was removed and the beads washed, three times, with 70% ethanol before being left to air-dry at room temperature for 20 minutes to remove any residual alcohol.

2.5.4. Ovation® SPIA® amplification

Amplification of the purified cDNA is achieved using SPIA®, an isothermal process using RNase H to digest RNA from within the DNA/RNA heteroduplex at the 5' end of the cDNA strand. This results in the exposure of cDNA that is available for binding the SPIA® primer. Once bound, DNA polymerase initiates replication at the 3' end of the primer, generating a new strand whilst displacing the existing forward strand. RNase H then removes the RNA portion of the newly synthesized strand and the process repeats, resulting in rapid accumulation of cDNA with a sequence complementary to the original strand.

40 µl SPIA® Master Mix was added to the beads and mixed thoroughly by pipetting. The sample was then heated to 47°C for 60 minutes, followed by 80°C for 20 minutes.

In order to prevent contamination with pre-amplification products, all further procedures were carried out in a separate laboratory work area.

Samples were positioned back on the magnetic stand to allow the beads to clear. Approximately 40 µl of the supernatent was transferred to a fresh tube ready to proceed with purification.

2.5.5. Ovation® cDNA purification

The amplified cDNA was purified using QIAquick PCR Purification Kit (Qiagen) according to the instructions provided in the Ovation® RNA-Seq System V2 user manual and not the manufacturers own. Briefly, 250 µl Buffer PB (supplied) was added to the sample before being transferred to a QIAquick Spin Column (supplied). Samples were centrifuged at 17,900 X g at room temperature for one minute then washed, twice, with 80% ethanol. Columns were again centrifuged at 17,900 X g for two minutes to ensure complete removal of ethanol. Purified cDNA was eluted in 30 µl Buffer EB (containing 10mM Tris-Cl) (supplied) and stored at -20°C until use.
Figure 2-3: Principle of Ovation® RNA amplification
Ribo-SPIA® technology was used to generate amplified cDNA from small starting samples of RNA previously extracted from LCM specimens. A DNA/RNA chimeric (SPIA®) primer binds either to the 5’ portion of the poly(A) sequence, or randomly across the RNA transcript, and reverse transcriptase generates a first-strand cDNA. Binding sites for DNA polymerase are created following the fragmentation of mRNA within the cDNA/mRNA complexes and a second cDNA strand is synthesized, resulting in a double-stranded cDNA molecule with a DNA/RNA heteroduplex at one end. RNase H is then added to the reaction to digest RNA from within the DNA/RNA heteroduplex. This exposes cDNA to which SPIA® primers can bind. A new cDNA strand is synthesized, displacing the previous, with the activity of DNA polymerase. RNase H digests the RNA-portion of the newly synthesized strand and the process repeats resulting in rapid accumulation of cDNA product.

2.6. RNA sequencing

RNA-sequencing (RNA-Seq) utilizes nucleotide specificity and PCR techniques to sequence the base order of DNA fragments based on fluorescent emission. As compared to the static genome, the transcriptome of a cell/tissue is dynamic; that is that the expression profile of different genes changes and can be profiled according to the expression of transcripts (e.g. mRNA) at a given time.
RNA-Seq is performed to identify what transcripts are present in samples of extracted RNA. In contrast to microarray analysis, RNA-Seq does not use probes and is therefore not limited by coverage and can identify novel transcripts and gene variants. Random fragmentation of cDNA and subsequent hybridisation onto an oligo-coated surface ensures unbiased sequencing of the cDNA across all regions of the template.

After collection, all samples were sent to the Genomic Technologies Core Facility, Faculty of Life Sciences, University of Manchester, UK for library preparation and RNA-Seq. All further procedures were therefore carried out by trained staff within the facility and not by myself.

2.6.1. Library preparation and QC

cDNA samples, following LCM collection (Section 2.3), RNA extraction (section 2.4) and RNA amplification (section 2.5) were prepared for RNA-seq using the TruSeq® DNA Sample Preparation Kit (Illumina Inc, San Diego, CA, USA). Briefly, this involved checking the concentrations, size and quality of the cDNA samples intended for RNA-Seq. Before loading into a ‘FlowCell’ compatible with the Illumina HiSeq® RNA-Seq platform, cDNA samples were fragmented, and oligo primers (adapters) annealed to each end of the fragment.

2.6.2. Illumina® RNA-seq

Following library preparation and quality checks, fragments cDNA samples, labeled with adapters, were processed for RNA-seq on the Illumina HiSeq® 2000 Sequencing platform (Illumina Inc, San Diego, CA, USA), according to the manufacturers instructions. Briefly DNA fragments are hybridized onto a surface coated with millions of oligos complementary to the adapter sequences. cDNA fragments bound to the oligos, are amplified by PCR reaction. The adapters also contain primer annealing sites for the “universal sequencing primer” which identifies cDNA fragments for the sequencing reaction to begin. A polymerase recognizes the universal sequencing primer and adds fluorescently-labelled ddNTPs to the growing nucleotide chain. As well as containing a fluorophore, the ddNTPs also contain a “terminator” which prevents the polymerase being able to add another nucleotide. A laser then excites the fluorophore and the emission colour is captured on a camera. The fluorophore and the “terminator” are then cleaved from the ddNTP, reverting the nucleotide to a natural form. The polymerase can subsequently bind to the universal sequencing primer once again and facilitate the addition of another ddNTP to the nucleotide chain. Each cycle begins with a new pool of ddNTPs. Software keeps track of each “cluster” of fragments bound to the reaction
surface and records what colour is emitted after fluorophore excitation with each cycle, thereby indicating which base is present (see figure 2-5).

Figure 2-5: Principle of Illumina HiSeq® RNA-Sequencing.
RNA is extracted from samples and converted to cDNA which is subsequently fragmented, allowing adapter oligo sequences to anneal to either end of the fragment. The adapters hybridize onto a reaction surface containing millions of complementary oligo sequences and cDNA fragments amplified by a process of PCR resulting in fragment clusters on the reaction surface. Each cluster is sequenced following the addition of modified nucleotides (ddNTPs) that are conjugated to a fluorophore and a terminator sequence. A universal sequencing primer anneals to the adapter on the cDNA fragment, recognized by the polymerase which adds the corresponding ddNTP. The fluorophore and terminator sequence are then cleaved from the ddNTP allowing the polymerase to bind again and repeating the cycle. Software detects the colour emitted by each cluster following each cycle and the base sequence is generated.

2.6.3. RNA-Seq mapping, quantification and analysis

Paired-end reads were mapped to the GENCODE 15 transcriptome (Harrow, et al. 2012) (http://www.gencodegenes.org/releases/15.html) using TopHat (Trapnell, et al. 2009) (version 1.4.1; parameters: ‘-r 60 -G [gtf file] --transcriptome-index= [folder] --solexa1.3-quals’). Gene-level transcription abundances (read counts and rpkm) were estimated
using the algorithm of [Xing et al, 2006], implemented in the Partek Genomics Suite (version 6.6 (6.12.1227); Partek Inc., St. Louis, MO, USA). Transcriptome expression data was uploaded on to the UCSC browser to allow for gene of interest expression analysis.

2.7. Polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect gene expression following the creation of cDNA from RNA (see section 2.4.). Quantitative RT-PCR (qRT-PCR) was used to accurately measure and compare specific gene transcripts in samples based on the detection of SYBR-Green fluorescence.

2.7.1. Primer design

Primers for specific use in qRT-PCR were, where possible, selected from previously published work. If suitable primers could not be identified by this method primers were designed using NCBI Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/Primer-blast). Briefly, the gene of interest sequence was uploaded into the software and suitable primers identified based on (where possible) specific filtering criteria, including; intron-spanning sequences, product size between 50-150 base pairs (bp) and primer size between 18-25 bp. Primer sequences were checked for validity using the UCSC in silico PCR programme (http://genome.ucsc.edu/cgi-bin/hgPcr). A list of primers used is provided in Table 2-3.
<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Fragment Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β ACTIN</td>
<td>NM_001101.3</td>
<td>CCAACCGCGAAGATG A</td>
<td>CCAGAGGCTACAGGG TAG</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>TGACCTGTTATATTTTG CATTACC</td>
<td>CGAGCAAGACGTCAGT CCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGACCACCTTTGCAAGCT CA</td>
<td>GGGTCTTACTCCTTGGAG GC</td>
<td>206</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAAGGCCAGTGCCAGG CGG</td>
<td>GCCGCGGCGTTGAGATGT ACT</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAAGATGGGAAAGGCAG AGC</td>
<td>GTACGTGTTTGCAGCTT CA</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTACCGCAGTTGCACAA C</td>
<td>TCGCTCTCGTTCAGA AGT</td>
<td>72</td>
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<tr>
<td></td>
<td></td>
<td>GCCCTGTACCCCTCATCA AG</td>
<td>TCCGGGAAAGTGGGTCG CTG</td>
<td>79</td>
</tr>
<tr>
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<td></td>
<td>GAGGCCTCGAAGGTTATG AGA</td>
<td>TGGTCATGTTTGCATTTC CTTTGT</td>
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<tr>
<td></td>
<td></td>
<td>AGAGCGAGAACTGCCAA CTC</td>
<td>GTACAGGTCCTCCTTCCC</td>
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</tr>
<tr>
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<td></td>
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<td>GATGTCCCTGGACCGAAAA CCC</td>
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<td>ATGGTGGAGTTGGGAGAGG AGG</td>
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<td></td>
<td>AGCCCGCCCTAAAGAGCGA GTT</td>
<td>TTGGTGAGCTTCCGC TGC</td>
<td>158</td>
</tr>
</tbody>
</table>

**Table 2-3: Details of primers used for qRT-PCR analysis**

Forwards and reverse primer sequences are given for each gene of interest analysed in qRT-PCR reactions, as well as the accession number for each and the size of fragment for the final product. All primers were designed around exon-exon junction wherever possible.
2.7.2. qRT-PCR

All samples were tested in triplicate using SYBR-Green reagents and in 96-well PCR-specific MicroAmp Fast Optical Reaction plates (Applied Biosystems). qRT-PCR experiments were performed using a StepOnePlus Real-Time PCR system (thermal cycler; Applied Biosystems) and StepOne analysis software V2.1 (Applied Biosystems).

2.7.2.1. Product amplification with SYBR-green

A Precision 2X Real-Time PCR Master Mix (containing SYBR-Green; Primer Design, Southampton, UK) was used for gene amplification. The master mix contains a thermo-stable TAQ polymerase, MgCl2 (at concentrations optimized for the enzymes), buffer, dNTPs and SYBR-Green I Fluorescent dye. Each well in the 96-well plate contains a 20 µl reaction containing;

- 10 µl SYBR-Green Master Mix
- 2 µl Forward Primer (5 µM)
- 2 µl Reverse Primer (5 µM)
- 1 µl cDNA
- 5 µl RNAse-free water

Each experiment was run, in triplicate, with two reference genes (A combination of either HPRT/ β-Actin/GAPDH) and a negative control for each sample (RNA from minus-RT reaction). If there was space on the plate a second, no-template control was run for each primer where RNAse-free water was substituted for the cDNA template. After set-up the plate was sealed and briefly centrifuged (1000 X g for 30 seconds) before being transferred to the thermal cycler. The PCR reaction was performed according to the manufacturers guidelines (see below), and a primer melt-curve was generated at the end of the cycle to confirm amplification of a single product.

**PCR Reaction**

- 50°C 2 minutes
- 95°C 2 minutes
- 40 cycles of:
  - 95°C 15 seconds
2.7.2.2. Analysis of amplification data with StepOne

Quantitative product amplification was determined following measurement of the fluorescent signal emitted by SYBR-Green I. SYBR-Green binds to double-stranded DNA and emits fluorescence upon excitation. As more cDNA is generated with each cycle of PCR, more SYBR-Green binds, increasing the intensity of the signal.

The threshold cycle (Ct) was calculated as the cycle number at which the fluorescence generated within a reaction crossed the fluorescence threshold (ie. Exceeds background fluorescence levels) and became exponential. Ct values were compared between the sample of interest and a control sample. The Ct values of both the control and the sample were normalized to housekeeper gene expression. Relative levels of gene expression were calculated as the difference in fold change between the sample of interest and the control sample, each normalized to the endogenous housekeeper gene expression.

Fold change = $2^{-\Delta\Delta Ct}$

Where $\Delta\Delta Ct = \Delta Ct_{\text{experimental}} - \Delta Ct_{\text{control}}$;

$[\Delta]\Delta Ct$ is the change in cycle number between the two conditions. The fold change is calculated by $2^{-[\Delta]\Delta Ct}$ which adjusts for the exponential increase between samples.

2.7.3. Primer Efficiencies

New primers were assessed for their binding efficiencies before use in qRT-PCR experiments. A series of five concentrations (2-16ng/µl) of cDNA were set up for each primer of interest, alongside two housekeeper genes (HPRT/ B-ACTIN) in a standard 96-well PCR-plate (Applied Biosystems). Each concentration was tested in duplicate and including a minus-RT control reaction for each primer.

qRT-PCR was performed as previously described (section 2.7.2.) and average Ct values recorded for each concentration and primer. The difference in gene expression of each concentration of sample between each primer of interest and housekeeping genes was calculated and entered into an Excel Spreadsheet alongside the corresponding log[concentration]. The slope was calculated for each primer. A slope of <0.1 is
considered to indicate good primer/cDNA pairing.
The binding efficiency was calculated from the slope of the standard curve using the formula:

\[
\text{Efficiency} = (10^{(-1/\text{slope})}-1) \times 100
\]

This identifies the increase in PCR product after each cycle. An ideal reaction has an efficiency of 90-100%.

2.8. Statistical analysis

All data were entered into Excel which was used to calculate averages, standard deviation and standard error as well as generating graphs and charts. SPSS was used for statistical analysis using a minimum n=3 determinant where possible. CHI-D, Control and fetal samples were compared using the Mann-Whitney U test and correlation assessed using the Spearman Rank Correlation Test. Group analysis was performed using the ANOVA test with Tukey post-hoc analysis.
Chapter 3- Characterising histology and key developmental transcription factor expression in CHI-D pancreas

3.1. Introduction

Congenital hyperinsulinism of infancy (CHI) is a rare, genetic disorder most commonly caused by mutations affecting the $K_{ATP}$ channel (Dunne et al., 2004). Before the genetic basis for CHI was discovered, research was focused on the histology of the disease and the association of perturbed glucose homeostasis with the pancreatic architecture of affected patients.

This chapter provides a detailed investigation of the histology related to CHI. Based on the historical association of CHI with nesidioblastosis, a hypothesis of islet neogenesis was explored with consideration for a recapitulation of the fetal endocrine differentiation programme being employed. The aim of the work was to extend on previous histological observations reported in the field, and to offer new insights and comparisons for the analysis of CHI. Investigations were focused solely on the diffuse form of the disease with five patient samples studied alongside age-matched controls, allowing for a comprehensive exploration of hormone expression and histological compositions. This chapter also presents a unique collaboration of CHI-D research with studies into human fetal development, investigating aspects of hormone expression, endocrine differentiation and islet cell maturity that has never, to our knowledge, been explored previously.

3.1.1. Nesidioblastosis in diffuse-CHI

Laidlaw, in 1938, appears to have provided the earliest description of hyperinsulinism; relating severe and recurrent episodes of hypoglycaemia to histological features that he termed “nesidioblastosis” (Laidlaw, 1938). For several years afterwards this term continued to be used describing, what was defined to be, the proliferation of islet structures from the ductal epithelium; a phenomenon characterized by the diffuse distribution of insulin-positive cells outside of defined islets, and their appearance to be “budding” from the ductal epithelium (Laidlaw, 1938, Rahier, 1981, Dahms et al., 1980, Heitz et al., 1977).

A similar process had also been described in fetal samples (Bensley 1911; Laidlaw 1938). Many authors tried to validate these observations of nesidioblastosis by analyzing CHI samples for their profiles of proliferation. In 1998, Sempoux et al., published the first investigation of $\beta$-cell proliferation in CHI and control tissue (Sempoux et al., 1998).
expression of Ki67 was not found to be significantly elevated in CHI-D samples as compared to age-matched controls, and the Ki67-labelling was not observed to be more obvious in either the isolated, or small clusters of β-cells, nor in those associated with exocrine ducts (Sempoux et al., 1998). As nesidioblastosis had already been reported by several authors to also be apparent in normoglycaemic neonates (Dahms et al. 1980; Goossens, et al. 1989; Goudswaard et al. 1986; Jaffé et al. 1980; Rahier 1989; Rahier et al. 1984; Sempoux et al. 1995), and proliferation in the CHI β-cells was not observed to be neither abnormal nor continuous (Sempoux et al., 1998), the significance of the histological presentation associated with nesidioblastosis was becoming less apparent.

However, in the campaign to define nesidioblastosis as a pathologic mechanism that underlies CHI, the expression of genes or markers involved in endocrine development of the pancreas do not appear to have been considered. The presence of insulin-positive cells emerging from ductal structures may not be related to a continuous proliferation of β-cells, but rather a recapitulation of the fetal development programme and a neogenesis of islet cells emerging from a progenitor population residing in the ducts.

3.1.2. Progenitor population within ductal epithelium

The concept of neogenesis has origins in the observations reported from various models and species that described the appearance of hormone-positive cells in, or budding from, the ductal epithelium. For many years the ductal epithelium has therefore been the focus for many researchers investigating the potential for pancreatic regeneration.

From as early as 1911 (Bensley, 1911) the observation of insulin-producing cells in, or close to, ductal structures has led to the suggestion of a ductal origin for the generation of islet cells in the postnatal pancreas, and there have been many that have advocated this idea since (Bonner-Weir et al. 1993; Bouwens and Pipeleers 1998; Bouwens and Rooman 2005; Finegood, et al. 1999; Kozawa, et al. 2005; Tokui, et al. 2006; Wang et al. 1995). Morphologically, the emergence of islets from ducts mirrors what is normally seen during fetal development and, when observed in the postnatal pancreas, represents an appealing assumption that fetal development can be recapitulated in the pancreas after birth.

Growing evidence supports a dynamic regulation of β-cell mass in the adult pancreas that can increase or decrease in both function and mass in order to maintain a constant level of glycaemic control. Possible mechanisms that could account for changes in β-cell mass include increases or decreases in proliferation and/or cell death rates, changes in β-cell volume, induction of a programme of neogenesis, or a combination of these factors.
Histologically the accumulation of islet cells around ducts during pancreatic regeneration has been extensively reported in the literature (Inada, et al. 2008; Rooman and Bouwens 2004; Xu et al. 2008). In vitro experiments culturing human ductal cell lines show potential for at least partial endocrine reprogramming (Bonner-Weir et al. 2000; Heremans, et al. 2002). However, the majority of data in this area come from animal studies and models of severe pancreatic damage.

Specific β-cell ablation following injection of streptazocin or alloxan is sufficient in mice to trigger regeneration, identified as the reappearance of β-cells (Finegood et al., 1999). This reappearance cannot otherwise be accounted for by the proliferation of escapee β-cells and are therefore thought to represent a new population of insulin-producing cells derived from a progenitor source.

In pancreatic duct ligation models, a ductal proliferation is observed alongside an increase in insulin-positive cells (Bouwens & Rooman, 2005). In a similar model, duct cells demonstrated an ability to differentiate into islet cells following the induction of NGN3—an essential factor involved in endocrine commitment (Xu et al., 2008). Overexpression of Ngn3 in pancreatic ductal cell lines has also shown success in initiating an endocrine differentiation programme (Gasa et al. 2004; Heremans et al. 2002), further evidence that the ductal cell population has the ability to change fate.

However, Ngn3 expression data in these models is not conclusive and conflicting reports are also represented in the literature (Lee et al. 2006). Several lineage tracing and genetic labeling experiments have also failed to demonstrate a direct contribution of duct cells to the regenerating β-cell population (Dor et al. 2004; Nir, et al. 2007; Solar, et al. 2009; Teta et al. 2005).

Despite extensive research and increasingly more sophisticated studies the mechanisms that underlie β-cell mass regulation are still inconclusive and the existence of a progenitor population within the ductal epithelium remains controversial.

### 3.1.2. Endocrine differentiation during human fetal development

All cell lineages of the pancreas, including the acinar cells, their associated ducts and the endocrine cells, derive from an original pool of apparently identical progenitor cells (Gu et al. 2002; Herrera 2000).

Briefly, the fetal programme of endocrine differentiation involves the commitment of SOX9- and PDX1-positive multipotent progenitors of the ductal epithelium towards an
endocrine fate following the transient expression of NGN3 (Akiyama et al. 2005; Gradwohl et al. 2000; Gu et al. 2002; Kawaguchi et al. 2002; Seymour et al. 2008). This initiates a complex and hierarchical cascade of TF expression, delineating the endocrine progenitors into the different islet-cell fates (Gasa et al. 2004; Habener et al. 2005; Heremans et al. 2002; Sosa-Pineda, et al. 1997) (Figure 3-1).

**Figure 3-1. Transcriptional regulation of β-cell development**

Schematic diagram showing some of the key transcription factors involved in regulating β-cell development in the fetal pancreas. Adapted from (Wilson et al. 2003)

The majority of knowledge of the transcriptional regulation has derived from extensive animal studies in which gene manipulation and loss-of-function phenotypes can provide valuable information on signaling pathways and determination of pancreatic cell fate (Review; Jensen, 2004). There have been several studies since that have attempted to validate these findings in early human tissue (Jennings, et al. 2013; Lyttle, et al. 2008; Piper et al. 2004; Sarkar, et al. 2008). The process of differentiation in both mouse and human involves the movement of cells out of the duct-like structures into the pancreatic mesenchymal stroma (Murtaugh 2007).

Endocrine differentiation from the ductal epithelium is initiated by the transient expression of NGN3 (Gradwohl et al., 2000). NGN3 is both necessary and sufficient to drive endocrine differentiation. Without it islets fail to develop (Gradwohl et al., 2000).

Downstream targets of Ngn3 include NeuroD1, Pax4 and Nkx2.2, all of which cause impaired β-cell differentiation when inactivated in rodent models (Gasa et al. 2004; Heremans et al. 2002; Huang, et al. 2000; Mellitzer, et al. 2006; Smith, et al. 2004; Sosa-Pineda et al. 1997; Sussel et al. 1998). Together with Pdx1, the co-ordinated expression of other TFs including Pax6, Nkx6.1 and MafB, all contribute to the regulation of endocrine differentiation (Habener et al., 2005).

Between 8-21 wpc in human fetal pancreas, insulin-positive cells can be seen to colocalise with NKX6.1, FOXA2, PDX1, ISL-1, NEUROD1, NKX2.2 and PAX6 (Jennings et al., 2013). At this time NKX2.2 expression is high in α-, β- and some δ- cell types, but NKX6.1 only colocalises in insulin-positive cells (Lyttle et al., 2008). The same coexpressions can also be observed in adult human islets (Lyttle et al., 2008). These observations suggest that these TFs may initially be involved in the differentiation
programme of endocrine cell types, but their persistence in the mature organ assumes an additional maintenance role for these factors.

3.1.3. Classification of CHI

Nesidioblastosis has subsequently been demonstrated as a common feature of normoglycaemic neonates and infants through to the first year or two of life (Goossens et al. 1989; Goudswaard et al. 1986; Jaffe et al. 1980; Kassem et al. 2000; Rahier 1989; Rahier et al. 1984; Sempoux et al. 1995). With just under two decades of researching the disease, Rahier et al., have studied almost 100 neonates and infants with CHI and have not been able to define a single morphological feature or pathological entity in the islets of these patients that are, or could be, attributable to the disease.

Histological investigation can, however, divide CHI cases into two major subtypes; diffuse and focal (Rahier et al. 1984; Goossens et al. 1989; Jaffe et al. 1980). Diffuse-CHI (CHI-D) is the more common of the two and presents as an abnormal function of all the β-cells throughout the pancreas. CHI-D cases are characterized by the nesidioblastosis that originally defined them, but also by the incidence of enlarged cell-nuclei occurring throughout tissue samples. However, there are no significant changes in β-cell mass (Rahier et al. 1984).

Although no longer used as a diagnostic or pathogenic observation for CHI-D, nesidioblastosis is still a characterizing feature in patient tissue, the significance of which is incompletely determined. Whilst poorly-defined islet structures with insulin-positive cells diffusely distributed and associated with ducts can also be observed in healthy newborns, it is a feature that normally “evolves” within the first year of life as the islets take on a more adult-type architecture (Goossens et al. 1989; Jaffe, et al. 1982; Kassem et al. 2000; Rahier et al. 1981). In CHI-D samples an immature-type β–cell distribution persists (Ariel, et al. 1988; Kassem et al. 2000; Rahier et al. 1984). Patients with CHI-D that are unresponsive to treatment, require a total or near-total pancreatectomy in order to treat the disease (Dillon, 2013; Glaser, 2011; Rahier et al. 1984).

Focal CHI (CHI-F) presents as a lesion, or several lesions that, although rarely visible macroscopically or with conventional H&E staining microscopically, when analysed by IHC contain aggregates of insulin-positive cells. The removal of these lesions would be sufficient to cure CHI-F patients without the need for a full pancreatectomy procedure (Rahier et al. 1984).
3.1.4. Mutations in $K_{\text{ATP}}$

Despite a genetic association now reported for the majority of CHI cases, offering mechanisms to account for the dysfunctional glucose-insulin coupling of $\beta$-cells, they provide little explanation for the histological characteristics observed in CHI samples.

The $K_{\text{ATP}}$ channel is a membrane-spanning protein complex that functions to couple metabolic state with cellular excitability (Yokoshiki, et al. 1998). Although it can be found in different compositions of inwardly-rectifying potassium ion channels and SUR1 subunits within other tissues, including the heart, muscle and brain, the pancreatic $\beta$-cell $K_{\text{ATP}}$ channel specifically, is composed of four subunits of Kir6.2 surrounded by four SUR1 subunits (Aguilar-Bryan, et al. 1995; Sakura, et al. 1995).

Under normal resting conditions the $\beta$-cell $K_{\text{ATP}}$ channels are open (Clark and Proks 2010). As glucose levels rise, glucose is transported into the $\beta$-cell via the GLUT2 transporter where it is metabolized by glucokinase (GCK) to generate ATP. ATP acts on the $K_{\text{ATP}}$ channel causing it to close, in turn causing membrane depolarisation and the opening of voltage-gated calcium ($Ca^{2+}$) channels. An influx of $Ca^{2+}$ triggers the release of insulin via the exocytosis of vesicles in which insulin is stored (Ashcroft, et al. 1984; Misler, et al. 1992).

Disabling mutations in the $K_{\text{ATP}}$ channel result in CHI when the channel remains closed or absent from the $\beta$-cell membrane. The unregulated influx of $Ca^{2+}$ that ensues causes unregulated insulin release, thereby uncoupling glucose sensing with insulin secretion. The result is defined as an inappropriate insulin release (Dunne et al. 2004). In the majority of CHI cases plasma insulin levels are not significantly elevated (Stanley 1997). CHI is therefore not the result of a hypersecretion of insulin but rather an inappropriate secretion in the presence of a low blood glucose concentration (Dunne et al. 2004).

Whilst mutations in $K_{\text{ATP}}$ channel genes can primarily account for the unregulated insulin release in CHI samples, the system in which the $K_{\text{ATP}}$ channel is involved is complex. It is possible that other factors contribute to the aetiology of CHI, consequential to the $K_{\text{ATP}}$ channel mutations. Abnormal development and dysregulated function of the islets may also contribute to the distinctive and prolonged histological features associated with CHI.
3.2. Aims

The work in this chapter was carried out to investigate the basic histology of CHI-D samples as a comparison with age-matched control and fetal samples.

3.2.1. To characterise hormone expression and islet architecture in CHI-D samples; A comparison with age-matched controls

Hormone expression was investigated by IHC in CHI-D and age-matched control pancreatic samples. Structures resembling those of mature islets of Langerhans were assessed by immunofluorescence for the endocrine cell organisation in samples of CHI-D and age-matched control pancreas and the theory of neogenesis presented as hormone-positive cells emerging from ductal structures was also explored histologically.

3.2.2. To Investigate a process of islet neogenesis in CHI-D; A comparison with normal human fetal development

Components of the β-cell differentiation programme normally represented during human fetal pancreas development were investigated in samples of CHI-D and age-matched control pancreas. Transcript levels were assessed using qRT-PCR techniques from pancreatic biopsy samples whilst protein expression was detected in tissue sections by virtue of IHC.

3.2.3. To determine whether CHI-D is consistent with a prolonged immaturity of islets

Characteristics usually representative of the fetal pancreas, including disrupted islet architecture, NKX2.2 colocalisation in SS-producing δ-cells and expression of the fetal hormone gastrin were all assessed in CHI-D samples to explore whether CHI-D pancreas resembles an immature organ that has failed to mature appropriately with postnatal development. TF expression for some key genes involved in β-cell maturity were also analysed by qRT-PCR.
3.3. Results

Research in CHI is lacking due to the scarcity of tissue and the limited availability of appropriate control samples with which to compare with. For several decades the disease was attributed to a proliferative phenomenon termed nesidioblastosis (McQuarrie, 1954) however, with the evidence that nesidioblastosis was in fact a feature of normal development in strictly normoglycaemic neonates (Gregg, et al. 2012; Jaffe et al. 1982; Rahier et al. 1984), further research was instead focused on understanding the pathogenesis of CHI. It is clear that no single morphological feature is pathognomic of CHI islets, but with the distinction of two forms of CHI identified; focal and diffuse, there was an increased interest in distinguishing these two subtypes morphologically as their treatment options could be very different (Rahier et al. 2000). With the progression in genetic and molecular biology investigation discovering mutations mapped to chromosome 11, and in particular to genes encoding subunits of the K\textsubscript{ATP} channel (Dunne et al. 1997; Nestorowicz et al. 1997; Otonkoski, et al. 1999; Thomas et al. 1995) over the years less histological investigations were being carried out, and where nesidioblastosis was once the focus of such research there now exists gaps in truly exploring this ascribing feature.

3.3.1. Characterising hormone expression and islet architecture in CHI-D and age-matched control samples

Although characteristics of the β-cell have been fairly well represented in the literature, the consequences of the other pancreatic cell types in CHI-D have been less-well explored. To determine whether the histology associated with CHI-D was insulin-specific the expression of other islet hormones in sequential sections of CHI-D tissue was analysed using IHC techniques. Consistent with published data in this field (Dahms et al. 1980; Rahier et al. 1984), insulin-positive cells were observed to be diffusely distributed throughout the pancreatic tissue, with observations of individual or small clusters of insulin-positive cells amongst the exocrine tissue, with poorly defined islet-structures, as well as the detection of insulin-positive cells associated with ductal structures (Figure 3-2).
Sequential staining with glucagon and SS revealed a similar diffuse distribution, not just for insulin-positive cells, but also for those containing other islet hormones; scattered expression with a reduced tendency to form islet aggregates (Figure 3-3). All three hormone cell-types localized to similar regions either as islets (arrows in Figure 3-3) or clustered in areas lacking mature islet structures. This was consistent in both CHI-D and neonatal control samples up to 6 months post-birth. Negligible extra islet PP-positive or ghrelin-positive cells were detected in these samples. In both control and CHI-D samples at or beyond one year of age, hormone staining was more noticeably restricted to islet structures, consistent with the convergence to a more mature, adult-type distribution where hormone-positive cells are predominantly seen clustering in large islet aggregates (Figure 3-4). Cells containing glucagon, SS and PP could also be observed in association with ductal structures, further evidence that “nesidioblastosis” is not specific to the β-cell population (Figure 3-5).
Figure 3-3. Hormone distribution in diffuse-CHI and age-matched controls
Brightfield images of 5 µm sections of CHI-D biopsies and age-matched control pancreas counterstained with Toluidine blue. For each sample (A-I) serial sections show immunohistochemical staining (brown) for insulin, glucagon and somatostatin respectively. Arrows (A, C, F, G, H, I) show islet structures. Scale bars represent 200 µm.
Figure 3-4. Hormone Expression in adult human islets
Brightfield images of 5 μm sections of adult pancreatic tissue showing sequential immunoreactivity (brown) of insulin (A), glucagon (B) and somatostatin (C) counterstained with Toluidine blue. Scale bar represents 100 μm.

Figure 3-5. Islet hormones in CHI-D ducts
Brightfield images of 5 μm sections from CHI-D biopsies counter-stained with Toluidine blue. Immunohistochemical staining (brown) for glucagon, somatostatin and PP shows their association with ductal structures. Scale bar represents 50 μm.

Although expression was predominantly diffuse throughout the pancreatic tissue in CHI-D samples, islet structures were still identified (arrows, Figure 3-3). Dual immunofluorescence in CHI-D samples revealed an intermingled distribution of glucagon-, and SS-positive cells amongst insulin-secreting cells throughout the islet, including at the centre, for all ages studied (Figure 3-6). This was in contrast to the typical distribution patterns observed in control islets which presented with a predominantly β-cell core with α- and δ-cell types around the periphery of the islets, as expected (Gregg et al. 2012). Ghrelin-positive cell types however, were only found peripherally in islets along with occasional PP-cells, in both CHI-D and control samples. In the older CHI-D patient (CHI-D 5, 12 mo), α- and δ-cell types tended to localize more peripherally in the islet cluster, surrounding clusters of β-cells. Interestingly, the islet aggregates that formed in CHI-D samples appeared unusual in structure. Larger islets were seen in the older CHI-D samples, resembling an accumulation of several islet-like clusters that aggregated to form a single large islet (Figure 3-6 F,H).
Figure 3-6. Islet composition in diffuse-CHI and age-matched controls

Dual immunofluorescence of 5 µm sections from CHI-D biopsies and age-matched control pancreas counter-stained with DAPI. For each sample (A-I) serial sections show insulin immunoreactivity (green) with glucagon, somatostatin, ghrelin and PP respectively (red). The inset box in (B,E,F) shows PP immunoreactivity within the same sample. Scale bars represent 100 µm.
IHC detection clearly showed the expression of ghrelin and PP in CHI-D and control samples. Others have shown an increase in the volume density of PP cells in cases of CHI-D (Rahier et al. 1984) however, the relative infrequency of these hormone-positive cell types made meaningful spatial analysis difficult, although no obvious variations in either abundance or distribution for any of the islet cell types investigated were observed between CHI-D and control samples in our experiments.

3.3.2. Investigating islet neogenesis in CHI-D through expression profiles of TFs in a comparison with age-matched controls

Given the altered islet structure and hormone-positive cells associated with ducts, we looked for evidence of ongoing or aberrant endocrine differentiation in neonatal and CHI-D samples. Our group has previously shown the onset of endocrine differentiation to commence in human fetal pancreas at approximately 8 wpc, although it remains unclear when the window of endocrine differentiation closes (if it does close) in human fetal or postnatal development (Jennings et al. 2013; Piper et al. 2004). The distribution of endocrine cells close to duct-like structures during the neonatal period, and the lack of mature islet structures, led us to hypothesize that β-cells might still be arising by neogenesis in the early neonatal period. NGN3 is a crucial TF involved in endocrine differentiation; without it islet cells do not form (Gradwohl et al. 2000). NGN3 expression within the ductal compartment, detected at 10 wpc in the human fetal pancreas, identifies which of the multipotent cells are destined to become hormone-secreting endocrine cell-types (Figure 3-7, A). These NGN3-positive cells emerge from the multipotent SOX9-positive population, subsequently losing the SOX9 expression as they commit to the endocrine lineage (Figure 3-7, B). By 15 wpc clusters of hormone-positive cells, suggestive of primitive islet structures, can be seen aggregating around the ducts (Figure 3-7, C).

Despite this, NGN3 transcripts were barely detected by qRT-PCR in either the CHI-D or age-matched control samples and expression was not statistically altered between the two groups (Figure 3-8). NGN3 protein-positive cells were also barely detected in the five CHI-D cases or age-matched controls, however rare and weakly immunoreactive were occasionally observed in all samples following IHC investigation (Figure 3-9).
Figure 3-7. NGN3 expression during human fetal development
Immunohistochemical staining in 5 μm sections of human fetal pancreas (11-15 wpc). (A) shows a brightfield image of NGN3 reactivity (brown) counter-stained with Toluidine blue. (B) shows omission of SOX9-reactivity (green) in NGN3-positive (red) cells, whilst (C) shows primitive islet clusters containing insulin (green) and glucagon (red) expression close to ductal structures. Scale bars represent 20 μm (A) and 10 μm (B,C). [B] Taken from Jennings et al. 2013.

Figure 3-8. NGN3 mRNA expression in CHI-D and age-matched controls
Quantitative gene expression for NGN3 in samples of CHI-D and age-matched controls relative to 14 wpc fetal specimens. N=3. Bars show mean error bars show standard error.
Figure 3-9. NGN3 immunoreactivity in samples of fetal and CHI-D pancreas

NGN3 protein was detected as strong nuclear staining in the ductal structures of fetal pancreas (arrows A, E). Rare cells in CHI-D and control samples showed weak brown nuclear staining, close to ductal structures (arrows B, C, D), shown at higher magnification in (F, G, H). Scale bars (A-H) represent 20 µm.

To further investigate whether a process of endocrine differentiation was occurring in the CHI-D and age-matched control samples, key TFs important for the differentiation and function of β-cells were examined at both the transcript and protein level.

The mean expression of PDX1, FOXA2, SOX9, NKX6.1 and MAFA as examined by qRT-PCR were all quantitatively no different in CHI-D samples compared to controls (n=3), whilst NEUROD1 showed a significant decrease overall in expression between CHI-D and age-matched controls (P<0.05)(Figure 3-10).

The pattern of TF expression during the development of the human fetal pancreas changes with the differentiation of the various pancreatic lineages. These developmental markers were first identified in human fetal samples by IHC (Figure 3-11). The early cells of the developing pancreas are positive for PDX1 and SOX9; the coexpression of these markers identifies a population or multipotent cells from which all the cells of the pancreas will emerge, including the hormone-secreting endocrine cells. As development progresses further TFs are expressed including GATA4 marking the acinar compartment and NKX6.1 in developing endocrine cells. FOXA2 can also be identified in the ductal and endocrine compartments during differentiation of the two lineages.
Figure 3-10. Expression of developmental transcription factor mRNA in CHI-D and age-matched controls
Quantitative gene expression of key transcription factors involved in pancreas development and beta cell maturity in samples of CHI –D relative to age-matched controls. N=3. Bars show mean, error bars show standard error. Statistical significance calculated using Mann-Whitney U test P<0.05.

Figure 3-11. Human pancreas formation and early lineage differentiation
(A) Human embryo at 28 dpc/CS13. (B) and (C) transverse 5 μm sections of human endoderm at 28 dpc stained by immunohistochemistry for SOX9 (B) or PDX1 (C) and counter stained with Toluidine blue. Outgrowth of the dorsal and ventral pancreatic buds are indicated in (B) with the labels (v) ventral bud and (d) dorsal bud. Panel (D) shows immunohistochemical staining for key transcription factors involved in normal development; SOX9, PDX1, NKX6.1, FOXA2, and GATA4. Different sections are shown in (D) to permit demonstration of immunoreactivity but are all taken from the same specimen. Scale bars represent 800 μm (A), 200 μm (B-C), 50 μm (D). [(A-C) Adapted from Jennings et al. 2013].
Profiling the same TFs in CHI-D tissue and corresponding controls revealed FOXA2, PDX1 and NKX6.1 all to be appropriately detected as nuclear proteins in the endocrine cells, with SOX9 detected in the ductal cells and GATA4 in the acinar compartment (Figure 3-12).

![Figure 3-12. Profiling transcription factor expression in CHI-D and control samples](image)

Brightfield images showing immunoreactivity (brown) for some of the key transcription factors involved in normal pancreatic development; SOX9, PDX1, FOXA2, NKX6.1 and GATA4 in 5 μm sequential sections of CHI-D (A-E) and age-matched controls (F-J) counter stained with Toluidine blue. Scale bars represent 100 μm.

3.3.3. CHI-D as a model of immature islets

Given previous suggestion of β-cell immaturity in CHI-D (Heitz et al. 1977; Rahier et al. 1984), we studied the potential coexpression of insulin with other islet hormones. During human fetal development coexpression of insulin occurs in approximately <10 % of glucagon-positive cells (Piper et al. 2004). Dual stained cells were not observed in any of the control samples. However, in two of the four CHI-D cases investigated, single cells containing both insulin and glucagon were detected, although their incidence was rare, occurring in approximately 2-5% of glucagon- and insulin-positive cells (Figure 3-13). Although this was superficially reminiscent of observations in fetal development, surprisingly, whereas in fetal cells costaining completely overlapped, in the two CHI-D samples insulin and glucagon appeared to localise discretely to opposite aspects of the same cell (Figure 3-14). Colocalisation was not observed between insulin and SS, insulin and ghrelin or insulin and PP in CHI-D samples.
Figure 3-13. Co-hormone expression in CHI-D

Dual immunofluorescence of 5 µm sections from CHI-D biopsies and 10 wpc fetal pancreas counter-stained with DAPI. Co-expressing cells for insulin (green) and glucagon (red) were identified in two CHI-D samples (A, B), a similar phenomenon to that seen during normal fetal pancreas development (C). Scale bar represents 50 µm.

Figure 3-14. Dual hormone expression in CHI-D islet cell

Dual immunofluorescence showing insulin (green) and glucagon (red) co expression in a CHI-D islet cell counter stained with DAPI. The Panel (A-H) represents progressive z-stack images taken through a 5 µm cross-section of a CHI-D sample. No scale bar available for this image.

To further investigate the authenticity of the insulin-secreting cells in CHI-D samples, markers of mature β-cells were examined. This was to rule out any processes of transdifferentiation from other pancreatic lineages, and to verify, as best as possible, that the insulin-producing cells present in CHI-D were bona fide β-cells. Sequential sections of pancreas, stained by IHC, showed a similar distribution of NKX2.2 and ISL-1 localising in areas that were also apparently positive for insulin in both CHI-D and control specimens (Figure 3-15). Additional IF experiments provided further investigation into the colocalisation of β-cell-specific markers with secreted insulin in samples of CHI-D, age-matched controls and fetal pancreatic tissue (Figure 3-16). In all samples investigated PDX1, FOXA2, NKX6.1 and NKX2.2 colocalised in the majority of insulin-expressing endocrine cells although insulin-positive cells that appeared to be absent of PDX1, NKX6.1 and NKX2.2 were observed in both CHI-D and age-matched control samples (arrows Figure 3-16).
Figure 3-15. Endocrine cell markers in clusters of insulin-positive cells in CHI-D and control samples

Brightfield images of 5 μm sections of CHI-D biopsies and age-matched control pancreas counter-stained with Toluidine blue. For each sample (CHI-D and Control) sequential sections show immunoreactivity (brown) for NKX2.2, ISL-1 and insulin. Scale bars represent 50 μm.

Figure 3-16. Coexpression of mature β-cell markers with insulin-positive cells in CHI-D, control and fetal samples

Dual immunofluorescence of 5 μm sections of CHI-D, age-matched control or fetal (11 wpc) counter-stained with DAPI, showing immunoreactivity for insulin (green) with nuclear PDX1, FOXA2, NKX6.1 and NKX2.2 (red). Sections are not sequential. Scale bars represent 50 μm. White arrows show apparently protein-negative insulin-positive cells.

Whilst investigating the expression profile of key TFs involved in fetal endocrine development, NKX2.2 expression was also explored. NKX2.2 is a homeodomain TF that is essential for the differentiation of islet α-, β- and PP- cell types (Sussel et al. 1998).
NOKX2.2 also plays a regulatory role in the function of adult β-cells related to insulin production and glucose-stimulated insulin secretion (Doyle and Sussel 2007).

Whilst levels of NOKX2.2 did not vary during the first year of postnatal life in the control samples, overall, the expression of NOKX2.2 transcripts tended to be higher in CHI-D samples relative to their age-matched controls, although only two out of five CHI-D samples, aged 2 and 6 months, reached statistical significance (P<0.05) (Figure 3-17). However, by 12 months of age NOKX2.2 expression was not different between the CHI-D and control samples.

![Figure 3-17. Expression of NOKX2.2 mRNA in cases of CHI-D relative to age-matched controls](image)

Quantitative gene expression of NOKX2.2 in CHI-D samples (2-13 months) relative to age-matched control pancreas (2-12 months). N=3. Bars show mean, error bars show standard error. Statistical significance calculated using Mann-Whitney U test P<0.05.

IF was used to determine if the increased transcript expression of NOKX2.2 could also be observed at the protein level (Figure 3-18). Nuclear NOKX2.2 was observed in insulin-, glucagon-, SS- and ghrelin- producing islet cell types at varying proportions in all samples studied. On sequential sections of CHI-D, neonatal control or fetal pancreas, the percentage of hormone-positive cells observed to have nuclear NOKX2.2 reactivity were counted for each sample (Figure 3-19). NOKX2.2 protein was found in 96% of insulin-positive, and 90% of glucagon-positive cells in CHI-D samples, with 86% of ghrelin-positive, and 65% of SS-positive cells also colocalising with NOKX2.2. In contrast, NOKX2.2 was absent from approximately 75% of SS-producing δ-cells in control samples whilst the same high percentage of β- and α- cells showed NOKX2.2 immunoreactivity (92% and 82% respectively). In human fetal pancreas NOKX2.2 has been quoted as present in all endocrine cell-types in the second trimester (Lyttle et al. 2008), although only α-, β-, δ-,...
and PP-cell types were explored. Here at 15 wpc, similar to the postnatal CHI-D samples, approximately two thirds of SS-producing δ-cells contained nuclear NKX2.2 (70%) whilst the majority of glucagon-, and insulin- positive cells were also NKX2.2-positive (97% and 80% respectively).

Figure 3-18. Expression of NKX2.2 in CHI-D, control and fetal pancreatic endocrine cells
Dual immunofluorescence of 5 µm sections from (A) CHI-D biopsies, (B) age-matched control and (C) 15 wpc fetal pancreas counter-stained with DAPI. Co-expression of NKX2.2 (red) with islet hormones insulin, glucagon, somatostatin and ghrelin (green) was assessed across the different samples. Scale bar represents 10 µm.

With a significant decrease in NKX2.2/SS colocalisation observed between fetal and neonatal control pancreatic samples (P<0.01), a further range of infant samples were investigated to determine whether NKX2.2 reactivity is progressively lost from δ-cells as the islets mature (Figure 3-20). Cells coexpressing NKX2.2 and SS were counted as a percentage of the total SS-producing cell population in samples of control pancreas ranging in age between 6 weeks and 4 years, 12-13 years, and adult samples. Although more δ-cells were observed to contain nuclear NKX2.2 in the older samples (12-13 years, and adult, 42.6 and 37.7 % respectively), the majority of SS-producing cells in all cases remained consistently negative for NKX2.2, a feature seemingly unchanged by islet maturity.
Figure 3-19. Quantification of endocrine cells displaying co-expression with NKX2.2

5 µm sections of CHI-D (2-6 months), fetal (11-15 wpc) and age-matched control samples were counted for dual immunoreactivity of NKX2.2 with each hormone; insulin, glucagon, somatostatin and ghrelin, counted across 10 fields of view and expressed as a percentage of the total hormone population for each. In total, 604 hormone-positive cells were counted in two fetal samples (11-15 wpc), 2651 hormone-positive cells across four CHI-D samples (2-6 months) and 2181 hormone-positive cells in age-matched control samples (2-6 months). Bars show mean, error bars show standard error. Statistical significance calculated using Mann-Whitney U test (*P<0.05, **P<0.01).
Figure 3-20. NNX2.2 expression in δ-cells in control postnatal and adult pancreas samples

The percentage of somatostatin-producing cells positive for NNX2.2 expression were counted as a proportion of the total δ-cell population in 5 µm sections of control pancreas ranging from 6 weeks – 4 years of age, then 12-13 years and adult specimens (A). By virtue of dual immunofluorescence NNX2.2-positive cells were identified as nuclear red staining, with somatostatin detected as green cytoplasmic staining, each counter-stained with DAPI (B). On average, 93 somatostatin-positive cells (±38) were counted across 11 different samples. Bars show mean, error bars show standard error (A). Scale bar represents 10 µm (B).

With the elevated expression of NNX2.2 transcripts in CHI-D biopsies and persistence of NNX2.2 protein in SS-producing δ-cells at a similar incidence to the fetal period, it was hypothesized that the CHI-D islets may be maintaining an immature phenotype, failing to mature appropriately in accordance with their age-matched control samples. To further investigate the developmental status of the CHI-D islets gastrin expression was explored in samples of CHI-D, fetal and neonatal pancreas (Figure 3-21). Gastrin is a peptide hormone mainly secreted within the gastric antrum to lower pH and increase stomach motility and accelerate food digestion (Baldwin 1995; Samuelson and Hinkle 2003). By virtue of IHC, gastrin-positive cells were observed in the epithelium of human fetal intestine but were not detected in fetal pancreas in the same section (Figure 3-21) or in
any other stages of fetal development explored. Likewise, gastrin immunoreactivity was not seen in any of the CHI-D samples, nor in any of the age-matched control specimens (Figure 3-21).

![Brightfield images of 5 µm sections of fetal and CHI-D samples. Positive Gastrin immunostaining can be seen in the intestine of 10 wpc fetal sample but is absent from the pancreas of the same specimen and not detected in any of the CHI-D samples or age-matched controls (data not shown). Scale bars represent 100 µm.](image)

**Figure 3-21. Gastrin expression in human fetal development and CHI-D**

Brightfield images of 5 µm sections of fetal and CHI-D samples. Positive Gastrin immunostaining can be seen in the intestine of 10 wpc fetal sample but is absent from the pancreas of the same specimen and not detected in any of the CHI-D samples or age-matched controls (data not shown). Scale bars represent 100 µm.

Finally, additional TFs involved in endocrine cell maturity and function were profiled for expression in CHI-D samples relative to their age-matched controls. Although overall mean expression of PAX4, PAX6, ARX and MAFB was decreased in CHI-D samples, one sample; CHI-D 4, had elevated MAFB and PAX6 transcripts compared to the control. However, PAX4 and ARX expression was consistently decreased in all five CHI-D samples (Figure 3-22).

![Expression of developmental marker mRNA in samples of CHI-D and age-matched controls](image)

**Figure 3-22. Expression of developmental marker mRNA in samples of CHI-D and age-matched controls**

Quantitative gene expression of key transcription factors involved in pancreas maturity and function in samples of CHI-D relative to age-matched controls. N=3. Bars show mean, error bars show standard error. Statistical significance calculated using Mann-Whitney U test P<0.01.
3.4. Discussion

Diffuse insulin expression and investigation of β-cell characteristics in CHI-D has been considerably represented in the literature, but to the best of our knowledge, the effect of CHI-D on other pancreatic-cell lineages has not been previously explored. We wanted to characterize these additional cell-types in CHI-D in order to determine a wider picture of the pathological aetiology of the disease, pursuing the hypothesis that it is not simply a β-cell specific entity but rather the result of a developmental disorder.

The driver for this work came from the observation of insulin-positive cells emerging from ductal structures, as well as their diffuse distribution and poorly defined islets throughout the pancreas of CHI-D patients (Rahier et al. 1984). Although attributed to a process of β-cell proliferation at the time, the association of hormone-positive cells with the ductal epithelium, outside of defined islets, shows intriguing resemblance to the process of endocrine differentiation in the developing fetal pancreas. Over numerous decades since, the ductal epithelium has been of considerable interest in the postnatal and mature pancreas as a possible source of endocrine progenitors and hope of an intrinsic capacity for β-cell regeneration (Bonner-Weir et al. 1993; Bouwens and Pipeleers, 1998; Bouwens and Rooman, 2005; Finegood et al. 1999; Wang et al. 1995).

Many research groups have tried a variety of investigative techniques, some with success, to induce β-cell regeneration in the pancreas. These were predominantly in rodent species and included streptozocin-induced β-cell ablation (Finegood et al. 1999), pancreatic duct ligation (Wang et al. 1995), and near-total pancreatectomy (Bonner-Weir et al. 1993). Although the data is somewhat inconsistent between groups and the evidence from genetic lineage studies as yet unable to identify the cell of origin for β-cell regeneration, a limited capacity for pancreatic regeneration, at least in the rodent, has been confirmed.

Primary data from human subjects has also proposed an intrinsic mechanism in the mature pancreas for regulating β-cell mass, evidenced in subjects with increased insulin requirement such as obesity or pregnancy (Blondeau, et al. 1999; Butler et al. 2010; Butler, et al. 2003; Meier, et al. 2006b). In these subjects increases in β-cell number were detected along with their increased association with ductal structures and scattered distribution in the pancreatic tissue (Butler et al. 2010; Meier et al. 2006). Further evidence that new β-cells may be forming from the differentiation of ductal epithelium cells, and that this can occur in the mature pancreas in response to physiological demands.
3.4.1. Islet structure and hormone colocalisation in CHI-D

Our first objective was to identify whether CHI-D resulted from the over exuberance of insulin-producing cells and whether the characteristics of nesidioblastosis described in CHI-D samples were related to the neogenesis of the islet population. Investigating the hormone expression, distribution and relative abundance of each islet cell type would determine whether the patterns of insulin localisation were specific to the β-cell population or if other islet hormones were involved too. We hypothesized, if this was a recapitulation of the fetal differentiation program from a source of endocrine progenitors, other islet hormones may also be generated by the same mechanism.

Initial IHC studies demonstrated characteristics in CHI-D samples consistent with previous publications, including the diffuse distribution of insulin-positive cells, as well as their association with the ductal compartment. These early experiments served to validate the samples and the techniques for antibody detection.

Microscopic bright-field analysis in CHI-D samples confirmed the scattered distribution of hormone-positive cells, for insulin-, as well as glucagon-, and SS-producing cells. In CHI-D pancreas, the majority of hormone expression was outside of defined islet structures, however, clusters of endocrine cells containing insulin-, glucagon- and SS-secreting cells were still observed in each of the CHI-D samples. Cells expressing ghrelin could be found in the majority of islet aggregates whilst PP expression was more rare. Concomitant investigation with neonatal control samples showed a similar expression profile for endocrine cells, where small clusters and individually hormone-positive cells could be observed in all ages studied (2-13 months). However, the prevalence of islet structures became more apparent with increasing age in both control and CHI-D pancreas, although they appeared larger and less well-organised in CHI-D in comparison. By one year of postnatal life the majority of hormone-expressing cells had evolved into a more adult-type formation within islet clusters that had a predominantly β-cell core, surrounded by a mantle of α- and δ-cells and a reduced frequency of extra-islet hormone-positive cells in both CHI-D and control specimens. These observations are consistent with those of other groups for control samples (Goossens et al. 1989; Jaffe et al. 1982; Rahier et al. 1981) however they are in apparent contrast to reports describing a persistence of immature endocrine phenotype in CHI-D samples (Ariel et al. 1988; Rahier et al. 1984). These patterns of observation have only been validated in a few samples (Ariel et al. 1988; Rahier et al. 1984) and with only one sample above the age of 6 months in our data set it is important to consider inter-patient variation as a contributor to the different results seen.

Organisation of the islet is likely a response to glucose or other islet hormones. Mice models from genetic studies show disrupted islet architecture in both mutant (affecting
only β-cells) and knock-out (affecting all endocrine cell-types) models for Kir6.2 K\textsubscript{ATP} channelopathies, as well as Sur1\textsubscript{-/-}, suggesting that organisation of the islet is affected directly as a result of β-cell dysfunction or abnormality (Marhfour, et al. 2009; Miki, et al. 1998; Miki, et al. 1997). Further evidence that the histopathology of CHI-D is a knock-on effect of the CHI-D-causing mutation comes from work on other CHI-related mutations. Of particular interest are mutations affecting glucokinase (CHI-GCK) in which patients present with large, hyperplastic islets (Kassem, et al. 2010), a stark contrast to the diffuse arrangement of islet cells in CHI-K\textsubscript{ATP}.

Although studying human patient samples for CHI-D is crucial to our understanding of β-cell physiology, the inherent differences that come with such samples can render the results difficult to interpret. All the patients used in these investigations were diagnosed with CHI-D at 1 month of postnatal age, however surgical treatment was carried out between two and 13 months. Differences in the maintenance of glycaemic control in these patients prior to surgery, as well as variations in diet and feeding patterns may influence how quickly and effectively the adult-like architecture is assumed. After diagnosis, treatment is required for CHI-D as soon as possible to prevent developmental problems. The older samples are therefore quite distinct and the data presented in them should therefore be handled with more caution as their disease management and treatment regime are not described prior to surgery, and therefore the effects of this are not fully understood. Consistent with this, since the mutations in these patients are naturally occurring it is unlikely that patients will have the same genetic basis underlying their disease. The different ages at the time of study, environmental circumstances to which they were exposed before surgery and variant mutations all likely contribute to the aetiology of the disease and inter-patient variability.

3.4.2. TF alterations in CHI-D; increased NKX2.2 in δ-cells during the first 6 months similar to fetal development

Over the last decade several TFs have been identified that are essential for islet cell development and differentiation. Unfortunately, many of these are also found in the adult pancreas where they are required for the regulation of endocrine-cell function. Whilst NGN3 expression is necessary for the promotion of endocrine commitment a further series of TF expression downstream specify the different endocrine subtypes as well as the function and survival of each islet cell type.

By virtue of IHC analysis, positive NGN3 staining was difficult to determine. Detection of weakly-stained brown nuclei were seen, albeit rarely, in CHI-D samples and also occasionally in controls. When compared to the strong nuclear staining observed during
human fetal development the presence of NGN3 protein in these samples is not fully substantiated, and this is supported by the qRT-PCR analysis which failed to amplify NGN3 transcripts in scraped tissue sections from CHI-D or control samples. This apparent absence of NGN3 implies that de novo endocrine differentiation is unlikely to be occurring during the first year of life in humans.

The expression of TFs involved in fetal endocrine differentiation were assessed in CHI-D samples, and compared to their respective controls. Across the majority of CHI-D samples, the expression of PDX1, SOX9, FOXA2 and NKX6.1 remained within the normal expression range detected in age-matched controls. IHC analysis of these TFs in CHI-D samples did not show any obvious discrepancies from the controls. Each TF was detected in accordance with the control profile and in-line with what was expected from published data; PDX1 in islets and weakly in ducts (Piper et al. 2004), FOXA2 in endocrine islets (Cockell, et al. 1995; Wu, et al. 1997), NKX6.1 in islets (Sander, et al. 2000) and GATA4 throughout the acinar tissue (Ritz-Laser, et al. 2005). SOX9 staining identified the ductal structures in all fetal, neonatal and adult samples as expected (Piper et al. 2004; Seymour et al. 2007).

NEUROD1 was down regulated in 3 out of 5 CHI-D samples, with CHI-D 1 (2 mo) and CHI-D 5 (13 mo) showing essentially normal levels of expression as per their age-matched controls. During development NEUROD1 is expressed downstream of NGN3 and is thought to regulate a similar set of pro-endocrine genes in order to maintain the process of endocrine differentiation once the transient NGN3 expression subsides (Gasa et al. 2004; Gasa, et al. 2008; Naya et al. 1997). The low expression of NEUROD1 in the majority of CHI-D samples would therefore follow with the low detection of NGN3 transcripts and suggestion that a fetal endocrine differentiation programme is not operating in these samples.

NEUROD1 is believed to function mostly in the maturation of the endocrine cells that have already committed to endocrine differentiation as functional endocrine cells are still generated in the mutant model (Naya et al. 1997). NEUROD1 has also been shown to be critical for insulin expression in vitro (Naya et al. 1997; Qiu, et al. 2002), although this is not a crucial role for the TF since NeuroD1-null mice still contain 10-15% of insulin levels typically detected in controls (Bonner-Weir et al. 1983). When NeuroD1 is deleted from the mature β-cell in mice, severe glucose intolerance ensues along with a reduced secretion of insulin (Gu, et al. 2010). The reduction in NEUROD1 expression detected in 3 of the 5 CHI-D samples may represent a role for NEUROD1 in glucose-stimulated insulin-secretion (GSIS). Since this pathway is disrupted in CHI-D samples as a result of K_{ATP} channel dysfunction, it could be that the expression of NEUROD1 is consequently reduced in accordance with decreased glucose responsiveness and unregulated insulin-
release. This decrease in \textit{NEUROD1} transcripts could then be responsible for reverting (or maintaining) CHI-D \(\beta\)-cells to an immature phenotype (Gu et al. 2010).

\textit{NKX6.1} is a homeodomain factor with an essential role in \(\beta\)-cell development, the cell-type in which it is specifically expressed in the mature pancreas (Jensen, et al. 1996; Oster, et al. 1998a). The near-normal expression of \textit{NKX6.1} in the CHI-D samples is in accordance with IHC data that do not appear to show the \(\beta\)-cell population to be elevated in the CHI-D samples (Our data; Rahier et al. 1984).

However \textit{MAFA}, a \(\beta\)-cell specific TF, was found to be increased in 3 out of 5 CHI-D samples. During development, \textit{MAFA} is detected in the first insulin-positive cells of the early pancreas, and its expression is maintained in \(\beta\)-cells through to adulthood where it is involved in activating the \textit{insulin} gene (Matsuoka et al. 2004; Oliver-Krasinski and Stoffers 2008). Unlike the other \textit{insulin}-transactivating genes, \textit{PDX1} and \textit{NEUROD1}, \textit{MAFA} is not required during development but is important for maintaining \(\beta\)-cell function by regulating the expression of genes involved in GSIS, including \textit{GLUT2} (Artner et al. 2006).

\textit{MAFA} is a potent transactivator of the insulin gene (Kataoka, et al. 2002; Matsuoka et al. 2004; Olbrot, et al. 2002). Its increase in expression in CHI-D samples could provide an explanation for the prolonged and inappropriate secretion of insulin in these specimens. Although \textit{PDX1} and \textit{NEUROD1} are not upregulated, their role in \textit{insulin}-gene activation may be different and dispensable to the system. The increase in \textit{MAFA} expression may also represent an attempt to regulate insulin-secretion through the unregulated GSIS pathway in CHI-D samples by upregulating the GSIS components.

Of particular relevance, however, is the discovery that \textit{MAFA} is regulated by \textit{NKX2.2}, and an increase in \textit{NKX2.2} expression can cause an increase in \textit{MAFA} (Doyle and Sussel 2007; Raum, et al. 2006).

\textit{NKX2.2} expression was elevated in CHI-D compared to age-matched controls and this was most obvious during the first 6 months after birth. IHC investigation revealed persistent expression of \textit{NKX2.2} in SS-producing cells, a feature reminiscent of fetal \(\delta\)-cells and in contrast to the low levels of coexpression observed in age-matched control samples.

During development, \textit{NKX2.2} is critical for the differentiation of \(\beta\)-cells and is one of few TFs to cause absolute loss of \(\beta\)-cells when ablated (Sussel et al. 1998). The \textit{Nkx2.2}\(^{-/-}\) phenotype also shows significant reduction in \(\alpha\)- and PP-cell numbers whilst the SS-producing \(\delta\)-cell population is unaffected (Sussel et al. 1998). This is consistent with \textit{NKX2.2}'s absence from the \(\delta\)-cell lineage in the mouse. However, our data on human fetal pancreas clearly shows \textit{NKX2.2} expression in the majority of SS-producing \(\delta\)-cells, and
this is in agreement with other groups (Lyttle et al. 2008). NKX2.2 expression in SS-positive cells persisted into the postnatal pancreas in healthy neonates and could also be observed in adult δ-cells, however their proportions were significantly lower than those observed in the fetal period with approximately 2/3 of SS-producing cells lacking nuclear NKX2.2. Why NKX2.2 is present in human δ-cells but not mouse is unclear, nor is the progressive loss of dual expression after birth. Jennings et al (2013) also found differences in NKX2.2 expression profiles between mouse and human samples with NKX2.2 not detected as protein in early pancreatic progenitor cells in humans (Jennings et al. 2013). These differences may suggest a variability in the role of this TF between the two species. The incidence of NKX2.2+/SS+ coexpression in CHI-D samples resembles those proportions observed in the fetal period (70%, 65%; fetal, CHI-D respectively). This may represent an immaturity of the δ-cell population in CHI-D samples, perhaps identifying their failure to commit to the mature state. This would fit with the partial-success in using SS analogs as a treatment option for CHI-D patients, and an explanation why SS in vivo fails to suppress or inhibit insulin secretion in these samples (Otonkoski, et al. 1993). Immaturity in the δ-cell lineage also revives old ideas that CHI-D could be at least in part a defect in the somatostatin-producing lineage (Rahier, et al. 1984).

SS+/NKX2.2+ cells may even represent a progenitor population in the CHI-D pancreas. In an experiment by Teitelman (1996), the destruction of β-cells by streptozocin treatment resulted in a regeneration of insulin- (only) positive cells, following the observation of increased SS+/PDX1+ population (Teitelman 1996).

Whilst MAFA is solely required for GSIS and not in islet cell development (Zhang, et al. 2005), MAFB is expressed earlier during development, activating insulin and glucagon genes in immature β- and α- cells before being restricted to a subset of α-cells in the adult (Artner et al. 2006; Nishimura, et al. 2006; Oliver-Krasinski and Stoffers 2008). When the MafB gene is knocked-down in mice, the principal defect observed is a reduction in insulin expression (Artner, et al. 2007). However, the total number of endocrine cells in these samples is unaffected, evidence that MafB is involved in the later stages of differentiation. Gene expression analysis supports this later role for MAFB since many of the genes involved in β-cell differentiation are unaffected in the MafB mutant, including Pax6, NeuroD1, Pax4 and Isl1, whilst genes important in the mature cell function, including Pdx1, Nkx6.1 and Glut2 are reduced (Artner, et al. 2007).

In 4 out of 5 CHI-D cases, MAFB expression was reduced below normal range of age-matched controls. A decrease in MAFB activity in CHI-D samples may be consistent with their failed glucose-response system. The unregulated insulin-secretion system as a result of KATP channel mutations may be potentiated in these samples by lack of genes important
for glucose sensing or β-cell function as a result of decreased MAFB activity during development.

However, CHI-D 4 (6 mo) showed significantly elevated MAFB expression as compared to the age-matched control. The persistent expression of MAFB in the CHI-D pancreas in this instance may represent a persistent immaturity of the pancreatic β-cells. After the differentiation of β-cells during development, there is a transition from MAFB to MAFA expression. This shift is critical for β-cell function (Nishimura, et al. 2006) since MAFA is required to maintain many of the genes that are first regulated by MAFB in the developing β-cells (Artner, et al. 2007) and permit appropriate β-cell function. Persistence of MAFB may therefore demonstrate dysfunction in the postnatal β-cell lineage. In a study by Kroon et al., (2008), whilst attempting to generate insulin-producing cells from human embryonic stem cells, primitive β-cells expressing MAFB+/insulin+ were dysfunctional until MAFA expression commenced (Kroon, et al. 2008).

A similar expression pattern was observed for PAX6; in 4 out of 5 CHI-D samples PAX6 expression was reduced, whilst CHI-D 4(6 mo) once again, showed elevated transcript levels compared to the age-matched control.

A reduced expression of PAX6 in CHI-D samples may represent a consequence of the hyper-activity of the β-cell. PAX6 is required for normal islet hormone gene transcription and has been found to bind with the promoter sequence on the glucagon and insulin genes, which share similarity with a binding region also found on the SS promoter (Knepel, et al. 1991; Sander, et al. 1997). Decreased expression of PAX6 will therefore negatively affect the transactivation of the islet hormone genes, resulting in a decrease in insulin, glucagon and SS expression. Mice containing homozyous null alleles for Pax6 lack the number of α-, β-, δ- and PP-cells, as well as the amount of hormones produced (Sander et al. 1997; St-Onge, et al. 1997).

Insulin is a paracrine regulator of glucagon synthesis and can inhibit its secretion by the α-cell. Interestingly, this activity has been associated with the binding sites for PAX6 (Philippe, et al. 1995; Sander et al. 1997) where increased levels of insulin have a negative effect on the expression of PAX6 in the other islet cell types. Prolonged insulin synthesis in CHI-D samples and local areas of hyperinsulinaemia could therefore cause downregulation of PAX6.

Interestingly, whilst inter-patient variations were apparent in the expression profiles of most of the TFs explored, both PAX4 and ARX were consistently downregulated across all five CHI-D samples.
PAX4 is expressed in the early pancreatic epithelium where it is necessary for β- and δ-cell differentiation. Later in development, and through to the adult pancreas, PAX4 is restricted specifically to the β-cell lineage (Dohrmann et al. 2000; Smith et al. 1999). The homozygous null mutant lacks mature β- and δ- cells whilst an increase in the number of α-cells is observed (Sander et al. 1997). If PAX4 expression was low during development, this may explain the immature phenotype of δ-cells in the CHI-D samples, characterized by their persistent expression of nuclear NKX2.2.

The expression of PAX4 has been described to be dependent on the concerted activity of NEUROD1 and PDX1 (Kanno, et al. 2006; Smith et al. 2004). Although PDX1 expression was essentially normal in CHI-D samples, NEUROD1 was decreased in the majority of CHI-D samples which could have a consequential effect on the PAX4 transcripts, accounting for the reduced expression in these samples.

ARX expression was also consistently decreased across all the CHI-D samples as compared to the controls. In normal pancreas, ARX is first detected at the onset of bud evagination and later becomes restricted to the islet cells (Collombat, et al. 2003). Gene K/O studies for Arx resulted in a decrease in SS- and insulin-producing cells (Collombat, et al. 2005). The presence of ARX in the endocrine progenitor population likely specifies which cells will adopt an α-cell lineage, with its absence favouring a β- or δ- cell fate (Collombat, et al. 2005).

If pancreatic endocrine progenitors are present in the CHI-D samples, inhibition of ARX may encourage these to adopt a β- (or δ-) cell fate. However, since there is no apparent increase in the expression of other genes involved in the β-cell differentiation programme in our data, including PDX1 NKX6.1, NEUROD1 or PAX4, it is unlikely that new β-cells are being generated by reinstatement of the fetal differentiation programme.

The expression of ARX is repressed through the tinman domain on NKX2.2 (Papizan et al., 2011). In the Nkx2.2/- model, there is a 5-fold increase in the detection of Arx transcripts. However, the Nkx2.2 gene is unaffected in the Arx/- (Collombat, et al. 2003). This positions Arx downstream of Nkx2.2 in the regulatory pathway and may go some way to explaining the reduced expression of ARX in the CHI-D samples that show elevated NKX2.2. These observations are also consistent with our observation of an unexpanded PP-cell population.

Of the factors involved specifically in cell lineage commitment steps, ARX and PAX4 were the only 2 genes to show significant reduction in expression across all five CHI-D samples. This suggests these 2 genes may play a role in the pathogenicity of this disease, unaffected by patient variability, age or genetic mutation. These changes in gene expression could result from the physiological symptoms of the disease, such as high
intracellular calcium concentrations, hypoglycaemia, or other mechanisms synonymous with the different patients. In addition, further validations of the qRT-PCR procedure would be warranted. Whilst primer designs were validated on the UCSC browser and single product amplification confirmed by melt curve analysis, agarose gel separation procedures would provide further validation of the products amplified in the qRT-PCR experiments for both –RT RNA and +RT cDNA samples. Further validation of the amplicons could be achieved by sequencing the product after qRT-PCR amplification.

Further work on cell populations would be interesting to decipher information gathered on gene expression in CHI-D samples. Reduction in certain islet cell populations would be indicative of gene expression changes during development and not just a loss of expression in the postnatal phenotype. These studies would help build on the preliminary observations identified in our CHI-D samples.

Significantly, many of the TFs explored in this investigation may also have roles in regulating β-cell mass. Mutations in NEUROD1, PDX1 and PAX4 have been identified to predispose patients to MODY and/or T2DM (Brun, et al. 2004; Maedler, et al. 2006; Malecki, et al. 1999). Since both MODY and T2DM are characterized by the gradual decline in β-cell function and/or number these TFs are likely to be involved in β-cell expansion and survival as well as mature function. Mutated forms of the PAX4 gene have shown an increased susceptibility to apoptosis and reduced rates of proliferation in β-cells (Maedler, et al. 2006; Sosa-Pineda, et al. 1997). In contrast, increased expression of PAX4 causes increased proliferation (Maedler, et al. 2006). NEUROD1 expression has also been associated with cell survival, where cells lacking NEUROD1 have been found to have increased rates of apoptosis (Miyata, et al. 1999; Naya et al. 1997). Likewise, their association with MODY demonstrates a critical role of these factors in the mature functioning of β-cells.

In the presence of a genetic mutation in the K\textsubscript{ATP} channels of pancreatic islet cells in CHI-D samples, along with the inappropriate secretion of insulin, the persistent hypoglycaemia that results, elevated intracellular calcium concentrations and prolonged membrane depolarisation, survival mechanisms of the cell may be downregulated in the affected cell types. Decreases in PAX4 and NEUROD1 transcripts would therefore render the cell susceptible to further driving apoptotic signals, resulting in increased cell death, which has previously been described in CHI-D samples (Kassem, et al. 2000; Chapter 4).
3.4.3. Prolonged immaturity of CHI-D islets

Whilst the observation of hormone co-expression in single cells within CHI-D samples is reminiscent of fetal development, instead of overlapping, production of each hormone appeared discretely localized to opposite ends of the same cell. This division has not been reported previously and therefore the implications are incompletely understood. Co-expression in CHI-D samples was only observed between insulin and glucagon and not insulin with any other islet hormone. The $\beta$- and $\alpha$- cells are similar cell types, deriving from a common ancestor and sharing many of the same components including machinery for sensing and metabolizing glucose, such as GCK and $K_{ATP}$ channels, as well as similar mechanisms for hormone secretion (Heimberg, et al. 1996; Heimberg, et al. 1995; Rorsman, et al. 2008). This therefore makes the $\alpha$-cell an appropriate candidate for reprogramming into a $\beta$-cell fate. In fact, several authors have reported transdifferentiation of the $\alpha$- to $\beta$-cell lineage following conditions of injury and $\beta$-cell loss. In these models $\alpha$-cells pass through a transitional stage where both glucagon and insulin hormones are produced (Chung and Levine 2010; Collombat and Mansouri 2009; Thorel et al. 2010).

Although dual expressing cells are observed in the human fetal pancreas, lineage tracing experiments suggest that mature insulin- and glucagon-producing cells in the adult pancreas have never expressed the other hormone (Herrera, 2000). These experiments propose that the coexpressing cells seen in the fetal period are a distinct cell type that disappears with further development and maturity (Herrera, 2000). In fact, between 9-21 wpc there is a 50% reduction in the prevalence of coexpressing cells, and these are apparently absent by birth (Riedel, et al. 2012).

Others have suggested that dual hormone expression represents a partially differentiated cell, not fully committed to either cell lineage (Alpert, et al. 1988; Herrera, et al. 1994). In an IHC investigation, Riedel and colleagues speculated these dual-hormone producing cells to be mature $\alpha$-cell precursors based on their TF profiles (Riedel, et al. 2012). The coexpressing cells detected in CHI-D samples may therefore be representative of a preserved fetal artifact, identifying a cell type that is either in transition from one fate to another, an immature endocrine cell, or a distinct cell population.

To further investigate the authenticity of the CHI-D $\beta$-cell, the expression of mature $\beta$-cell markers was explored, including PDX1, FOXA2, NKX6.1 and NKX2.2. In-line with observations in control and also fetal samples, each protein was appropriately expressed in the majority of insulin-producing cells in CHI-D samples.

However, these markers are not distinct to maturity, as each is also involved in the development and differentiation of the endocrine populations. Occasionally cells were
observed to be insulin+ but absent of PDX1 or NKX6.1 reactivity, and these were observed in both CHI-D and control samples. These may be experimental artifacts, where proteins have escaped detection by IHC or that the signal produced was too low for detection by this method. Also, as samples were cut at 5 µm thickness, without confocal microscopy, it is not possible to rule out that insulin reactivity may be localized to a cell behind the plane of focus on traditional microscopy methods. Without more sophisticated cell lineage tracing experiments, including the tracing of cells through development, it is not possible to conclusively verify bona-fide β-cells in the CHI-D pancreas. However, IF confirmed the majority of insulin-producing cells to contain markers that would be expected in genuine and mature β-cells.

Finally, the expression of gastrin was also explored in the CHI-D pancreas. Although the exact expression of gastrin is incompletely qualified, historically, gastrin has been reported in the human fetal pancreas (Bardram 1990) as well as rodent species (Gittes, et al. 1993; Suissa, et al. 2013; Takaishi, et al. 2011). Expression of gastrin in these cases disappeared at birth, however malignant neuroendocrine tumours known as gastrinomas, can reappear in the adult (Bertolino, et al. 2003). A reappearance of gastrin has also been reported in the islets of patients with T2DM where its presence was ascribed to a process of de-differentiation (Dor, et al. 2013-Unpublished observations).

The gastrin antibody used in these experiments was previously described in a publication by Suissa et al (2013). Although cross-reactivity with CCK, a similar hormone also described to be present in the embryonic pancreas, has been reported for the antibody, we did not detect any pancreatic staining for the antibody in any of the fetal samples investigated. In a report by Bardram and colleagues, both gastrin and progastrin were detected in the human adult and fetal pancreas. However, the combined concentrations of gastrin molecules detected in the samples were significantly lower in the human samples compared to the rat fetal and adult pancreas samples (Bardram 1990). It is therefore possible that gastrin peptides do exist in the human pancreas, but are present at concentrations that are insufficient to detect using IHC techniques.

Validation of the antibody was performed in sections of human fetal intestine, where G-cells of the intestinal epithelium secrete gastrin that could be detected by IHC. However, pancreatic regions in the same samples did not show immunoreactivity for the protein. Likewise, gastrin was not detected in CHI-D, age-matched controls, or adult pancreatic sections.
3.5. Summary

The results presented in this chapter have offered novel insights into the pathophysiology of CHI-D, building on observations described by others that imply a more diverse contribution to the aetiology of disease that involves other pancreatic cell types.

We have shown that CHI-D is not a β-cell specific entity, and that other pancreatic cell types also appear to show abnormalities in their maturity and/or function. Observations of diffuse insulin expression were extended to show these areas to also contain scattered glucagon-, and SS-producing cells and that these were also seen associated with ducts.

The realisation that diffuse insulin-positive clusters associated with ducts and poorly-formed islets are also apparent in the normal neonatal period has reframed the historic interpretation of CHI-D away from the idea of nesidioblastosis. Extending our observations beyond the β-cell we show for the first time that endocrine cell organisation within CHI-D islet structures is less well-organized, and that occasionally cells expressing both insulin and glucagon could be observed which were absent from postnatal control samples. Of particular intrigue was the persistence of nuclear NKX2.2 expression in the SS-producing δ-cell population, a characteristic reminiscent of fetal δ-cells but contrary to neonatal control samples in which approximately 75% of SS+ cells were lacking NKX2.2 colocalisation.

The effect of CHI-D on other pancreatic cell-lineages may be a direct result of abnormal β-cells since the activity of islet endocrine cells is often influenced by the paracrine interactions of the other cell lineages (Jain and Lammert 2009). Disruptions in the activity of the β-cell may therefore have knock-on effects on the other pancreatic cell-types. Alternatively, the onset of CHI-D may be a developmental disorder affecting the endocrine progenitor cell population rather than a functional β-cell-specific anomaly. It is difficult to conceive that the abnormalities extending beyond the β-cell in CHI-D are solely the results of excessive or inappropriate insulin secretion. K_{ATP} channels are present in other islet-cell types (Ashcroft and Rorsman 2013) and it therefore follows that these may also be affected by CHI-D-associated mutations thereby also affecting the α-, and δ-cell phenotype, function and/or maturity.

Whilst the appearance of hormone-positive cells emerging from the ductal epithelium were encouraging of an islet neogenic process, we were unable to provide convincing evidence for ongoing or aberrant endocrine differentiation based on the programme of fetal development. The relative lack of NGN3 transcripts detected in CHI-D or control samples implies the endocrine differentiation programme is not maintained into the postnatal period and the absence of enhanced expression in any of the downstream targets for NGN3,
including *PAX4, ARX, PAX6, NEUROD1* and *NKK6.1* provides further evidence that *de novo* endocrine generation is unlikely to be occurring in CHI-D.

Likewise, comparative expression of *INSULIN* and *NKK6.1* in CHI-D and age-matched controls suggests that the β-cell mass is unlikely to be increased in CHI-D tissues, indeed an excess of insulin-staining following IHC investigation was not apparent, supporting a mechanism of β-cell over-activity rather than an over-representation as a contributor to the aetiology of the disease.

Overall our work provides new insights into the pathological mechanisms underlying CHI-D where a loss of *KATP* channel activity affects other pancreatic islet cell-types, and not just β-cells, in hitherto unappreciated ways.

The work presented in this chapter supports a more widespread and diverse involvement of other pancreatic cells in CHI-D either as a knock-on effect of persistent insulin secretion or a direct contribution of the *KATP* channel mutations.
Chapter 4- Proliferation and cell cycle regulation in CHI-D and age-matched controls

4.1. Introduction

CHI is a rare genetic disorder, usually arising from defects in the \( K_{\text{ATP}} \) channel genes; \( ABCC8/KCNJ11 \) (Dunne et al. 2004; Dunne et al. 1997; Glaser et al. 1994). CHI occurs when inappropriate insulin release from hyperfunctional pancreatic \( \beta \)-cells results in persistent or recurrent episodes of hypoglycaemia (Dunne et al. 2004). CHI-D is associated with defects in all \( K_{\text{ATP}} \)-expressing cells whereas CHI-F results from \( \beta \)-cell specific defects in localized regions of the pancreas (Rahier et al. 1998). In its diffuse form insulin-positive cells are widespread and often observed associated with ducts (Dunne et al. 1997; Kassem et al. 2000; Rahier et al. 2000).

In the previous chapter we showed that new \( \beta \)-cells were unlikely to be arising from a process of neogenesis in CHI-D samples, despite their appearance to be budding from ductal structures; similar to their differentiation in the fetal period. In this chapter we look into the possibility of enhanced proliferation in the \( \beta \)-cell lineage that could account for increased insulin secretion and persistent hypoglycaemia.

Whilst CHI-D is known to be associated with unregulated insulin release, the role of cell proliferation is less well represented in the literature. This is partly accountable by the variable results that present in samples when proliferation is explored. However, for the most part, only proliferation in the \( \beta \)-cell compartment has been described (Kassem et al. 2000; Lovisolo, et al. 2010; Sempoux et al. 1998a), which is understandable since this is the apparently dysfunctional cell type in CHI-D to which the majority of symptoms are associated.

Surprisingly, little is known about the molecular control of the cell cycle events in pancreatic \( \beta \)-cells. For a long time it was believed that \( \beta \)-cells do not replicate, but evidence has since emerged demonstrating that human \( \beta \)-cells can and do replicate \textit{in vivo} (albeit very slowly), and most noticeably in response to changing metabolic pressures or injuries, such as obesity or pregnancy (Butler et al. 2003; Kassem et al. 2000; Meier, et al. 2008). Understanding the regulation of \( \beta \)-cell mass is important for understanding the pathogenesis of endocrine-related diseases, including diabetes, insulinomas and CHI associated with the ability of the endocrine pancreas to compensate for changing insulin demands (Bonner-Weir 2000b; Butler et al. 2003).
A principal goal of many research groups and institutions is to develop viable therapies that can induce β-cell replication for the treatment of T1DM/T2DM, where there is a shortfall in functional β-cell mass. However, whilst various mitogens, growth factors and nutrients can induce expansion in rodent models, limited effects are observed in human β-cells (Kulkarni, et al. 2012). Part of this resistance to replication in human β-cells is likely due to age-related impacts, since proliferation has been reported in human embryonic as well as neonatal β-cells (Bouwens, et al. 1997; Kassem et al. 2000; Meier et al. 2008) but is less apparent in older, adult tissue. Even in these samples, observed proliferation rates are still substantially lower than in their rodent counterparts.

During development, the pancreas and its components must increase in size to support the growing metabolic and digestive demands of the body. Whilst β-cell expansion in the fetal period is predominantly a mechanism of differentiation, after birth replication of pre-existing β-cells has been reported as the primary mechanism subserving this expansion (Meier et al. 2008). The human pancreas demonstrates a linear increase in volume from birth until around 20 years of age, after which point the mass remains steady (Saisho, et al. 2007). Most rapid growth of the β-cell compartment occurs within the first couple of years of postnatal life (Meier et al. 2008) reflective of an early capacity for β-cell replication that decreases with each year thereafter. Even during the first year of life β-cell proliferation can be seen to steadily decline, almost approaching 0 by the age of 6 months (Kassem et al. 2000).

Despite initial interpretations of nesidioblastosis (the diffuse distribution of β-cells and their budding from ducts), as being a disseminated proliferation of islet cells (Aynsley-Green 1981; Dahms et al. 1980; Heitz et al. 1977; Yakovac, et al. 1971), the overall volume density of β-cells is not significantly changed in patients with CHI-D compared to controls (Rahier et al. 1984). This is also consistent for α-cells, whilst δ-cells show a tendency for reduced representation in CHI-D samples (Rahier et al. 1984). These data ruled out nesidioblastosis as a causative feature of CHI-D since excessive proliferation was expected to cause an increased population of β-cells which was not seen in any of the CHI-D cases (Rahier et al. 1984). This was further supported in a follow-up study in 1998 where Ki67 expression was explored in seven CHI-D patients. Although high inter-patient variability was observed, the overall rate of proliferation in CHI-D β-cells was not significantly different to the age-matched controls (Sempoux et al. 1998). However, a more recent report using the same techniques were able to show a significant increase in β-cell proliferation in CHI-D patients, based on investigation of 14 individuals and
compared to a group of age-matched controls (Kassem et al. 2000). Similar observations were also observed in a Brazilian cohort of CHI-D patients (Lovisolo et al. 2010).

Taken together, these data support a tendency for β-cell proliferation to be increased in patients with CHI-D, although the mechanisms underlying this capacity for replication are less well understood.

In all the studies mentioned above, whilst proliferation in CHI-D β-cells was at best modest in comparison to age-matched controls, lesions associated with CHI-F showed a significant frequency of β-cell replication in comparison (Kassem et al. 2000; Lovisolo et al. 2010; Sempoux et al. 1998). The mechanisms underlying proliferation in β-cells can be partially explained from the genetic basis that causes CHI-F rather than CHI-D. Both CHI-F and CHI-D are most commonly caused by mutations in genes encoding subunits of the $K_{ATP}$ channel (Thomas, et al. 1996; Thomas et al. 1995), however, the inheritance patterns for these gene mutations determines the disease subtype. Whilst CHI-D is caused by the recessive inheritance of genetic mutations on both the paternal and maternal alleles, CHI-F is caused by the initial inheritance of a mutation on the paternal allele, followed by the somatic loss of a portion of the maternal chromosome (chromosome 11) containing the same gene, in a subset of β-cells or β-cell precursors (Fournet, et al. 1998). In losing part of this maternal chromosome containing the $K_{ATP}$ channel genes, the expression of imprinted genes in the same region are also lost. These include genes important for cell cycle regulation, including P57$^{KIP2}$, H19 and IGF-II (Fournet, et al. 2000; Fournet et al. 1998; Kassem et al. 2001).

P57$^{KIP2}$ (P57) is an important cell cycle inhibitor, binding with cyclin and CDK complexes to inhibit their activity (Lee, et al. 1995; Matsuoka, et al. 1995). In healthy control samples, P57 is found in the nucleus of approximately 30-40 % of islet β-cells (Fiaschi-Taesch, et al. 2013a; Kassem et al. 2001), its presence complementing the low proliferation rates observed in these cell types. The loss of P57 has been associated with a variety of cancers (Bourcigaux, et al. 2000; Kondo, et al. 1996; Thompson, et al. 1996) the hallmarks of which include an increased capacity for cell proliferation and a resistance to cell death. Crucially, P57 is absent from β-cells in CHI-F lesions (Kassem et al. 2001), supporting its imprinted expression on the maternal chromosome, and also an involvement in permitting β-cell proliferation. However, even in the absence of the P57 inhibitor protein, proliferation in CHI-F β-cells is still only ~6 % (Kassem et al. 2000), implicating other mechanisms to be involved in regulating β-cell replication.
4.1.2. Regulation of the cell cycle in pancreatic β-cells

There are several molecular targets involved in the cell cycle that have been identified in pancreatic β-cells of both mouse and human islets. These include cyclin-proteins, E2F transcription factors and cyclin-dependent kinases (CDKs), as well as the retinoblastoma protein (pRb) and cyclin-kinase inhibitors; each with their own role in regulating cell turnover.

The cell cycle is a series of events that occur in the cell leading to its division. It consists of 4 phases; G1, S-phase, G2, M-phase. When a cell stops dividing it is said to enter a state of quiescence, known as G0 (Figure 4-1).

**Figure 4-1. Phases of the cell cycle**

The cell cycle is a series of events within a cell leading to its division and replication. Outside of the cell cycle, a quiescent cell is in G0. On receipt of mitogenic stimulus the cell enters G1, with the G1/S checkpoint representing a critical transition for driving cell cycle proliferation.

The G1, S-phase and G2 collectively make up what is known as interphase. This is when the cell is prepared ready to enter into the process of mitosis (M-phase). During M-phase the cell separates into two distinct and genetically identical daughter cells.

G1 represents the gap between the end of mitosis and the next round of synthesis (S-phase) in preparation for another cycle of cell division. During G1 the proteins and enzymes necessary for DNA replication are synthesized.

S-phase starts when DNA replication commences and is complete when every chromosome has a duplicate copy.

G2 phase is the gap between synthesis and mitosis when the cell continues to grow, taking in nutrients and making sure everything is ready for cell division.
Whilst growth factors and other mitogenic signals might influence β-cell replication at the G2/M checkpoint, the pathway controlling the G1/S checkpoint, governed by pRb, is likely to be central in controlling β-cell proliferation (Cozar-Castellano, et al. 2006a).

pRb is a principal check-point protein for the G1/S phase transition and its activity is regulated by D-Cyclin and CDK complexes. pRb is one of a family of proteins called the pocket proteins which includes p107 and p130. pRb is the most ubiquitously expressed of the pocket proteins and as a result has been extensively studied in many different cell types (Berns 2003; Chau and Wang 2003). It is believed to play a central role as the molecular brakes on cell cycle progression, arresting cells at the G1/S transition. pRb binds to a family of transcription factors, E2Fs, repressing their transcriptional activity and therefore preventing cell cycle progression.

Two regulatory molecules also involved in monitoring a cell's progression into the cell cycle are D-cyclin and CDK proteins. CDKs are inactive unless they are in a complex with the cyclin proteins. CDK4 and CDK6 are found in human islets along with cyclin-D1 and cyclin-D3 (Cozar-Castellano, et al. 2004). When bound together these proteins act in concert to stimulate cell cycle progression by phosphorylating pRb and releasing the brakes on the transcriptional activity of the E2Fs. CDK4/6 and Cyclin-D1/3 are synthesized in the cytoplasm and this is where they reside in human β-cells (Fiaschi-Taesch, et al. 2013a). It is not known whether these proteins have cell cycle regulatory properties whilst in the cytoplasm, but an additional role for the CDK4-pRB pathway in insulin secretion has also been speculated (Annicotte, et al. 2009). When CDK6/Cyclin-D3 are overexpressed in human β-cells there is an increase in β-cell proliferation, however, overexpression of CDK6 alone is also effective at driving β-cell replication (Fiaschi-Taesch, et al. 2010).

P21CIP, P27KIP1 and P57KIP2 are widely expressed proteins generally viewed as inhibitors of cell cycle progression (Pestell, et al. 1999). These proteins bind to CDK4/6-Cyclin-D complexes, preventing them from performing their kinase roles (and phosphorylating pRb). However, evidence shows that the effects of these proteins can actually be bifunctional (Cheng, et al. 1999; Sherr and Roberts 1999). P27 and P21 act as molecular chaperones and contain nuclear localisation signals (NLS) which means after helping to assemble the CDK4/6 and Cyclin-D complexes, P27 and P21 can also help transport them to the nucleus (Sherr and Roberts 1999).

P27 appears to have a role in mediating mitogenic stimuli and regulating β-cell mass. In humans P27 is predominantly cytoplasmic in β-cells but shuttles to the nucleus when cell cycle progression is activated (Fiaschi-Taesch, et al. 2013b). In contrast, nuclear accumulation of P27 has been demonstrated in β-cells of T2DM mice, whilst deletion of P27 is associated with increased β-cell mass (Uchida, et al. 2005).
Even though β-cells appear to contain essentially every possible cell cycle inhibitory protein (Cozar-Castellano, et al. 2006b; Fiaschi-Taesch et al. 2013a), the loss of these brakes does not cause cell cycle progression and increased proliferation. Further factors are still necessary once the brakes have been removed (Cozar-Castellano et al. 2006b).

4.1.2. Programmed cell death regulates tissue dynamics

The population of cells within a multicellular organism is controlled by an intrinsic balance of cell proliferation and programmed cell death. The role of apoptosis is critical throughout most stages of an organism’s life; from tissue sculpting and remodelling during early development, through to maintenance of physiological turnover, as well as the removal of unwanted or harmful cells throughout the lifecycle of the organism (Jacobson 1997; Thompson 1995). Whilst we know that cell proliferation is a highly regulated and complex process resulting from the divergence of various positive and inhibitory pathways, it is only recently beginning to be appreciated that regulation of cell death is an equally complex process (Ellis, et al. 1991).

In contrast to necrosis which is a rapid form of cell death usually the result of acute injury, apoptotic cell death is a controlled process usually initiated by the cell itself (Jacobson, et al. 1994). Although various intrinsic and extrinsic signals can feed into the apoptosis programme, the ultimate role of apoptosis in regulating normal tissue dynamics utilizes the ability to eliminate cells that have been produced in excess, cells that have failed to develop appropriately, or that contain genetic defects (Thompson 1995).

During normal neonatal growth, an initial accumulation of β-cell mass is followed by a transient increase in β-cell death (Bonner-Weir 2000a; Kassem et al. 2000). However, with increasing age, the activity of these mechanisms balances, such that a steady β-cell mass is maintained assuming consistent conditions are kept (Bonner-Weir 2000b).

In samples of CHI-D, the increased proliferation documented within the β-cell population are believed to arise through a mechanism of replacement rather than new growth, and this accounts for the stability observed in β-cell mass between CHI-D and control samples (Rahier et al. 1984). This therefore implicates a role for apoptosis in the management of β-cell mass in patients with CHI-D, accounting for a loss of β-cells to which increased proliferation replaces them.
Animal models expressing β-cell mutations in K$_{ATP}$ channel genes show characteristic hyperinsulinism with associated hypoglycaemia during the neonatal period. However, as the animal develops, a state of hyperglycaemia due to a decrease in insulin secretion ensues, resulting from an increased frequency of apoptotic β-cells (Miki, et al. 1999; Miki et al. 1997). These data show concordance with the clinical course of CHI-D in patients, indicating a net increase in β-cell death. Over time, the glycaemic state of patients slowly subsides, with patients either entering clinical remission or developing insulin-dependent diabetes (Beltrand, et al. 2012; Glaser et al. 1999; Leibowitz, et al. 1995). Indeed, when rates of apoptosis were investigated in CHI-D β-cells, there was a tendency for increased β-cell death in neonatal CHI-D samples compared to age-matched controls which was maintained in the older CHI-D samples (Kassem et al. 2000).

Overall, the histological profile of patients with CHI-D, along with the physiological changes in β-cell dynamics, strongly implicates K$_{ATP}$ channel mutations in the enhancement of both β-cell proliferation and β-cell death. Complexity in cell-cycle regulation in CHI-D patients seems likely given the stark differences in histology observed between the different causative mutations; where CHI-K$_{ATP}$ results in scattered insulin-positive clusters, and CHI-GCK produces large hyperplastic islets (Cuesta-Muñoz, et al. 2004; Kassem et al. 2010).

At a time when an inventory for cell cycle regulatory proteins is just starting to emerge, CHI-D presents as a unique in vivo physiological model for studying cell cycle modulations in a background of increased β-cell proliferation and programmed cell death that is relevant to the human. Studies of this nature offer potential to better understand those mechanisms leading to β-cell proliferation that can hopefully be translated into viable and effective therapies for the expansion of β-cell mass for the treatment of diabetes mellitus.
4.2. Aims

The work in this chapter was carried out to investigate the profiles of proliferation and apoptosis in CHI-D samples, and to relate these to changes in cell cycle regulation.

4.2.1. To investigate proliferation in CHI-D pancreatic cell types, including the analysis of samples collected following a second operation; A comparison with age-matched controls

Ki67 was used to identify proliferating cells in the CHI-D pancreas alongside age-matched controls and fetal specimens. Total proliferating cells were counted for each sample across several different regions of tissue and the frequency of Ki67-positive cells within different pancreatic compartments; exocrine (acinar and duct) and endocrine (β- and α-cells) was also explored. The significance of β-cell proliferation was determined by calculating the number of proliferating cells observed within the insulin-positive population.

4.2.2. To investigate the expression and cellular localisation of G1/S cell cycle components; A comparison with neonatal controls and fetal development

A recent publication cataloguing the expression and cellular localisation of G1/S cell cycle components in human adult β-cells (Fiaschi-Taesch et al. 2013a) provided a reference for the investigation of some of these proteins in CHI-D samples. Using IHC and IF techniques the expression profiles of CDK6, pRb and P27 were explored in samples of CHI-D, alongside age-matched controls and fetal samples.

4.2.3. To determine whether apoptosis is enhanced in CHI-D samples, and whether the β-cell population is involved; A comparison with age-matched controls

Cells undergoing programmed cell death were identified using an in situ TUNEL detection kit in CHI-D, fetal and neonatal control samples, whilst dual staining with insulin identified the proportion of apoptotic cells occurring in the β-cell population.
4.3. Results

Having been unable to verify whether a process of endocrine differentiation was occurring in CHI-D pancreas, the focus of the study was instead shifted to investigate the regulation of pancreatic mass in these samples.

Pancreatic growth in the human postnatal pancreas is notoriously difficult to study. Whilst the rodent pancreas seems to maintain a heightened capacity for proliferation after birth, the mechanisms involved in human maturity are less well understood. The β-cell mass has been described as dynamic, demonstrating an inherent ability to adapt to changing conditions and insulin demands. Although, compared to the mouse, these adaptations are still slight in comparison.

Some, but not all groups have reported an increase in β-cell proliferation in cases of CHI-D (Kassem et al. 2000; Loviso et al. 2010; Sempoux et al. 1998), although overall an increase in β-cell mass has not been observed (Rahier et al. 1984). Whilst loss of imprinted genes controlling cell cycle regulation in the focal form of CHI β-cells can explain why β-cell proliferation is enhanced in these samples (Kassem et al. 2001), mechanisms regulating CHI-D β-cell expansion are less clear.

4.3.1. Investigating proliferation in CHI-D and age-matched controls

With varying accounts of β-cell proliferation across CHI-D samples available, and the relatively small number of cases studied, the expression of Ki67 was further studied in our samples of CHI-D biopsies. Five patients, each with an identified mutation affecting the KATP channel were analysed for Ki67 expression using IHC. Immunoreactivity for the Ki67 antigen was readily detected as brown nuclear staining in both CHI-D and age-matched control samples, as well as in human fetal pancreas (Figure 4-2). Surprisingly, Ki67 staining was considerably more prevalent in CHI-D than control samples.

Following cell counting of Ki67-positive nuclei across 10 random fields of view in each CHI-D sample (2-13 mo) and control sample (2-13 mo), and in three different positions within each biopsy, significantly more Ki67-positive cells were observed in CHI-D samples compared to age-matched controls (Figure 4-3). Ki67 immunoreactivity in CHI-D samples occurred at more than 6 times the frequency as in control samples during the first year of life. This was surprising since the data on β-cell proliferation is so variable (and the values comparatively low) but the widespread incidence of Ki67 outside of the endocrine lineage in CHI-D samples has not been reported previously. Spatial analysis of whole sections of pancreatic biopsy stained for Ki67, showed approximately 5 % of all pancreatic cells to be proliferating in CHI-D pancreas (Salisbury et al., in press-observations made in lab group).
Since total islet mass is estimated to occupy approximately 1-2% of total pancreatic mass in the mature pancreas (Naya et al. 1997), it is unlikely that only β-cell proliferation is increased in CHI-D samples.

**Figure 4-2. Cell proliferation in CHI-D, age-matched control and fetal pancreas**
Brightfield images of 5 µm sections of CHI-D, age-matched control and fetal pancreas showing immunohistochemical staining (brown) for Ki67, and counter-stained with Toluidine blue. Arrows show the few Ki67-positive cells in control samples whilst widespread staining could be seen in CHI-D and fetal samples. Scale bar represents 100 µm.

**Figure 4-3. Frequency of proliferating cells in CHI-D and age-matched controls**
The average number of Ki67-positive cells per field of view at 200X magnification was counted across 10 fields of view and 3 different regions of CHI-D (2-13mo) and age-matched control pancreas (2-13mo). Error bars show standard error. Statistical significance calculated using Mann-Whitney U test (P<0.01).

We therefore wanted to look at characterising the cell types involved in the excessive proliferation occurring in CHI-D samples. Whilst wanting to study the β-cell population in particular to contribute to the relatively sparse data available on this feature in CHI-D β-cells, since we have shown previously that many features of CHI-D, including the diffuse distribution of insulin, its association with ducts and disorganisation of islet structures
extend beyond the β-cell, it was important to consider alterations in other pancreatic lineages.

Using dual IF the detection of proliferating cells within different pancreatic lineages was explored. Ki67 was analysed for coexpression with insulin and glucagon (β- and α- cell-types), SOX9 (ductal cells) and GATA4 (acinar compartment)(Figure 4-4). Nuclear Ki67-positive cells were observed within each compartment for each sample studied.

Figure 4-4. Characterising proliferating cell types in CHI-D, control and fetal pancreas
Dual immunofluorescence of 5 μm sections from (A) CHI-D, (B) neonatal control and (C) 15 wpc fetal. Co-expression of Ki67 (red) with insulin, glucagon, SOX9 and GATA4 (green) was assessed across the different samples. Arrows show Ki67-positive cells within each compartment. Scale bar represents 20 μm.

During human postnatal development there is a rapid increase in total pancreatic mass within the first few years of life, continuing in a linear correlation until adolescence (Saisho et al., 2007).

Analysing samples of postnatal control pancreas between 6 weeks – 12 months of age, including adult samples, total proliferation across all pancreatic lineages was correlated inversely with age ($r^2=0.929$, $P<0.01$) (Figure 4-5). Since levels of proliferation within the endocrine compartment were low across all samples, the decreases in proliferation with increasing age were largely due to a reduction in acinar (GATA4) proliferation. In adult samples very few Ki67-positive cells were detected in either of the compartments.
Figure 4-5. Decreasing proliferation in aging postnatal and adult control pancreas

Proliferation in the normal pancreas during the first year after birth and adulthood. Average counts (+/− S.E) for Ki67-positive cells dual stained for markers of different pancreatic lineages (β-cells, insulin; α-cells, glucagon; ductal cells, SOX9; acinar cells, GATA4; other endocrine lineages had negligible rates of proliferation).

The same analysis was performed on CHI-D samples, with the numbers of proliferating cells counted in each compartment expressed relative to the counts in age-matched control samples (Figure 4-6).

Whilst Ki67 staining during the first year of life has been shown to be approximately 2-3 fold higher in CHI-D β-cells than in controls (Kassem et al. 2000), our data showed only two out of the five CHI-D samples to have significantly elevated β-cell proliferation compared to their age matched controls (CHI-D 1, CHI-D 4). This was the same number of samples that had significantly more α-cell proliferation (CHI-D 1, CHI-D 2). In contrast, the majority of CHI-D samples had significantly elevated ductal proliferation, identified by coexpression of SOX9 and Ki67 (CHI-D 2-5). This increase in ductal proliferation varied from 5- to 11- fold between CHI-D samples relative to their age-matched controls, although these rates were not related to age of sample. All five CHI-D samples showed significantly increased proliferation in acinar cell populations compared to their age-matched controls. In contrast to the SOX9+/Ki67+ counting data, the incidence of GATA4+/Ki67+ did appear to advance with increasing age of specimen, from a 7-fold increase in 2 month CHI-D up to a 47-fold increase in 12 month CHI-D compared to their respective controls.
Relative average counts of Ki67-positive cells in five CHI-D samples compared to their age-matched controls (2-13 mo). Each bar stacks the fold increase (+/- S.E) in proliferation within each pancreatic cell lineage (β-cells, insulin; α-cells, glucagon; ductal cells, SOX9; acinar cells, GATA4; other endocrine lineages had negligible rates of proliferation). Statistical analysis performed using Mann-Whitney U test; * P<0.05, **P<0.01.

These data demonstrate that whilst rates of β- and α- cell proliferation are variable between CHI-D samples, the most consistent increases in proliferation are seen in the ductal and acinar cell compartments. These data are consistent with similar data obtained in the human fetal pancreas, in which the ductal and acinar cell-types also represent the most proliferative populations (Bouwens et al. 1997; Piper Hanley, et al. 2010).

Since our data were derived as counts per field of view data were also collected to identify what proportion of the insulin-expressing population were proliferative. This is the format in which the majority of the published data is presented and therefore permits more direct comparisons to be made (Figure 4-7).

In CHI-D samples less than 3 months of age, proliferation in the β-cell lineage was significantly greater than control samples of the same age (4.7 fold increase). Above 3
months of age, CHI-D β-cells were also significantly more proliferative than their age-appropriate controls (3.5 fold increase), although these samples showed a reduced proportion of insulin+/Ki67+ in comparison to their younger CHI-D counterparts. These data are consistent with the data published by Kassem and colleagues who showed 2.13% β-cells to be proliferative in CHI-D <3mo, whilst approximately 1% costained with Ki67 > 3mo (Kassem et al. 2000).

**Figure 4-7. Enhanced proliferation in CHI-D β-cells**

CHI-D and age-matched control β-cells were counted for dual reactivity with Ki67 and the average proliferating β-cells expressed as a percentage of the insulin-producing population in CHI-D and age-matching control pancreas (+/- S.E). An average of 632 insulin-positive cells (±236) were counted per section with three sections from each sample. Five different CHI-D samples (2-13 months) and six age-matched control samples (2-13 months) were analysed. Statistical significance was calculated using Mann-Whitney U test p<0.01.

For each of the samples studied for Ki67 expression, including CHI-D and age-matched control specimens, there were a number of Ki67-positive cells that did not appear to colocalise in any of the cell-compartments explored. These unidentified Ki67-positive cell types were significantly more prevalent in CHI-D samples than the neonatal controls (P<0.05)(Figure 4.8).

Going back through the digital images captured for each CHI-D sample, the location of each Ki67-positive cell was recorded based on visual assessment of histologic compartments; islet, acinar, ducts or mesenchyme.
Figure 4-8. Increased incidence of unidentified proliferating cells in CHI-D

The average number of Ki67-positive cells occurring per x200 field of view that did not colocalise with insulin, glucagon, SOX9 or GATA4 in CHI-D or age-matched control samples (2-13 mo). On average 42 (±23) Ki67-positive cells were counted per field of view in CHI-D samples (2-13 months), with an average 7 (±5) Ki67-positive cells in age-matched controls (2-13 months). Error bars show standard error. Statistical significance was calculated using Mann-Whitney U test P<0.05.

Although not an accurate technique, the intention was to rule out additional Ki67-positive cells located in the mesenchyme of the samples, identified as loosely associated, elongated cells. The data obtained from this post-hoc analysis reflected what had been previously discovered (Figure 4-9); the acinar compartment contained the most Ki67-positive cells, whilst islet cells contributed approximately 7% of all Ki67-positive cells observed. Ductal cells represented approximately 15% of the Ki67-positive cells in CHI-D samples however this value is likely to be an underestimation since ductal structures are much more difficult to identify without positive staining or the presence of a lumen.

Since approximately 7% of Ki67-positive cells were observed within islet structures, but only approximately 2% counted by insulin and glucagon staining, coexpression of Ki67 with somatostatin, ghrelin and PP was also explored. However, very few cells were observed to be Ki67-positive in any of these hormone-secreting cell-types, and therefore offered negligible contribution to the islet cell proliferation rates described.
Figure 4-9. Histological examination of proliferating cells

The location of Ki67-positive cells within each CHI-D sample was recorded as either acinar, ductal, islet or mesenchyme depending on the histological localisation in the tissue. The majority of proliferating cells were confirmed in the acinar compartment with very few Ki67-positive cells detected in the mesenchyme lineage. Error bars show standard error.

A couple of CHI-D samples used in this study underwent a second operation to remove more of the pancreas since the first pancreatectomy had been unsuccessful in treating the symptoms of CHI-D. The necessity for a second operation has also been described in the literature, although the samples were mostly included in grouped data and have rarely been analysed separately (Aynsley-Green 1981; Kassem et al. 2000; Moazam, et al. 1982; Rahier et al. 1984).

Within our samples, patients CHI-D 3 (3 mo) and CHI-D 5 (13 mo) both had a second operation at the ages of 31- and 34- months respectively. At the time of surgery during the second operation, it was reported that the surgeon observed a re-growth of the pancreas. Although it had not performed full regeneration, the new growth appeared to represent normal pancreatic architecture, with the organ maintaining a lobular appearance.

We therefore repeated the IHC Ki67 experiments in the re-operated samples, making comparisons with the first surgical sample for each CHI-D case as well as an age-matched control (Figure 4-10).

Surprisingly, the extent of Ki67-positive staining in the CHI-DR samples appeared considerably reduced compared to the first operation in each case. This observation was quantified by cell counting (Figure 4-11). Whilst proliferating cells were significantly elevated in the first biopsy sample from CHI-D 3 and CHI-D 5, relative to their age-matched controls, the proliferation appears to have been suppressed between the first
operation and the second, since both CHI-D 3R and CHI-D 5R maintained proliferation at a rate consistent with the age-matched control (3 years).

![Figure 4-10. Rates of proliferation returned to normal levels in CHI-DR](image)

Brightfield images of 5 μm sections of two CHI-D samples that underwent a second operation to remove more pancreatic tissue in order to treat the persistent symptoms of hyperinsulinism compared to the original CHI-D biopsy and age-matched controls. Immunohistochemical staining (brown) for Ki67, counter-stained with Toluidine blue shows a high prevalence of Ki67-positive in the original biopsy (CHI-D 3, CHI-D 5) compared to their age-matched controls whilst few Ki67-positive cells were observed in CHI-DR biopsies, similar to the 3 year control. Scale bars represent 50 μm.

Even though the number of Ki67-positive cells had returned to an apparently 'normal' level, dual IF of Ki67 with insulin was performed to confirm how many of these Ki67-positive cells were β-cells. These experiments showed that Ki67-positive cells were rarely insulin-positive in CHI-DR samples (Figure 4-12).
Figure 4-11. Quantification of Ki67-positive cells in CHI-D, their second biopsy and controls

Ki67-positive cells identified by immunohistochemical staining were counted per x200 field of view in at least 10 different fields of view across three sections of CHI-D pancreas, their corresponding second biopsy and age-matching controls. An average total of 572 (±276) Ki67-positive cells were counted in CHI-D samples, with 39 (±4) in CHI-DR samples and 30 (no standard deviation) in age-matched control. Error bars show standard error. Statistical significance calculated using Mann-Whitney U test (P<0.01).

Figure 4-12. Proliferation in CHI-D reoperation samples

Dual immunofluorescence of 5 μm sections from two CHI-D samples that underwent a second pancreatectomy operation to remove more pancreatic tissue in order to treat the symptoms of hyperinsulinism. Dual staining of Ki67 (red) with insulin (green) shows most proliferating cells to be outside of β-cell lineage, demonstrated in three different regions of each sample. Scale bars represent 50 μm.
4.3.2. Expression and cellular localisation of G1/S cell cycle components

Recently, a comprehensive study of cell cycle regulation in the human pancreatic β-cell has devised an atlas of G1/S cell cycle marker expression. Surprisingly, these studies identified a previously unappreciated cytoplasmic localisation for many of these molecules, including the CDKs and Cyclin proteins in the human β-cell (Fiaschi-Taesch et al. 2013a). Figure 4-13 shows the most recent model for the cell cycle network in human β-cells compared to the older version that assumed a nuclear localisation for most of the factors.

Figure 4-13. Cell cycle network
A schematic model of cell cycle regulation in the quiescent human pancreatic β-cell showing the old model (A) and the revised newer model based on the cytoplasmic localisation for many of the components (B). Red arrows indicate inhibition, green arrows show activation. Most G1/S molecules are cytoplasmic, apart from the cell cycle inhibitors pRb, P57 and P21. CDK6 and Cyclin D3 shuttle to the nucleus upon induction of β-cell proliferation. (Adapted from Fiaschi-Taesch et al., 2013b).

Since the human β-cell is notoriously refractory in its ability to proliferate, the cytoplasmic localisation of many of the G1/S components can be assumed to reflect the quiescence of these cell types (Fiaschi-Taesch et al. 2013a; Fiaschi-Taesch et al. 2013b). However, it is possible to induce a modest increase in β-cell proliferation in adult preparations by overexpressing CDK6 alone, or in combination with Cyclin D3 (Fiaschi-Taesch, et al. 2009). Upon induction, many G1/S components, previously cytoplasmic in location, including CDK6, P27 and P21, can be seen to shuttle to the nucleus of β-cells (Fiaschi-Taesch et al. 2013b) (Table 4-1). The cellular distribution of these cell cycle markers may therefore provide an indicator for the capacity of a cell to proliferate, ie. if the cell is “poised” in a state ready to drive cells into s-phase upon further mitogenic stimulus. By virtue of IHC experimentation, we investigated the expression of several cell cycle markers in samples of CHI-D pancreas, making comparisons with age-matched control pancreas as well as human fetal samples.
<table>
<thead>
<tr>
<th>Cell Cycle Molecule</th>
<th>Mouse Islets</th>
<th>Human Islets</th>
<th>Quiescent β Cells (adult human)</th>
<th>Overexpressed</th>
<th>Induced β cell proliferation (overexpression of CDK6/CyclinD3)</th>
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<td>E2F1</td>
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<td>+</td>
<td>Cytoplasmic</td>
<td>Cytoplasmic</td>
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<tr>
<td>E2F2</td>
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<td>Cytoplasmic</td>
<td>Cytoplasmic/ some nuclear</td>
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<tr>
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<td>Cytoplasmic</td>
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<tr>
<td>E2F4</td>
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<td>Cytoplasmic/Some nuclear</td>
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<td></td>
<td></td>
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<tr>
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<td>+</td>
<td>Cytoplasmic (also α, δ cells)</td>
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<tr>
<td>Cyclin D1</td>
<td>+</td>
<td>+</td>
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<td>Nuclear</td>
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<td>V Low detection, if at all</td>
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</tbody>
</table>

Table 4-1. Location of cell cycle molecules in quiescent and proliferating islet cells
Cell cycle molecules in mouse and human islets. + indicates the protein (or RNA) is present and – indicates that protein (or RNA) is absent.
We first looked at CDK6 (Figure 4-14). Consistent with other observations in human islets (Fiaschi-Taesch et al. 2013a; Fiaschi-Taesch et al. 2010), CDK6 was detected in the cytoplasm of control islets in both neonatal and fetal samples, where it was observed to colocalise predominantly with insulin (Also Appendix 4-1).

Since CDK6 is capable of driving β-cell proliferation (Fiaschi-Taesch et al. 2010) its absence from the nuclear compartment in neonatal samples is in-keeping with the low proliferation rates detected in these samples.

In contrast, CDK6 was less noticeable in the cytoplasm of CHI-D islets, but was clearly nuclear in a small proportion of insulin-positive cells (Figure 4-14, arrowhead C, D). Widespread CDK6 expression was detected, however, in the acinar tissue, where clear nuclear staining could be seen following both bright-field and IF investigations (Figure 4-14, C). Nuclear CDK6 was also observed within the CK19-positive ductal cells (Figure 4-14, D). This is consistent with the increased proliferation observed in these compartments.

Figure 4-14. Expression of CDK6 in CHI-D, age-matched control and fetal pancreatic samples

Brightfield immunohistochemistry counterstained with Toluidine blue and dual immunofluorescence counterstained with DAPI for CDK6 (red) and insulin (green)(A-D) and CK19 (green)(D) in 5 μm sections of normal control pancreas, fetal pancreas and CHI-D. Hatched lines in brightfield image encircles an islet. Arrowhead in (A) points to insulin-positive cells in which CDK6 localizes to both cytoplasm and nucleus. In (C) arrow points to cytoplasmic CDK6 whilst arrowhead in (C) and (D) points to clear nuclear staining in insulin-positive cells. Scale bars represent 50 μm (A,C), 20 μm (B) and10 μm (D).
A similar investigation was carried out to explore the expression of retinoblastoma protein (pRb) in the same samples. Fiaschi-Taesch and colleagues have reported pRb to be in the nucleus of human β-cells, a finding they confirmed by both IHC and subcellular fractionation in isolated islet preparations and fixed tissue samples (Fiaschi-Taesch et al. 2013a). However, pRb was not detected either in the cytoplasm or nucleus of any cell types in the control samples (Figure 4-15, A). In contrast, cytoplasmic pRb staining was seen in the ductal epithelium of fetal samples, but did not localize in regions of insulin-immunoreactivity (Figure 4-15, B). In CHI-D samples, pRb protein was seen in a similar distribution pattern to CDK6, with widespread expression in the acinar cells, almost exclusively outside of islet structures, by both bright-field and IF detection (Figure 4-15, C, Appendix 4-2). Although apparently nuclear in acinar cell-types, pRb expression was predominantly cytoplasmic in CK19-positive ductal cells (Figure 4-15, D), an observation similar to the fetal expression profile.

**Figure 4-15. Expression of pRb in CHI-D, age-matched control and fetal pancreatic samples**

Brightfield immunohistochemistry counterstained with Toluidine blue and dual immunofluorescence counterstained with DAPI for pRb (red) with insulin (green) (A-C) and CK19 (green) (D) in 5 μm sections of normal control pancreas, fetal pancreas and CHI-D. No pRb staining was detected in control (A), whilst cytoplasmic staining was observed in fetal ductal epithelium (B). Widespread nuclear pRb staining was seen outside of islets in CHI-D (C) whilst cytoplasmic expression was detected in the ducts (D). Scale bars represent 20 μm (A-C) and 10 μm (D).
To gain further insight into possible mechanisms for adjusted cell proliferation in CHI-D, network analysis was performed on a microarray dataset of CHI and control samples (Using GSE32610 from gene Expression Omnibus, GEO: http://www.ncbi.nlm.nih.gov/geo/) (Michelsen, et al. 2011) allowing for the identification of altered gene expression between the two sample groups.

Using this approach, 1288 genes were identified as being significantly changed in CHI tissue (P<0.05, ANOVA), and 140 functional modules were established based on similarities in gene function and interactions (Appendix 4-3). Interestingly, when these modules were ranked according to their index of centrality, the module to which the gene CDKN1B was most central, was ranked third highest (Appendix 4-4).

CDKN1B encodes the cyclin-dependent kinase inhibitor P27Kip1 (P27). P27 is a component of the G1/S cell cycle regulation programme, but actually appears to have a diverse function. P27 is involved in the progression of cell cycle, acting as a chaperone for the transport of CDK6-Cyclin complexes into the nucleus, but also has a role as a member of inhibitory complexes, preventing the progression of the cell cycle (Cheng et al. 1999; Fiaschi-Taesch et al. 2013b; Fiaschi-Taesch et al. 2010; Kulkarni et al. 2012; Pestell et al. 1999; Sherr and Roberts 1999).

qRT-PCR experimentation validated the network analysis, confirming a >7-fold increase in CDKN1B transcripts in CHI-D samples, compared to their age-matched controls (Figure 4-16, A). Like CDK6, P27 was almost entirely expressed in the cytoplasm of control islets where it again showed colocalisation with insulin (Figure 4-16, B) consistent with previous publications (Fiaschi-Taesch et al. 2013a). In contrast P27 was extensively nuclear in both fetal and CHI-D β-cells (Figure 4-16, C-D) and could also be detected as a nuclear protein in a small subset of ductal cells in the fetal and CHI-D samples colocalising with CK19 (Figure 4-16, D, E).

Having detected an altered localisation of these factors in the nucleus instead of the cytoplasm of CHI-D β-cells, we explored whether these nuclear G1/S molecules indicated a proliferating cell. Using dual IF techniques, the colocalisation of CDK6, pRb and P27 with Ki67 was analysed in fetal samples (Figure 4-17). Nuclear CDK6 colocalised in some, but not all Ki67-positive cells, and not all CDK6-positive cells were Ki67-positive. pRb was predominantly cytoplasmic in fetal samples and this expression coincided with nuclear expression of Ki67 in some proliferating cells. However, once again, pRb was not seen in the cytoplasm of all Ki67-positive cells, and cytoplasmic pRb expression was much more widespread than just the Ki67-positive cells.

In contrast, P27 was not observed in the nucleus of any proliferating cells.
Figure 4-16. Expression of P27 in CHI-D and age-matched controls

(A) qRT-PCR showing increased CDKN1B expression in CHI-D (mean +/- S.E across five CHI-D cases) compared to age-matched controls (n=3) *P<0.05 by Mann-Whitney U test. (B-E) Brightfield immunohistochemistry counterstained with Toluidine blue and dual immunofluorescence counterstained with DAPI for insulin (green) (B-E) and CK19 (green) (E) in 5 μm sections of normal control (B), fetal (C) and CHI-D (D-E) samples. Hatched lines in brightfield images encircle an islet. Scale bars represent 10 μm.

Figure 4-17. Cell cycle markers in proliferating cells

Dual immunofluorescence of cell cycle components CDK6, pRb, P27 (red) with Ki67 (green) in 5 μm sections of fetal pancreas, counterstained with DAPI. Arrows point to dual expressing cells where CDK6 is nuclear in Ki67-positive cells and pRb is cytoplasmic in some proliferating cells. P27 did not colocalise in any Ki67-positive cells. Scale bars represent 20 μm.
Components of the G1/S cell cycle were also studied in the samples of CHI-D tissue obtained following a second pancreatectomy operation necessary to treat the persistent hypoglycaemia (Figure 4-18).

**Figure 4-18. Expression of cell cycle components in CHI-D re-operated samples**

Brightfield immunohistochemistry counterstained with Toluidine blue of CDK6, pRb and P27 (brown) in 5 μm sections of CHI-D re-op and normal control samples. CDK6 was apparent in the cytoplasm of control samples but absent from CHI-DR whilst pRb was not detected in either sample. P27 showed weak cytoplasmic staining in CHI-DR but absent from control. Scale bars represent 50 μm.

Consistent with the decreased proliferation observed in the CHI-DR samples, CDK6 and pRb did not show the same extensive nuclear staining in either acinar or ductal cell compartments as seen in the first CHI-D biopsies. Whilst cytoplasmic CDK6 was readily detected in the control (3 year) islets, this apparent coexpression with insulin was once again absent from the CHI-DR islets, consistent with observations in CHI-D samples.

pRb was not detected in any of the samples; CHI-DR or controls, as either nuclear or cytoplasmic staining. However, P27 was detected as weak cytoplasmic immunoreactivity in the islets of CHI-DR, although apparently absent from the age-matched control. This is in contradiction to the previous analysis of neonatal samples (2-6 months) that showed cytoplasmic localisation of P27.

**4.3.3. Investigating apoptosis in CHI-D and age-matched control pancreas**

Finally, rates of apoptosis were investigated in the CHI-D tissues. Although the data on β-cell proliferation in CHI-D is relatively variable in the literature, the β-cell mass is generally considered unexpanded in these samples, consistent with our observation that neither INSULIN nor NKX6.1 transcripts were significantly elevated in our samples (Chapter 3).
(Rahier et al. 1984). It is assumed that the β-cell population in CHI-D samples is maintained at a steady population due to a balance of β-cell proliferation offset by an increased rate of apoptosis (Kassem et al. 2000).

Terminal deoxynucleotidyl transferase-mediated dUTP-X 3' nick-end labeling (TUNEL) reactions detect fragmented DNA in tissue samples, identifying cells undergoing a process of apoptosis (Gavrieli, et al. 1992).

An in-situ TUNEL detection kit was used on samples of CHI-D (2-4 months), in age-matched controls and samples of fetal pancreas. Control experiments were run for each investigation, using DNase I as a positive control for the detection kit, and omitting the TACS enzyme from the staining procedure as a negative control (see section 2.2.3/Appendix 4-5). Apoptotic cells were readily detected by nuclear staining (Figure 4-19) whilst dual IF with insulin identified which of these apoptotic cells were β-cells. Regions of tissue damage or necrosis (including the periphery of the tissue sections) were excluded from frequency analysis, and only cells with clear nuclear TUNEL and cytoplasmic insulin staining were counted.

![Figure 4-19. Apoptosis in CHI-D, age-matched control and fetal β-cells](image)

Dual immunofluorescence of 5 μm sections from two CHI-D samples (A,B), age-matched controls (C) and fetal (D) pancreas, counterstained with DAPI for TUNEL (green) and insulin (red). Arrows show apoptotic β-cells. No TUNEL-positive cells were observed in fetal samples. Scale bars represent 100 μm.
Overall, the number of TUNEL-positive cells detected per field of view in CHI-D was not significantly different to those in control pancreas (39 ± 6.2, 27 ± 3.3 respectively). However, when the frequency of TUNEL-positive cells costaining with insulin were counted as a proportion of total insulin-positive cells, CHI-D samples contained significantly more TUNEL+/Insulin+ than the control (P<0.05) (Figure 4-20).

Of note, no TUNEL-positive cells were detected in the fetal samples assessed (11-15 wpc). This is consistent with data published by Kassem and colleagues who observed very few TUNEL+/Insulin+ between 17-32 weeks of gestation (0.6% of β-cells) (Kassem et al. 2000).

![Figure 4-20. Quantification of apoptotic β-cells in CHI-D and age-matched controls](image)

CHI-D (2-3 mo), normal control (2-3 mo) and fetal (11-15 wpc) were counted for dual immunoreactivity of TUNEL with insulin-positive cells, counted across 10 fields of view and expressed as a percentage of the total insulin-secreting population for each. Bars show mean, error bars show standard error. Statistical significance calculated using Mann-Whitney U test p<0.05.
4.4 Discussion

CHI-D due to inactivating mutations affecting subunits of the $K_{ATP}$ channel is the most common form of CHI. Many of these patients do not respond to medical therapy and therefore require partial- or near-total pancreatectomy to treat severe hypoglycaemia and prevent irreversible brain damage. Whilst presenting with features of electrophysiological β-cell over-activity, including persistent β-cell depolarisation, elevated intracellular Ca$^{2+}$ concentrations and inappropriate insulin secretion (Ashcroft and Rorsman 2013; Kane et al. 1996), patients also display an increased abundance of extra-islet β-cell clusters with poorly formed islets and β-cells associated with ducts (Heitz et al. 1977; Rahier et al. 1984). Although histologically similar to the pancreas of neonatal control samples, these features appear to persist longer in CHI-D samples (Heitz et al. 1977; Rahier et al. 1984).

Over time, medically-treated CHI-D patients who do not undergo surgery appear to enter clinical remission, with a tendency to develop a degree of glucose intolerance later in life, whilst patients who did have a pancreatectomy procedure often subside to a state of insulin-dependent diabetes during the second or third decade of life (Beltrand et al. 2012; Leibowitz et al. 1995). The clinical course of CHI-D seems to suggest an adjusted balance of the β-cell turnover dynamics.

There is strong evidence to suggest mammalian β-cell mass can adapt throughout life based on changing metabolic needs, and this has been conclusively shown in rodents. However, data are more limited in humans. To date there have been a handful of studies exploring the dynamics of β-cell mass in CHI-D subjects. An elevated rate of β-cell proliferation was observed in most of these studies (Kassem et al. 2000; Lovisolo et al. 2010), whilst a modest but statistically non-significant increase in β-cell proliferation was observed in others (Sempoux et al. 1998).

In the human adult pancreas the β-cell is widely considered as quiescent with very low, if any, replication occurring. A comprehensive inventory of cell cycle proteins in the mature human β-cell has been described, detailing a unique distribution of cell cycle components. Surprisingly, it would appear that every cell cycle inhibitor that could be present is present in the human β-cell, identifying a cell-type that really doesn’t want to proliferate (Fiaschi-Taesch et al. 2013a).

Given the altered tendency for proliferation and apoptosis that seems to be apparent in CHI-D samples, generating a profile of cell cycle components in the samples would help advance our understanding of the mechanisms involved in the control of β-cell turnover. This is especially pertinent given the recent breakthroughs in identifying the GSIS

Similarly, since recognizing in the previous chapter (Chapter 3) that many of the features of CHI-D extended beyond the β-cell lineage, the proliferation rates in other pancreatic cell types was also explored.

4.4.1. Enhanced cell proliferation in CHI-D

Proliferation in the β-cell compartment was subtle in our samples with only 2 out of 5 of the CHI-D samples showing statistically enhanced β-cell proliferation. These data were collected as the total number of Ki67-positive cells co-staining with insulin and counted per field of view (X200 magnification), averaged across several different fields of view and in at least 3 different regions of the pancreatic biopsy. This was to ensure that counting data were as representative of the pancreas as possible and not biased by the position (ie head or tail of the pancreas). Counting per field of view presented as the best method for counting at the time, accounting for the high Ki67-positive cells detected in each sample and also avoiding having to count large populations of cells (ie acinar or ductal populations) if Ki67-reactivity were to be expressed as a proportion of total population for each cell-type. Conventionally, providing data as a proportion of a total population typically involves counting approximately 1000 cells to which the proportion is expressed. Counting by field of view in our samples allowed a greater area of tissue to be covered for each experiment including the analysis of approximately 10,000-15,000 cells. Unfortunately this method of counting meant that the results obtained were not directly comparable with other studies. However, the data clearly shows a previously unappreciated proliferation in the acinar compartment across all the CHI-D samples, as well as an expansion in the ductal cell population based on nuclear Ki67 immunoreactivity.

Proliferation in the exocrine compartments may be simply explained by the local hyperinsulinaemic conditions, based on the persistent secretion of insulin from the CHI-D β-cells diffusely distributed throughout the acinar tissue. Insulin is a potent growth factor, the effects of which are most apparent in the fetal period. As such, infants with CHI are often born with an increased birth weight, indicating an over-secretion of insulin throughout most of the gestational period (Arnoux, et al. 2010; Aynsley-Green, et al. 2000; de Lonlay-Debeney, et al. 1999; Dunne et al. 2004; Rahier et al. 2011). Such macrosomia is also apparent in babies born to diabetic mothers as a result of fetal hyperinsulinaemia, whilst fetal hypoinsulinaemia invariably causes restriction of fetal growth (Aynsley-Green, et al. 2000).
The insulin receptor is ubiquitously expressed, but mainly found in metabolically active cells, and as such is also found in the pancreatic acinar cells (Korc, et al. 1978; Mössner, et al. 1984). Insulin can therefore react with these receptors and cause an increase in proliferation and cell growth in acinar cell types. Proliferation in the acinar compartment was correlated with age, supporting the insulin-driven mechanisms for cell growth, in which the older samples have been exposed to elevated insulin concentrations over a longer period, resulting in a more profound effect on cell replication.

Proliferation in the ductal cell lineage was also significantly elevated in 4 out of 5 CHI-D samples, although the incidence did not appear to show correlation with the age of the patient. It is unknown at this stage whether this increase in SOX9-positive cell proliferation is an expansion of the progenitor cell population. Certainly, this would have an appealing hypothesis, given the frequency of endocrine cells associating in these structures, as well as scattered extra-islet hormone-positive cells throughout the pancreas. However, since conclusive evidence was not obtained to support a process of neogenesis in CHI-D samples, any hypotheses would be unsubstantiated at this time.

As an endocrine precursor cell has never been isolated from the ductal compartment, and therefore the genetic profile of these cells not identified, it would be difficult to try and distinguish these cells by genetic profiling alone. However, since the ductal compartment is thought to harbour such elusive pancreatic stem cells, further characterisation of these proliferating duct cells would be justified. Loss of mature ductal markers such as carbonic anhydrase II (CAII) or mucin 5AC may be one possibility to identify an immature ductal phenotype (Bonner-Weir, et al. 2008), or the use of more general progenitor markers such as IDX1 or ISL1. In the mean time, the enhanced ductal proliferation observed in CHI-D samples can be assumed another feature of the prolonged insulin secretion and hypoglycaemia resulting from hyperfunctional β-cells.

Proliferation in β-cells has been shown to be less pronounced in CHI-D samples compared to lesions in CHI-F (Kassem et al. 2000; Lovisolo et al. 2010; Sempoux et al. 1998) and this is consistent with the genetic background underlying each type. Although both CHI-D and CHI-F are most commonly caused by mutations affecting K_ATP channels, CHI-F represents a more complex pattern of maternal inheritance and somatic loss of chromosome 11, which means the expression of imprinted genes found around the lost region on chromosome 11 are also lost. These include genes involved in the cell cycle, including P57, H19 and IGF-II (Kassem et al. 2001; Suchi, et al. 2006), each of which have been demonstrated to increase rates of proliferation when knocked-out (Kassem et al. 2001; Matsumoto, et al. 2011; Sempoux, et al. 2001).

This is further supported by the proliferation rates outside of the affected region(s) in CHI-F that do not deviate significantly from those of normoglycaemic controls. Whilst the
increased proliferation in CHI-F samples is therefore justified genetically, the mechanisms underlying CHI-D proliferation are less well understood.

During postnatal development the pancreas must continue to grow in functional mass in order to meet growing metabolic and digestive demands of the body. In a study by Saisho et al., total pancreatic volume, as measured by CT scan analysis, increased in a linear fashion between birth and 20 years of age (Saisho et al. 2007). After the rapid increases seen in childhood, the rate of growth plateaus, with little change in mass between 20-60 years before starting to decline beyond 60 years of age (Saisho et al. 2007). Studying Ki67 expression in neonatal samples we show a substantial decline in overall proliferation from 6 weeks to 1 year of postnatal age, suggesting most rapid growth to have occurred within the first 6 months of life. The very low observation of Ki67-positive cells in the adult tissue supports the suppressed growth in older samples.

However, in this study overall only a small area of pancreas was examined for proliferation, and this became increasingly unrepresentative of the entire sample as the total pancreatic volume increased. In younger, and therefore smaller samples, 3 separate 5 μm cross sections of pancreas can represent a larger proportion of the whole organ compared to older samples with substantially larger pancreata. In these specimens, 5 μm sections become increasingly less representative and the number of Ki67-positive cells per field of view is diluted by the increasing pancreatic mass.

During fetal development proliferation in CK19-positive cells is statistically higher than in synaptophysin-identified endocrine cells at all stages of development (Bouwens et al. 1997). We show here that this trend appears to continue in the postnatal pancreas, with SOX9+/Ki67+ being more represented than α- or β-cell Ki67+ (with the exception of 3-6 mo and 12-13 mo).

From the first few weeks of birth until late adulthood, the pancreatic β-cell mass increases by approximately 30.5 fold (Meier et al., 2008), with the most pronounced increase occurring in the youngest age group (< 2 years) and decreasing each year thereafter (Meier et al. 2008).

By virtue of histological analysis (number of islets and size of islets) as well as Ki67-immunoreactivity, approximately 88 % of β-cell mass increases were attributed to β-cell replication, whilst 12 % assumed from a non-islet source (Meier et al. 2008). Our data showed a steady rate of endocrine proliferation in the majority of samples between 6 weeks to 12-13 months, which was abolished in adult samples. It is likely that the β-cell mass is established early in infancy, at which point there is a surplus of β-cells relative to their functional requirements. However, these become more closely matched to the body’s
glycaemic requirements by adulthood (Meier et al. 2008). Our data support this early endocrine expansion.

When β-cell proliferation data were expressed as a proportion of the insulin-positive population, the significance of the increased β-cell replication became more pronounced. The samples were grouped by age in accordance with the age categories used by Kassem and colleagues (2000). In grouping the samples in this way it was apparent that the percentage of proliferating β-cells in both CHI-D and control samples <3 months of age was higher than in their counterparts at >3 months of age. These data mirror almost exactly the data collected in the most recent and inclusive study of proliferating β-cells so far (Kassem et al. 2000), providing confidence and reproducibility in the results presented herein.

Significance of β-cell proliferation was not as apparent in CHI-D samples when Ki67-positive cells occurring in the β-cell compartment were counted in a given area, rather than as a proportion of the insulin-secreting population. This could be for a variety of reasons; in particularly the distribution of the endocrine population between the CHI-D and control samples. Although diffuse insulin expression was observed in both CHI-D and control samples at all ages (see chapter 3), there was a higher prevalence of islet structures in controls overall than in the CHI-D, especially at the younger ages. Within a given area, islet structures therefore present a compact accumulation of insulin-positive cells, likely representing a bigger population than the scattered extra-islet β-cells within the same area. Therefore differences in Ki67-reactivity between samples within a given area were not as apparent until expressed as a percentage of the total population.

Time limitations in this instance prevented the analysis of proliferation in all compartments to be assessed using this method, as it is likely that this represents a more accurate interpretation of the rate of proliferation occurring in each pancreatic sample, correcting for differences in tissue size and composition in each individual case. Recent work carried out within the group has been focused on analyzing whole pancreatic sections of CHI-D and control samples stained by IHC for Ki67, for overall rates of proliferation. Preliminary data have estimated approximately 5 % of the total pancreatic mass to be proliferating in CHI-D samples, approximately 6-fold greater than proliferation in age-matched control samples (Han et al., unpublished).

Since both types of diabetes mellitus result from an insufficient functional mass of β-cells (Butler et al. 2003), the proliferation observed in CHI-D β-cells may offer important insights
into the mechanisms involved in regulating β-cell mass and in identifying new targets for the treatment of diabetes.

Although data is limited in humans (Butler et al. 2003; Meier et al. 2008), adult mice can generate new β-cells either as a mechanism of normal growth or in response to diabetogenic injury, predominantly from the replication of pre-existing β-cells (Dor et al. 2004; Meier et al. 2008; Nir et al. 2007; Teta, et al. 2007). As well as diabetic insults, food intakes and glucose infusion can also induce β-cell replication in mice (Alonso, et al. 2007; Bonner-Weir, et al. 1989). In fact, whilst the apoptotic cells and disrupted islet architecture can play a minor role in inducing β-cell replication, the main factor appears to be glucose, and specifically, glucose metabolism (Porat et al. 2011).

In a study by Porat and associates, it was shown that the rate of glycolysis, independent of circulating glucose levels, could determine β-cell replication and this was predominantly via its effects on membrane depolarisation (Porat et al. 2011). This represents an unusual system in which the factor causing insulin secretion can also induce β-cell replication. But replication rates are low in rodent β-cells and even more rare in human β-cells. Porat et al. also considered this idea and speculated that the effects of glycolysis are temporally controlled and prolonged or persistent activation of the glucose-metabolizing pathway would stimulate replication, whilst short activation triggered by the onset of meal digestion results in insulin secretion (Porat et al. 2011).

This offers an appealing mechanism for the enhanced β-cell proliferation seen in CHI-D samples. KATP channel mutations cause prolonged membrane depolarisation and therefore β-cell replication. The involvement of the glucose metabolism pathway is also evidenced by the presence of large and hyperplastic islets in GCK-activating mutations found in CHI-GCK patients (Cuesta-Muñoz et al. 2004; Kassem et al. 2010). Consistent with this hypothesis, when GCK is knocked-down in β-cells, the rate of β-cell proliferation also decreases. This could be partially recovered with the administration of sulphonylureas, a diabetic drug targeting the KATP channel to depolarize the β-cell membrane (Dadon et al. 2012; Porat et al. 2011). This is intriguing, since the partial recovery of β-cell replication suggests that membrane depolarisation alone is not sufficient to promote maximal proliferation. This fits with the profiles observed in CHI-D samples, where the level of proliferation observed in CHI-D β-cells, resulting from KATP channel mutations and prolonged membrane depolarisation, are not as severe as those seen in CHI-GCK (Cuesta-Muñoz et al. 2004; Kassem et al. 2010; Kassem et al. 2000).
By counting the total number of Ki67-positive cells in each sample and then counting how many of these occurred in each of the four pancreatic compartments explored, subtraction of these “identified” cell-types from the total Ki67 counts yielded a population of “unidentified” Ki67-positive cells. This discrepancy in Ki67-counts was more pronounced in CHI-D than control samples, with approximately 17 Ki67-positive cells not matching to either acinar-, duct-, β-, or α-cell types per field of view in CHI-D, and only approximately 1-2 cells unaccounted for in control samples within the same area.

The much higher number of Ki67-positive cells occurring in CHI-D samples in the first instance is likely a contributing factor to these differences as the chances of these then occurring in another cell type are also increased. Mesenchymal proliferation was superficially ruled out by histological examination and the majority of proliferating cells were confirmed to be in acinar clusters outside of islets and in ductal structures.

Failed identification of these “unidentified” Ki67-positive cells could be for a variety of reasons, including technical variabilities and quality of staining following the IF procedure. IHC provides a well-established and reliable method for the identification of proteins in fixed tissue samples however, variability in the intensity of staining can vary throughout a tissue sample. For the purpose of this study only strongly staining cells were included in counting analysis, weaker staining cells may therefore have avoided detection and therefore been excluded in the final counting data for that compartment.

As it was not possible to stain all the pancreatic compartments and Ki67 in a single section of pancreas, 4 consecutive sections were used for each. The Ki67-positive cells seen in the first section are likely to be a different population of cells detected in the 3rd or 4th section, 10-15 μm away. This may contribute to variances in the number of Ki67-positive cells counted in each compartment since the Ki67+/GATA4+ and Ki67+/SOX9+ cells cannot be counted when Ki67+/Insulin+ cells are being counted (etc). However, these differences are not expected to contribute substantially to the increased incidence of unidentified Ki67 in each case, and do not justify the significant differences observed between CHI-D and control samples.

A higher number of Ki67-positive cells seemed to be apparent in endocrine clusters (based on histological detection and insulin/glucagon-staining) than were accounted for by insulin/glucagon-staining alone. Although their relative infrequency in CHI-D samples meant counting was maybe not as reliable than β-/α-cell types, we did not observe proliferation in SS- or PP-producing cells in CHI-D pancreas. This increased incidence of islet proliferation that is not accounted for by α, β, δ, or PP cells shares similarities with an investigation by Bouwens et al, studying proliferation in the human fetal pancreas. They showed a statistically higher proportion of Ki67-positive cells staining for the endocrine
marker synaptophysin, than were accounted for by α- or β-cell labeling indices (Bouwens et al. 1997). They too did not detect proliferation in either the PP- or δ-cell lineages, concluding that a subpopulation of synaptophysin-positive, hormone-negative cells likely exists, displaying a higher proliferative activity than surrounding differentiated hormone-producing cells (Bouwens et al. 1997). Since CHI-D results from a persistent secretion of insulin, the "unidentified" islet+/Ki67+ cells may represent a degranulated endocrine cell, or a primitive cell-type that has not yet started producing hormones.

As with all studies seeking to investigate the cell turnover in human tissues, we are confronted with various limitations. The fixed-nature of the tissue makes characterising specific cell types very difficult and, by definition, the results presented are cross-sectional of proliferation in each sample at a given time.

In-keeping with previous reports (Kassem et al. 2000; Lovisolo et al. 2010), we have shown a significant increase in the frequency of proliferating β-cells in CHI-D samples, compared to their age-matched controls. Inter-sample variation has been reported by others (Lovisolo et al. 2010; Meier et al. 2008; Sempoux et al. 1998), and was also apparent in our samples. A greater number of samples in our study would therefore help balance these variations. However, patient variance in this instance did not appear to interfere with our results too much since the data were representative of those published by previous groups (Bouwens et al. 1997; Kassem et al. 2000; Lovisolo et al. 2010; Meier et al. 2008). Similarly, for the purpose of fair analysis, it is important to note that the control samples and CHI-D samples were obtained from two different sources, and can therefore be exposed to different fixation procedures which might affect the quality and accuracy of the staining obtained by IHC. Indeed, both CHI-D and control samples required longer boil times for antigen retrieval steps in IHC protocol as compared to processing the fetal samples. However, the effect of tissue-fixing on the expression of Ki67 has been extensively studied (Hendricks and Wilkinson 1994) and shown that the quality of the Ki67 staining is not affected as long as samples are fixed within 24 hours of death. All our samples met these criteria.

### 4.4.2. Profiling cell cycle components in CHI-D and age-matched controls

There has been extensive research over the last decade to demonstrate that rodent β-cells can and do replicate (Dor et al. 2004; Nir et al. 2007) and that the G1/S pathway is critical in regulating this ability (Cozar-Castellano et al. 2006a). However, regulation of the human β-cell is less comprehensively understood. Despite this, there has been a recent surge of data emerge on G1/S components of the human β-cell, following extensive
efforts to identify the intrinsic brakes that prevent β-cell replication in humans, at least to the same extent as seen in mice (Fiaschi-Taesch et al. 2013a).

Whilst it has been shown that adult human β-cells, unlike rodent β-cells, do not respond to growth signals such as growth factors or nutrients, they can be robustly stimulated to proliferate following the overexpression of various G1/S cell cycle components either independently or in combination (Fiaschi-Taesch et al. 2009; Fiaschi-Taesch et al. 2010). This ability to induce proliferation in the human β-cell by rates of up to 10-15% provides evidence that the cell cycle machinery is not compromised in human β-cells, however the right signal is needed to activate them and drive cell cycle progression.

Surprisingly, the majority of cell cycle components are found predominantly in the cytoplasm of β-cells, despite their largely nuclear function driving or inhibiting transcriptional activity.

When CDK6 is overexpressed in human islet preparations, the protein translocates to the nucleus where it can drive proliferation (Fiaschi-Taesch et al. 2013b). However, for the most part, CDK6 is cytoplasmic and colocalises specifically with insulin (Fiaschi-Taesch et al. 2013a), a finding we too observed across all neonatal and adult samples studied (Appendix 4-1).

This specific localisation for CDK6, which was apparently absent from CHI-D islets (and CHI-DR islets), suggests an additional role for this protein in the β-cell that is disrupted or non-functional in the pathology of CHI-D. In a study by Annicotte and colleagues it was demonstrated that CDK4 and pRb, along with E2F1, were involved in a metabolic pathway to regulate the secretion of insulin via mechanisms controlling membrane depolarisation, and in particularly the $K_{ATP}$ channel (Annicotte et al. 2009). Since mice express CDK4 and lack CDK6, but CDK6 is the predominant functioning cyclin kinase in human islets (Fiaschi-Taesch et al. 2009), we hypothesise that the expression of CDK6 in the cytoplasm of control β-cells is evidence of a function in the GSIS pathway. Further to this, since the GSIS pathway is dysfunctional in CHI-D β-cells, this offers an explanation for the absence of this protein in the cytoplasm of CHI-D islets.

During cell cycle progression, complexes of CDKs and Cyclin molecules phosphorylate pRb, releasing the brakes from E2F activity and permitting the expression of genes necessary for cell expansion.

The work by Stewart and colleagues identified pRb in the nuclear compartment of pancreatic β-cells, however the protein was never detected in any of our control samples (6 weeks-adult) (Fiaschi-Taesch et al. 2013a). This is likely due to differences in protein structure to which the antibodies were developed since our group and Stewart’s group
used different preparations. Although both antibodies were designed to detect the unphosphorylated (hypophosphorylated) form of pRb, the antibody used in this study was designed around the serine 780 site on the protein. However, this would not rule out phosphorylation at other sites on the pRb protein. CDK6 phosphorylates pRb at threonine 821, whilst CDK4 phosphorylates pRb at threonine 826 (Grossel and Hinds 2006). Since neither of these sites are detected by our antibody we were unable to verify the activity of CDK6 on cell cycle regulation through the E2F/E2F1/pRb pathway.

Interestingly, pRb (unphosphorylated Ser780), although apparently nuclear in the acinar compartment of CHI-D samples, was observed predominantly in the cytoplasm of CK19-positive ductal cells in both fetal and CHI-D pancreas. Positive pRb staining with our antibody could identify unphosphorylated pRb at Serine 780 residue but does not rule out phosphorylation at other residues on the pRb protein. When dualled with Ki67, pRb could be observed in the cytoplasm of some, but not all, Ki67-positive proliferating cells. This supports a cytoplasmic localisation for pRb to drive cell cycle progression, which seems to contradict the acinar cells representing the most proliferative compartment. However, since Ki67 detects cells at any stage of the cell cycle (G1 to S-phase) (Gerdes et al., 1983), the nuclear and cytoplasmic localisation for pRb may reflect different stages. Since we can’t confirm the exact phosphorylation state of pRb at each stage and in each cell type, the data in this instance are difficult to interpret.

Dual expression experiments with CDK6 and pRb may be able to disentangle some of this information regarding pRb localisation, however, since the antibodies were produced in the same species, this was not possible in our experiments.

Given the complexity of pathways and interactions apparent within the G1/S network, further work would be necessary to clarify the mechanisms of cell cycle regulation operating in CHI-D samples. In particular, a larger catalogue of cell components should be explored, including the investigation of expression and localisation patterns for the cyclin and other CDK molecules, as well as the E2F transcription factors. In addition, a range of antibodies designed against different phosphorylation residues are commercially available and would help shed light on the pathways of pRb phosphorylation in the human β-cell in both normal and disease states. Since it has been shown that nuclear CDK6 expression in the proliferation-induced β-cell is more widespread than the number of cells that actually commit to replicate, there must be additional features within the β-cells that control the progression into G1/S phase (Fiaschi-Taesch et al. 2013b).

Completing the profile of cell cycle components would provide important insights and possible associations to generate potential pathways and mechanisms that permit cell cycle progression in a physiological setting.
The alterations in cell cycle were explored in a more wide-spread analysis using microarray data from CHI and neonatal tissue samples (Michelsen et al. 2011). Although CDK6 and pRb were not identified as central genes in the network analysis, another gene linked to the cell cycle was; CDKN1B, encoding the P27 protein. We confirmed an upregulation of this gene in CHI-D samples and identified a predominantly nuclear expression of the protein in CHI-D islets, contradictory to the G1/S atlas showing P27 in the cytoplasm of human adult β-cells (Fiaschi-Taesch et al. 2013a).

P27 is an intriguing protein since it seems to have conflicting roles in the regulation of cell cycle progression. Historically, P27 has been classified as a cell cycle inhibitor, forming complexes with cyclin and CDKs to prevent their activity (Pestell et al. 1999). Paradoxically, P27 can also serve as a chaperone to shuttle these complexes into the nucleus where they can drive cell cycle progression (Cheng et al. 1999; Sherr and Roberts 1999). A balance in the amount of P27 present in the β-cell likely determines whether that cell is proliferating or quiescent.

P27 was predominantly observed in the nuclei of CHI-D islet cells, whilst we, and others, showed a cytoplasmic localisation within islets of neonatal and mature controls (Fiaschi-Taesch et al. 2013a). When proliferation was induced in human β-cells by overexpression of CDK6 and Cyclin D3, P27 shuttles from the cytoplasm to the nucleus of β-cells (Fiaschi-Taesch et al. 2013b). However we, and others, have shown that P27 never colocalises with Ki67. These data are compatible with a dual role for P27, chaperoning CDK6 cargo into the nucleus whilst also inhibiting cell cycle progression. It is likely that P27 provides a titration mechanism for controlling proliferation according to its levels of expression, although how it executes its pro- or anti-proliferation functions are not clear. Interestingly, following CDK6 and Cyclin D3 overexpression in β-cells, P27 was only observed in approximately 11 % of β-cell nuclei. In CHI-D samples P27 is found in the majority of β-cell nuclei. This might be important since, despite the changes in cell cycle molecules we have shown in this study, CHI-D β-cells still rarely proliferate. An over-representation of P27 in CHI-D islet cells may be the result of a convergence of mitogenic factors including local hyperinsulinaemia, hypoglycaemia and persistent membrane depolarisation as described earlier, or the result of a more chronic exposure to such signals. Adenovirus delivery of CDK6 and Cyclin D3 in the β-cell induction model developed by Fiaschi-Taesch and colleagues only delivered a short-sharp stimulus which was monitored in β-cells for 72 hours, during which time many of the cell cycle molecules showed transient nuclear expression before returning to their cytoplasmic locations (Fiaschi-Taesch et al. 2013b). Since nuclear P27-positive cells do not appear to
proliferate, this molecule may represent a defense mechanism of the β-cell to maintain quiescence despite these pro-proliferative influences. In fact, accumulation of P27 in β-cells has been associated with their quiescence, and disabling P27 activity resulted in the ability of the β-cell to divide (Zhong, et al. 2007). More specifically, in a study by Uchida et al., P27 was seen to accumulate in the nuclei of mouse β-cells that lacked either the insulin receptor substrate 2 (Irs2) or leptin receptor (Lepr), components of the insulin-signalling pathway. In fact, nuclear exclusion of P27 was specifically identified in wild-type mice in which the GSIS was activated (Uchida et al. 2005). These experiments, although studied in mice and therefore may not directly extrapolate to the human, demonstrate a regulatory role for P27 in which a nuclear presence inhibits proliferation. GSIS pathway dysfunction in CHI-D patients may therefore account for the nuclear accumulation of P27 in these samples and contribute to the clinical course of CHI-D in which β-cells mass is lost and not replenished over time. Interestingly in CHI-DR samples, P27 was no longer pronounced in the nuclear compartment but was instead identified in the cytoplasm of the islets. This is consistent with the absence of a persistent driver for proliferation in these samples since CDK6 and pRb expression were also reduced and Ki67-reactivity low. This would suggest that whilst GSIS activation may have some influence on P27 activity, it is likely that other pathways also impose on this system.

A microarray data set has been published by Bensellam et al, investigating the effects of differing concentrations of glucose on various gene expressions in the rat (Bensellam, et al. 2009). Searching the raw data published we were able to identify gene expression changes for P27 in changing concentrations of glucose (Figure 4-21). Although not published in the original manuscript, their data suggest a regulation of the P27 gene by levels of glucose. These data show a decreasing expression of CDKN1B with increasing concentrations of glucose. This mechanism for CDKN1B regulation would fit with an increase in P27 expression in conditions of hypoglycaemia, a well-recognized symptom of CHI-D.
**Figure 4-21. Regulation of Cdkn1b expression by glucose**
Box-whisker plot showing average expression of Cdkn1b in increasing concentrations of glucose. The box demonstrates the first and third quartiles, with the band inside showing the median. Error bars show minimum and maximum values. Adapted from Bensellam et al. 2009.

A proposed mechanism for cell cycle dysregulation, where expression of CDKN1B is central to the regulation of proliferation is outlined below (Figure 4-22). Although the mechanism is likely more complex than is represented since changes in Ca$^{2+}$ signaling and hypoglycaemia levels are also likely to contribute to a stimulatory pathway driving cell cycle proliferation in the CHI-D β-cell.

**Figure 4-22. Mechanisms for cell cycle regulation in CHI-D**
Schematic diagram showing possible mechanisms causing cell cycle alterations in CHI-D samples caused by genetic mutations in the $K_{ATP}$ channel genes and resulting in an upregulation of P27.
Proliferation in the CHI-D β-cell was only modestly increased, and although some changes in cell cycle expression were seen in CHI-D islets (occasional CDK6 and pRb nuclear expression, P27 nuclear expression and reduced cytoplasmic CDK6) the most apparent differences were in the acinar and ductal compartments, consistent with these representing the highest frequency of proliferating cells.

The mechanisms for β-cell proliferation are therefore likely far more complex than simply an on/off ability. Whilst the atlas described by Fiaschi-Taesch and colleagues provides a comprehensive catalogue of cell cycle components, their prevalence and cellular location, it cannot provide details on interactions and regulatory networks. The effect of $\text{K}_{\text{ATP}}$ channel dysfunction on the expression and distribution of cell cycle components has not been reported previously. Whilst preliminary in our data, the results presented in this report describe alterations in the regulation of some of these G1/S components and as such warrants further investigation.

4.4.3. Cell cycle changes in CHI-D patients undergoing a second pancreatectomy operation

Occasionally the symptoms of CHI-D are not alleviated by near-total pancreatectomy. This was apparent in two of our CHI-D samples, and has also been described by other groups (Aynsley-Green 1981; Kramer, et al. 1982; Moazam et al. 1982; Rahier et al. 1984). However, the histology of the second pancreatic resection has not been described previously. We therefore studied these samples, initially to investigate proliferation rates and then further to investigate cell cycle component expression.

Surprisingly, in each of the re-operation biopsies from CHI-D samples, proliferation had significantly subsided and was maintained at a level consistent with those seen in the age-matched controls. Proliferation in insulin-secreting β-cells was not observed. In-line with these observations, the expression of cell-cycle markers also appeared to return to a near-normal distribution, where CDK6 and pRb were not observed throughout the exocrine tissue and P27 was predominantly in the cytoplasm of the islets. Interestingly, cytoplasmic CDK6 was still absent from the islet structures in the CHI-DR samples. This provides further evidence for a role of CDK6 in GSIS as, even though proliferation was no longer elevated in these samples, and CDK6 was not over-expressed in the remaining pancreatic tissue, CDK6 was consistently absent from the cytoplasm in both CHI-D and CHI-DR samples.

Although early data in CHI-D samples suggests a modest β-cell hyperplasia occurring in these samples, the fact that some patients required a second operation for the treatment
of continued hypoglycaemia, even after an ~80% pancreatectomy, proves that symptoms of CHI-D are not caused by an over-representation of β-cells, but is more likely the result of a hyperactivity of their function. Further to this, changes in proliferation rates and cell cycle regulation between the two operations suggests a more complicated involvement of mechanisms causing increased proliferation and cell cycle alteration in CHI-D samples. Since considerable time had passed between the first and second operation in the CHI-DR samples (22-27 months), and the patients continued with the symptoms of hypoglycaemia, it is unlikely that the mechanisms assumed to be driving proliferation in the first instance have been reduced (membrane depolarisation, hyperinsulinism, hypoglycaemia). This puts further weight on the idea that CHI-D results from a developmental dysfunction, or at least that processes during development offer significant contribution to the aetiology of the disease in early infancy. However, without knowing the clinical interventions received by each CHI-DR patient between operations, including diet management and treatment regimes, it is not possible to comment on the effect of these on pancreatic histology following the first operation.

4.4.4. Increased apoptosis in CHI-D compared to age-matched controls

Beta-cell mass is considered normal in CHI-D patients (Rahier et al. 1984), because increased proliferation appears to be offset by an increased rate of apoptosis (Kassem et al. 2000).

In-keeping with other studies, we could not detect apoptotic cells in fetal samples (Kassem et al. 2000), however TUNEL-positive cells were detected in control samples and may play a role in the pancreatic remodeling observed during the postnatal period. There was a higher incidence of apoptotic cells in CHI-D samples and a higher percentage of these in the insulin-producing compartment compared to age-matched controls.

The mechanisms causing apoptosis in CHI-D samples are not known, however persistent membrane depolarisation and elevated intracellular Ca\textsuperscript{2+} concentrations as a result of mutations in the \(K_{\text{ATP}}\) channel subunits may precipitate activation of the apoptotic cascade. Whilst it has been shown that both increased glucose metabolism and prolonged β-cell membrane depolarisation can enhance β-cell proliferation, the same mechanisms likely also have a role in regulating β-cell death (Dadon et al. 2012). However, whereas membrane depolarisation appears to cause submaximal induction of β-cell replication as compared to GCK-activation, patients with CHI-GCK do not present with the same clinical course of disease as those with \(K_{\text{ATP}}\) channel mutations (Cuesta-Muñoz et al. 2004;
Kassem et al. 2010). This would position severe-membrane depolarisation as the predominant mechanism inducing β-cell apoptosis.

The clinical course of CHI-D in patients that are not surgically treated for the condition suggests an imbalance in the apoptosis/proliferation rates later in life, with a preference for apoptosis and a reduced capacity for β-cell replacement (Leibowitz et al. 1995).

The TUNEL protocol was confirmed in our samples using both positive and negative control sections in each experiment to rule out false-positive results or technical errors in the procedure. Only cells showing clear positive nuclear staining for TUNEL were included in the counting experiments, whilst clear cytoplasmic staining for insulin identified the β-cell population. Cells around the periphery of the tissue were not included in the counting data since they show signs of trauma and therefore a high relative abundance of TUNEL-positive cells as a result of the tissue fixing and sectioning procedures.

Rates of apoptosis in the β-cells of control samples were similar between our data and those published by Kassem and colleagues. However, the values calculated for CHI-D β-cell apoptosis were higher in our samples than those previously published by the same group (Kassem et al. 2000). The differences in the numbers presented here are likely due to patient variation since the rates of proliferation between the two CHI-D samples showed differing proportions. Only two different CHI-D cases were investigated in this experiment, and therefore the inter-patient differences are not reasonably averaged between the two. Kassem and colleagues used considerably more samples in their analysis, and the rate of apoptosis described in their paper represents an average of these samples, therefore accounting for the inter-patient differences (Kassem et al. 2000). Also, the time-course for the process of apoptosis to complete in β-cells is not known. Since the use of archival, fixed-tissue for the analysis provides only a ‘snap-shot’ of the processes occurring in the samples at a single time, the differences in frequency of TUNEL-positive cells may represent differences in age and extended exposure to apoptotic signals, with an inability to differentiate between newly apoptotic cells and those that have been committed to the programme for longer.
4.5. Summary

In summary, these data extend on the observations presented in the literature for cell proliferation, confirming significant but modest increases in β-cell proliferation in CHI-D samples compared to their age-matched controls in-line with previous authors (Kassem et al. 2000; Lovisolo et al. 2010). However, we also show that the acinar and ductal compartments are the most proliferative cell type in CHI-D. These data follow on from those findings presented in the previous chapter, identifying alterations in CHI-D that lie outside of the β-cell lineage. The preliminary studies into cell cycle regulation presented here offer a previously unexplored insight into the mechanisms underlying the increased proliferation observed in each compartment and the dynamics of the pancreatic tissue in CHI-D samples, to which further work focused on providing a more comprehensive analysis of the components involved and teasing apart the intrinsic mechanisms would be warranted. The recent detailed study of G1/S cell cycle components in the pancreatic β-cell provides a fantastic reference for designing future experiments and exploring differences in cases of CHI-D (Fiaschi-Taesch et al. 2013a).

Since human tissue is notoriously limited in terms of the investigations that can be performed and the data that can be generated, CHI-D samples provide a unique system in which the effects of changes in metabolic systems on cell cycle components can be explored. The work presented in this chapter have provided further evidence that the mechanisms regulating β-cell proliferation are not simply the result of pRb phosphorylation from an increased expression of CDK6 and Cyclin D3 (Fiaschi-Taesch et al. 2010). What’s more, it is likely that epigenetic regulation of cell cycle components is just as important as transcriptional control in activating cell cycle progression.

We have made progress in trying to determine pathways that may go some way to providing a mechanism by which cell proliferation can be enhanced in CHI-D through the mediatory activity of P27.

The β-cell contains every cell cycle inhibitor that has been identified, identifying a cell-type that really doesn’t want to proliferate and has almost every possible system in place to prevent it. Understanding why CHI-D β-cells have an enhanced ability to proliferate and how they execute this function could offer unequivocal insights into human β-cell dynamics that could be exploited for the potential treatment of diabetes mellitus as well as providing insights into the mechanisms of disease.

Given the differences exhibited between rodent and human β-cells throughout their respective life cycles, with particular regard to their capacity to proliferate, experiments in human β-cells are invaluable to further our understanding of our species.
Chapter 5- Characterising nucleomegaly in CHI-D

5.1. Introduction

Since it was first described almost 80 years ago, CHI has been ascribed to various morphological abnormalities describing a heterogeneous endocrine cell disorder which is unlikely to be caused by a single pathological entity. In fact, despite being attributed to nesidioblastosis for many decades, a term that describes the “budding” of islet cells from the ductal epithelium, it has since been discovered that the same feature is observed in normoglycaemic neonatal controls and therefore not necessarily pathogenic of CHI. Indeed, upon comprehensive analysis by Rahier and co-workers, CHI could not be credited to nesidioblastosis, increased β-cell mass or decreased SS-producing cell mass. As a result it was hypothesized that CHI might result from an abnormality in β-cell function (Rahier et al. 1984).

Over the years there have been various reports investigating the morphology of CHI samples, and the presence of enlarged nuclei is one such observation that has been repeatedly reported in these samples since as early as 1974 (Alexandrescu et al. 2010; Ariel et al. 1988; Goossens et al. 1989; Grampa, et al. 1974; Jack et al. 2000; Jaffe et al. 1980; Lovisolo et al. 2010; Rahier et al. 1984; Smith, et al. 2001; Suchi, et al. 2004).

This chapter offers a more comprehensive insight into this morphological feature of CHI-D, exploring possible causes or nucleomegalic cells and their role in the pathology of the disease. The aim of the work was to characterise the cell types with enlarged nuclei in CHI-D samples and determine their cell cycle status.

5.1.1. Role of nucleomegalic cells

Enlarged nuclei are thought to represent a hyperfunctional cell (Bowen and Swartz 1976; Hellman and Hellerstrom 1959; Kompmann, et al. 1966; Kracht 1958), and have also been shown to correlate with DNA content and an increase in secretory function (Wagner and Richart 1968).

Although it is not clear exactly what function these enlarged nuclei have in CHI samples, nor whether they are a primary abnormality of the CHI β-cells or secondary to the disease, they are particularly prevalent in CHI samples and rarely observed in the neonatal control pancreas (de Lonlay-Debeney et al. 1999; Lovisolo et al. 2010; Rahier et al. 1984; Sempoux, et al. 1998b; Suchi et al. 2004).
The presence of enlarged nuclei within pancreatic islets could therefore serve as a simple morphologic criterion for diagnosing CHI, but more specifically, may also be able to distinguish between the subtypes.

Since the general consensus that two classifications of CHI do exist; CHI-D characterized by a diffuse $\beta$-cell abnormality throughout the pancreas and CHI-F containing a focal lesion (or lesions) of adenomatous hyperplasia, there have been considerable efforts to distinguish between the two subtypes prior to surgery. This is important given the different extents of pancreatectomy necessary to treat each type. In CHI-D a near-total or total pancreatectomy is usually required to relieve symptoms of hypoglycaemia, however, a partial resection limited to the affected lesion(s) in CHI-F is adequate to restore normoglycaemia in these patients (de Lonlay-Debeney et al. 1999; Rahier et al. 2000).

Unlike insulinomas or other adult insular adenomas, the lesions in CHI-F are caused by the confluence of hyperplastic but otherwise apparently normal islets, separated by exocrine acinar cells, therefore maintaining a lobular architecture throughout the pancreas and rendering the lesion(s) difficult to identify macroscopically (Sempoux et al. 2003; Sempoux et al. 2001). As such, other distinguishing features are necessary to guide clinicians during surgery. Whilst Ki67-immunoreactivity has been shown to be elevated in CHI-F lesions compared to $\beta$-cells outside of the lesions or in CHI-D or control pancreatic samples (Kassem et al. 2000; Sempoux et al. 1998), the protocol for such investigations is not appropriate for pre-surgical analysis. A quicker process for differentiation is therefore required.

In a differential analysis by Sempoux and colleagues, nucleomegaly was confirmed as a specific marker to distinguish CHI-D from CHI-F, since hypertrophied nuclei were statistically larger and more frequent in samples of CHI-D compared to lesions of CHI-F, whilst nucleomegalic cells were not observed outside of the lesion(s) or in controls (Sempoux et al. 1998) and this has been confirmed by other groups since (Jack et al. 2000; Smith et al. 2001; Suchi et al. 2004).

Although this technique may not be sufficient to accurately identify the boundary of the lesion(s) within CHI-F, the frequency of large nuclei within the lesion compared to their absence outside provides sufficient criteria for diagnosing 100% of cases correctly (Sempoux et al. 1998). Likewise, the prevalence of hypertrophied nuclei in CHI-D islets throughout the pancreas permits approximately 91-94% of CHI-D cases to be accurately diagnosed from frozen intraoperative sections (Sempoux et al. 1998; Suchi et al. 2004).
5.1.2. Nucleomegalic cell phenotypes

Whilst techniques to differentiate the two subtypes of CHI have progressed significantly with the introduction of $^{18}$F-DOPA PET scans, islet cell nucleomegaly remains a distinct feature of CHI patients and particularly those with CHI-D. However characterisation of these cells has rarely been described previously.

Enlarged nuclei are predominantly observed within the islets of CHI samples, which either by assumption or IHC staining, have been mostly believed to reside within the $\beta$-cell lineage. Indeed, $\alpha$-cell nuclei have never been observed to be enlarged, nor have cells of the exocrine compartment, whilst $\delta$-cells are rarely observed with large nuclei (Rahier et al. 1984).

Likewise, although nuclear size has been associated with DNA content and therefore regulation of the cell cycle (Ariel et al. 1988; Ehrie and Swartz 1974; Zerbini, et al. 1992) markers of cell cycle regulation in these nucleomegalic cells has not been previously explored.

In this chapter we therefore sought to better define the population of cells displaying enlarged nuclei, and to determine their proliferative state based on expression of cell cycle markers and Ki67.
5.2. Aims

The work in this chapter was carried out to investigate the occurrence and phenotype of cells with enlarged nuclei in CHI-D and age-matched control samples.

5.2.1. To determine if nucleomegaly is a consistent feature in CHI-D patients; A comparison with age-matched controls

H&E-stained sections of CHI-D and neonatal control pancreas were digitized and analysed using Pannoramic software (3DHISTECH) to identify and measure the diameter of large nuclei. Total numbers of nucleomegalic cells were counted to investigate rates of prevalence between the two sample groups.

5.2.2. To characterise the cell-types displaying nucleomegalic properties

IHC and IF techniques were employed to identify hormone expression within the population of cells displaying enlarged nuclei as well as the investigation of endocrine and exocrine cell markers.

5.2.3. To determine whether enlarged nuclei are associated with proliferation and stages of the cell cycle

Ki67 IHC and IF was used to identify proliferating cells in CHI-D samples with previously employed analysis techniques used to measure their cell diameters. Markers of the cell cycle were also investigated for their presence in nucleomegalic cells.
5.3. Results

5.3.1. Nucleomegalic cells in CHI-D; A comparison with age-matched controls

The observation of large cell nuclei is a feature that has been well documented in cases of CHI and has even been used as diagnostic criteria to distinguish between CHI-D and CHI-F lesions by several groups (Jack et al. 2000; Lovisolo et al. 2010; Rahier et al. 1998). Large cell nuclei were readily seen in CHI-D samples, predominantly within the islet structures, but were rarely detected in neonatal control samples (Figure 5-1).

![Image of CHI (2 mo) and Control (2 mo)](image)

**Figure 5-1. Histology of CHI-D and control pancreas shows enlarged cell nuclei**

H&E staining of 5 μm sections of CHI-D and age-matched control pancreas show the presence of enlarged nuclei in CHI-D islets that are not apparent in control. Scale bar represents 50 μm.

Large islet cell nuclei were detected in approximately 90% of islets investigated across five separate cases of CHI-D (Figure 5-2A). However, the frequency of large cell nuclei occurring within each of these islets varied between cases. For example; hypertrophied nuclei were readily detected in CHI-D 1(2 mo) where in almost every islet cell cluster there were on average 4 enlarged nuclei defined as being >50% the size of surrounding islet cell nuclei (Figure 5-2B). Although enlarged nuclei were observed in the majority of islets in the other CHI-D samples, they occurred at a lower rate of incidence with, on average, <2 cells per islet containing nuclei with >50% diameter than that of surrounding islet cell nuclei. The extent of enlargement in the cell nuclei was similar across all samples (average 1.8 fold larger than surrounding islet cells) (Figure 5-2C) therefore it was only the frequency of these cells that appeared to be elevated in CHI-D 1 (2 mo). Of note, sample CHI-D 4 (6 mo) had a relatively smaller overall size of enlarged nuclei compared to the enlarged nuclei in the other CHI-D samples (9.01 μm Vs average 10.70 μm for CHI-D 1-3 & 5). However, since the surrounding islet nuclei were also of a reduced size in CHI-D 4, the fold increase remained consistent across all samples.
Figure 5-2. Enlarged cell nuclei in CHI-D islets

10 islets counted for each CHI-D sample. For each islet the number of large nuclei (defined as being at least 50% larger in diameter than the average diameter of surrounding cell nuclei within the same islet) was recorded along with the fold increase of each enlarged nuclei compared to the average diameter for the surrounding cells in each islet. (A) The proportion of islets observed to contain at least one enlarged nuclei in 5 studied CHI-D cases ranging in age from 2-13 mo. (B) The number of enlarged nuclei found in each islet. (C) Fold increase in the diameter of the nuclei in cells considered to have an enlarged nucleus and compared to the average diameter recorded from the surrounding islet cell nuclei in each islet. Error bars show standard error. (B) P<0.01 using ANOVA test with Tukey post-hoc analysis.

Although assumed a distinctive feature of CHI, enlarged islet cell nuclei were observed in control samples (9 wk-10 mo) (Figure 5-3). Nuclear diameter was measured using Pannoramic Viewer Software (3DHISTECH) following digitisation of H&E-stained samples using the Pannoramic 250 Flash II Digital Slide Scanner (3DHISTECH). As such, measurements made in the software were consistently accurate regardless of the resolution and magnification in which the measurements were taken. Large cell nuclei were measured in the first instance, followed by the measurements of 10 surrounding cell nuclei repeated across 10 different islets in each sample. An example of these annotations for CHI-D and control samples is shown below (Figure 5-4). Nucleomegalic cells in neonatal control samples occurred at a lower frequency than in CHI-D samples,
with approximately 30% of islets containing enlarged nuclei (defined as >50% size of average surrounding islet cell nuclei) (Figure 5-5A). Of the islets containing enlarged cell nuclei in control samples, only 1 nucleus on average could typically be identified as enlarged.

**Figure 5-3. Enlarged cell nuclei observed in CHI-D and control samples**
Digitized 5 μm sections of CHI-D and control pancreas following H&E staining were analysed for the presence of enlarged cell nuclei. Arrows show nucleomegalic cells apparent in CHI-D and to a lesser extent, in age-matched controls. Scale bars represent 20 μm.

**Figure 5-4. Annotation of cell nuclear diameters in CHI-D and control pancreas**
Digitized 5 μm sections of CHI-D and age-matched controls samples were analysed following H&E staining for cell nuclear diameter of enlarged nuclei and 10 surrounding cell nuclei within the same islet for each sample, and across 10 different islets. Each panel shows the annotations provided by the 3DHISTECH software for measuring cell nuclear diameter of nucleomegalic cells and normal surrounding cells.
The average increase in size of the enlarged nuclei was similar between CHI-D and control samples (1.8, 1.6 fold respectively) but due to the frequency, this was statistically different between the two (P<0.01) (Figure 5-5B). Whilst the distribution curve showing the frequency of cells occurring at each fold-increase interval was shifted towards larger diameters in CHI-D samples (Figure 5-5C).

Overall, the average nuclear diameter of all islet cells counted in CHI-D samples was significantly larger than those counted in age-matched controls (6.7 ±0.45 μm; 5.0 ±0.07 μm, p<0.05 respectively) (Figure 5-5D). The frequency histogram showing all islet cell diameters measured in CHI-D and neonatal controls shows a right shift towards larger diameters in CHI-D, with a clear subset of islet cells apparent above 8 μm in diameter in CHI-D samples, which are identified as nucleomegalic (Figure 5-5D). In contrast, no cells were detected above 10 μm in nuclear diameter in any of the control samples.

When the 50 largest nuclei were averaged in CHI-D and control samples in order to avoid influence from the frequency in each sample, nuclear diameter in CHI-D islet cells was 12.2 ± 1.4 μm (mean ± std. dev), whilst the largest nuclei in control samples averaged 6.7 ± 0.8 μm. Overall, approximately 4 % (± 1.9 %) of counted islet-cell nuclei had a diameter more than twice the mean of a ‘normal’ surrounding islet cell in CHI-D, whilst none of the control nuclei were observed at this magnitude.

These data show that whilst there was a higher frequency of large nuclei identified in CHI-D islet clusters, a higher proportion of these cells also had a nuclear diameter >10 μm (Figure 5-5C,D).
5.3.2. Cell types with enlarged nuclei

In the literature enlarged nuclei are often referred to as “hyperfunctional” or “hypertrophic” β-cells, with the giant nuclei thought to reflect an increased cell function (Krafft 1958). These nucleomegalic cells have also been described as being “polyploid” - which is a characteristic that has also been observed in adult pancreas (Ehrie and Swartz 1974) and is often also correlated with an increase in secretory function (Wagner and Richart 1968). It is therefore plausible that the enlarged nuclei seen in CHI samples are indicative of polyploid β-cells that are hypersecreting. These cells have always been assumed to be β-cells but this has rarely been validated. We therefore wanted to look closer at characterising these cell types in more detail.
The same five CHI-D samples (2-13 mo) were examined to identify large cell nuclei by virtue of DAPI staining in dual hormone IF experiments (insulin with either glucagon, SS, ghrelin or PP). Once large nuclei were identified filters on the other colour channels permitted identification of hormone secretion (Figure 5-6).

**Figure 5-6. Hormone expression in large nuclei in CHI-D samples**
Dual immunofluorescence of 5 μm sections of CHI-D pancreas for insulin (green) and glucagon, somatostatin, ghrelin or PP (red) counterstained with DAPI. Arrows show enlarged nuclei. Most nucleomegalic cells contained insulin, whilst some were also apparent with somatostatin immunoreactivity but were never observed in the glucagon-, ghrelin- or PP-secreting cell types. Scale bar represents 10 μm.

The total number of large cell nuclei was counted, and the proportion secreting each hormone type was calculated as a percentage of this total for each CHI-D sample (2-13 mo). Large nuclei were never seen in glucagon, ghrelin or PP-producing cells however a subset of SS-secreting cells were identified with large nuclei (6.5% ± 1.9) (Figures 5-6, 5-7). The majority of large nuclei were insulin-positive (72.4% ± 4.7) (Figures 5-6, 5-7) however there were some cells with hypertrophied nuclei that were detected within islets that were not identified by hormone staining (21% ± 4.6) (Figure 5-7).

**Figure 5-7. Quantification of nucleomegalic cells in the different endocrine compartments**
Approximately 60 large nuclei, on average, were counted for each CHI-D sample (2-13 mo) following dual immunofluorescence to detect hormone expression. The relative proportions for each hormone-expressing cell with an enlarged nucleus was calculated as a percentage of the total enlarged nuclei counted in each sample.
The endocrine, and predominantly β-cell, phenotype of the enlarged nuclei was confirmed with the nuclear expression of β-cell specific transcription factors; NKX6.1, PDX1 and endocrine markers NKX2.2, ISL1 and FOXA2 (black arrowheads Figure 5-8). However, PDX1 was observed to be absent from some of the nucleomegalic cells (white arrowhead Figure 5-8).

**Figure 5-8. Characterising enlarged nuclei with endocrine phenotype**

Immunohistochemistry on 5 μm sections of CHI-D shows positive immunoreactivity for the β-cell markers PDX1 and NKX6.1 as well as the endocrine cell marker NKX2.2, ISL1 and FOXA2 (brown) in enlarged cell nuclei (black arrows) counterstained with Toluidine blue. The white arrow in PDX1 experiment shows a large cell nucleus absent of PDX1 immunoreactivity. This observation is consistent with previous results that indicate not all enlarged nuclei are β-cells. Scale bar represents 10 μm.

Enlarged nuclei were not observed in the exocrine compartment, represented by the absence of either GATA4 or SOX9 immunoreactivity (Figure 5-9).

**Figure 5-9. Localisation of nucleomegalic cells in CHI-D samples**

Immunohistochemistry of 5 μm sections of CHI-D pancreas for exocrine markers GATA4 and SOX9 (brown) counter-stained with Toluidine blue. Nucleomegalic cells were identified in CHI-D samples but did not contain positive staining for either acinar or ductal markers, evidence of their predominantly endocrine nature. Scale bar represents 10 μm.

5.3.3. **Proliferation and cell cycle activity in nucleomegalic cells**

It is likely that the size of a cell’s nucleus is important for the cell function but the mechanisms underlying the regulation of cell nuclear size remains unconfirmed. It is therefore important to continue trying to characterise these cells in CHI-D samples with giant nuclei in order to discover whether any of these systems are altered in pathological conditions.
The expression of the proliferation-associated Ki67 protein was investigated in the nucleomegalic cell populations. Ki67 can detect all active phases of the cell cycle including G1, S-phase, G2 and mitosis (Gerdes, et al. 1983). Observations showed that some Ki67-positive cells were apparent with large nuclei, but this was not consistent in all nucleomegalic cells (Figure 5-10). The diameter of more than 300 cell nuclei for each individual case of CHI-D (average 566 cells, n=5) were measured and compared to >300 Ki67-negative cells (Figure 5-11). Ki67-positive cells were significantly larger on average than Ki67-negative cells (p<0.01) (Figure 5-11A) however there was a significant difference observed for the average size of Ki67-positive cells between CHI-D samples (Figure 5-11B ANOVA p<0.01) with CHI-4 having the smallest diameters recorded overall (p<0.01) and CHI-3 the largest.

Figure 5-10. Nucleomegalism in proliferating cells
Immunofluorescence of Ki67 (red) counterstained with DAPI and brightfield images showing Ki67 immunoreactivity (brown) counterstained with Toluidine blue in 5 µm sections of CHI-D pancreas. Arrows point to nucleomegalic cells. Some Ki67-positive cells contain enlarged nuclei but many are also apparent that are not actively proliferating. Scale bar represents 10 µm (top row) and 20 µm (bottom row).

When plotted as a frequency curve, the values for cell nuclear diameters amongst the CHI-D samples were distributed in an approximately Gaussian pattern but with the Ki67-positive population demonstrating a right shift towards the larger diameters compared to the Ki67-negative cell nuclei (Figure 5-12A). When these data were merged a greater spread in the frequency curve for cell nuclear size in Ki67-positive cells was apparent as compared to Ki67-negative cells although the trend remained the same and there was a tendency towards the larger diameters in the Ki67-positive cell population (Figure 5-12B). Whilst the nuclear diameter of Ki67-negative cells ranged from 2.73-7.8 µm, the Ki67-positive population showed a variation between 2.31 – 12.12 µm with a shoulder apparent on the Ki67-positive curve representing an additional population of cells with cell nuclear diameters typically between 9-10 µm.
Figure 5-11. Quantification of proliferating cell nuclei diameters
Nuclear diameters of Ki67-positive and Ki67-negative cells were measured using 3DHISTECH software following digitalisation of 5 μm sections of CHI-D samples (2-13 mo) previously stained for Ki67 immunoreactivity. (A) Ki67-positive nuclei tended to be larger than Ki67-negative cells as counted in samples of CHI-D tissue (2-13 mo). Approximately 524 (±279) Ki67-positive or Ki67-negative cells were counted per CHI-D sample. Statistical significance calculated with Mann-Whitney U test P<0.01. (B) The average diameter of Ki67-positive cell nuclei between CHI-D samples (2-13 mo). Proliferating cells in CHI-D 4 were significantly smaller than the other CHI-D samples (ANOVA, Tukey p<0.01). Bars show mean error bars show standard error.

Figure 5-12. Distribution of nuclear diameters in proliferating cells in CHI-D samples
Nuclear diameters of Ki67-positive and Ki67-negative cells were measured using 3DHISTECH software following digitalisation of 5 μm sections of CHI-D samples (2-13 mo) previously stained for Ki67 immunoreactivity. (A) Shows the frequency histogram for individual CHI-D sample data whilst (B) shows the frequency histogram for the average nuclear diameters recorded for Ki67-positive and Ki67-negative cells across all CHI-D samples.

The data collected for the Ki67-positive cell nuclei diameters were merged with the measurements recorded in CHI–D islets (Figure 5-13). A population of cells with nuclear diameters >10 μm that had been identified in the CHI-D islet cell measurements was apparent, that could not be accounted for within the Ki67-positive population (shaded area, Figure 5-13).
Figure 5-13. Frequency histogram for proliferating and nucleomegalic cells in CHI-D samples

Histogram showing the nuclear diameter of cells counted in CHI-D samples (2-13 mo) that were either Ki67-positive (Blue) or that were within an islet (red) including those cells identified as having enlarged nuclei. Where the two graphs cross the hatched area shows the proportion of cells, as identified in the CHI-D islets that were larger than any Ki67-positive cell nuclei measured. These data suggest that a large proportion of the enlarged nuclei observed in CHI-D islets are not Ki67-positive.

Since we have already shown that the majority of nucleomegalic cells were insulin-positive, and that overall, very few insulin-positive cells were proliferative in CHI-D (approximately 2%)(Chapter 4), dual IF experiments were performed to investigate whether the enlarged insulin-cell nuclei were also the proliferating cell nuclei observed in these samples. The number of large Ki67+/Insulin+ cells was counted as a proportion of the total large insulin-positive population in all five samples of CHI-D (2-13 mo).

Overall, <10% of enlarged insulin-positive nuclei were Ki67-positive (Figure 5-14). However one CHI-D sample [CHI 1 (2 mo)], showed a high incidence of Ki67+/Insulin+ colocalisation in the enlarged nuclei (31%) as compared to the other samples that showed a much lower incidence (average 4.5%). Sample CHI-D 1 (2 mo) was also noted previously for its much higher occurrence of enlarged nuclei and interestingly also showed the highest and most significant rates of insulin-cell proliferation in the previous chapter (Chapter 4, Figure 4-6).
Quantifying proliferative β-cells with enlarged nuclei

Dual immunofluorescence was performed on 5 μm sections of CHI-D pancreas for insulin and Ki67 and counterstained with DAPI. The proportion of enlarged nuclei that were Ki67+/Insulin+ within CHI-D samples (2-13 mo) was counted across 10 different fields of view for each sample and expressed as a percentage of total insulin-positive cells with enlarged nuclei observed in each sample. Approximately 29 (±10) insulin-positive nucleomegalic cells were counted per CHI-D sample. Bars show mean, error bars show standard error. Statistical significance calculated with ANOVA test and post-hoc Tukey analysis P<0.01.

Figure 5-14. Quantifying proliferative β-cells with enlarged nuclei

Given that the majority of enlarged nuclei did not appear to be actively proliferating (Ki67-positive), the expression of components of the cell cycle was explored to determine if the capacity for proliferation is altered in nucleomegalic cells.

There has been a lot of interest in recent years about the cell cycle regulation of β-cells, and the mechanisms that regulate β-cell mass which were explored in more detail in Chapter 4.

In the previous chapter we showed a predominantly nuclear expression for P27 in CHI-D islets which was in apparent contrast to its localisation in the cytoplasm of neonatal controls and adult human islets (Fiaschi-Taesch et al. 2013a). In the nuclei of CHI-D islets, P27 can be detected in the majority of nucleomegalic cells (White arrows Figure 5-15). However, there were occasional cells with enlarged nuclei that did not show P27 immunoreactivity (black arrows Figure 5-15). Similarly, although pRb and CDK6 were also detected predominantly in the exocrine compartment of CHI-D pancreas, occasional nucleomegalic cells were observed to have CDK6 or pRb nuclear reactivity (White arrows Figure 5-15), whilst the majority of insulin-positive cells with enlarged nuclei were negative for CDK6 or pRb expression (Black arrows Figure 5-15). Interestingly, not all pRb- or CDK6-positive cells within the islet were nucleomegalic, as demonstrated in the lower pRb panel (Figure 5-15).
Figure 5-15. Cell cycle components in nucleomegalic CHI-D islet cells
Dual Immunofluorescence of 5 μm sections of CHI-D showing CDK6, pRB or P27 reactivity (red) with insulin (green) and counterstained with DAPI. White arrows identify nucleomegalic β-cells with cell cycle component reactivity whilst black arrows identify β-cells with enlarged nuclei but absent of cell cycle markers. Scale bar represents 10 μm.
5.4. Discussion

Most eukaryotic cells contain a single nucleus that is relatively regular and spherical in morphology. The size of the nucleus can vary across different tissue and cell types although the mechanisms that determine or regulate its size are less clear. The significance of nuclear size is also incompletely understood. Nucleomegalic cells within a population of otherwise normal-sized nuclei are frequently observed in a variety of tissues including thyroid gland cells, acinar breast tissue, liver parenchyma and myocardium. Interestingly, many of these tissues have a predominantly metabolic function, commonly involved in producing, storing and/or secreting proteins (Ehrie and Swartz 1974). This is consistent with the idea that the presence of enlarged nuclei reflects a hyperactive cell and nuclear size has been correlated by some groups to increasing RNA transcripts and protein production (Sato, et al. 1994; Schmidt and Schibler 1995). Another mechanism proposed for regulating cell nuclear size involves the activity of the cell cycle. Indeed, an increased prevalence of nucleomegalic cells are often observed in pathologic conditions including certain cancers, the hallmarks of which include unregulated cell growth (Chow, et al. 2012; Ehrie and Swartz 1974; Webster, et al. 2009). Finally, the amount of DNA content within a cell has also been implicated in determining cell nuclear size. Following experiments in budding yeast, an increase in cell size was observed as the most consistent consequence of an increase in genetic ploidy (Epstein 1967; Mortimer 1958). Increases in DNA content can result from various mechanisms, including cell fusion, endoreduplication and disruptions in cell cycle (Storchova and Pellman 2004).

5.4.1. Cells with enlarged nuclei frequently seen in CHI-D islets

Nucleomegalic cells have been described previously in CHI pancreas, and are strongly associated with the pathology of the disease. Before the availability and widespread use of the $^{18}$F-DOPA PET scan and genetic investigations, the presence of enlarged nuclei was used to diagnose the different types of CHI as well as identifying CHI-F lesions since nucleomegalic cells are not observed outside of affected region(s) (Rahier et al. 1984; Sempoux et al. 1998; Suchi et al. 2004). There have been few efforts to try and identify these cell types in CHI samples since an early study confirmed their localisation in insulin-positive cells (Rahier et al. 1984). The appearance of nucleomegalic cells in the insulin-producing compartment in CHI-D is in-keeping with the idea of a hyperactivity in these cell types, resulting in the characteristic hypoglycaemia and inappropriate insulin secretion. A closer investigation of these nucleomegalic cells in CHI-D, in a comparison with age-matched controls, was attempted.
to shed more light on their phenotype, characteristics and contribution to the pathology of CHI-D.

Cells with enlarged nuclei were readily detected in all the CHI-D samples studied, however the frequency and extent of nucleomegalism in cells varied between patients. This inter-patient variability has also been reported by others studying enlarged nuclei in CHI samples (Rahier et al. 1984; Sempoux et al. 1998; Suchi et al. 2004). Whilst the presence of large nuclei was the most consistent histological feature of CHI patients, only approximately 91-94 % of patients could be correctly classified as having CHI-D based on the analysis of nucleomegalic cells alone (Jack et al. 2010; Sempoux et al. 1998; Suchi et al. 2004).

In our data set CHI-D 1 (2 mo) in particular seemed to have a higher abundance of nucleomegalic cells throughout the pancreas, with significantly more occurring in each islet compared to any of the other CHI-D samples. However, overall, the size of the enlarged nuclei did not vary significantly between samples, with the average nucleomegalic cell being approximately 1.8 fold larger than surrounding islet cell nuclei in each CHI-D sample.

Rahier and colleagues also measured the radius of islet cell nuclei in CHI samples and observed an approximate 40% increase in the size of the enlarged nuclei in CHI samples (Rahier et al. 1984). In our experiment enlarged nuclei were defined as being >50% larger than surrounding islet cell nuclei. These cells were identified initially by eye on the microscope and then confirmed by measuring the diameter. Based on the frequency histogram showing all the cell measurements recorded in the experiment (including all ‘normal’ and ‘enlarged’ measurements), the average cell nucleus in CHI-D samples was approximately 6 µm, but there was a clear presence of a nucleomegalic population with diameters greater than 9 µm, 50% bigger (Figure 5-5D). The largest nuclear diameter recorded in our samples was 17.9 µm, almost three times the width of the average islet cell nucleus. This is consistent with previous data that have described β-cells as having nuclei up to 19 µm in diameter (de Lonlay-Debeney et al. 1999; Suchi et al. 2004). This measurement appears larger than those reported by either of Rahier’s groups however, who measured the radius of β-cell nuclei (Rahier et al. 1984; Sempoux et al. 1998). In these studies the 50 largest β-cell nuclei were reported as having an average radius of 4.25 ± 0.43 µm, which equates to approximately 8.5 µm in diameter. In comparison, the 50 largest nuclei measured in our CHI-D samples averaged 12.2 ± 1.41 µm in diameter. Whilst our data seem to be larger than those presented by Rahier and colleagues, the mean nuclear radius (MNR) recorded in their control nuclei (surrounding ‘normal’ sized β-cells) is consistent with ours; MNR 2.68 ± 0.23 µm Vs 5.11 µm diameter in our samples.
(Rahier et al. 1984). This demonstrates that both methods of measuring cell radius/diameter are accurate and the results comparable. The differences are therefore likely the product of patient and tissue variability.

Approximately 4% of islet cells in our CHI-D samples had a nuclear diameter more than double that of the average surrounding nuclei, whilst Rahier’s group calculated a frequency of 1.46% in cases of CHI without a focal lesion (Rahier et al. 1984). The reason for this difference is unclear. There was a large variability in our samples, since CHI-D 1 (2 mo) showed an approximate 10% occurrence of islet cells with a nuclear diameter >2 times the average surrounding cells, far greater than any other CHI-D sample. Omitting this sample revealed an average 2.5% rate in the remaining CHI-D samples in our data set, still larger than previously described (Rahier et al. 1984). Since Rahier’s group studied 12 individual samples, the average value is likely to be less affected by patient variability and more representative of the diseased-population. Including more samples in our study would therefore verify our findings. Rahier’s frequency was also reported as a β-cell specific count, whilst we focussed on the islet-cell population as whole, based on H&E staining. Since we have shown that other islet nuclei, including SS-producing cells, can also display nucleomegaly our value is inclusive of these additional cell types and therefore may explain why a larger proportion of cells was observed with giant nuclei in our experiment compared to previously published data (Rahier et al. 1984; Sempoux et al. 1998).

When large nuclear diameters were plotted along with all the nuclear diameters for the surrounding islet nuclei, a clear subset of cells with nucleomegaly, typically with diameters >9 μm were apparent which were not seen in control samples (Figure 5-5,D). In fact, whilst Rahier and colleagues found 0.02% of β-cells in control samples to have a nuclear radius more than double that of surrounding β-cell nuclei (Rahier et al.1984), we did not observe any cells that matched these criteria in any of our controls. The average fold increase in our postnatal controls (9 wk- 10 mo) was 1.6 fold greater than the average surrounding islet-cell nuclei, and they did not tend to deviate significantly from this value (Std. Dev; 0.09 μm). These enlarged nuclei were observed in approximately 30% of islets but never more than one large nucleus was seen within each.

**5.4.2. Nucleomegalic cells were mostly insulin-secreting with a subset detected in the δ-cell lineage**

The size of the nucleus was classically believed to reflect function of the cell (Kracht, 1958). However whether this is achieved from an increased genetic transcription due to an over-exuberance of DNA, or simply a proportional response of the nucleus to increasing cytoplasmic volume resulting from elevated metabolic demand and hormone
production is unknown. This correlation in cytoplasmic and nuclear volume has been described previously, defined as the karyoplasmic ratio, and is thought to represent a primary determinant of nuclear cell size (Gregory 2005). The size of the β-cell nuclei in CHI-D patients was found to correlate with an increase in DNA content based on absorption data and comparisons with age-matched controls (Ariel et al. 1988). Polyploid cells in a tissue may provide a short-term benefit to increasing metabolic load or chronic stimulation, resulting in an increase in genetic machinery and more efficient transcription to synthesise proteins. This has also been associated with increased RNA transcripts (Sato et al. 1994; Schmidt and Schibler, 1995).

Using IF detection, we confirmed that the majority of nucleomegalic cells were insulin-secreting (approximately 72.5 %). However, approximately 6.5 % were identified in the SS-producing population, whilst approximately 21 % did not appear to be hormone-secreting in our experiment.

Rahier and colleagues also found enlarged nuclei to be predominantly within insulin-secreting cells but never in glucagon-secreting α-cells. However δ-cells were described as rarely containing enlarged nuclei, which suggests that the occasional hypertrophied nucleus may have been observed in these cell types. In-line with our findings, these were less frequent than the insulin-positive cells (Rahier et al. 1984). It is likely experimental error meant we were unable to characterise all the large nuclei in our CHI-D samples.

Other researchers have reported what is likely to be underestimations in their data when only the β-cell population has been explored. Particularly since we have demonstrated that almost a third of nucleomegalic cells were observed outside of the β-cell lineage. This could be further justified given the assumed hyperactivity of the nucleomegalic cells. If a hypersecretion is occurring, there may be absent or reduced levels of hormone stored in the cytoplasm that is below the detection threshold using IF techniques. Large nuclei therefore appear in cells without hormone expression. Of course there is also a technical discrimination since only two hormones could be observed in any one section. However since we never detected nucleomegalic cells in the α-, ε-, or PP- cell populations it is unlikely these represent an alternate endocrine cell type other than β- or δ-cells. We can therefore conclude the nucleomegalic cells that could not be identified by hormone staining are probably degranulated cells, or that the level of hormone expression is below the detection threshold for IHC staining.

IHC staining also proved the endocrine nature of the large nuclei, with PDX1, NKX2.2, NKX6.1, ISL1 and FOXA2 immunoreactivity detected in nucleomegalic cells. In contrast, GATA4 or SOX9 immunoreactivity were never observed in these cell types, consistent with the absence of large nuclei in the exocrine compartment (Rahier et al. 1984).
Occasionally PDX1-negative cells with enlarged nuclei were also observed. Since PDX1 is found exclusively in insulin-producing cells after birth where it is required for insulin gene transcription (Oster, et al. 1998b; Sander et al. 2000; Schwitzgebel 2001) it is possible that these represent the SS-producing nucleomegalic cells that were also identified in our experiment.

Due to time limitations we were not able to identify the cell types with hypertrophied nuclei in the control samples, although we can confirm they were only ever present in the islets. Previous studies have also observed enlarged nuclei in adult human islets of Langerhans and identified these in the β- but not α- or δ- cell lineages (Ehrie and Swartz, 1974).

The significance of hypertrophied nuclei in β-, δ-, or other islet cell types is not entirely understood. The size of the nucleus was classically believed to reflect function of the cell (Kracht, 1958) or polyplody, which has also been correlated with an increase in secretory function (Wagner and Richart, 1968). Increases in secretory function seem to make sense in the setting of CHI-D, since the disease presents as an unregulated and persistent secretion of insulin due to the uncoupling of glucose-sensing. Mutations in the $K_{\text{ATP}}$ channel therefore cause a persistent depolarisation of the β-cell membrane and a hyperstimulation of the secretory pathway. In this setting it is therefore plausible that other cell lineages may be involved; not least because $K_{\text{ATP}}$ channels have also been identified in other islet cells types (Ashcroft and Rorsman 2013). The role of these channels in the various hormone-producing islet cells is likely to be different however, since the clinical features of CHI are predominantly associated with insulin activity. For example, $K_{\text{ATP}}$ channel activation in α-cells can reduce rather than increase electrical activity and hormone secretion due to the presence of different combinations of voltage-gated ion channels (Gromada, et al. 2004).

Given that SS has an antagonistic role towards insulin secretion, it is not surprising that nucleomegalic δ-cells would also be apparent in CHI-D samples, indicating a hyperfunction associated with increased SS-secretion in an attempt to counteract the hyperinsulin-secretion (Alberti, et al. 1973; Hauge-Evans, et al. 2009). SS secretion from pancreatic δ-cells involves a variety of intra-islet paracrine interactions in order to regulate the secretion of other islet hormones and maintain normoglycaemia. This includes an ability of the δ-cell to respond to extracellular glucose concentrations whilst also being regulated by local insulin levels (Hauge-Evans, et al. 2009). Chronic insulin secretion and resultant hypoglycaemia could therefore represent an increased metabolic load to which the δ-cell responds by increasing its activity and, in some cells, nuclear size. Alternatively it may be a more complex involvement of the dysfunctional $K_{\text{ATP}}$ channel in these cell types. These findings support earlier hypotheses generated in previous chapters (Chapter
3, Chapter 4), that the pathology of CHI-D extends beyond abnormalities in the β-cell lineage exclusively.

5.4.3. Ki67-positive cells tended to be larger than quiescent cells but did not account for nucleomegalic cells in CHI-D

Having confirmed the β-cell population to contain significantly more proliferating cells in CHI-D samples than their age-matched controls (Chapter 4; Kassem et al. 2000; Lovisolo et al. 2010), we used Ki67 IHC to investigate whether the enlarged β-cell nuclei identified in these samples were proliferating cells.

Some, but not all, Ki67-positive cells displayed enlarged nuclei. In fact, when the diameter of Ki67-positive cell nuclei was compared to Ki67-negative cell nuclei, although they were significantly larger on average in the Ki67-positive population, the nuclear diameter was rarely measured above 10 μm. As such when the CHI-D Ki67-positive nuclear diameter measurements were plotted along with islet-cell measurements from the same samples, a population of cells greater than 10 μm in nuclear width was apparent in CHI-D islets that were not accounted for in the Ki67-positive population. Indeed, the average diameter of the largest 50 Ki67-positive nuclei was 10.41 ± 0.64 μm compared to 12.2 ± 1.4 μm in CHI-D islets. Although β-cell nucleomegaly is a clear characteristic of CHI-D pancreas (Lovisolo et al. 2010; Rahier et al. 1984; Sempoux et al. 1998; Suchi et al. 2004) these cells are not simply a reflection of increased proliferation. This is not entirely surprising given that the most proliferative cells were observed in the acinar and ductal lineages (Chapter 4) and these cell types were devoid of such nucleomegalic cells.

The effect of DNA content on nuclear size is a contentious area, and as such, there are various hypothesis within the literature regarding how cell nuclear size is controlled. Most eukaryotic cells are diploid containing 2 paired chromosomes. However as cell cycle proceeds through G1-G2, the cell and nuclear size can be seen to increase concomitantly (Jorgensen, et al. 2007; Neumann and Nurse 2007). Likewise, when polyploidy was explored, a linear increase in cell volume was observed with each extra complement of chromosomes (Mortimer, 1958; Epstein, 1967). In general, increased nuclear size is the most obvious and consistent consequence of increases in DNA content. Polyploid cells can occur either as a result of abnormal cell division or aborted cell proliferation (Storchova and Pellman, 2004). The extra energy required by the cell to produce and secrete hormones may therefore occur at the expense of complete cell proliferation.
5.4.4. P27 accumulation in the nucleus of islet cells may cause cell cycle arrest and contribute to development of enlarged nuclei

Since Ki67 identifies cells at all active stages of proliferation it will also be expected to detect cells that are still progressing in the cell cycle but have not completely divided, therefore containing additional genetic material. Given more time we would have also attempted to investigate the DNA content of the nucleomegalic cells present in CHI-D samples to confirm whether an increase in ploidy is responsible for the increase nuclear size, therefore providing insight into some of the mechanisms involved in their generation. Various techniques have been employed by others including the Feulgen technique which measures absorbance based on DNA content, or fluorescence in situ hybridisation (FISH) which offers a more specific protocol to identify which chromosomes are over represented.

A variety of defects during the cell cycle can result in inefficient cytokinesis and/or, multiple rounds of DNA replication without the appropriate rate of mitosis. Uncoordinated cell cycle mechanisms often occur in pathological situations (Gorla, et al. 2001) and the work presented in the previous chapter have already postulated an alteration in cell cycle regulation in CHI-D samples compared to their age-corresponding controls.

If a cell loses its ability to respond to mitogenic signals the process of mitosis cannot be completed. If this occurs after DNA synthesis, polyploid cells can be generated. Mechanisms controlling mitogenic response are believed to involve components of the G1-S checkpoint, including P53, P21 and pRb (Borel, et al. 2002; Meraldi, et al. 2002). We therefore explored the expression of components of G1 cell cycle in nucleomegalic CHI-D islet cells.

We showed previously that CDK6, a pro-proliferation marker predominantly seen in the cytoplasm of control samples, had widespread nuclear expression in acinar and ductal cells (Chapter 4). CDK6-positive cells were infrequently observed in islet cell nuclei. When these CDK6-positive cells were studied to see if their expression coincided with hypertrophied nuclei we found that whilst some of the CDK6-positive cells had enlarged nuclei, the majority of nucleomegalic cells were not CDK6-positive. The same pattern was observed in pRb-positive cells. However, expression of both CDK6 and pRb was apparent in ‘normal’ nuclei within the islet cells showing that these cell cycle markers are not specifically expressed in nucleomegalic cells. As such, it is unlikely these cell cycle components have a consistent role in the development or maintenance of nucleomegalic cells.

P27 on the other hand is seen in the majority of islet cell nuclei and as such is found frequently in enlarged nuclei. P27 in adult human β-cells is usually found in the cytoplasm.
However, whilst human β-cells are typically considered quiescent, accumulation of P27 in the nuclei has also been implicated in preventing cell cycle progression (Georgia and Bhushan 2006). Zhong and coworkers found an increase in the prevalence of endoreduplication following accumulation of nuclear P27 in islet β-cells, suggesting that the nuclear presence of P27 is associated with an interruption in cell cycle and a halted proliferation around the stage of mitosis (Zhong et al. 2007). Molecular responses within the cell cycle can not only block cell cycle progression but also trigger apoptosis in some cells (Storchova and Pellman, 2004). We showed previously that the number of apoptotic cells were increased in CHI-D β-cell population compared to controls and, although these were rarely observed to contain giant nuclei (own observations), it is further evidence of an involvement of cell cycle machinery in CHI-D pathology.

It is therefore possible that CHI-D islets are responding to increased mitogenic signals, such as local insulin concentrations and membrane depolarisation by upregulating the expression of P27. Whilst increased P27 can facilitate the transport of CDK6 into the nucleus to drive proliferation in some cells, its paradoxical role means it also blocks cell cycle progression in others (Cheng et al. 1999; Kulkarni et al. 2012; Pestell et al. 1999; Sherr and Roberts 1999).

Occasionally large nuclei were observed to be absent of P27-staining. There is evidence to suggest that cell cycle block might only produce a transient delay in most cells therefore allowing some cells to ‘slip’ past the arrest and proliferate (Elledge 1996). Since P27 is never found in Ki67-positive cells (Chapter 4; Fiaschi-Taesch et al. 2013b), these P27-negative cells may represent a population that have progressed past the block and are actively proliferating and are therefore included in the population of enlarged Ki67-positive cell nuclei that were also detected in these experiments.

There is still a lot more to be learnt regarding mechanisms of cell cycle and its involvement in nucleomegalism. The extensive expression of P27 in hypertrophied cell nuclei in CHI-D samples is consistent with a mechanism for endoreduplication as a possible cause of nucleomegalism in CHI-D. Although we alluded in the previous chapter to a mechanism of glucose concentration regulating $CDKN1B$ gene activity, the elevated insulin concentrations and persistent membrane depolarisation may also contribute to the phenotype; driving proliferation in otherwise quiescent β-cells that subsequently respond with an effort to block these signals by upregulating P27.

Cancer cells also often show evidence of polyploidy and are characterised by their unregulated growth and uncoordinated cell cycle activity that occurs in pathological situations. This therefore provides a link between unregulated cell cycle and DNA polyploidy and likely implicates more components of the cell cycle in this mechanism.
beyond what we are able to demonstrate in this report. We are also unable to confirm whether alterations in the expression of G1 components are a primary or secondary response to the pathological disease.

5.5. Summary

In summary, we confirm the prevalence of nucleomegalic cells in islet cells as a histopathological characteristic of CHI-D, and further identified that, whilst the majority were insulin-positive, approximately 6.5% were also detected in the SS-producing δ-cell lineage.

By measuring the diameter of these hypertrophied islet cell nuclei, in comparison to their surrounding ‘normal’ counterparts, a clear subset of nucleomegalic CHI-D endocrine cells positioned right of the normal distribution were observed. Although Ki67-positive cells were often found to be larger in size than the Ki67-negative population, these proliferating cells did not account for a substantial proportion of nucleomegalic cells in CHI-D samples which measured > 10 μm in diameter. These data prove that β-cell nucleomegaly is not simply a reflection of cell proliferation, but that further mechanisms likely feed into the overall regulation of cell nuclear size. We have shown previously an alteration in G1-S cell cycle molecule expression in CHI-D islets compared to their age-matched controls, and demonstrated a likely involvement of this system in disrupted cell development, growth and division. We further showed here that disruptions in the cell cycle machinery may provide a mechanism for the development of polyploid nuclei although the exact mechanisms of this are not clear.

Taken together we hypothesize that the nucleomegalic cells in CHI-D samples represent a hyperfunctional cell with an uncoordinated cell cycle regulation, either as a causative or consequential effect of the pathological situation. We propose that the genetic machinery is increased in a subset of islet cells which subsequently exhibit enlarged nuclei as a result of an over-activity of hormone production and secretion. K\text{ATP} channel mutations result in a persistent membrane depolarisation, similar to chronic metabolic demand that would occur in the presence of elevated glucose concentrations. The β- (and δ-) cells respond by increasing their hormone output. The increase in nuclear size seen in some β- and δ- cells may represent the cells that ‘escaped’ cell cycle arrest, and underwent DNA replication before being halted from further cell cycle progression by additional mechanisms. Polyploid cells have a higher abundance of genetic material and therefore a greater efficiency of genetic transcription and increased secretory function. Whether increased protein production and storage causes increased cell cytoplasmic volume which
in turn influences the nuclear volume as a primary cause of nucleomegaly or whether it is a combination of prolonged β-cell activation and a change in cell cycle activity and DNA replication that causes this distinct phenotype is unknown. More work would therefore be required to further characterise and determine the significance of the giant nuclei in CHI and other pathological-associated cell types.
Chapter 6- The profile and phenotype of NGN3-expressing cells in human fetal development

6.1. Introduction

Given that each of the three pancreatic lineages; ductal, acinar and endocrine, are specified during embryonic development, understanding the normal course of differentiation during this time is important to help facilitate the discovery of future therapies focussed on pancreatic regeneration.

In particular, knowledge of the differentiation programme for β-cell generation during human fetal development is important for appreciating how the insulin-cell mass is generated and maintained in postnatal life. This is especially pertinent given the low rates of proliferation observed in the human endocrine compartment (Bouwens et al. 1997; Chapter 4.).

This chapter will explore some of the crucial aspects of endocrine differentiation previously identified in the rodent pancreas, in the context of human development. Studies in human fetal pancreas are rare and this paucity of information is important as it prevents insight into when the ability for endocrine commitment ceases in human.

The aim of the work was to identify a period of competence during human fetal development within which endocrine differentiation occurs and after which further expansion is the result of proliferation of either pre-existing progenitor cells or differentiated islet cells.

Whilst longitudinally tracing Neurogenin3 (NGN3) expression throughout human development can identify cells undergoing transition from a multipotent epithelial progenitor to a hormone-secreting endocrine cell, further analysis was focussed on deciphering the phenotype of these cells; studying both transcript and protein expression profiles once endocrine commitment has commenced.

Understanding the process of endocrine differentiation and the closure of endocrine commitment in the human fetus represents an important gap in knowledge, where insights could help target regeneration programs towards the production of β-cells from alternative sources for the treatment of T1DM.

6.1.1. The window of endocrine differentiation

Most of the knowledge of embryogenesis has derived predominantly from studies on rodent and chick species which have conclusively demonstrated a necessary and
sufficient requirement for the basic helix-loop-helix (bHLH) factor Neurogenin3 (NGN3) in endocrine commitment (Apelqvist et al. 1999; Gradwohl et al. 2000; Grapin-Botton, et al. 2001; Gu et al. 2002; Schwitzgebel et al. 2000). The pancreas of Ngn3-deficient mice lack hormone-producing endocrine cells during development and after birth whilst the acinar and ductal compartments develop normally (Gradwohl et al. 2000).

Expression of Ngn3 occurs within a population of progenitor cells and is regulated by a complex network involving sex-determining region Y-box 9 (Sox9) and pancreas and duodenal homebox 1 (Pdx1) that regulate a further cascade of TFs to activate Ngn3 (Lynn, et al. 2007; Oliver-Krasinski and Stoffers 2008). Interestingly, after the initiation of NGN3-expression in SOX9- and PDX1-positive progenitors, both TFs are quickly downregulated, suggesting that Ngn3 may play a role in repressing the progenitor gene programme (Nelson, et al. 2007; Schwitzgebel et al. 2000; Seymour et al. 2007).

NGN3 is detected in two phases during mouse development. The first NGN3-positive cells are detected at embryonic day (E) 8.5, peaking at E9.5 and then reducing by E11. This is followed by the so-called ‘secondary transition’ where NGN3-positive cells reappear at E12 until E18, with a peak in expression at E15 (Gradwohl et al. 2000; Jensen et al. 2000; Schwitzgebel et al. 2000).

The expression profile of NGN3 in the developing human pancreas is less well understood. This is at least in part due to difficulties accessing fetal tissue, including ethical constraints and limited availability. There is no first phase of NGN3 expression to coincide with the first NGN3 detection in the mouse, instead, both transcript and protein for NGN3 become progressively more detectable immediately after the embryonic period, from 8 wpc (approximately E17-18 in mouse) (Castaing, et al. 2005; Jennings et al. 2013; Lyttle et al. 2008; Piper et al. 2004). Expression at this time coincides with the appearance of the first insulin-secreting cells (Castaing, et al. 2005; Piper et al. 2004).

The presence of NGN3 has been reported in the human fetal pancreas until mid-gestation (Jeon, et al. 2009; Lyttle et al. 2008; Piper Hanley et al. 2010; Sugiyama, et al. 2007), however it is not clear if and/or when NGN3 becomes extinguished during development.

6.1.2. Regulation of the differentiation programme

It is well established, at least in mouse, that Ngn3 has an essential role in the differentiation of the endocrine cell lineage in the pancreas. However, the genetic programme regulating the induction of Ngn3 expression and the subsequent cascade of genes that are activated downstream are incompletely defined.

Our current understanding of pancreatic endocrine cell differentiation positions Ngn3 at the top of the TF hierarchy where its forced expression has been shown to induce non-
endocrine, or even non-pancreatic tissues to become hormone-secreting (Gasa et al. 2004; Heremans et al. 2002; Mellitzer et al. 2006). NGN3 also appears important for β-cell regeneration in adult mice (Xu et al. 2008).

Direct genetic targets of Ngn3 have been identified using microarray analysis following ectopic expression of Ngn3 in cell lines and mouse knock-out models. These downstream targets include NeuroD1, Pax4, Nkx2.2 and Ia1, all of which impair β-cell differentiation when knocked-out in mice (Gasa et al. 2004; Heremans et al. 2002; Huang et al. 2000; Mellitzer et al. 2006; Smith et al. 2004; Sosa-Pineda et al. 1997; Sussel et al. 1998).

Of these downstream targets NeuroD1 is believed to regulate a similar set of genes to Ngn3. Shortly after NeuroD1 and Pax6 are expressed Ngn3 is down-regulated (Gu et al. 2002; Smith et al. 2004). As such, NeuroD1 can function to maintain the differentiation programme initiated by Ngn3 in the developing endocrine pancreas (Gasa et al. 2004; Gasa et al. 2008; Naya et al. 1997).

Of intrigue is the comparison in expression profile for Ngn3 transcript with that of the NGN3 protein. It has been observed previously that Ngn3 transcript expression is more widespread than the protein which is instead restricted to individual scattered cells (Villasenor, et al. 2008). Whilst it is possible that the two methods used to identify either the protein or transcript levels could be more sensitive for the latter, it is also conceivable that a secondary level of Ngn3 regulation is important during endocrine differentiation. This could be either as post-transcriptional modifications to Ngn3 or that a threshold level exists for the transcript, beyond which the protein accumulates.

Varying degrees of NGN3 protein-expressing cells have been reported following IHC detection; with some cells showing strong nuclear staining and others appearing much weaker (NGN3high, NGN3low, respectively) (Villasenor, et al. 2008). These could either represent different stages of what is apparently transient Ngn3 expression, or is further evidence for a threshold concept which facilitates the selection of cells based on their strongest expression, that subsequently progress towards an endocrine cell fate. It is then possible that NGN3low expressing cells are consequently unable to sufficiently differentiate into hormone-producing endocrine cells and instead adopt an acinar or ductal cell fate. This is supported by lineage tracing experiments in which the majority of Ngn3-expressing cells become endocrine cells, but a small proportion of the exocrine population show evidence for previous Ngn3 expression at some point during development (Schonhoff, et al. 2004).

Unfortunately, less is known about the NGN3 programme in human pancreatic development due to the inability to dynamically trace human β-cell development from the progenitor state to the differentiated hormone-secreting cell. Although many aspects of
pancreas and islet development appear conserved between rodent and human species there are differences. Significantly, α-cells are the first hormone-secreting cell type detected in mouse development and remain the most prominent lineage. In humans this role is fulfilled by the β-cell (Castaing et al. 2005; Piper et al. 2004).

There are also subtle differences in the development process. Islet structures are not formed until birth in mouse, whilst multi-hormone clusters are observed from approximately 14 wpc in humans (Piper et al. 2004). Also, NKK2.2 expression is widespread in early rodent progenitor cells but is absent at the equivalent time point in humans (Jennings et al. 2013). These differences may influence the development and maintenance of the endocrine cell types in the two species and knowledge that can be gained relevant to the human are therefore important in order to consolidate the rodent data and understand islet cell neogeneisis relevant to the human.

6.1.3. Endocrine cell specification

Endocrine cells within the islet structures originate from a number of progenitors (Scharfmann, et al. 2008). Since NGN3-positive cells are believed to exit the cell cycle (Gradwohl et al. 2000; Gu et al. 2002; Jensen et al. 2000; Maestro, et al. 2003; Schwitzgebel et al. 2000) expansion of the endocrine pool either results from the proliferation of their progenitors (SOX9+/PDX1+) or of the differentiated hormone-secreting population (Scharfmann, et al. 2008).

Although well regarded as an endocrine progenitor necessary for islet cell differentiation, it is not entirely understood whether each NGN3-positive cell represents a multipotent progenitor cell able to give rise to each of the different islet cell types, or whether they are in fact unipotent, with each NGN3-positive cell pre-committed to a specific endocrine cell fate. Emerging data suggests the latter, where early factors and conditions pre-determine the hormone-secreting cell fate prior to Ngn3-expression (Desgraz and Herrera 2009).

This is in support of previous work in which an early potential for α-cell development was apparent following forced expression of Ngn3 in the early pancreatic epithelium, which becomes unrestricted, giving rise to the different endocrine cells later in development. This work proposed a temporal window of expression for NGN3-progenitors in favour of the different endocrine cell types (Johansson et al. 2007).

The mechanisms or conditions restricting the endocrine differentiation throughout pancreas development are not clear. However, understanding what factors are necessary to favourably yield a β-cell population are obviously desirable for the future of stem cell differentiation protocols and treatments for diabetes.
Whilst crucial factors in the differentiation of the β-cell lineage have been discovered, including PAX4, PDX1 and NNX6.1 (Collombat et al. 2003; Gannon, et al. 2008; Henseleit, et al. 2005; Holland, et al. 2002; Sosa-Pineda et al. 1997) simply forcing the expression of these does not generate a β-cell. Even the most sophisticated differentiation protocols have failed to produce fully functional, glucose-responsive β-cells (Collombat and Mansouri 2009; D'Amour, et al. 2006; Meier, et al. 2006a).

Generation of the β-cell likely requires a complex interplay of gene dosage and temporal expression profiles, as well as signal contributions from the extracellular matrix and mesenchyme. Vascularisation of the islet is also likely to play a role in the development of the β-cell along with the proximity of each of the endocrine cells to the blood vessels during development.

Downstream expression profiles certainly affect the differentiation and stability of the developing endocrine lineages after Ngn3 expression (Olinger-Krasinski and Stoffers, 2008). Forced expression of TFs such as Pax4, Pdx1 and Nkx6.1 in endocrine precursors has been shown to result in a predominantly β-cell population at the expense of α-cells (Collombat and Mansouri 2009). In contrast, overexpression of Arx in the same progenitor population results in an abundance of α-cells (Collombat, et al. 2007).

In recent studies however, upstream factors have also been identified that can influence the resultant fate of NGN3-positive progenitors. The Nkx6.1 K/O mouse has a reduced number of β-cells. In this model, induced expression of Nkx6.1 in the PDX1-positive progenitor population is sufficient to restore the β-cell population, however the same recovery was not observed when Nkx6.1 was induced in the NGN3-positive population. This suggests an upstream requirement for Nkx6.1 in β-cell determination prior to Ngn3 expression (Nelson et al. 2007).

Interestingly, this profile of early Nkx6.1 expression also fits with Johansson’s model of temporal endocrine development, where early expression of NGN3 favours an α-cell development with β-cells appearing after E11.5 (Johansson et al. 2007). This window of β-cell competence correlates with NKX6.1 activity that peaks between E11.5-E13 (Henseleit et al. 2005; Pedersen, et al. 2005).

This window also correlates with the expression profile of NKX2.2. During mouse development, NKX2.2 is initially observed as widespread expression in the pancreatic epithelium, later becoming restricted to the endocrine population (excluding the δ-cell lineage) (Sussel et al. 1998). In human development, although early expression patterns in human are not consistent with those in mouse (Jennings et al. 2013) NKX2.2-positive, endocrine-negative cells are observed within the same window of NGN3 expression.
These NKX2.2+/Endocrine- cells identify a pool of endocrine-competent progenitors which appears to be maintained for a longer period in human development than in mouse (Capito et al. 2013; Sussel et al. 1998).

Of interest is the interplay between various TFs during endocrine specification and development. If NGN3-positive cells are unipotent, then the expression of TFs responsible for differentiation of each hormone-secreting cell type must be established prior to Ngn3 expression.

NKX2.2 has been shown to adopt different regulatory roles in endocrine progenitors depending on the cell type being specified. In β-cells, NKX2.2 recruits a repressor complex which binds to the promoter region on Arx, preventing its expression. Lack of this complex permits Arx gene transcription establishing α-cell differentiation (Papizan, et al. 2011).

Nkx2.2 has also been shown to function with NeuroD1. Deletion of NeuroD1 in the mouse does not affect the formation of α- or β-cells but rather their survival, as both populations are reduced by birth (Naya et al. 1997). When Nkx2.2 is knocked-out there is an absolute loss of β-cells and reduced α- and PP-cell populations (Sussel et al. 1998). However, when both NeuroD1 and Nkx2.2 are deleted simultaneously a recovery of the α- and PP-cell numbers is observed (Chao, et al. 2007). Further work demonstrated an early requirement for NeuroD1-exclusion in the PDX1-positive progenitor population, prior to Ngn3 expression, to specify the α-cell lineage, whilst expression of both NKX2.2 and NEUROD1 in the PDX1-positive population would promote a β-cell fate (Mastracci, et al. 2013).

These studies have begun to decipher the transcriptional background necessary for each of the endocrine cell differentiation pathways. They provide initial support to the early work presented by Johansson’s group, demonstrating a reduced competence for α-cell differentiation as development progresses (Johansson et al. 2007). Collectively this work has reframed the programme of endocrine differentiation beyond the induction of Ngn3-expression and the downstream effectors, to include the regulatory network upstream of this crucial factor. It is ultimately the timing and specific location of TF expression and function that determines the appropriate differentiation of each of the pancreatic endocrine cells.
6.2. Aims

The work in this chapter was carried out to investigate the period for endocrine differentiation in the developing human pancreas by characterising the expression of NGN3 as well as attempting to characterise these cell types in terms of their genetic profile and cell cycle regulation.

6.2.1. To define the window period for NGN3 expression in the developing human fetal pancreas

IHC and cell counting techniques allowed identification and quantification of NGN3-expressing cells within the developing epithelium of aging fetal samples. qRT-PCR analysis supported these observations at the transcript level to investigate peak activity for endocrine differentiation in human.

6.2.2. To determine the genetic profile of NGN3-positive cells

Revolutionary LCM techniques were employed to isolate NGN3-expressing cells and process them for RNAseq analysis in an attempt to decipher the transcriptomic profile of NGN3-positive cells as a comparison with surrounding cell nuclei that did not show NGN3 immunoreactivity.

6.2.3. To characterise fetal endocrine progenitors

Recent work suggests NGN3-positive cells may represent a unipotent rather than multipotent endocrine progenitor. Using IHC and IF analysis the profile of key TF expression around the time of endocrine differentiation was explored whilst also determining the mitotic state of endocrine progenitors.
6.3. Results

6.3.1. The window of NGN3-expression in human development

Our group is fortunate to have a large library of embryonic and fetal tissue samples that can be accessed in an attempt to establish a comprehensive analysis of the window period for NGN3 expression. The process of endocrine differentiation is of significant interest regarding the potential for regeneration for the treatment of diabetes and the process is well characterised in the rodent (Gradwohl et al. 2000; Gu et al. 2002; Schwitzgebel et al. 2000). Data in humans is, however, lacking.

NGN3 and SOX9 IHC was performed on a series of human fetal pancreas, ranging in age from CS18 to 41 wpc (n=32) (Figure 6-1). Each sample had at least 3 different sections of pancreas analysed for NGN3 reactivity with the fourth for SOX9. Designing the experiment this way meant that SOX9 reactivity on the fourth consecutive section confirmed positive staining for the IHC experiment whilst also identifying a population of progenitor cells from which the NGN3-positive cells could emerge and from which to reference the NGN3 counts.

![Figure 6-1. NGN3 expression during development](image)

Immunohistochemistry of 5 μm sections of fetal pancreas of increasing age for NGN3 (brown) counter-stained with Toluidine blue. Inset boxes show positive SOX9 immunoreactivity within the same sample. Scale bar represents 50 μm.

NGN3-protein was first detected in human fetal pancreas at 8 wpc as individual, scattered cells within the ductal epithelium. Where NGN3-positive cells were not detected (<8 wpc
and >35 wpc) SOX9-positive reactivity within the same sample confirmed integrity of the experiment (Inset boxes Figure 6-1). NGN3-positive cells were counted across 10 random fields of view, or the entire pancreatic section for smaller samples, on 3 consecutive sections of tissue for each sample. These counts were averaged and expressed as a percentage of the total SOX9-positive cell counts across the same area (Figure 6-2).

![Figure 6-2. Frequency of NGN3-positive cells throughout human fetal development](image)

The average number of NGN3-positive cells counted in at least three different sections per sample and expressed as a percentage of the total SOX9-positive population in the same area. In total, approximately 3111 (±1380) SOX9-positive cells were counted per sample. Bars show mean, error bars show standard error. Statistical significance calculated using ANOVA with post-hoc Tukey test *P<0.05, **P<0.01.

Exocrine differentiation has already commenced before NGN3-reactivity is first detected in the fetal pancreas (Jennings et al. 2013), and is the cell lineage largely responsible for the increase in pancreatic mass observed during development. Since NGN3-positive cells emerge from the SOX9-positive progenitor population in the ductal epithelium, this population provides the most consistent reference with which to compare NGN3 positive proportions between aging samples. As such other groups have also published with this technique (Villasenor et al., 2008). Simply counting NGN3-positive cells as a proportion of the total cell counts would be diluted as the samples get older and the acinar compartment larger, therefore under-representing the true NGN3 content.

Consistent with previous observations from both our group and others (Castaing et al. 2005; Jennings et al. 2013; Piper et al. 2004), NGN3-positive cells were not detected prior to 8 wpc in human fetal pancreas, but became progressively more apparent in samples 8-
9 wpc and 10-11 wpc. Prevalence of NGN3-positive nuclei was maintained in samples between 12-17 wpc, representing approximately 3.5-4% of the SOX9-positive cell population, before declining, such that no NGN3-positive cells were detected in term specimens (35-41 wpc).

The expression of NGN3-transcripts was also assessed across a subset of samples included in the previous counting experiments, ranging in age from 8-41 wpc using qRT-PCR techniques (Figure 6-3). These data corroborated those data previously obtained for protein expression, showing a peak in NGN3-transcripts between 12-14 wpc which demonstrated a significant decline by term (P<0.05).

Figure 6-3. Expression of NGN3 mRNA transcripts during human fetal development
qRT-PCR showing NGN3 mRNA expression in human fetal pancreas tissue from 8 – 41 wpc. NGN3 expression peaks at 12-14 wpc in human pancreas development and declines to very low levels by term. Bars show mean, error bars show standard error. Statistical significance calculated using Mann-Whitney U test (*P<0.05).

Taken together these data suggest a peak in NGN3 expression and therefore endocrine differentiation between 10-17 wpc, which is severely diminished by 35-41 wpc of development in the human.

6.3.2. Transcriptome of the NGN3-positive progenitor cell

NGN3-protein positive cells were visualized following IHC in a 15 wpc sample of fetal pancreas, and isolated using LCM techniques (Figure 6-4). Up to 10,500 cells were
captured and processed for RNA extraction followed by RNA amplification to ensure adequate material for downstream RNA-sequencing analysis. Alongside NGN3-positive cells, surrounding cells in the ductal epithelium that were negative for NGN3-protein were also captured as a reference sample. Amplification was confirmed following qRT-PCR analysis with a cDNA sample of LCM-captured cells before and after the amplification procedure (Pre-Amp, Post-Amp, Figure 6-5). For both GAPDH and NGN3 transcripts, a left shift in the amplification plot was observed in the Post-Amp sample showing an increase in DNA content.

![Image](image_url)

**Figure 6-4. Laser capture microdissection to isolate and collect NGN3-expressing cells**

Immunohistochemistry of 5 μm sections of 15 wpc human fetal pancreas was performed to identify NGN3-expressing cells (brown) counter-stained with Toluidine blue. Laser capture microdissection techniques were used to identify (middle panel, blue circles) and then capture (right panel) NGN3-protein-positive cells into a collection cap ready for RNA extraction and downstream RNA-sequencing applications. Scale bar represents 20 μm.

Following amplification, samples were sent for RNA-sequencing. Data received back was mapped onto the UCSC browser based on the number of reads recorded for each sample (NGN3-positive and NGN3-negative), and their alignment with the human genome. Once the data were uploaded onto the UCSC browser individual genes or positions in the genome could be searched to determine whether there was any gene expression in those regions of interest (Figure 6-6). Unfortunately RNA yield from our experiment was very fragmented and as a result the read-counts were relatively low (compared to successful experiments within the lab group) and did not map to the genome very efficiently.
**Figure 6-5. qRT-PCR analysis to confirm amplification of RNA product**

qRT-PCR analysis was performed on samples of NGN3-positive cells previously isolated following laser microdissection. For each gene of interest the two annotated results represent a sample of RNA before the amplification procedure (Pre-Amp) and a sample of the same RNA that had undergone amplification using the Ovation V2 Kit (Post-Amp). A left shift can be seen in the amplification plot for each gene of interest in the post-amplification sample.

**Figure 6-6. Fragments of RNA identified from RNA-seq analysis mapped onto genome browser**

Transcripts present within a sample of NGN3-positive or NGN3-negative cells as collected using laser capture microdissection techniques were mapped onto the human genome browser (UCSC) according to the number of transcripts present at each site (reads). For each of the transcription factors; NGN3, NEUROD1, PAX4, NKX2.2, SOX9 and PDX1, the top chart shows the NGN3-positive population and the bottom chart shows the NGN3-negative population. When fragments of RNA in the sample match the gene sequence a peak is observed on the chart.
The first thing to notice when assessing the mapped data is that \textit{NGN3} reads were not recorded in either of the \textit{NGN3}-positive or \textit{NGN3}-negative samples. However, there were readings in other pancreas-specific genes, such as SOX9 and PDX1 in both samples (top line shows \textit{NGN3}-positive, bottom line shows \textit{NGN3}-negative, Figure 6-6).

Downstream targets of \textit{NGN3} including \textit{PAX4} and \textit{NKX2.2} were also observed in the \textit{NGN3}-positive samples. With negligible reads recorded in the \textit{NGN3}-negative samples, whilst \textit{NEUROD1} was observed to have slightly higher reads in the \textit{NGN3}-negative compared to the positive population.

To corroborate these data, genes with the highest read counts in each sample were compared with the other. Whilst many of the genes involved in endocrine differentiation, including the NKX and PAX factors were missing from the formulated table, presumably because their relative read counts were too low, several genes of interest were represented (Figure 6-7). Consistent with the UCSC data, \textit{NEUROD1} reads were relatively lower in \textit{NGN3}-positive compared to \textit{NGN3}-negative cell samples. In contrast, \textit{PDX1} shared equivalent expression between the two samples, as did \textit{SS}, whilst \textit{SOX9} was substantially elevated in the \textit{NGN3}-positive cells. Meanwhile, both \textit{insulin} and \textit{glucagon} transcripts were underrepresented in \textit{NGN3}-positive compared to \textit{NGN3}-negative cells.

![Figure 6-7. Relative read counts of gene transcripts between NGN3-positive and NGN3-negative laser microdissected cell populations](image)

Relative read counts for the gene transcripts \textit{NEUROD1}, \textit{PDX1}, \textit{SOX9}, \textit{Insulin}, \textit{Glucagon} and \textit{Somatostatin} in \textit{NGN3}-positive and \textit{NGN3}-negative cells, each captured by laser capture microdissection. Bars show equivalent expression of transcripts mapping to the gene of interest between the two samples.
6.3.4. Characterising fetal endocrine progenitor cells

Over the last several years there has been data emerging to suggest the ultimate fate of endocrine progenitors to be predetermined prior to the onset of NGN3 expression. A number of factors have been identified that appear to affect the endocrine subtype that eventually delineates from the NGN3-progenitor, based on their early expression in the PDX1-positive progenitor population, including NKX6.1, NEUROD1 and NKX2.2 (Mastracci et al. 2013; Nelson et al. 2007).

During mouse pancreas development the peak NKX6.1 activity is observed between E11.5-E13 (Henseleit et al. 2005). Around the same time is the emergence of the β-cell lineage which is secondary to α-cell differentiation which occurs from approximately E10.5 (Herrera et al., 1991). Since β-cells are the first islet cell type to differentiate in human fetal pancreas we used IHC to look at the expression profile for NKX6.1 during the development period (Figure 6-8).

Figure 6-8. NKX6.1 expression during human fetal development and postnatal pancreas
Immunohistochemistry of 5 μm sections of fetal (CS20-37 wpc), postnatal (6 months) and adult pancreas for NKX6.1 (brown) counter-stained with Toluidine blue. During early fetal stages NKX6.1 expression is widespread in the ductal epithelium, becoming restricted to the islet compartment with increasing development and maturity. Scale bar represents 50 μm.
Initially in the early pancreas (CS20-8 wpc) NNX6.1 is widespread throughout the epithelium. By 11 wpc this expression becomes more restricted to scattered nuclei within the ductal epithelium as well as clustered in small structures assumptive of primitive islets. This is apparent until approximately 21 wpc in our samples. By term NNX6.1 expression was mostly confined to the islet structures where it is maintained in neonatal and adult samples, consistent with its location in β-cells in adult pancreas (Jensen et al. 1996; Oster et al. 1998). Dual IF experiments further showed the expression of nuclear NNX6.1 in insulin-positive cell clusters, whilst the scattered NNX6.1-positive cells within the ductal epithelium were absent for SOX9-immunoreactivity (Figure 6-9). Once again, NNX6.1-positive cells outside of insulin-positive clusters were observed up to 21 wpc.

In mouse, NNX2.2 is first detected throughout multipotent progenitors in the pancreatic epithelium and later becomes restricted to β-, α- and PP-cell types. In the previous chapter (Chapter 3), we showed that NNX2.2 was also apparent in human fetal δ-cells despite its apparent absence in this lineage in mouse. NNX2.2 expression in the SS-positive lineage was downregulated after birth but persisted in approximately 1/3rd of SS-positive cells postnatally. Our group has also previously shown an absence of NNX2.2 in the early stages of human pancreas development, with the first cells positive for NNX2.2 nuclear staining not detected until 8 wpc, and by 10 wpc were largely restricted to the β-cell lineage (Jennings et al. 2013). In 15 wpc pancreas NNX2.2-positive cells could be observed within each of the hormone-secreting lineages but also scattered in the ductal epithelium (Figure 6-10), possibly identifying an endocrine progenitor population.
Figure 6-9. NKX6.1 expression outside of insulin-positive cells
Dual immunofluorescence of 5 μm sections of human fetal pancreas (8-21 wpc) for NKX6.1 (red) with SOX9 or Insulin (green) counter-stained with DAPI. Arrows point to NKX6.1 immunoreactive cells whilst dual staining with SOX9 confirms their distinction from the ductal lineage and expression with insulin identifies differentiated beta cells. Scale bars represent 20 μm.

Figure 6-10. NKX2.2 expression outside of hormone-secreting endocrine cells
Dual immunofluorescence of 5 μm sections of 15 wpc human fetal pancreas for NKX2.2 (red) with Insulin, Glucagon, Somatostatin and Ghrelin (green) counter-stained with DAPI. NKX2.2 immunoreactivity can be observed within each of the hormone-positive lineages whilst also scattered amongst the ductal epithelium. Scale bar represents 20 μm.
Insulin is the first hormone detected in the human fetal pancreas at approximately 52 dpc (Piper et al. 2004). At this stage both glucagon and SS were not detected. Consistent with this work, and others, we showed both insulin and glucagon staining by 8 wpc in human fetal pancreas, whilst SS remained absent (Clark and Grant 1983; Piper et al. 2004; Stefan, et al. 1983) (Figure 6-11). SS is produced shortly after insulin and glucagon (Piper et al. 2004) and we showed SS-positive cells in 10 wpc pancreas, around the same time that clusters of endocrine cells were starting to form. Initially these clusters contained predominantly insulin-positive cells, but by 13 wpc aggregations of multihormone cell types were apparent. Throughout development insulin remained the most abundant hormone.

In the postnatal pancreas P27 is observed in the cytoplasm of islets (Chapter 4, Fiaschi-Taesch et al. 2013a). However, in fetal samples, P27 was detected as a nuclear protein in the majority of insulin-positive cells as well as in scattered cells within ductal structures (Chapter 4). P27 was found to colocalise quite specifically with NKX2.2-positive cells within the epithelium (Figure 6-12), although both proteins were also apparent without costaining with the other.

Interestingly P27 also colocalised in the majority of NGN3-positive cells (Figure 6-13).
Figure 6-11. Hormone expression in developing human pancreas
Immunohistochemistry of 5 μm sections of human fetal pancreas (8 – 37 wpc) for islet hormones insulin, glucagon or somatostatin (brown), counter-stained with Toluidine blue. Each panel shows consecutive sections demonstrating multihormone islet clusters and prevalence of insulin at each stage of development. 8 wpc sample is not consecutive to show positive staining. Scale bar represents 50 μm.

Figure 6-12. P27 expression in NKX2.2 endocrine progenitor cells
Dual immunofluorescence of a 5 μm section of 10 wpc human fetal pancreas showing p27 (red) with NKX2.2 (green) counter-stained with DAPI. Arrows point to dual expressing cells whilst P27-positive and NKX2.2-positive cells are also detected without coexpression. Scale bar represents 10 μm.
Figure 6-13. P27 expression in NGN3 endocrine progenitor cells
Dual immunofluorescence of a 5 μm section of 10 wpc human fetal pancreas showing p27 (red) with NGN3 (green) counter-stained with DAPI. Arrows point to dual expressing cells. The majority of NGN3-positive cells co-express nuclear P27. Scale bar represents 20 μm.

We have shown previously, and demonstrate again here, that P27-positive nuclei are not proliferative (Figure 6-14, Chapter 4). This is consistent with NGN3-positive endocrine progenitors not proliferating, as demonstrated by the separate expression of NGN3 and proliferating cell nuclear antigen (PCNA) in dual IF (Figure 6-15).

Figure 6-14. P27-positive cells are not proliferative
Dual immunofluorescence of a 5 μm section of 10 wpc human fetal pancreas showing p27 (red) with Ki67 (green) counter-stained with DAPI. P27-positive cells do not co-express the proliferation marker Ki67. Scale bar represents 20 μm.
Figure 6-15. NGN3 cells are not proliferative

Dual immunofluorescence of a 5 μm section of 10 wpc human fetal pancreas showing NGN3 (red) with PCNA (green). Arrows point to PCNA-positive cells whilst arrowheads identify NGN3-positive cells. The two proteins are not found to coexpress (Adapted from Salisbury et al. 2014 –in press).

6.4. Discussion

Since the endocrine pancreas represents a major regulator of glucose homeostasis, loss or disruption of the insulin-producing β-cell compartment can result in diabetes. In this setting, repopulating the β-cells would offer significant promise to the treatment of the disease. Extensive research efforts have been focused on developing protocols to differentiate progenitor cells into β-cells (Meier et al. 2005), whilst others have focused on the cell cycle as a way of expanding the insulin-cell mass (Kulkarni et al. 2012). However, these strategies rely on a thorough understanding of pancreas development, the potential for islet progenitors and their genetic profile.

An important step in understanding the development of pancreatic islet cells has been the identification of NGN3 as an islet cell progenitor marker. Indeed, all five endocrine cell types have been shown to differentiate from NGN3-positive cells in vivo and deletion of this factor results in the absence of the islet cell compartment (Gradwohl et al. 2000; Gu et al. 2002; Suissa et al. 2013).

Whilst there has been major progress over the past few years in understanding mouse islet-cell development, information on human pancreas development remains scarce.

6.4.1. The NGN3-expression window peaks between 10-17 wpc in human fetal pancreas

Over the last few years there has been a lot of work reporting on human β-cell number, proliferation and apoptosis as crucial factors maintaining β-cell mass postnatally (Bonner-Weir et al. 2000; Kassem et al. 2000). Whilst these data contribute significant insight into the dynamics of the human β-cell population, there exists little data on the competence for
endocrine differentiation during human development, if this window closes and, if so, when.

We used IHC, cell counting and qRT-PCR techniques in an attempt to define the profile for NGN3-expression in the developing human pancreas as a means of defining levels of endocrine commitment at various stages of development.

Our data on the onset of NGN3-expression confirmed those previously published from our own group and others (Jennings et al. 2013; Lyttle et al. 2008; Piper et al. 2004; Sugiyama et al. 2007). NGN3-protein was first detected in human pancreas at 8 wpc, having been absent at previous time points. This marks the end of the embryonic period and correlates with the appearance of the first hormone-secreting cell types, confirmed by our data (Figure 6-11) and others (Piper et al. 2004).

Extending on this we observed a peak in NGN3-expression between 10-17 wpc, both by total NGN3-positive cell counts (data not shown), and when expressed as a proportion of the SOX9-positive cell population, and this was corroborated by qRT-PCR analysis. No NGN3-protein, and very low levels of NGN3 transcript were detected in samples by term. These data are inline with those reported previously, where, using qRT-PCR techniques, Jeon and colleagues found low levels of NGN3 prior to 9 wpc, with peak expression at 15 wpc which was maintained until 17 wpc before declining (Jeon et al. 2009). A similar pattern was also observed by Lyttle and coworkers who also reported a peak in NGN3 transcripts at 10 wpc which significantly decreased as development progressed (up to 21 wpc). By virtue of IHC detection, NGN3 protein was observed in the later stages (19-21 wpc) of human fetal development by Lyttle and colleagues, but by this time positive-staining cells were rare (Lyttle et al. 2008).

It is important to point out, however, that the qRT-PCR data, including our own, are likely affected by the growing pancreatic organ and in particular the proliferating acinar compartment. The exocrine lineage is the first to differentiate in the developing human pancreas and as such has already commenced prior to the first detection of NGN3 immunoreactivity (Jennings et al. 2013). The acinar cells are also highly proliferative and therefore contribute to the majority of the pancreatic mass increases observed during the fetal period (Jensen 2004). Since whole pancreatic sections were used for each sample to collect RNA, and equal amounts of total cDNA then inputted into the qRT-PCR reaction, the data obtained are likely to be an underestimation of the NGN3 transcripts present, due to dilution by the growing acinar tissue surrounding them. However, our IHC data up to 21 wpc and term confirm that NGN3 protein levels are certainly reduced by this time, and in-situ hybridisation studies up to 22 wpc pancreas also confirm their decrease in prevalence by this time (Jeon et al. 2009).
Taken together our data with others point to the window of endocrine commitment closing between 21-35 wpc. However, we can’t rule out that low-levels of NGN3-protein may have appeared negative due to the sensitivity of IHC methods. Interestingly however, in a recent study in which human fetal pancreas aged 7-9 wpc was engrafted under the mouse muscle capsule, NGN3 immunoreactivity could be traced throughout different stages of development (Capito et al. 2013). This method of engrafting has been previously validated to confirm its conformity with the human development programme (Castaing, et al. 2001).

In this model, NGN3 immunoreactivity was observed up to 19 weeks post-engraftment (approximately 26-28 wpc), but absent at the next time point studied; 37 weeks post-engraftment (approximately 44-46 wpc). Whilst caution is needed to extrapolate these results from human allografts towards normal human gestation, pooling our results which appear to be concordant with theirs, would narrow the window for endocrine commitment to close between 26-35 wpc. Obviously this conclusion relies upon NGN3-expression representing the sole route for endocrine differentiation; a conclusion that has been well established in mouse (Apelqvist et al. 1999; Gradwohl et al. 2000; Gu et al. 2002; Schwitzgebel et al. 2000), but is somewhat incomplete in human development. This is further evidenced by a series of recent case studies describing the first known clinical presentations of homozygous NGN3-mutations in human.

In all the patients identified (n=4) with NGN3 homozygous mutations, the initial and most severe manifestation was malabsorptive diarrhoea which commenced within the first few days (Pinney, et al. 2011; Wang, et al. 2006). Due to less invasive methods available, a biopsy was taken of the intestine in each case, revealing an absence of enteroendocrine cells in the intestinal mucosa (Pinney, et al. 2011; Wang, et al. 2006). In most cases, a diagnosis of childhood-onset diabetes was also made. In two of the four cases, patients presented with hyperglycaemia around 8 years of age (Wang, et al. 2006), whilst another patient showed persistent hyperglycaemia by 5.5 months of age, and was started on insulin soon after (Pinney, et al. 2011) (there is no data yet on the fourth patient).

There were also reports of a low birth weight in one of the patients, which would suggest a deficiency of insulin during fetal development (Pinney, et al. 2011). Whilst these data provide evidence for a crucial role for NGN3 in the development of enteroendocrine cells in the intestine, its importance or necessity in human pancreatic endocrine cell differentiation is perhaps less defined. Unfortunately, biopsy samples of pancreas are not available for any of the patients to confirm the presence or absence of endocrine cells in the pancreatic tissues. The clinical course suggests that ultimately NGN3 does have a role in establishing sufficient β-cell mass to maintain normoglycaemia. However, whether other pathways for endocrine differentiation can be activated in the absence of NGN3 are yet to be determined. As such, whilst our data suggests a termination of NGN3-dependent
endocrine differentiation by term in human fetal development, this does not preclude further increases in endocrine cell mass by alternative pathways of neogenesis.

6.4.2. Defining the NGN3-positive cell transcriptome

In the most widely accepted model of pancreatic endocrine differentiation, Ngn3 is positioned at the top of the hierarchy of TFs whose orchestrated activation ultimately results in the generation of one of five islet cell types. As such, direct genetic targets for Ngn3 have been identified including NeuroD1, Pax4, Nkx2.2 and Ia1 (Gasa et al. 2004; Heremans et al. 2002; Huang et al. 2000; Mellitzer et al. 2006; Smith et al. 2004; Sosa-Pineda et al. 1997; Sussel et al. 1998). Factors affecting the regulation of Ngn3 are also starting to be deciphered. These include components of the Notch signaling pathway whose inhibition in select cells results in the activation of Ngn3 (Apelqvist et al. 1999). With its transient expression in both mouse and human pancreas (Gradwohl et al. 2000; Jensen et al. 2000; Jeon et al. 2009; Lyttle et al. 2008; Schwitzgebel et al. 2000; Sugiyama et al. 2007) the elusive NGN3-positive cell type is not well characterized in either species. We therefore sought to discover the phenotype of an NGN3-expressing cell.

Within the group a protocol has been developed using laser-capture microdissection to isolate regions of interest from archival fixed-tissue samples, and process them for downstream applications such as RNA-sequencing. This has been previously completed with some success. The same techniques were used with the additional step of IHC to identify and ultimately isolate NGN3-positive cells from human fetal pancreas.

Initially the process appeared to have worked with some success collecting a large number of NGN3-positive and neighbouring NGN3-negative cells using LCM. Processing for RNA extraction and cDNA synthesis showed reasonable amplification for GAPDH housekeeper gene using qRT-PCR, however this yield was improved following an amplification process. However, even at this stage NGN3 transcript detection was low by qRT-PCR. RNA quality was also confirmed using the Nanodrop spectrophotometer as described in the methods (section 2.4).

Amplified samples were processed for RNA-sequencing, a technology that has revolutionized transcriptome profiling (Wang, et al. 2009b). The process allows for the quantification of differential gene expression between two or more samples, based on both protein-coding and non-protein coding genes. Unfortunately the data we received back from the RNA-seq analysis were fairly damaged and not clean, and as a result it is difficult to be confident about any of the readings received. Consistent with this, a second
and even third repeat dataset would have been advantageous to decipher what is the true genetic profile for NGN3-positive cells, and what is experimental artifact, however, due to time restrictions and tissue availability this was not possible within this project.

The first warning sign for the quality of the LCM and RNA-seq experiment was the absence of NGN3 transcripts in the qRT-PCR data, which was also apparent in the mapped data viewed on the UCSC browser where no reads were observed to map to any of the genetic sequence for the length of the NGN3 gene. However, Ngn3 transcripts are only expressed for approximately 2 hours during development (Thummel, et al. 2005) and as such few transcripts may still only be present when the protein is detected (Desgraz and Herrera, 2009). Lack of NGN3 transcripts in the NGN3-protein-positive cells may simply reflect the longer half-life of the transcribed protein. Downstream targets of NGN3 were therefore also explored, including NEUROD1, PAX4 and NKX2.2. Consistent with the idea that NGN3-transcript has been expressed in the NGN3-protein-positive captured cells, a number of transcript reads had mapped to each of the downstream target genes in the NGN3-positive LCM sample. Although the frequencies were relatively low, this identifies the presence of transcripts for these genes in the NGN3-positive samples, assumptive that an endocrine differentiation programme downstream of NGN3 expression had been activated in these cells. SOX9 and PDX1 expression in both NGN3-positive and NGN3-negative samples confirms the pancreatic origin of these cells.

From a database of the highest expressing genes in each sample, the expression of selected transcripts was compared between the two LCM sample groups. As this was a statistically generated dataset, many of the genes of interest were not represented due to low or negligible read counts. However, some genes were listed, including PDX1 which had similar expression across the two positive and negative samples, SOX9 which was elevated in NGN3-positive cells and some of the hormones; insulin and glucagon which were also reduced in NGN3-positive cells whilst SS had equivalent expression in both. NEUROD1 was also present, and relative read-count expression between the two LCM samples showed a higher frequency of NEUROD1 transcripts mapping to the NEUROD1 gene in the NGN3-negative samples compared to NGN3-positive. This is somewhat confusing since NEUROD1 has been extensively described as a direct target of NGN3 and persists in the endocrine cell after NGN3-expression subsides to maintain the endocrine differentiation programme (Gasa et al. 2004; Gasa et al. 2008; Naya et al. 1997). However, there is also evidence to suggest an early requirement for NEUROD1 transcripts before NGN3-expression to delineate the α-cell lineage in the early pancreatic progenitor population (Mastracci et al. 2013).

Caution should be taken when interpreting these results however. Even when the reads are equivalent between the two samples does not necessarily mean that gene is highly
expressed. Low expression could be observed in both samples. Likewise, due to the relatively low read counts overall, a large relative increase in expression between the two samples (as observed in SOX9) could result from very low or absent readings in one sample compared to the other.

Since PDX1 is in the progenitor population and has also been shown to be coexpressed in NGN3-positive cells in human fetal pancreas (Lyttle et al. 2008), the equivalent expression observed between the NGN3-positive and NGN3-negative LCM samples might be appropriate. Likewise, since we have shown that NGN3-positive cells have initiated an endocrine differentiation programme with the onset of NEUROD1, PAX4 and NKX2.2, hormone expression would not be expected at this early stages, and this is consistent with the absence of hormone-positive staining in NGN3-positive cells (Apelqvist et al. 1999; Gradwohl et al. 2000; Jensen et al. 2000; Schwitzegebel et al. 2000) and the low expression of Insulin and Glucagon transcripts in the LCM NGN3-positive samples. The relatively higher expression of Insulin and Glucagon in the Ngn3-negative cells might represent a population of cells that were captured in the surrounding ductal epithelium, absent of NGN3-positive staining but representative of a later stage of endocrine-committed cells.

The aim of this work was to try and establish whether a threshold for NGN3 was necessary for NGN3-protein expression. Following IHC analysis, it is apparent that differing levels of NGN3-expression exist in cells; some showing strong positive staining, whilst in others, the staining is much weaker. It has already been shown that the NGN3 transcript is expressed more widely in the ductal epithelium than the protein, and therefore supports the hypothesis that a threshold level for NGN3-transcript must be exceeded in order for the protein to accumulate and drive the endocrine differentiation process (Villasenor et al. 2008).

Due to the inability to use a coverslip on the LCM material, it was very difficult to distinguish between varying degrees of positive staining. As such, only strongly NGN3-staining cells were captured in the NGN3-positive LCM collection. There is a chance however, that the NGN3-negative sample contained a heterogenous population of weak NGN3-positive cells, NGN3-negative and post-NGN3-expressing cells. Ideally the experiment would have focused on the NGN3<sup>high</sup> and NGN3<sup>low</sup> populations, to investigate if they contain the same transcriptomic profile, and will eventually reach the same fate. Unfortunately this was not possible with our protocol.

It is also likely that the protocol itself was incompatible with the LCM analysis. The process of fixing and embedding fetal tissue inadvertently results in a degree of RNA degradation (Benchekroun, et al. 2004) and it is likely that additional processing in the IHC protocol further reduced the quality of the RNA. Whilst our analysis at Nanodrop, qRT-
PCR and in-house quality control during the RNA-seq stage confirmed sufficient quality, the data we received were highly fragmented making it difficult to accurately map the fragments onto the human genome, or derive any meaningful information from them. Unfortunately at this stage there is no other way or identifying NGN3-positive cells, at least in human fetal pancreas, and as a result the experiment was terminated.

6.4.3. Further factors may be involved in endocrine specification

NGN3 is a key driver of endocrine differentiation and has been shown to not only induce premature endocrine commitment when early expression is forced in pancreatic progenitors, but also has shown some success in inducing non-pancreatic tissues towards pancreatic islet cell fates (Gasa et al. 2004; Heremans et al. 2002; Mellitzer et al. 2006).

In an era where diabetes is becoming a worldwide epidemic, it is becoming increasingly pertinent to understand the origins of the β-cell, with the promise that this knowledge will guide techniques towards yielding large, sustainable quantities for the treatment of this growing disease. At the same time, whilst NGN3 is certainly important for the effective differentiation of β-cells, there is emerging evidence to suggest a level of regulation above NGN3 which determines the cell fate before NGN3 is induced. This additional level of control would then render the NGN3-positive cell as a unipotent precursor to a specific hormone-secreting cell type (Nelson et al. 2007). Focus has now shifted to understanding what additional factors could influence the differentiation outcome prior to NGN3.

In mouse, premature expression of Ngn3 results in an abundance of glucagon-producing cells (Apelqvist et al. 1999; Schwitzgebel et al. 2000). It was later shown that expressing Ngn3 at different time points during development resulted in the differentiation of different cell populations at each stage. In this system, early expression results in the differentiation of α-cells only, whilst the other endocrine cell types are generated at later stages (Johansson et al. 2007). This suggests the existence of a temporal window during development within which each cell lineage can be produced. Changing conditions at various stages of fetal development could therefore impose restrictions on the NGN3-positive population and affect the cell-type they are competent to differentiate in to.

Whilst some of these conditions have not been conclusively explored, including signals from the mesenchyme or extracellular matrix, or the effects of islet vascularisation on the endocrine cell outcome, the influence of a number of TFs upstream of Ngn3 expression have been studied. In mouse, Nkx6.1 is initially expressed throughout the pancreatic epithelium from approximately E11 (Oster et al. 1998). The first insulin-positive cells appear around E13.5 and colocalise with NKX6.1 at this time (Oster et al. 1998).
However, between E15.5-E17.5, as the insulin-producing population is increasing, Nkx6.1-positive/hormone-negative cells are still observed in the ductal epithelium. After E18.5, Nkx6.1 is rarely seen in hormone-negative cells (Oster et al. 1998). This expression pattern fits with an early requirement for Nkx6.1 in endocrine progenitors that pre-determine the \( \beta \)-cell population as its expression does not coincide with the onset of glucagon staining which commences by E11 (Oster et al. 1998).

In our data, similarly to mouse, NKX6.1 expression is widespread in the CS20 human pancreas and becomes progressively restricted to individual ductal cells as development proceeds, an expression pattern not dissimilar to NGN3. Consistent with its course in mouse, by term in human pancreas NKX6.1 is restricted to the islet structures where it is maintained into adulthood. Upon closer analysis, from 8 wpc NKX6.1-positive cells could be observed in the epithelium that were negative for insulin reactivity as well as SOX9 immunoreactivity. This may be evidence for the commitment to the endocrine lineage. Even up until 21 wpc, NKX6.1-positive, Insulin-negative cells could be observed in the epithelium. However, without dynamic tracing studies it is not possible to determine whether any of the NKX6.1 expression observed outside of the differentiated endocrine lineage occurs in cells prior to the onset of NGN3. It is also not possible to determine the fate of these cells, and whether the presence of NKX6.1 in early progenitors identifies a cell destined for a specific fate. A pre-requisite for Nkx6.1, prior to Ngn3 has been shown to have an important role in the specification of the \( \beta \)-cell lineage in mice. In these experiments Nkx6.1 expressed in the PDX1-positive domain was able to restore \( \beta \)-cell numbers in an Nkx6.1/- background (Nelson et al. 2007).

In a separate study, a pre-Ngn3 requirement was also demonstrated for Nkx2.2 and NeuroD1 to direct endocrine differentiation (Mastracci et al. 2013). Nkx2.2 is an interesting TF as it is the only one to cause absolute \( \beta \)-cell loss when deleted (Sussel et al. 1998). Nkx2.2 has an early role in mouse pancreas development, where it has widespread expression in the early progenitor population and later becomes restricted to the endocrine (except \( \delta \)) cell lineage as islets develop (Sussel et al. 1998). During the human fetal pancreas grafting experiments, NKX2.2 was observed to have a similar window of expression as NGN3 (Capito et al. 2013), suggesting an additional role for the TF in identifying endocrine-competent progenitors. However, unlike NKX6.1 which appears to have a specific \( \beta \)-cell selection role, additional factors are involved in the specification process with NKX2.2. If both Nkx2.2 and NeuroD1 are expressed in Pdx1-positive progenitors in mouse, that cell is more likely to become a \( \beta \)-cell. If NeuroD1 is deactivated in the progenitor cell and instead Arx is activated, the Nkx2.2-positive/Pdx1-positive progenitor is likely to become an \( \alpha \)-cell (Mastracci et al. 2013).
Whilst NKX2.2 expression is not observed in human pancreas until 8 wpc (Jennings et al. 2013), by 15 wpc scattered NKX2.2 immunoreactivity was observed throughout the ductal epithelium, outside of hormone-producing cells. Consistent with the peak in NGN3 expression at this age, these hormone-negative, NKX2.2-positive cells could represent an endocrine progenitor, although again, limitations mentioned previously prevent conclusive definitions for the role of these cell types in human development.

Taken together this work demonstrates that additional factors, alongside NGN3, may identify a competence of the epithelium to permit differentiation into different endocrine cell types. Further analysis is necessary to conclusively define this process which is unfortunately limited in human samples. Further work would be focused on the PDX1-positive population and expression patterns in the early progenitor cells. Tracing expression patterns of NKK6.1, NKX2.2 and NEUROD1 throughout fetal development might help better characterize the process of differentiation throughout the NGN3-competent window.

Islets are made up of five endocrine cell types and the cell populations within them have been shown to be polyclonal (Scharffman et al. 2008). This means that each hormone-secreting cell has differentiated from a separate progenitor cell.

In the postnatal pancreas P27 is found in the cytoplasm of islets. However, during fetal development and in certain pathological situations, P27 can be seen within the nucleus of some insulin-positive cells and a subset of ductal cells (Chapter 4). P27 has a dual role in both promoting and preventing cell cycle progression, presumably another feature reliant on threshold requirements and post-translational modifications (Kulkarni et al. 2012). Regardless, in the pancreas when a β-cell is induced to proliferate P27 shuttles to the nucleus (Fiaschi-Taesch et al. 2013a).

During fetal development P27 was observed to colocalise quite specifically with NKX2.2 in both islet structures (Chapter 4) and in the ductal structures. In keeping with its presence in endocrine progenitors, P27 was also observed in the majority of NGN3-positive cells at the same age.

We and others have previously shown that P27-positive cells do not proliferate (Fiaschi-Taesch et al., 2013a) and this is consistent with the absence of PCNA from the NGN3-positive population in human fetal pancreas. NGN3-positive cells have a limited mitotic potential and have been shown to not proliferate at the time of Ngn3 expression (Gradwohl et al. 2000; Gu et al. 2002; Jensen et al. 2000; Maestro et al. 2003; Schwitzgebel et al. 2000). Likewise, differentiated endocrine cells have also been shown to have a limited proliferation capability (Bouwens et al. 1997). As such, some groups have concluded that β-cell mass is set early in life, and before birth (Dor et al. 2004).
However, we can’t rule out the reinstatement of a neogenic programme from a progenitor population postnataally and the prevalence of NGN3 in the adult pancreas remains contentious (Bonner-Weir et al. 2000; Gradwohl et al. 2000; Gu et al. 2002; Xu et al. 2008).

6.5. Summary

The results presented in this chapter disseminate for the first time the expression profile of NGN3 during human fetal development, including when it declines and disappears. A comprehensive investigation of this kind has not been done previously, largely in part due to the limited availability of human tissue.

Using IHC and cell counting analysis, as well as supporting qRT-PCR data, we have shown a peak expression for the pro-endocrine TF NGN3 between 10-17 wpc in human fetal development. Although both NGN3 protein and transcript were identified until 21 wpc, by term NGN3 protein was no longer observed and very low levels of transcript were detected, suggesting the window for endocrine differentiation had closed by this time. Our data are in line with those published in a recent study on human fetal allografts (Capito et al. 2013), and when the data are combined, both studies propose a closure for NGN3-dependent endocrine differentiation window between 26-35 wpc. After closure, increases in β-cell mass would rely on the balance of proliferation and apoptosis.

Of course, these data do not rule out the idea of postnatal remodeling and reappearance of NGN3-progenitors after birth, however our previous investigations in neonatal and early infant samples failed to show credible NGN3 protein staining or transcript levels at this age. As with all IHC techniques however, the sensitivity of the technique must also be considered since low levels of protein may have escaped detection.

Further to this window of differentiation we showed that NGN3-positive cells were non-proliferative in human fetal samples, and this coincided with a nuclear expression of P27 in NGN3-positive cells.

Attempts to determine whether an early expression profile for NKX6.1 and NKX2.2 in the developing pancreas specify distinct endocrine cell fates prior to NGN3 were also explored but without conclusive results.

Limited by the static tissue dynamics consistent with the use of fixed tissue, it was not possible to longitudinally trace progenitor cells to their ultimate cell fate. However, similar expression profiles coincident with the NGN3 expression window are supportive for a role for NKX2.2 and NKX6.1 in identifying an endocrine-competent progenitor pool at this time.
Emerging data proposing a unipotent potential for NGN3 progenitors has re-framed the endocrine differentiation pathway to include factors before the onset of NGN3 expression. Further work is therefore important to understanding the genetic programme that establishes the pancreatic β-cell, with the promise that advances in this understanding would help guide differentiation protocols for the production of sufficient quantities of functional β-cells for the treatment of diabetes.
Chapter 7- Discussion and future work

The overarching aim of this project was to explore the potential for β-cell regeneration based on our knowledge of β-cell development, and whether this is altered in pathological conditions. In doing this we also sought to characterise tissue samples of CHI-D in contribution to the growing knowledge in this disease area and to understanding its pathogenecity.

7.1. Congenital hyperinsulinism of infancy

To date there has been little consideration of CHI as a prerequisite to islet generation and the relative rarity of the disease means the literature in this area is also limited.

We have provided a detailed histological investigation of CHI-D, reporting on alterations not just within the β-cell lineage, but also in the wider pancreatic domain. This included an altered phenotype in the δ-cell population, where NKX2.2 expression persisted in the nuclear domain and nucleomegalic cells were also apparent.

Despite not being able to conclusively verify whether a process of endocrine differentiation, reminiscent of the fetal development programme, was occurring in the CHI-D pancreas, we did show an altered potential for cell proliferation across the whole organ.

We demonstrated that whilst enhanced proliferation in the endocrine lineage was subtle, more obvious increases were observed in the ductal and acinar cell compartments. Analysis of G1/S cell cycle components in CHI-D revealed, for the first time, an altered mitotic potential for CHI-D β-cells, based on the nuclear accumulation of P27 in islet cells and a shifted nuclear expression of CDK6 in some endocrine cells, in contrast to its cytoplasmic location in controls.

Despite the limited availability of CHI-D and reliable postnatal control tissue the work presented in this thesis was able to validate previous publications in the field in a new cohort of patients whilst also expanding on observations and providing new insights into possible mechanisms underlying the aetiology of the disease.

Indeed, genetic mutations have been identified for the majority of CHI-D cases, and the patients involved in this study all had a CHI-related mutation affecting the K_ATP channel. However, we have provided evidence to show that histological features of the disease likely extend beyond the underlying genetics.

Unfortunately work in this area is limited by the scarcity of tissue samples, and the extent of experimental techniques that can be employed to study this disease are restricted by
the fixed state in which the tissue is received. Animal models have been indispensable in generating some insight into the genetics and electrophysiology of CHI-D samples, however the translational significance of these experiments is let down by the differing features and clinical course of the disease between the two species. This makes studies on human tissue even more valuable.

Inter-patient variability was apparent between CHI-D samples, especially when qRT-PCR analysis was performed. It is known that CHI-D is a heterogenous disorder, the characteristics of which are unlikely to be caused by a single pathological entity. Unfortunately the low sample numbers and variances in age and genetic mutations between the samples makes it difficult to substantiate these differences and determine what is truly pathogenic of disease and what is normal variation. To over-come this problem, age-matched control samples were used in each experiment as a reference to which comparisons between CHI-D and “normoglycaemia” could be made. However, these samples are also rare and difficult to obtain and qualification of results described in this thesis would be of a significantly higher power with access to more samples.

Considering the limitations in this field, opportunities for further work would be involved in the investigation of developmental pathways, especially TF expression in mice models. These could provide greater detail and lineage tracing mechanisms in order to decipher if the loss of $K_{ATP}$ channel function affects their roles and expression in differentiating endocrine cells. It is anticipated that further work in this area will focus on teasing apart intrinsic defects resulting from CHI-D compared to those features that might be secondary to $\beta$-cell dysfunction. A greater dissection of abnormalities outside of the $\beta$-cell is also warranted to generate additional clues that could go some way to explaining the inappropriate insulin release in CHI-D.

Attempts were made within the group to isolate and culture CHI-D islets following surgical pancreatectomy. However, due to their diffuse distribution in the tissue, accuracy of extraction and ultimate yield of competent sample were not possible. Likewise, further efforts were employed to culture isolated adult islets in various conditions in an attempt to explore the mechanisms for cell cycle activation, based on knowledge of CHI-D. However, protocols were not sufficient to sustain the experiments, with too much tissue being lost between media changes. As such the tiny islet structures were too difficult to work with and produce meaningful results.

The majority of information on CHI-D comes from human tissue which in itself generates inherent variability since it is unlikely any two patients have the same genetic mutation, or have been exposed to the same environment during development and after birth. These additional factors need to be considered when trying to discern the aetiology of the disease.
This work has provided novel insights into the characteristics of the disease and its effect, whether primary or secondary, on the wider pancreatic populations. The early studies into cell cycle regulation in CHI-D have opened up further areas to focus research efforts including the investigation of possible pathways involved, such as hypoglycaemia, local elevations of insulin levels, high intracellular calcium concentrations or chronic membrane depolarisation. Further work would therefore be focused on exploring these mechanisms and the effects of exposing islets to these conditions and the expression and location of cell cycle markers.

A more detailed description of the β- or islet-cell phenotype in CHI-D samples, compared to controls, may also be valuable. Using techniques previously described in this thesis such as LCM, CHI-islets could be accurately isolated from fixed tissue samples and then processed for downstream applications such as microarray analysis or RNA-sequencing. This would provide a more reliable database of genetic expression profiles in a specific cell population. Problems with the current database available for CHI samples concern its reliability since the data contained within it are not ‘clean’. The CHI sample consists of a heterogenous pool of CHI-patient data which contains information from whole pancreas sections and are not lineage specific.

The role of nucleomegalic cells in CHI-D would also be of importance. Given their increased prevalence in pathologic samples, a more detailed analysis of their phenotype would be warranted. This could include FISH techniques to assess DNA content.

7.2. Human development

Diabetes mellitus is characterized by hyperglycaemia secondary to insufficient insulin activity, either due to a short-fall in β-cell mass, a resistance to insulin, or a combination of both. It is a chronic condition affecting approximately 3.9 million people in the UK and the impacts both on health and socioeconomic costs are substantial. Whilst insulin therapy permits a relatively normal life to be continued, there is currently no cure. With prevalence of diabetes mellitus reaching epidemic levels worldwide, there is a significant requirement for both sustainable and effective therapies for these patients.

Protocols for the differentiation of stem cells into functional β-cells offer the greatest promise for restoring insulin-cell mass in both T1DM and T2DM. A better understanding of normal β-cell development and function, however, will help facilitate progress in this area. Studies on human islet development have identified differences with the rodent species and as such, further research into the human-specific differentiation programme are of substantial importance.
In this thesis we have identified a window within which NGN3 is expressed, therefore identifying a competence period for endocrine differentiation which appears to be diminished by term. Whilst transient expression has also been demonstrated in the mouse, a comprehensive analysis of NGN3 expression has not been previously performed in human tissue. Although we are unable to confirm the significance of NGN3 in fetal development, and its importance in the differentiation pathway, further research in this area would be warranted. This is especially pertinent given the relatively late onset of hyperglycaemic symptoms that are apparent in patients with homozygous NGN3 mutations, suggesting the possibility of an alternate pathway for endocrine cell generation in this situation, or a redundant role for NGN3 in the differentiation of human pancreatic endocrine cells.

The idea that NGN3-positive cells are unipotent is also an intriguing concept and one that warrants further investigation. Provisional experiments were performed in an attempt to characterize the additional requirement of factors prior to NGN3 expression. These were based on previous publications that had identified NKX2.2, NKX6.1 and NEUROD1 as having a role in the pre-determination of endocrine cell fate in PDX1-positive progenitors before the onset of NGN3-expression in mouse. However, the results of our investigation were far from conclusive. This is in part due to the static nature of the tissue and the inability to trace cells dynamically to their ultimate fate, or to label them to assess whether their expression is before or after NGN3 expression in human samples. It is also not a complete piece of work; further investigations focused on dual expressing cell profiles including coexpression with PDX1 and NGN3, other TFs involved in the differentiation pathway and also islet hormones may have been beneficial in identifying cells that are early in the differentiation programme and those that are already committed.

An effective marker for pancreatic progenitor cells would also be advantageous, since both PDX1 and SOX9, whilst identifying multipotent progenitor cells in the early epithelium, have additional roles in the differentiated pancreas and therefore present an ambiguous cell type in IHC and IF experiments.

Unfortunately, working on fixed-tissue samples and relying on IHC experiments, we are limited by the availability of antibodies against specific antigens. As such various TFs that would have been interesting to investigate were not possible, such as NEUROD1, PAX4, PAX6, MAFA, MAFB, to name a few.

Techniques developed in LCM are revolutionizing research, providing a platform for detailed genetic characterisation of specific cell populations. Although unsuccessful on this occasion, other work within the group has produced encouraging results and we remain hopeful that this procedure can be optimized to produce genetic profiles for the NGN3-positive human endocrine progenitor cell in the future.
7.3 Concluding remarks

Work presented in this chapter has offered novel insights into the pathogenecity of the rare genetic disease, CHI-D. Whilst limited by the experimental procedures that could be exploited in this investigation, histological studies have identified previously unappreciated aspects of the disease involving additional pancreatic lineages outside of the insulin-secreting β-cell. Clues from this research were further used to investigate cell cycle regulation in human pancreatic progenitor populations. A peak expression of NGN3 within human pancreas development identified a window within which endocrine commitment was most prominent and its absence by birth and postnatally emphasises the importance of this critical period in the generation of hormone-secreting cells. Whilst further validation for the importance of this factor in the differentiation programme in human are necessary, we have provided new evidence to imply a mitotically inactive phenotype of the endocrine progenitor with new insights into cell cycle control mechanisms in these populations. New clues have been explored to investigate the potency of NGN3-expressing progenitor cells however the profile is one-dimensional and incomplete and therefore warrants further investigation. Radical techniques in LCM are generating novel advances in the field providing new techniques for harvesting a wealth of data from individual cell populations using tissue that was otherwise limited in experimental properties.

Whilst CHI does not appear to present a pathological model for the regeneration of β-cells, we have discovered a previously unappreciated involvement of other pancreatic lineages in the aetiology of the disease, including the SS-producing δ-cell. As such, we have shed light on some of the mechanisms that may be involved in CHI-D and the potential mechanisms for cell proliferation based on the cellular expression and location of cell cycle components. This work has built on the observations of others whilst presenting new theories and directions for ongoing research to explore. As such, aspects of the cell cycle regulation programme in CHI-D and postnatal control have been taken on by other members of the group with the hope of delineating mechanisms involved in driving cell cycle progression in the pancreatic islets. There are also plans to continue the LCM work on CHI-D islets and extend observations made in this thesis to include other types of CHI, such as CHI-F, both lesion and non-lesion, as well as atypical cases.

The work on human β-cell development and the role of NGN3 is certainly ongoing in the research group. Recent data from other LCM experiments have generated a set of new targets involved in the specification of the human pancreas, and this will no doubt generate further components to be explored in fetal samples.
Likewise, with the success of the human allograft experiments to model human pancreas development in a dynamic system, future collaborations between our two groups may help better define the window for endocrine cell generation whilst also studying expression profiles for other TFs involved in the differentiation programme.

Overall the results presented in this thesis, whilst the majority being descriptive, are novel and offer intriguing insights into the underlying pathology of CHI-D and the competency for endocrine differentiation throughout development and after birth.
Chapter 8- References


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29 April 2013

Prof Neil Harley
University of Manchester
AV Hill Building
Oxford Road
M13 9PT

Dear Prof Hanley

Title of the Research Tissue Bank: Early Pregnancy Tissue Collection-
(RESPNOW/0205)

REC reference: 13/NW/0205
Designated individual: Prof Giorgio Terenghi
IRAS project ID: 118755

Thank you for your letter of 22 April 2013, responding to the Committee’s request for further
information on the above research tissue bank and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair and
the study Lead-Reviewer.

We plan to publish your research summary wording for the above study on the NRES website,
together with your contact details, unless you expressly withhold permission to do so.
Publication will be no earlier than three months from the date of this favourable opinion letter.
Should you wish to provide a substitute contact point, require further information, or wish to
withhold permission to publish, please contact the Co-ordinator Mrs Firan. Jblj,
nrescommittee.northwest-haydock@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion of the above
research tissue bank on the basis described in the application form and supporting
documentation as revised.

The Committee has also confirmed that the favourable ethical opinion applies to all research
projects conducted in the UK using tissue or data supplied by the tissue bank, provided that the
release of tissue or data complies with the attached conditions. It will not be necessary for these
collectors to make project-based applications for ethical approval. They will be deemed to
have ethical approval from this committee. You should provide the researcher with a copy of this
letter as confirmation of this.

The Committee should be notified of all projects receiving tissue and data from this tissue bank
by means of an annual report.

A Research Ethics Committee established by the Health Research Authority
Duration of ethical opinion

The favourable opinion is given for a period of five years from the date of this letter and provided that you comply with the standard conditions of ethical approval for Research Tissue Banks set out in the attached document. You are advised to study the conditions carefully. The opinion may be renewed for a further period of up to five years on receipt of a fresh application. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

Additional conditions of approval

In addition to the standard conditions attached, ethical approval is subject to the following:

- On the PIS in the section headed Stem Cell, the sentence which makes reference to "very rarely using laboratory rats and mice for testing" should be removed.

Approved documents

The documents reviewed and approved at the meeting were:

<table>
<thead>
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<th>Document</th>
<th>Version</th>
<th>Date</th>
</tr>
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<tr>
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<td></td>
<td>06 March 2013</td>
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<td>25 February 2008</td>
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<tr>
<td>Other: Patient Details Sheet</td>
<td>1</td>
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<td>22 April 2013</td>
</tr>
<tr>
<td>Participant Information Sheet: Brief</td>
<td>3</td>
<td>22 April 2013</td>
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<td>Participant Information Sheet</td>
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<td>Protocol for Management of the Tissue Bank</td>
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<td>06 March 2013</td>
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<tr>
<td>Response to Request for Further Information</td>
<td>email</td>
<td>22 April 2013</td>
</tr>
</tbody>
</table>

Licence from the Human Tissue Authority

Thank you for providing a copy of the above licence.

Research governance

A copy of this letter is being sent to the R&D office responsible for Central Manchester University Hospitals NHS Foundation Trust. You are advised to check their requirements for approval of the research tissue bank.

Under the Research Governance Framework (RGF), there is no requirement for NHS research permission for the establishment of research tissue banks in the NHS. Applications to NHS R&D offices through IRAS are not required as all NHS organisations are expected to have

A Research Ethics Committee established by the Health Research Authority
included management review in the process of establishing the research tissue bank.

Research permission is also not required by collaborators at tissue collection centres (TCCs) who provide tissue or data under the terms of a supply agreement between the organisation and the research tissue bank. TCCs are not research sites for the purposes of the RGF.

Research tissue bank managers are advised to provide R&D offices at all TCCs with a copy of the REC application for information, together with a copy of the favourable opinion letter when available. All TCCs should be listed in Part C of the REC application.

NHS researchers undertaking specific research projects using tissue or data supplied by a research tissue bank must apply for permission to R&D offices at all organisations where the research is conducted, whether or not the research tissue bank has ethical approval.

Site-specific assessment (SSA) is not a requirement for ethical review of research tissue banks. There is no need to inform Local Research Ethics Committees.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

Here you will find links to the following:

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

b) Annual Reports. Please refer to the attached conditions of approval.

c) Amendments. Please refer to the attached conditions of approval.

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/hra-training/

13/NW/0205 Please quote this number on all correspondence

Yours sincerely

Signed on behalf of:
Dr Tim Sprosen
Vice-Chair
E-mail nrescommittee.northwest@haydock@nhs.net

Enclosures: Standard approval conditions
Copy to: Prof Giorgio Terenghi, University of Manchester
        Central Manchester University Hospitals NHS Foundation Trust

A Research Ethics Committee established by the Health Research Authority
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<td>Research Tissue Bank: Central Manchester University Hospitals NHS Foundation Trust</td>
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<td>REC reference number: 13/NW/0205</td>
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<tr>
<td>Name of applicant: Professor Neil Harvey</td>
</tr>
<tr>
<td>Date of approval: 20 April 2013</td>
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Ethical approval is given to the Research Tissue Bank ("the Bank") by the Research Ethics Committee ("the Committee") subject to the following conditions.

1. **Further communications with the Committee**
   1.1 Further communications with the Committee are the personal responsibility of the applicant.

2. **Duration of approval**
   2.1 Approval is given for a period of 5 years, which may be renewed on consideration of a new application by the Committee, taking account of developments in legislation, policy and guidance in the interim. New applications should include relevant changes of policy or practice made by the Bank since the original approval together with any proposed new developments.

3. **Licensing**
   3.1 A copy of the Licence from the Human Tissue Authority (HTA) should be provided when available (if not already submitted).
   3.2 The Committee should be notified if the Authority revokes the licence, varies the licensing conditions or revokes the Licence, or of any change of Designated Individual. If the Licence is revoked, ethical approval would be terminated.

4. **Generic ethical approval for projects receiving tissue**
   4.1 Samples of human tissue or other biological material may be supplied and used in a Research Ethics Committee established by the Health Research Authority.
research projects to be conducted within the establishment responsible for the Bank and/or by researchers and research institutions external to the Bank within the UK and in other countries in accordance with the following conditions.

4.1.1 The research project should be within the fields of medical or biomedical research described in the approved application form.

4.1.2 The Bank should be satisfied that the research has been subject to scientific critique, is appropriately designed in relation to its objectives and (with the exception of student research below doctoral level) is likely to add something useful to existing knowledge.

4.1.3 Where tissue samples have been donated with informed consent for use in future research ("broad consent"), the Bank should be satisfied that the use of the samples complies with the terms of the donor consent.

4.1.4 All samples and any associated clinical information must be non-identifiable to the researcher at the point of release (i.e. anonymised or linked anonymised).

4.1.5 Samples will not be released to any project requiring further data or tissue from donors or involving any other research procedures. Any contact with donors must be confined to ethically approved arrangements for the feedback of clinically significant information.

4.1.6 A supply agreement must be in place with the researcher to ensure storage, use and disposal of the samples in accordance with the HTA Codes of Practice, the terms of the ethical approval and any other conditions required by the Bank.

4.2 A research project in the UK using tissue provided by a Bank in accordance with these conditions will be considered to have ethical approval from the Committee under the terms of this approval. In England, Wales and Northern Ireland this means that the researcher will not require a licence from the Human Tissue Authority for storage of the tissue for use in relation to this project.

4.3 The Bank may require any researcher to seek specific ethical approval for their project. Such applications should normally be made to the Committee and booked via the NRES Central Allocation System.

4.4 A Notice of Amendment form should be submitted to seek the Committee’s agreement to change the conditions of generic approval.

5. Records

5.1 The Bank should maintain a record of all research projects to which tissue has been supplied. The record should contain at least the full title of the project, a summary of its purpose, the name of the Chief Investigator, the sponsor, the location of the research, the date on which the project was approved by the Bank, details of the tissue released and any relevant reference numbers.

5.2 The Committee may request access to these records at any time.

6. Annual reports

6.1 An annual report should be provided to the Committee listing all projects for which tissue has been released in the previous year. The list should give the full title of each project,

A Research Ethics Committee established by the Health Research Authority
the name of the Chief investigator, the sponsor, the location of the research and the date of approval by the Bank. The report is due on the anniversary of the date on which ethical approval for the Bank was given.

6.2 The Committee may request additional reports on the management of the Bank at any time.

7. **Substantial amendments**

Substantial amendments should be notified to the Committee and ethical approval sought before implementing the amendment. A substantial amendment generally means any significant change to the arrangements for the management of the Bank as described in the application to the Committee and supporting documentation.

The NRES Notice of Amendment form should be used to seek approval. The form is available at [http://www.nres.npsa.nhs.uk/applicants/after-ethical-review/amendments/#NoticesofSubstantialAmendment](http://www.nres.npsa.nhs.uk/applicants/after-ethical-review/amendments/#NoticesofSubstantialAmendment).

7.1 The following changes should always be notified as substantial amendments:

7.1.1 Any significant change to the policy for use of the tissue in research, including changes to the types of research to be undertaken or supported by the Bank.

7.1.2 Any significant change to the types of biological material to be collected and stored, or the circumstances of collection.

7.1.3 Any significant change to informed consent arrangements, including new/modified information sheets and consent forms.

7.1.4 A change to the conditions of generic approval.

7.1.5 Any other significant change to the governance of the RTB.

8. **Serious adverse events**

8.1 The Committee should be notified as soon as possible of any serious adverse event or reaction, any serious breach of security or confidentiality, or any other incident that could undermine public confidence in the ethical management of the tissue. The criteria for notifying the Committee will be the same as those for notifying the Human Tissue Authority in the case of research tissue banks in England, Wales and Northern Ireland.

9. **Other information to be notified**

9.1 The Committee should be notified of any change in the contact details for the applicant or where the applicant hands over responsibility for communication with the Committee to another person at the establishment.

10. **Closure of the Bank**

10.1 Any plans to close the Bank should be notified to the Committee as early as possible and at least two months before closure. The Committee should be informed what arrangements are to be made for disposal of the tissue or transfer to another research tissue bank.

10.2 Where tissue is transferred to another research tissue bank, the ethical approval for the

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A Research Ethics Committee established by the Health Research Authority
Bank is not transferable. Where the second bank is ethically approved, it should notify the responsible Research Ethics Committee. The terms of its own ethical approval would apply to any tissue it receives.

11. **Breaches of approval conditions**

11.1 The Committee should be notified as soon as possible of any breach of these approval conditions.

11.2 Where serious breaches occur, the Committee may review its ethical approval and may, exceptionally, suspend or terminate the approval.
A4-1. Coexpression of CDK6 with insulin in control islets

Dual immunofluorescence of 5 μm sections of human postnatal and adult pancreas (7 weeks- 10 months, adult) for CDK6 (red) and insulin (green) counter-stained with DAPI. Panels show islet structures where CDK6 is coexpressed with insulin. Scale bars represent 100 μm (7 weeks-10 months) and 10 μm (adult).

A4-2. Expression profiles for CDK6 and pRb in CHI-D and control pancreas
Bright field images of 5 µm sections of CHI-D and control pancreas (2-6 months) for CDK6 and pRb (brown) on sequential sections, counter-stained with Toluidine blue. Expression profiles within exocrine tissue are similar for CDK6 and pRb in CHI-D samples whilst CDK6 is cytoplasmic in the islets of postnatal control pancreas and pRb staining absent. Scale bars represent 50 µm.

A4-3. Network analysis and interactome model for CHI
(A) Interactome model derived from the published CHI database (Michelsen et al. 2011) showing significantly altered gene expression in CHI tissue. Cluster modules (coloured) were identified within the interactome model using Moduland algorithm. (B) Network of the cluster modules showing the most central gene to each one (Summarised in A4-4).
## A4-4. Highest expressing genes in CHI network analysis

The most central gene in the top 20 modules is shown in bold whilst other genes expressed within the top 10 are shown in brackets. These functional modules were ranked according to their index of centrality based on network analysis of the CHI database (Michelsen et al. 2011).

<table>
<thead>
<tr>
<th>Module rank</th>
<th>Most central gene in the module (others genes in the top 10)</th>
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<tbody>
<tr>
<td>1</td>
<td><strong>EP300</strong> (UBD, SIRT7, KAT2B, TP53, FN1, PRMT1, ARRB1, VCAM1, TRIM28)</td>
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<tr>
<td>2</td>
<td><strong>PML</strong> (UBD, DAXX, SUMO2, TRIM28, FN1, SUMO1, PIAS1, ARRB1, VCAM1)</td>
</tr>
<tr>
<td>3</td>
<td><strong>CDKN1B</strong> (CDK2, CDK7, CDK4, SKP2, CCT8, UBD, CCNE1, COPS5, VCAM1)</td>
</tr>
<tr>
<td>4</td>
<td><strong>NCOA2</strong> (ESR1, EP300, AR, GNAI2, PRMT1, FN1, SIRT7, SAFB, ACTR2)</td>
</tr>
<tr>
<td>5</td>
<td><strong>CAND1</strong> (CUL1, COPS3, FN1, CUL3, PHKG2, CUL4A, RNF7, SIRT7, UBD)</td>
</tr>
<tr>
<td>6</td>
<td><strong>PIK3R1</strong> (CRK, FYN, CBL, VAV1, ITSNI1, ERBB3, GRB2, EGFR, BCAR1)</td>
</tr>
<tr>
<td>7</td>
<td><strong>TBP</strong> (TAF9, TAF6, TAF10, TAF5, TCEA1, TAF1, TAF4, ELAVL1, GTF2B)</td>
</tr>
<tr>
<td>8</td>
<td><strong>HDAC5</strong> (AR, UBD, TBLIXR1, NCO1, PHKG2, ZBTB16, ARRB1, HNF4A, HSP90AA1)</td>
</tr>
<tr>
<td>9</td>
<td><strong>CDK7</strong> (TCEA1, CCNH, GTF2H2, POLR2A, SIRT7, ERCC3, MNTA1, GTF2H1, APP)</td>
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<tr>
<td>10</td>
<td><strong>COPS3</strong> (COPS5, COPS6, ERCC8, TK1, SIRT7, CUL3, DDB1, CUL4B, TOR1AIP2)</td>
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<tr>
<td>11</td>
<td><strong>MRE11A</strong> (SIRT7, RAD50, NBN, DDX1, TERF1, BRCA1, NRF1, HNRNPD, VCAM1)</td>
</tr>
<tr>
<td>12</td>
<td><strong>ZBTB16</strong> (HDAC1, HDAC5, TRIM28, FN1, PHB, DNMT3B, TK1, EHMT2, VCAM1)</td>
</tr>
<tr>
<td>13</td>
<td><strong>SMURF1</strong> (SMAD1, SMAD7, SMAD5, STRAP, NEDD4, APP, DCTN2, PHKG2, UBE2D3)</td>
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<tr>
<td>14</td>
<td><strong>FN1</strong> (SIRT7, HNRNPD, VCAM1, GAPDH, VHL, TK1, PHKG2, CCT8, ELAVL1)</td>
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<td>15</td>
<td><strong>BAG1</strong> (HSPA8, HSPA4, TTC1, ARRB1, NRF1, PHKG2, STUB1, TERF1, ACTR2)</td>
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<td>16</td>
<td><strong>NEDD4</strong> (UBE4B, UBE2D2, UBE2D3, UBE2L3, LAPTM5, MKRN3, MGRN1, ARRB1, UBE2D1)</td>
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<tr>
<td>17</td>
<td><strong>UBE2M</strong> (APP, NEDD8, PDA3, NRF1, RBX1, CLU, NDUFS6, DCUN1D1, UBA3)</td>
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<tr>
<td>18</td>
<td><strong>PSMA6</strong> (PSMA5, UBD, VCAM1, PHKG2, FKBP8, FN1, PSMA2, PSMA3, STK4)</td>
</tr>
<tr>
<td>19</td>
<td><strong>HEXIM1</strong> (BRD4, CDK9, CCNT1, EAF1, TERF1, RN7SK, NRF1MED12, STK4)</td>
</tr>
<tr>
<td>20</td>
<td><strong>RING1</strong> (BMI1, PHC1, RNF2, PCGF2, PHC2, INTS6, CBX4, TERF1, APP)</td>
</tr>
</tbody>
</table>
A4-5. Specificity of TUNEL staining in CHI-D samples

Dual immunofluorescence of 5 μm sections of CHI-D pancreas showing (top row) positive nuclear TUNEL staining (green), with insulin (red) and counter-stained with DAPI. The negative control shows absence of positive TUNEL staining when the TACs enzyme was omitted, proving specificity of the TUNEL staining. DNase was used to fragment DNA in the same tissue samples as a positive control, showing positive TUNEL staining in all cells. Arrows point to dual expressing apoptotic β-cells.

![Image of TUNEL staining and insulin expression in CHI-D samples](image-url)