Congenital Hyperinsulinism: a Unique Model to Study Pancreatic Islet Cell Development and Maintenance

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2013

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Abstract

Diabetes mellitus is a disease usually associated with insulin deprivation, insulin resistance and disordered insulin secretion. Conventional treatments can only control disease progression but do not have the ability to cure diabetes. Cell therapy has provided us with an alternative and better way to fight the disease. However, extreme shortage of islet cell surrogates is one of the obstacles that have been hampering the application of the technology. Congenital hyperinsulinism (CHI), a potentially fatal disease that has been characterised as “inappropriate release of insulin for the level of glycaemia”, has offered us a unique model for studying pancreatic islet cell development and maintenance. In this thesis, I aimed to investigate morphological and physiological alterations associated with CHI pathogenesis so that novel diagnostic tools and therapeutic options for CHI treatment could be developed.

By studying the architecture of the islets from CHI patients as well as a mouse model of CHI (SUR1-KO), I discovered that CHI was associated with morphological changes in islet structure and delay in islet cell maturation. This provided us with valuable information to advance our knowledge on CHI pathogenesis and in the diagnosis. Also, significantly elevated serum GLP-1:GIP ratio was noticed in atypical CHI patients, which suggests that serum incretin ratio could also assist the diagnosis and differentiation of atypical CHI.

Currently, treatment options available for CHI are limited due to adverse effects and limited efficacy. As a result, pancreatectomy is normally required in severe cases. Therefore, I intended to explore new treatment options for CHI. Currently available somatostatin receptor (SSTR) agonists mainly target SSTR2 and are widely used in CHI treatment to inhibit insulin release. However, there are concerns regarding techyphylaxis and the loss of natural counterregulatory response. Through immunohistochemical staining, I found that the loss of SSTR2 expression was evident in diffuse and atypical patients which could explain the sudden decrease in responsiveness to medication. After pancreatectomy and withdrawal of the SST analogue octreotide, SSTR2 expression recovered. SSTR2 expression was present in α-cells in almost all the patients, which questioned the usefulness of SSTR2-targeting SST analogues. Fortunately, SST analogue SOM230, which mainly targets SSTR1, 3 and 5, was found to be able to inhibit insulin release at a similar level to that of octreotide, indicating its potential in CHI treatment. Another medicine I investigated was GABA\textsubscript{B} receptor agonist baclofen. However, it could only suppress insulin secretion if there was a large enough elevation of insulin release which does not happen often in CHI.

In the present thesis, I have investigated morphological and physiological changes associated with CHI and evaluated the potential of new medical treatment options for CHI, which has implications in CHI management and our understanding on postnatal pancreatic development and maintenance.
Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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Acknowledgement

I would like to thank my supervisor Prof. Mark Dunne and Dr. Karen Cosgrove for firstly giving me the opportunity to work on this interesting but also labour intensive project. The constant support, suggestion and advice on the project that you have given me are invaluable and I am especially grateful for them. I would also like to thank my advisor Prof. Alison Gurney for priceless advice and comments throughout my PhD training.

I would like to express my appreciation to all former and present members in Dunne and Cosgrove lab, including Dr. Leon Adams, Dr. Lauren Eastwood, Dr. Georgios Anyfantis, Dr. Abu Sajib, Dr. Mars Skae, Dr. Anastasia Tsakmaki, Amna Gsour and Helga Palma Gutierrez, for your advice, help and, of course, funny jokes!

Throughout my entire PhD, I have worked closely with doctors and nurses at the Royal Manchester Children’s Hospital. Therefore, I’d like to show my sincere gratitude to all the people who have supported me for my work and research.

Finally, I want to thank my parents for financially and mentally supporting my PhD training. Without you, I would never have had the opportunity to become a doctor!
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>3-hydroxyacyl-CoA dehydrogenase</td>
<td>HDAH</td>
</tr>
<tr>
<td>3-Phosphoinositide–dependent protein kinase 1</td>
<td>PDK1</td>
</tr>
<tr>
<td>ATP-sensitive potassium</td>
<td>$K_{ATP}$</td>
</tr>
<tr>
<td>calpain-10</td>
<td>CAPN10</td>
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<tr>
<td>central nervous system</td>
<td>CNS</td>
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<tr>
<td>complementary DNA</td>
<td>cDNA</td>
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<tr>
<td>congenital hyperinsulinism</td>
<td>CHI</td>
</tr>
<tr>
<td>double-distilled water</td>
<td>ddH$_2$O</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay</td>
<td>ELISA</td>
</tr>
<tr>
<td>fish skin gelatin</td>
<td>FSG</td>
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<tr>
<td>fluorine-18 L-3,4-dihydroxyphenylalanine positron emission tomography</td>
<td>[$^{18}$F]-DOPA PET</td>
</tr>
<tr>
<td>genomic DNA</td>
<td>gDNA</td>
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<tr>
<td>glicentin-related pancreatic polypeptide</td>
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</tr>
<tr>
<td>GLP-1 receptor</td>
<td>GLP-1R</td>
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<tr>
<td>Glucagon-like peptide-1</td>
<td>GLP-1</td>
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<td>glucokinase</td>
<td>GCK</td>
</tr>
<tr>
<td>glucose-dependent insulinoieptropic polypeptide</td>
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</tr>
<tr>
<td>glucose-induced insulin secretion</td>
<td>GSIS</td>
</tr>
<tr>
<td>glucose transporter 4</td>
<td>GLUT4</td>
</tr>
<tr>
<td>glutamate dehydrogenase 1</td>
<td>GLUD1</td>
</tr>
<tr>
<td>hepatocyte nuclear factor 1α</td>
<td>HNF1A</td>
</tr>
<tr>
<td>hepatocyte nuclear factor 4α</td>
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</tr>
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<td>Term</td>
<td>Abbreviation</td>
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<tr>
<td>------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>human embryonic stem cells</td>
<td>hESC</td>
</tr>
<tr>
<td>human somatostatin receptor</td>
<td>hSSTR</td>
</tr>
<tr>
<td>induced pluripotent stem cells</td>
<td>iPSC</td>
</tr>
<tr>
<td>industrial methylated spirit</td>
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</tr>
<tr>
<td>insulin receptor substrates</td>
<td>IRS</td>
</tr>
<tr>
<td>insulin response substrate 2</td>
<td>IRS2</td>
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<tr>
<td>insulin-like growth factor-1</td>
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<tr>
<td>interventing peptide</td>
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<tr>
<td>inward rectifier potassium channel</td>
<td>Kir6.2</td>
</tr>
<tr>
<td>islet equivalents</td>
<td>IEQ</td>
</tr>
<tr>
<td>Kir6.2 knockout</td>
<td>Kir6.2-KO</td>
</tr>
<tr>
<td>Krebs ringer phosphate HEPES</td>
<td>KRH</td>
</tr>
<tr>
<td>low-$K_m$ hexokinase I</td>
<td>HK-I</td>
</tr>
<tr>
<td>major proglucagon fragment</td>
<td>MPGF</td>
</tr>
<tr>
<td>mesenchymal stromal cells</td>
<td>MSC</td>
</tr>
<tr>
<td>monocarboxylate transporter 1</td>
<td>SLC16A1</td>
</tr>
<tr>
<td>National Center for Biotechnology Information</td>
<td>NCBI</td>
</tr>
<tr>
<td>Neurogenin3</td>
<td>Ngn3</td>
</tr>
<tr>
<td>nonobese diabetic</td>
<td>NOD</td>
</tr>
<tr>
<td>non-significant</td>
<td>ns</td>
</tr>
<tr>
<td>Not determined</td>
<td>ND</td>
</tr>
<tr>
<td>Octamer-binding transcription factor-4</td>
<td>Oct4</td>
</tr>
<tr>
<td>oral glucose tolerance test</td>
<td>OGTT</td>
</tr>
<tr>
<td>Pancreatic/duodenal homeobox 1</td>
<td>PDX1</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
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<td>--------------</td>
</tr>
<tr>
<td>paraformaldehyde</td>
<td>PFA</td>
</tr>
<tr>
<td>PBS with 0.05% (v/v) Tween 20</td>
<td>PBST</td>
</tr>
<tr>
<td>peroxisome proliferator-activated receptor gamma</td>
<td>PPARγ</td>
</tr>
<tr>
<td>phosphate buffer saline</td>
<td>PBS</td>
</tr>
<tr>
<td>prohormone convertase</td>
<td>PC</td>
</tr>
<tr>
<td>readily releasable pool</td>
<td>RRP</td>
</tr>
<tr>
<td>Reverse Transcription- Polymerase Chain Reaction</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>somatostatin receptor</td>
<td>SSTR</td>
</tr>
<tr>
<td>Sry/HMG box transcription factor 9</td>
<td>Sox9</td>
</tr>
<tr>
<td>streptozotocin</td>
<td>STZ</td>
</tr>
<tr>
<td>sulfonylurea receptor 1</td>
<td>SUR1</td>
</tr>
<tr>
<td>SUR1 knockout</td>
<td>SUR1-KO</td>
</tr>
<tr>
<td>transcription factor-7-like 2</td>
<td>TCF7L2</td>
</tr>
<tr>
<td>Tris base, acetic acid and EDTA</td>
<td>TAE</td>
</tr>
<tr>
<td>Type 1 diabetes mellitus</td>
<td>T1D</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus</td>
<td>T2D</td>
</tr>
<tr>
<td>uncoupling protein 2</td>
<td>UCP2</td>
</tr>
<tr>
<td>v-maf musculoaponeurotic fibrosarcoma oncogene homologue A</td>
<td>MafA</td>
</tr>
<tr>
<td>weeks post-conception</td>
<td>w.p.c.</td>
</tr>
<tr>
<td>wild type</td>
<td>WT</td>
</tr>
<tr>
<td>β-cell-specific PDK1 deficient</td>
<td>βPDK1−/−</td>
</tr>
<tr>
<td>γ-aminobutyric acid</td>
<td>GABA</td>
</tr>
</tbody>
</table>
Chapter 1 Thesis organisation and structure

1.1 Rationale for submitting in alternative format

My PhD research involved several inter-related but individual projects and they all have generated potentially publishable data. Although manuscripts may require additional experimental work, presenting my thesis in alternative format has given me the chance to prepare potential publications at the same time as completing my thesis writing tasks. I believe this is an important opportunity to develop skills in writing manuscripts.

1.2 Structure of the thesis

The current thesis is written in alternative format following the instructions in the “Presentation of Thesis Policy” provided by the University of Manchester. The thesis is divided into 7 major chapters: Chapter 1 provides the outline of the thesis; Chapter 2 reviews previous research in the relevant field; Chapter 3-6 are “Results and discussion” chapters formatted suitable for publication; Chapter 7 is the summary that brings together all the outcomes to form a coherent story and points out future research directions. Since each “Results and discussion” chapter is presented in publishable format, it contains “Abstract”, “Introduction”, “Materials and methods”, “Discussion”, “Acknowledgement” and “Reference”. References from Chapter 2 and Chapter 7 are separately listed after Chapter 7. Detailed methodology employed during the research is included in Appendix A at the end of the thesis. Please notice that none of the “Results and discussion” chapter has actually been published.
1.3 Contribution of authors

The contribution of authors to each “Results and discussion” chapter is as follows:

Chapter 3: Differential Presentation of Pancreatic Islet Architecture in Congenital Hyperinsulinism.
Contribution: YS, MS and MD designed the experiments; YS and MS researched the data (MS only initiated the study); YS wrote the manuscript; BA and IB are clinicians who took care of the patients; NH and PC provided some research materials; KC contributed to the discussion; MD contributed to the discussion and reviewed the manuscript.

Chapter 4: Novel Variants of Congenital Hyperinsulinism Detected by Measurements of Serum GLP-1 (7-36) / GIP.
Contribution: KC initiated the study; IB, MS, PC, MD and KC designed experiments; YS, BA, and MS performed data collection; MN, LR provided histological data and analysis; IB, MS, RP and LR are clinicians who took care of the patients; Data were analysed by YS, BA, and KC; YS, MD and KC wrote the paper with contributions and discussion from all authors.

Chapter 5: Differential Inhibitory Effects of GABA_B Receptor Agonist Baclofen on Insulin Secretion in Isolated Islets from Mouse and Patients with Congenital Hyperinsulinism.
Contribution: YS and KC designed the experiments; YS, BA and LA researched the data; YS wrote the manuscript; BA, MS, and IB are clinician who took care of the patients; NH and PC provided some research materials; KC contributed to the discussion; MD contributed to the discussion and reviewed the manuscript.

Chapter 6: Expression of Somatostatin Receptors in Congenital Hyperinsulinism: SOM230 as a Potential Replacement for Octreotide.


Contribution: YS and MD designed the experiments and researched the data; YS wrote the manuscript; IB, BA, and MS are clinician who took care of the patients; NH and PC provided some research materials; KC contributed to the discussion; MD contributed to the discussion and reviewed the manuscript.
Chapter 2 Introduction

2.1 Diabetes mellitus

2.1.1 Diabetes epidemic

Diabetes mellitus, a disease that has a significant impact on public health globally, is considered one of the most serious threats to mankind. Surprisingly, as a non-communicable disease, diabetes mellitus is currently estimated to affect more than 200 million people and this number is projected to double by 2030 (Amos et al., 1997, Wild et al., 2004). Without proper blood glucose management, various complications resulted from long-term hyperglycaemia are well documented after years of research and clinical observation. Common complications consist of microvascular diseases (including diabetic neuropathy, nephropathy and retinopathy), macrovascular diseases (including coronary heart disease, stroke or cerebrovascular disease, and peripheral vascular disease) and many others (Amos et al., 1997). As a result, in the year 2000, more than 5% of “excess global mortality” was estimated to be attributable to diabetes and its complications, although this figure varies depending on region and age-group, with higher rates in more developed countries and middle-aged people (Roglic et al., 2005).

2.1.2 Current knowledge on diabetes

There are two major forms of diabetes mellitus: Type 1 diabetes mellitus (T1D) and Type 2 diabetes mellitus (T2D). T1D represents about 10% of diabetes cases and usually has an early onset but can occur at any age. In most cases, T1D is characterised as an autoimmune
destruction of pancreatic islet β-cells leading to a significant or complete insulin deprivation, which is likely caused by insulitis (inflammation of the pancreas associated with immunoregulatory T cell infiltration) (Kukreja et al., 2002). However, T1D also includes idiopathic cases and the patients are prone to ketoacidosis. The aetiology and pathogenesis of these idiopathic cases still remain undiscovered (Alberti and Zimmet, 1998). The treatment for T1D, on the other hand, is less complicated: insulin therapy is still currently the most favourite and effective treatment but cannot cure the disease; however, several other approaches have been used to assist or replace it and seem promising, including islet transplantation (Froud et al., 2005, Eisenbarth, 2007).

The second major form of diabetes is T2D, which is commonly recognised as either a disorder of insulin secretion, insulin resistance or both. Since T2D affects over 90% of the diabetic population (Zimmet et al., 2001), researchers all around the world are now putting more and more effort into investigation of the mechanisms behind T2D pathogenesis and the development of efficient treatments for T2D. So far, multiple contributors have been directly linked to T2D pathogenesis, including obesity leading to excessive accumulation of fatty acid metabolites, insulin receptor substrates (IRS) phosphorylation reduction, downregulation of glucose transporter 4 (GLUT4) expression, increased β cell apoptosis, reduced β-cell mass etc. (Fig. 2.1) (Froud et al., 2005, Stumvoll et al., 2005, Lin and Sun, 2010). In addition, genetic components have also been playing important roles in triggering T2D. For example, monozygotic twins have 2 to 2.5-fold concordance rate for diabetes compared to dizygotic twins (Ridderstrale and Groop, 2009). Also, the lifetime risk of developing T2D is about 40% higher in offspring who have a diabetic parent and this figure is calculated to be over 60% if both parents are diabetic (Stumvoll et al., 2005, Ridderstrale and Groop, 2009). So far, at least 19 candidate genes have been identified,
**Fig. 2.1** Current theories of T2D pathogenesis. Multiple factors have been identified so far to act as contributors to T2D, which include insulin resistance in multiple organs, adipose tissue dysregulation, β cell dysfunction, genetic components, and abnormal neurological responses (Stumvoll *et al.*, 2005, Lin and Sun, 2010).

Including peroxisome proliferator-activated receptor gamma (*PPARγ*), calpain-10 (*CAPN10*), transcription factor-7-like 2 (*TCF7L2*), ATP-sensitive potassium (*K\text{ATP}* ) channel subunit Kir 6.2 (*KCNJ11*) and others. These genes have been reported to be candidate genes for T2D based on whole genome association studies or candidate gene approaches, although the disease mechanisms have not been fully uncovered (Stumvoll *et al.*, 2005, O'Rahilly, 2009, Ridderstrale and Groop, 2009).
2.1.3 Cell therapy for the treatment of diabetes

As mentioned above, although insulin therapy has been widely used currently and shows good efficacy in controlling the disease and complication progression, it not only does not cure diabetes but often induces hypoglycaemia (Steffes et al., 2003). β-cell replacement therapies, including whole pancreas and islet cell transplantation, have been utilised by many clinical researchers and show evidence of normalisation of metabolism (Ricordi and Strom, 2004, Ren et al., 2007). The technique of pancreas transplantation was attempted as early as 1893 by the English surgeon Watson Williams, who attempted to replace the pancreas of a 15-year-old boy suffering from terminal diabetes, with a sheep pancreas fragment. Unfortunately, the boy died a few days after the operation due to immune rejection (Ricordi and Strom, 2004). It was not until nearly a century later people, for the first time, successfully reversed diabetes for a long term in clinical trials by using islet transplantation (Ricordi et al., 1992). Despite the excitement this breakthrough brings to us, limitations are severely hampering the application of this technique, among which immune rejection and lack of islet cell surrogates are the most outstanding.

When diabetic patients are transplanted with pancreases from sources other than the recipients, immunosuppression therapy would be required to protect these xenogeneic or allogeneic pancreases from being attacked by recipients’ immune system. Furthermore, autoimmunity that initially destroyed islet β-cells further complicates the situation (Huang et al., 2008). However, though patients who received pancreas transplantation no longer require exogenous insulin infusion for controlling glucose level, there has been no concrete evidence so far to prove that the treatment can reverse diabetes-induced complications. In fact, immunosuppressive drugs can be more nephrotoxic than diabetes itself. Indeed, transplant recipient survival may even be worse compared to insulin therapy treated
patients (Ren et al., 2007). Moreover, immunosuppressive drugs are associated with insulin resistance, inhibitory effect on insulin secretion (indicating β-cell toxicity) and possibly malignancy (Halban et al., 2010).

Islet cell transplantation received more attention than pancreas transplantation recently simply because it does not require major surgery and less immune rejection is expected due to the use of smaller volume of tissue in comparison to whole pancreas transplantation (Ren et al., 2007, Huang et al., 2008). In 2000, a group from University of Alberta, Edmonton reported their protocol (known as the “Edmonton protocol”) for islet transplantation and immunosuppression, which dramatically improved islet graft function. This protocol requires using high quality islets from two or more donors and steroid-free immunosuppression regimen (including anti-IL-2 receptor antibody (daclizumab) and chronic immunosuppression sirolimus (rapamycin) and tacrolimus (FK-506)) (Ren et al., 2007). However, in a recent international clinical trial, only 44% of diabetic patients receiving islet transplantation using Edmonton protocol reached primary insulin independence and, without obvious explanation, only 14% maintained this insulin-independent state two years after the transplantation (Shapiro et al., 2006).

To fulfil the requirement of islet transplantation, a huge amount of islet cells (over 10,000 islet equivalents (IEQ)/kg recipient body weight) is required (Huang et al., 2008). This results in severe shortage of islet resources due to imbalance between donors and patient numbers. Therefore, alternative islet cell surrogate sources are urgently needed to meet the demand. Islet cells extracted from pig has already been proved to be effective in diabetic non-human primates and have a relatively long survival time after xenotransplantation, although more research needs to be conducted before clinical trials on human patients can
be started (Dufrane and Gianello, 2009). Moreover, insulin-producing cells can be generated by differentiating human embryonic stem cells (hESC), pancreatic stem/progenitor cells, induced pluripotent stem cells (iPSC) and mesenchymal stromal cells (Schulz et al., 1998). Also, liver and intestinal cell trans-differentiation into functional β cells is another area that is under intensive investigation (Ling and Pipeleers, 1996, Aguayo-Mazzucato and Bonner-Weir, 2010). Besides, *ex vivo* human β-cell expansion is attracting many scientists due to the success on rodent models; however, low replication rate of human β-cells has been hampering its application. A process that involves de-differentiation, expansion and re-differentiation of β-cells has been reported to be capable of expanding β-cells efficiently, but low insulin expression level was obstructing further progress (Lechner *et al.*, 2005, Ouziel-Yahalom *et al.*, 2006). Nevertheless, recently, Russ and colleagues (2011) have successively expanded and re-differentiated β-cell-derived cells into insulin-producing β-cells *in vitro* using a cocktail of soluble factors, including glucose, N2 and B27 supplements, nicotinamide, exendin-4 and activin A.

### 2.2 Functional and developmental aspects of the pancreas

#### 2.2.1 The anatomy of the pancreas

Human pancreas consists of both exocrine and endocrine components. Exocrine acinar cells and duct cells are responsible for secretion and transport of digestive enzymes to the intestine respectively, whereas endocrine cells, which make up only a small proportion of the pancreas and form small clusters, called islets, secrete and transport hormones into the
bloodstream (Slack, 1995, Edlund, 2002). Islets are mainly composed of four types of cells: insulin-producing β cells, glucagon-producing α cells, somatostatin-producing δ cells and pancreatic-polypeptide-producing PP cells. Of these hormones, insulin and glucagon exhibit opposite effects on glucose homeostasis, and somatostatin and pancreatic polypeptide exert inhibitory effects on both endocrine and exocrine secretion (Edlund, 2002). In addition to these well-known cell types and their products, a fifth peptide hormone, called ghrelin, has been identified to be present in the islets. The expression of ghrelin, however, remains somewhat controversial. So far, it has been reported that this hormone is expressed in α-cells (Date et al., 2002), β cells (Volante et al., 2002) and a novel cell type – ε-cells and the function of this hormone in the islets has not been fully understood (Wierup et al., 2002, Prado et al., 2004). However, more recent findings have already suggested that ghrelin plays important roles in glucose homeostasis, as it regulates (possibly inhibits but still controversial) insulin secretion from β-cells and exerts protective effects on pancreatic islets and β-cell derived cell lines (Granata et al., 2010, Sangiao-Alvarellos and Cordido, 2010).

Islet architecture and composition varies among different species (Fig. 2.2). In mouse, α-cells and δ-cells, which represent a small proportion of the islet cells, reside at the periphery of islets whereas β cells present a core localisation. In pig pancreas, islets seem to be formed by small subunits that are similar to mouse islet organisation pattern (Cabrera et al., 2006). On the other hand, monkey and human islets have a distinctive islet composition. α- and β-cells in human and non-human primate islets are numerous and dispersed throughout the islets, while δ-cells and PP cells constitute a minor proportion of the islet cells (Brissova et al., 2005, Cabrera et al., 2006). Also, in human pancreas, the ratio of β-cells to α-cells has been found to be higher in the core area than the mantle.
position and decreases along with islet diameter increase. Furthermore, while whether all islet cells are adjacent to blood vessels remains controversial, evidence of heterologous contact between β-cells and α-cells and homologous contact among β-cells has been reported (Cabrera et al., 2006, Bosco et al., 2010).

Fig. 2.2 Cytoarchitecture of islets in different species. Significant interspecies differences of islet organisation have been observed among human (A), monkey (B), mouse (C) and pig (D). Images taken from (Cabrera et al., 2006).

2.2.2 Postnatal islet mass remodelling

2.2.2.1 Islet mass reduction causes diabetes

After years of intensive investigation, people have come to the consensus that, in T1D patients, severe or even complete islet cell loss (80-100% in recent onset patients and >99% in long-standing patients) due to autoimmunity is the major contributor to disease
aetiology (Mathis et al., 2001, Meier et al., 2005, Butler et al., 2007, Matveyenko and Butler, 2008, Meier, 2008). By using nonobese diabetic (NOD) mice (which mimics T1D), Sreenan et al. (1999) were able to demonstrate that progressive reduction of β-cell mass starts shortly after the onset of insulitis (4 week old). Subsequently, compared to age-matched control NOD/Scid mice (which have the same genetic background as NOD mice but do not develop diabetes due to severe immunodeficiency), at 13 weeks of age, a significant decrease of 42% of the β-cell mass was observed in non-diabetic NOD mice, whereas, at 18 weeks of age, a dramatic reduction of 73% of the β-cell mass is evident in NOD mice. This indicates that gradual islet mass loss is highly related to and even initiates disease onset in mouse, and these are consistent with the symptoms observed in human patients (Matveyenko and Butler, 2008).

The situation of T2D in humans, on the other hand, is more debatable since contradictory evidence has been reported (Butler et al., 2003). Yoon et al. (2003) reported an over 50% loss of islet mass in a Korean population compared to Body Mass Index (BMI) -matched control subjects, while a 30% decrease of total β-cell mass was identified in Japanese T2D patients (Sakuraba et al., 2002). On the contrary, one study reported that mean total β-cell mass was not significantly reduced in T2D patients, although dramatically lowered pancreatic weight was noted (Guiot et al., 2001). However, in 2008, the same group reported that European T2D subjects have significantly lower (approximately 39%) average β-cell mass compared to non-diabetic control subjects (Rahier et al., 2008).

Although people have not been able to elucidate all the controlling mechanisms that are involved in islet mass regulation, by using rodent models of T2D, a signalling pathway initiated by insulin or insulin-like growth factor-1 (IGF-1) has been suggested to be important. For example, 3-Phosphoinositide–dependent protein kinase 1 (PDK1) is a
downstream activator of PI 3-kinase. β-cell-specific PDK1 deficient (βPDK1−/−) mice has been shown to exhibit type 2 diabetic phenotype caused by significant loss of β cell mass, decrease in β-cell size and increase in β cell apoptosis (Hashimoto et al., 2006). Similarly, β-cell mass reduction in mice induced by insulin response substrate 2 (IRS2) knockout has been directly connected to the development of type 2 diabetic phenotype (Withers et al., 1998).

2.2.2.2 β-cell mass maintenance and expansion: cell division vs. differentiation of progenitor cells

β cell mass turnover and expansion have long been observed in laboratory rodents. During foetal and neonatal development, body mass increase and pregnancy, β cell mass have been reported to expand to either fulfil the need of development or compensate for the increase of insulin demand. It is important for the β-cell population to be closely regulated under the control of glucose homeostasis and hormone production; failure to adapt to changes in these aspects would result in chronic hyperglycaemia or diabetes (Bouwens and Rooman, 2005). Due to the obvious difficulty of replicating these studies in human subjects, there are only a limited number of circumstances which support the argument that β-cell mass can be increased in human (Butler et al., 2003, Meier et al., 2008). Although evidence from animal models, predominantly rodent models, suggest that islet cell numbers increase under various physiological conditions (Bernard-Kargar and Ktorza, 2001, Flier et al., 2001, Steil et al., 2001, Meier et al., 2008), we should notice that genetic and physiological differences between animal models and humans might cause incompatibility of the data (i.e. experimental results obtained by using animal models could lead to false assumptions, and conflicts could appear when we try to compare the data to those from human subjects). For example, while partial pancreatectomy usually triggers islet mass regrowth in rodents
(Finegood et al., 1999, Dor et al., 2004), it is sometimes not the case in adult humans, who show no sign of increased β-cell proliferation and deterioration in glucose control (Menge et al., 2008).

How could islet cell mass be remodelled in the pancreas? Do new islet cells come from cell proliferation or progenitor/stem cell differentiation? Massive efforts have been made by generations of investigators in order to address these questions; as a result, several different theories have been raised. One theory is that, the predominant source of new islet cells comes from replication of existing cells without the contribution from stem/progenitor cells. Indeed, there is good evidence to support this theory, since several groups have now reported that partial pancreatectomy (50-70%) fails to lead to the formation of new islet cell from stem/progenitor cells. These studies are largely based on lineage-tracing techniques which provide strong evidence to support the idea that β-cell replication is the major contributor to change in islet mass (Dor et al., 2004, Lee et al., 2006, Teta et al., 2007). However, one could suspect that such evidence cannot exclude the possibility that there is just not enough damage to initiate the participation of precursor cells. In fact, Finegood et al. (1999) found that, by treating pancreas with β-cell toxin streptozotocin (STZ), islet regeneration was still not inhibited after 90% pancreatectomy in rats. Moreover, they showed that the regeneration starts from the ductal area of the pancreas, which is consistent with another popular theory that ductal tissue is enriched with adult pancreatic progenitor cells (Bonner-Weir et al., 2000, Bonner-Weir et al., 2004, Bonner-Weir et al., 2008). Nevertheless, these precursor cells might need to undergo transient dedifferentiation before they can fully differentiate into islets or acini (Bonner-Weir and Weir, 2005). Furthermore, acinar cells have also been considered to be a progenitor cell pool for new islet cell formation, because they possess enough capacity of
acino-ductal metaplasia under the control of the oncogene \textit{Kras}. However, though transcription factor \textit{Neurogenin3 (Ngn3)} (also recognised as islet progenitor cell marker), Pancreatic/duodenal homeobox 1 (\textit{PDX1}) and \textit{v-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MafA)} expression can result in acino-insular metaplasia, there has been no evidence so far that this transdifferentiation really occurs after partial pancreatectomy, duct ligation or chemical-induced pancreatitis (Baeyens and Bouwens, 2009).

2.2.2.3 \textit{Pancreatic progenitor cells}

Pancreatic progenitor cells represent a group of precursor cells that have the capacity to be differentiated into pancreatic cell types. As mentioned above, their presence in the ductal area and acini of the pancreas has been confirmed (Bonner-Weir and Weir, 2005). However, it was unclear whether these pluripotent cells actually contribute to islet cell regeneration. Lee \textit{et al.} (2006) did not find Ngn3 expression in adult mouse pancreas and they claimed that ~50\% pancreatectomy did not induce Ngn3 expression. On the contrary, Xu \textit{et al.} (2008) demonstrated that, in mouse, upon partial pancreatic duct ligation, progenitor cells were activated to generate β-cells. These cells originated from areas near the pancreatic duct and a significant decrease of β-cell regeneration was noticed when Ngn3 was knocked down by interfering RNAs, indicating: 1) progenitor cells are important contributors to β-cell regeneration; 2) new β-cell formation after partial duct ligation is at least partly dependent on Ngn3 activation. Besides, they were able to extract those progenitor cells from the pancreas and culture and differentiate them into functional islet cells \textit{in vitro}. Indeed, Ngn3 expression is critical for pancreatic progenitor cell differentiation and specification during pancreas development (Apelqvist \textit{et al.}, 1999). Moreover, Wang \textit{et al.} (2009) reported that Ngn3 expression was sustained at a low level (compared to embryonic
endocrine progenitors) in adult mouse hormone-producing endocrine cells. Also, they found that this low level of Ngn3 expression is critical to mature β-cell function and maintenance but not cell division or cell death. The inactivation of Ngn3 resulted in down-regulation of a few genes that are important to endocrine cell differentiation and function, including insulin and MafA.

During the human foetal stage of human pancreatic development, another important stem/progenitor cell transcription factor - Octamer-binding transcription factor-4 (Oct4) has been found to be co-expressed in Nestin positive cells. Nestin is recognised as a stem/progenitor cell marker in the central nervous system development, and Nestin-positive cells isolated from mouse embryonic stem cells and human foetal pancreas are capable of differentiating into insulin-producing cells and other endocrine cell types (Wang et al., 2009). Apart from Ngn3 and Oct4, other progenitor cell regulators have been discovered in pancreas. For example, in mouse developing pancreas, Sry/HMG box transcription factor 9 (Sox9) was found to be crucial to pancreatic progenitor cell maintenance, proliferation, survival, persistency in undifferentiated state and pancreas organogenesis (Lynn et al., 2007, Seymour et al., 2007).

2.2.2.4 Glucagon-like peptide-1 (GLP-1) and islet mass
GLP-1 belongs to the glycoincretin family and is mainly expressed in intestine epithelium L-cells as a result of glucagon gene expression in response to food intake (Holst et al., 2008, Tomas and Habener, 2010). Recent findings suggest that secretion of GLP-1 by L-cells is regulated by 2 glucose-sensing mechanisms: one involves K_{ATP} channel closure and the other relies on sodium-glucose co-transporter (Reimann and Gribble, 2002, Gribble et al., 2003, Reimann et al., 2008). GLP-1 is one of the peptide products resulting from
Proglucagon processing; other major products include glucagon, GLP-2 and glicentin-related pancreatic polypeptide (GRPP) (Fig. 2.3). The GLP-1 hormones are composed of a complex group of peptides ranging from 28 to 37 amino acids, each of which has its own biological significance (Tomas and Habener, 2010). Although it is generally accepted that GLP-1 localisation is absent in pancreas, a few studies directly or indirectly discovered GLP-1 expression in pancreatic α cells (Heller and Aponte, 1995, Masur et al., 2005, Thyssen et al., 2006). Nevertheless, these results, to some extent, are debatable since, when immunohistochemical or immunofluorescent techniques are used, the selectivity of the antibodies directed against GLP-1 is a major factor that influences the accuracy of the data. In fact, the selectivity of the GLP-1 antibodies used is questionable due to the existence of GLP-1 precursors in α-cells (Patzelt et al., 1979, Patzelt and Schiltz, 1984, Deacon and Holst, 2009). However, it is too early to draw any conclusions since the presence of PC1/3 in α-cells in human also implicates GLP-1 expression (Portela-Gomes et al., 2008).

**Fig. 2.3 Proglucagon processing in pancreas and intestine.** Proglucagon is selectively cleaved by either prohormone convertase (PC) PC2 or PC1/3. PC2 cleavage results in free glucagon and major proglucagon fragment (MPGF); whereas PC1/3 cleavage leads to glicentin, GLP-1 and GLP-2 (Patzelt and Schiltz, 1984, Tomas and Habener, 2010). GRRP: glicentin-related pancreatic polypeptide; IP-1: intervening peptide-1; IP-2: intervening peptide-2; K: lysine; R: arginine. Picture adapted from (Tomas and Habener, 2010).
The GLP-1 receptor (GLP-1R) belongs to the G protein-coupled receptor family and is 64 kDa in size. GLP-1R has seven transmembrane domains and was first cloned from rat by Thorens in 1992 (Thorens, 1992). The localisation of GLP-1R in the pancreas has been somewhat controversial since evidence that supports either side keeps accumulating in the literature. GLP-1R expression in the islets was first identified to be present in several β-cell lines and a δ-cell line (Goke and Conlon, 1988, Fehmann and Habener, 1991). Subsequently, Heller and Aponte (1995) observed and Tornehave et al. (2008) further confirmed GLP-1R expression in α-, β-, and δ-cells in rat in vitro. On the other hand, GLP-1R mRNA expression has been found in β-cells but not in α- and δ-cells. Moreover, effects of GLP-1 on α-, β- and δ-cells in humans and rodents have been gradually demonstrated (Schmid et al., 1990, Fehmann and Habener, 1991, Heller and Aponte, 1995, Heller et al., 1997, Lupi et al., 2010). More importantly, Tornehave and colleagues (2008) proved that GLP-1R mRNA and mature protein expression are restricted to β-cells in mouse, rat and human fixed pancreatic tissue (different from using single cells as used by previous studies). Also, they found that GLP-1R is localised to large pancreatic ducts in all three species, which could correlate with the effect of GLP-1 on pancreatic progenitor cell differentiation in the duct (Doyle and Egan, 2007).

Actions of GLP-1 in pancreas are well-established. In short, GLP-1 is responsible for enhancing insulin secretion, β-cell proliferation and neogenesis, and inhibiting β-cell apoptosis through multiple downstream pathways of the cAMP signalling pathway, including second messenger PKA dependent pathways, PI3-Kinase dependent pathways and Epac pathway. Moreover, GLP-1 has been reported to affect insulin exocytosis, KATP channel closure, intracellular calcium release, insulin transcription, β cell apoptosis and mitosis (Doyle and Egan, 2007, Vilsboll, 2009). However, the correlation between GLP-1
expression and the pathogenesis of T2D is somewhat debatable, as, compared to non-diabetic control subjects, normal, elevated and reduced level of GLP-1 expression in T2D patients have been observed in different studies (Diab and D’Alessio, 2010). Also, variation of GLP-1 secretion has been observed in both T2D patients and healthy controls, suggesting that GLP-1 secretion is highly variable between individuals (Meier and Nauck, 2010). Thus, it seems the variation of GLP-1 expression is rather a consequence of T2D than a contributor to the disease onset (Meier and Nauck, 2008). Therefore, GLP-1 and its stable analogue Exendin-4 (Exendin-4 is more favourable, as GLP-1 has a very short half-life in the circulation upon secretion ($t_{1/2} = 1\text{-}2$ min) and is rapidly degraded in vivo by dipeptidyl peptidase-IV (DPP-4) (Holst et al., 2008, Tomas and Habener, 2010)) have already been used to treat T2D diabetes (Kolterman et al., 2003).

2.3 Congenital Hyperinsulinism (CHI)

2.3.1 Current Knowledge on CHI

George Laidlaw first described CHI as “nesidioblastosis” in 1938 (Laidlaw, 1938). From then on, CHI has been referred to idiopathic hypoglycemia of infancy, leucine-sensitive hypoglycemia, islet dysregulation syndrome, hyperinsulinism in infancy and persistent hyperinsulinemic hypoglycemia of infancy (Dunne et al., 2004, De León and Stanley, 2007). CHI is a rare and potentially lethal pancreatic β-cell disorder. It presents as a severe heterogeneous disease in infants and children, which is best described as “inappropriate insulin release for the level of glycaemia” (Dunne et al., 2004). CHI is associated with brain injury and mental retardation in a large number of patients (De León and Stanley, 2007). The incidence of CHI varies from 1 in 2,500 to 1 in 50,000 depending on
consanguinity in the society (James et al., 2009). Pathologically, CHI can be divided into two groups: “channelopathies” and “metabolopathies”. Channelopathies means that the disease is caused by a dysregulation of insulin secretion as a result of defects in the $K_{ATP}$ channel in pancreatic β cell $K_{ATP}$ channel (Fig. 2.4); metabolopathies refers to either intracellular signaling molecule concentration abnormalities or accumulation of intermediary metabolites (Hussain, 2008).

![Fig. 2.4 K$_{ATP}$ channel dysfunction leads to CHI.](image)

(A) In healthy pancreatic β-cells, glucose stimulation raises ATP / ADP ratio, which in turn closes $K_{ATP}$ channel and result in cell membrane depolarization, opening of voltage-gated $Ca^{2+}$ channels and exocytosis of insulin granules. (B) Loss of function mutations in $K_{ATP}$ channel in β-cells cause persistent cell membrane depolarization, constant $Ca^{2+}$ influx and uncontrolled insulin release (Dunne et al., 2004). Picture gifted from Mark Dunne.

Approximately half of the cases present as transient form of CHI (Banerjee et al., 2011) and little is known about the mechanisms of this particular form of CHI and how it is spontaneously resolved, although patients often present with long term abnormal neurological outcomes (Avatapalle et al., 2013). For patients with persistent CHI, on the other hand, the known molecular causes of CHI vary between 55% and 88% of the patients.
(Banerjee et al., 2011, Kapoor et al., 2013, Snider et al., 2013). Nonetheless, mutations in \( \text{K}_{\text{ATP}} \) channel genes (\( \text{ABCC8} \) and \( \text{KCNJ11} \)) have been suggested to lead to defects in channel production, protein folding and assembly, trafficking and function (Dunne et al., 2004). As the most common cause of CHI, so far, more than 150 mutations in these two genes have been identified in both recessive and dominant forms of CHI (James et al., 2009). Other candidate genes include: \( \text{HNF1A} \) (hepatocyte nuclear factor 1α), \( \text{NADH} \) (3-hydroxyacyl-coenzyme A dehydrogenase), \( \text{GLUD1} \) (glutamate dehydrogenase 1), \( \text{HNF4A} \) (hepatocyte nuclear factor 4α), \( \text{UCP2} \) (uncoupling protein 2), \( \text{GCK} \) (glucokinase) and \( \text{SLC16A1} \) (monocarboxylate transporter 1) (James et al., 2009).

Histologically, CHI can be classified into three sub-groups: diffuse, focal and atypical. The focal form of CHI is sporadic while diffuse CHI is inherited in an autosomal recessive or dominant manner (James et al., 2009). Diffuse CHI is usually associated with whole pancreas dysfunction and enlarged nucleus. Focal CHI is characterised as adenomatous islet cell hyperplasia and sometimes enlarged nuclei in the lesion, but normal morphology is expected in the surrounding tissue (Giurgea et al., 2006). Multiple studies have shown increases of islet cell mass in focal CHI (de Lonlay-Debeney et al., 1999, Kloppel et al., 1999, Kassem et al., 2000, Fournet et al., 2001, Kassem et al., 2001). However, this could not be explained by an elevated proliferation rate in the lesion since the apoptosis rate was also increased, and a correlation could not be determined (Kassem et al., 2000). Nevertheless, it is suggested that islet cell hyperplasia in focal CHI is associated with somatic loss of maternal imprinting which leads to unbalanced expression of several genes that regulate cell proliferation, including \( \text{p57KIP2} \), \( \text{IGF2} \) and \( \text{H19} \) (James et al., 2009). A third type of CHI has been identified, and genetic, morphological and immunohistochemical analysis revealed its distinctive features: lack of genetic mutation in
ABCC8, KCNJ11 and GCK; late onset (up to several months after birth); morphological mosaicism of the islets; loss of responsiveness to medication after medical treatment and a requirement of surgical intervention to regain euglycaemia (Sempoux et al., 2011). More recently, this rare form of CHI has been found to associate with excessive expression of low-\(K_m\) hexokinase I (HK-I) or a GCK activating mutation in hyperactive islets while resting islets appeared to be “normal” (Henquin et al., 2012).

2.3.3 Diagnosis of CHI

CHI typically presents within a few days after birth (Giurgea et al., 2006, Hussain, 2008, Arnoux et al., 2010). Half of the patients manifest severe symptoms such as seizure and coma while others might present with non-typical symptoms like hypotonia, poor feeding, cyanosis, hypothermia, abnormal movements, lethargy or irritability (de Lonlay-Debeney et al., 1999, Aynsley-Green et al., 2000, de Lonlay et al., 2002, Hussain, 2008, Kapoor et al., 2009). The majority of the patients are macrosomic, which indicates prenatal hyperinsulinaemia; however, the absence of macrosomia does not rule out CHI (Aynsley-Green et al., 2000, Hussain, 2008, Arnoux et al., 2010). Some patients with CHI may have mild facial dysmorphism such as a high forehead, a small nasal tip, and short columella with a square face. Some patients are discovered during routine blood glucose screening (de Lonlay et al., 2002, Giurgea et al., 2006). CHI patients with late onset hypoglycaemia (1–12 months of age) have similar clinical features, but usually require lower rates of glucose administration to maintain euglycaemia (de Lonlay et al., 2002, Giurgea et al., 2006).

Once persistent hypoglycaemia has been discovered, measurements of the following
hormones and metabolites should be completed immediately during hypoglycaemic episodes: blood glucose, lactate/pyruvate, ketone bodies, free fatty acids, amino acids, ammonia, total/free carnitine, acylcarnitine profile, insulin/C-peptide, cortisol/growth hormone, and urine ketones, reducing substances, organic acids (Aynsley-Green et al., 2000, Hussain, 2008). The diagnostic criteria of CHI include: (1) blood glucose ≤ 3 mmol/l; (2) plasma [insulin] > 1mU/l; (3) glucose infusion rate >8mg/kg/min; (4) glycaemic response to glucagon injection; (5) hypoketonaemia; (6) low fatty acids; and (7) hyperammonaemia (only happens in patients with GLUD1 mutations) (Aynsley-Green et al., 2000, Hussain, 2008, Arnoux et al., 2010). Once CHI is confirmed, further investigation should commence immediately, along with specific medical treatments, to determine the cause of the disease. With recent advances in imaging technology and molecular biology, genotyping of regular suspects (ABCC8 and KCNJ11 etc.) (Banerjee et al., 2011), fluorine-18 L-3,4-dihydroxyphenylalanine positron emission tomography computed tomography ([18F]-DOPA PET-CT) (Otonkoski et al., 2006) and post-operative histological examination of the pancreas should be used to make a definitive diagnosis.

2.3.3 Therapeutic options for CHI

Once the patients have been diagnosed for CHI, various acute treatments including constant glucose infusion (to prevent neurological damage caused by persistent hypoglycaemia), continuous intravenous infusion of glucagon and K<sub>ATP</sub> channel inhibitor diazoxide are administered. Depending on patients’ responses after the treatment, subsequent medical or surgical approaches are applied (Dunne et al., 2004, De León and Stanley, 2007, Hussain, 2008, Arnoux et al., 2010). However, patients who are responsive to diazoxide treatment are at high risks of various side effects, including sodium and water
retention, edema, pulmonary hypertension, heart failure and, most commonly, hypertrichosis (Dunne et al., 2004, Arnoux et al., 2010). While focal CHI patients can often be cured by limited resection of the lesion in the pancreas, the situation in diffuse CHI patients is more complicated as unresponsiveness or poor response to diazoxide and other drugs often leads to subsequent subtotal to near-total pancreatectomy, which usually causes pancreatic insufficiency and high incidence of diabetes mellitus in the late ages. Also, sometimes re-operation is required for some of the patients because of persistent hypoglycaemia (Meissner et al., 2003, Dunne et al., 2004, Arnoux et al., 2010).

For patients who are unresponsive to diazoxide, apart from pancreatectomy, there might still be some other therapeutic options available. Human somatostatin receptor (SSTR) are cell membrane G-protein-coupled receptors and have five subtypes SSTR1-SSTR5, with highest sequence identity between SSTR1 and SSTR4 (Patel and Srikant, 1997). The expression profiles of SSTRs in human are somewhat controversial. Kumar et al. (1999) reported that, in healthy human islets: (1) β-cells are enriched with SSTR1, 2 and 5, whereas SSTR3 and 4 are relatively poorly expressed; (2) SSTR2 is the dominant SSTR expressed in α-cells followed by SSTR5, 1 and 3 while SSTR4 is absent; (3) δ-cells predominantly express SSTR5, and SSTR1-3 were found in a small population of cells, while SSTR4 is also absent. However, by using receptor autoradiography with subtype-selective ligands, Reubi et al. (2001) only found SSTR2 to be present in the pancreatic islets. Recently, Portela-Gomes and colleagues (2010) claimed that SSTR1-5 are all present in α-, β-, δ- and PP cells, with the exception that SSTR5 was not detected in PP cells. These discrepancies could well be caused by differences in the tissue, antibodies, ligands and detection methods used.
SST analogues octreotide and lanreotide have been a potent drug in treating CHI patients due to its ability to mimic actions of SST by inhibit insulin release through the activation of SSTRs. Multiple mechanisms have been suggested to be involved in the inhibitory effects of SST on insulin secretion in rodents, including: inducing β-cell membrane potential hyperpolarisation and therefore averting Ca$^{2+}$ influx, inhibiting voltage-gated Ca$^{2+}$ channels, suppressing secretory granule release, etc. (Dunne et al., 2004, Strowski and Blake, 2008). The effects of octreotide in suppressing insulin secretion in CHI patients are well documented (Mazor-Aronovitch et al., 2007, Sherif et al., 2010, Loechner et al., 2011). Although not been confirmed in CHI, it is proved that octreotide’s effects on cells are predominantly through SSTR2 activation and internalization both in vitro and in vivo (Lesche et al., 2009, Reubi et al., 2010). Pasireotide (SOM230), another multi-receptor binding SST analogue, has been developed to treat patients suffered from acromegaly, Cushing’s disease and gastroenteropancreatic neuroendocrine tumors, which cannot be well controlled by octreotide. Compared to octreotide, the binding affinity and functional activity of SOM230 are much higher on SSTR1, 3 and 5 but lower on SSTR2 (Schmid, 2008). Since SSTR2 is highly likely expressed in α-cells (Kumar et al., 1999, Reubi et al., 2001, Portela-Gomes et al., 2010), SOM230 seems to be a better therapeutic option than octreotide when used to treat CHI.

γ-aminobutyric acid (GABA) is a major neurotransmitter in the central nervous system (CNS) and also regulates hormone secretion and islet cell function (Franklin and Wollheim, 2004, Ligon et al., 2007). In the pancreas, GABA is released from β-cells and modulate the secretion of insulin via metabotropic GABA$_B$ receptor (Brice et al., 2002, Braun et al., 2004, Braun et al., 2010) and glucagon through ionotropic GABA$_A$ receptor (Rorsman et al., 1989, Wendt et al., 2004, Xu et al., 2006). GABA$_B$ receptor agonist baclofen has been
previously shown to inhibit glucose-stimulated insulin secretion (GSIS) in MIN6 cells (Brice et al., 2002), isolated rat islets (Gu et al., 1993, Brice et al., 2002, Braun et al., 2004) and isolated mouse islets (Bonaventura et al., 2012). Therefore, baclofen seems to be a suitable novel candidate for CHI medical management.

2.4 Aims and objectives

As the most promising way of treating T1D is islet transplantation, it is critical to explore new avenues for ex vivo islet cultivation. Thus, CHI has provided us with a unique opportunity to study islet cell generation, development, maintenance and function. After years of research, CHI is well characterised as an insulin secretion disorder and often associated with β cell hyperplasia (Dunne et al., 2004). However, mechanisms underlying CHI pathophysiology have not been fully uncovered. Moreover, current treatment options are limited in severe cases due to lack of efficacy and strong adverse effects of the medication. Furthermore, present diagnostic methods are not fully capable of the early detection of CHI and rapid diagnosis and differentiation of the disease. Therefore, the aims and objectives of this thesis are:

(1) Chapter 3: to investigate the morphological changes of the islet structure in CHI and their significance to disease pathogenesis and progression by examining the distribution patterns of α- and β-cells in CHI patients and SUR1-KO mice;
(2) Chapter 4: to test the hypothesis that serum levels of enteroendocrine incretins GLP-1 and GIP vary among different forms of CHI and to see if this can be used as a biomarker for the diagnosis of CHI;
(3) Chapter 5: to study the efficacy and mechanism(s) of GABA\(_B\) receptor agonist baclofen in suppressing insulin secretion in CHI;
(4) Chapter 6: to examine the pancreatic expression of SSTRs in CHI and to evaluate and compare the efficacy of SST analogues octreotide and SOM230 in inhibiting insulin release.
Chapter 3: Differential Presentation of Pancreatic Islet Architecture in Congenital Hyperinsulinism

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3.1 Abstract

Congenital hyperinsulinism (CHI) is a potentially lethal pancreatic insulin secretion disorder that predominantly manifests during infancy. For about 50% of the cases, genetic mutations in genes encoding ATP-sensitive potassium channels and other β-cell metabolism regulatory genes have been identified. Disease pathogenesis has been associated with β-cell hypertrophy, hyperplasia and hyperfunction. Histologically, the disease is classified into diffuse, focal and atypical forms. Counterregulatory response (i.e. secretion of various hormones to form a primary defence against hypoglycaemia) has been
found to be blunted in CHI but the underlying mechanism is poorly understood. The
collection of glucagon-producing α-cells to disease progression and severity remains
elusive. In this study, we have examined insulin and glucagon expression in all three forms
of CHI and compared as well as normal human controls and mouse model of CHI for
comparison. Results suggest that, while diffuse CHI had normal islet formation, focal and
atypical CHI presented with abnormal islet architecture. Also, delayed postnatal islet
development, as indicated by co-expression of insulin and glucagon in a subpopulation of
islets, was demonstrated in all three forms of CHI. Our findings have provided useful
information to assist the diagnosis of CHI and have furthered our understanding on disease
pathogenesis and postnatal islet development.
3.2 Introduction

CHI is the most common cause for hypoglycaemia in children (De León and Stanley, 2007). This potentially lethal pancreatic β-cell disorder is characterised as inappropriate release of insulin for the level of glycaemia (Dunne et al., 2004). Molecular causes in approximately half of the CHI cases have been identified and the majority can be attributed to the loss of function mutations in ATP-sensitive potassium (K$_{ATP}$) channel subunit encoding genes- $ABCC8$ (sulfonylurea receptor 1 subunit (SUR1)) and $KCNJ11$ (inward rectifier potassium channel subunit (Kir6.2)), both of which are located in the 11p15.1 region. Rarer forms CHI have been found to be associated with genetic mutations in $GCK$ (glucokinase), $GLUD1$ (glutamate dehydrogenase 1), $HNF4A$ (hepatocyte nuclear factor 4α), $SLC16A1$ (monocarboxylate transporter 1), $HNF1A$ (hepatocyte nuclear factor 1α), $UCP2$ (uncoupling protein 2) and $HDAH$ (3-hydroxyacyl-CoA dehydrogenase) (Gonzalez-Barroso et al., 2008, James et al., 2009, Stanescu et al., 2012). The diagnosis of CHI is usually based on multiple criteria via serology (serum levels of glucose, insulin and/or C-peptide, ammonia, ketone bodies, fatty acids), urinary biochemistry (levels of acylcarnitines and/or organic acids), fluorine-18 L-3,4-dihydroxyphenylalanine positron emission tomography ([$^{18}$F]-DOPA-PET) scan, genotyping and post- or intra-operative histological examination of the morphology of the islets (De León and Stanley, 2007, Hussain, 2008, Kapoor et al., 2009).

Histologically, two major histological forms, diffuse and focal, of CHI have been recognised. Diffuse CHI affects the entire pancreas and usually presents as enlarged nucleus in the islets, indicating β-cell hyperactivity. Focal CHI, on the other hand, is often localised to a discrete region of the pancreas and usually presents as adenomatous hyperplasia. Outside the focal lesion, islets are small and resting with collapsed cytoplasm
The formation of such lesions in focal CHI is considered to be associated with somatic loss of heterozygosity in the 11p15 region due to uniparental isodisomy. Loss of maternal imprint leads to the unbalanced expression of several cell proliferation regulatory genes including p57KIP2, IGF2 and H19 (James et al., 2009). A few years back, a third type of CHI has been identified and genetic, morphological and immunohistochemical analysis revealed its distinctive features that are different from the two major forms: (a) no mutation in the ABCC8, KCNJ11 and GCK (encoding glucokinase); (b) late onset (5-6months old or later) and (c) morphological mosaicism of the islets (Sempoux et al., 2011). Recently, this rare form of CHI has been found to link to excessive expression of low-K_m hexokinase I (HK-I) or a GCK activating mutation in hyperactive islets while hypofunctional islets appeared to be unaffected (Henquin et al., 2012).

While research regarding CHI has been focusing on the genetic, morphological, and physiological abnormalities of the β-cells, little is known about changes of the counterregulatory machinery in CHI. In CHI patients, including diffuse, focal and diazoxide-responsive forms of CHI, glucagon response to hypoglycaemia is blunted compared to non-hyperinsulinimic hypoglycaemic patients (Hussain et al., 2005). The precise mechanism responsible for this decreased glucagon response could not be determined but prolonged exposure to uncontrolled insulin secretion was speculated to be attributable. This hypothesis is supported by the proposed microcirculation diagram (β→α→δ) in human islets (Stagner and Samols, 1992), and evidence suggests that glucagon response is suppressed by intra-islet hyperinsulinaemia during hypoglycaemic episodes in young healthy adult human subjects (Banar et al., 2002). Upon further research, an “intra-islet insulin hypothesis” (also called ‘switch-off” theory) was raised to
explain the loss of glucagon release in response to hypoglycaemia in type 1 diabetes, which emphasises the importance of decrement of insulin in mediating glucagon release (Raju and Cryer, 2005). More recent studies have revealed that it is zinc, a co-secretory product with insulin, but not insulin itself that regulates glucagon secretion (Zhou et al., 2007, Slucca et al., 2010). These studies have plausibly explained the loss of glucagon response in CHI; however, the ‘switch-off’ theory, in which $K_{ATP}$ channel plays a vital role in glucagon secretion (Gromada et al., 2004, Franklin et al., 2005, Leung et al., 2006, MacDonald et al., 2007, Slucca et al., 2010), cannot fully explain the consistent loss of glucagon response in different forms of CHI patients, whose islet cells have partial, complete or no loss of $K_{ATP}$ activity (Hussain et al., 2005). Therefore, we propose that some other factors might contribute to the blunted glucagon response in CHI.

During human foetal pancreatic development, the expansion of the endocrine via proliferation and neogenesis is accompanied by a concomitant process involving the specification of cell lineage, the increment in islet cell number, the change in the relative proportion of different endocrine cell types and the gradual clustering and re-organisation of the islet cells (Stefan et al., 1983, Bouwens et al., 1997, Piper et al., 2004, Jeon et al., 2009, Vignjevic et al., 2012). The subtlety and complexity of this process makes it vulnerable to pathological or physiological events such as hyperinsulinism triggered by $K_{ATP}$ channel defects or other molecular causes. In rodents, postnatal SUR1 knockout (SUR1-KO) and Kir6.2 knockout (Kir6.2-KO) mice exhibit increased proportion and altered distribution of $\alpha$-cells along with the increase of age (Winarto et al., 2001, Marhfour et al., 2009). However, little is known regarding the morphological changes of the islet structure in CHI and their significance to disease pathogenesis and progression. Hence, in the current study, we have examined the distribution patterns of $\alpha$- and $\beta$-cells in
diffuse, focal and atypical CHI patients as well as SUR1-KO mice. Insulin and glucagon colocalisation is also reported. The significance of our findings in the context of postnatal pancreatic development and CHI diagnosis and pathogenesis are discussed.

### 3.3 Materials and methods

**Demographics of CHI patients and controls**

CHI patients were diagnosed and treated by the Northern Congenital Hyperinsulinism Service at the Royal Manchester Children's Hospital (Manchester, UK). Patients were recruited retrospectively from 1989 to 2010 and prospectively from 2010 to 2012. Local ethical approvals and parental consents were obtained for the study. In all cases, wherever possible and appropriate, diagnosis was made using combinations of serology, genetic screening, \(^{18}\text{F}\)-DOPA-PET scan or post-operative histological examination according to accepted diagnostic criteria (Dunne *et al.*, 2004, Hussain, 2008, Arnoux *et al.*, 2010, Banerjee *et al.*, 2011).

Table 3.1 is a list of the clinical characteristics of the CHI patients. Of all 15 patients recruited, 10 are male and 5 are female. Seven patients have diffuse form CHI, 5 have focal CHI and 3 failed fall into either category and are therefore classified as suspected diffuse-atypical CHI (abbreviated as atypical below). Diffuse patients 3, 4 and 5 each underwent two separate pancreatectomies due to post-operative glycaemic control. Tissue samples from both surgeries were analysed in the current study. Because it was over two decades ago when patient 14 was diagnosed, genotyping and PET-CT scan were unavailable at the time, and therefore the CHI diagnosis was made based on serology, diazoxide responsiveness and post-operative histological examination. During
lesionectomies of the 5 focal patients, patient 8 and 10-12 had tissue samples removed outside the focal lesion besides the focal domain. Specimens from the focal lesion and surrounding tissue were analysed comparatively with the exception of patient 8 whose non-lesion tissue was omitted from the study because the tissue was too small to contain enough endocrine detail for examination.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at operation (months)</th>
<th>Diagnosis</th>
<th>Gene</th>
<th>Mutation (Paternal / Maternal)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>M</td>
<td>3</td>
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</tr>
<tr>
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<td>F</td>
<td>2</td>
<td></td>
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</tr>
<tr>
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<td>M</td>
<td>4</td>
<td></td>
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<td>F</td>
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<td>Diffuse</td>
<td>ABCC8</td>
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<tr>
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<td>M</td>
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<tr>
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<tr>
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<td>M</td>
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<tr>
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<td>10</td>
<td></td>
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<tr>
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<tr>
<td>12</td>
<td>F</td>
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<td>ABCC8</td>
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<tr>
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<td>M</td>
<td>12</td>
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</tr>
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</table>

Table 3.1 Demographics of CHI patients.
Pancreatic tissue harvested from 2 adult and 1 3-year-old donors during post-mortem autopsy served as controls. The causes of death of all three donors were not related to pancreatic disease, furthermore pancreata used were not suitable for transplantation. Representative pancreatic tissue samples from the donors were examined and normal morphology and good tissue preservation was confirmed. Information of the donors was anonymised due to ethical reasons.

**Murine pancreatic tissue**

Twelve mouse pancreata used in the study were kindly gifted from Prof. Gisela Dews (University of Tübingen, Tübingen, Germany). Pancreata were harvested from 4-5 months old adult female wild type (WT) and SUR1-KO C57/BL6 mice, 6 each. The generation and characteristics of these SUR1-KO mice have been described previously (Seghers *et al.*, 2000b).

**Immunohistochemistry**

All pancreatic tissue samples were fixed at 4°C overnight in 4% paraformaldehyde, dehydrated through ethanol gradient, embedded in paraffin and sectioned at 5µm of thickness. Pancreatic sections were dewaxed in xylene and rehydrated through an ethanol gradient and multiple PBS/H₂O washes. Antigen retrieval was performed by microwave boiling in 10 mM citric acid buffer (pH 6.0; supplemented with 0.05% (v/v) Tween-20) for 10 minutes. Permeablisation with 0.1% (v/v) Triton-X100 and blocking with 5% (w/v) fish skin gelatin to prevent non-specific binding were for 1 hour each at room temperature. Sections were incubated with insulin (guinea pig anti-pig, 7.5 µg/mL; Life Technologies Ltd, Paisley, UK) and glucagon (mouse anti-pig; 1:1000 dilution; Sigma-Aldrich Co. Ltd., Dorset, UK) antibodies for overnight at 4°C followed by multiple washes in 0.05% (v/v)
 Tween-20 supplemented PBS (PBST). Subsequently, DyLight 488-conjugated goat anti-guinea pig, Cy3-conjugated donkey anti-mouse secondary antibodies (both purchased from Stratech Scientific Ltd. (Suffolk, UK) and used at 7.5 µg/mL) and 2.5µg/mL of 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Co. Ltd. (Gillingham, UK)) were applied to the sections for 1 hour at room temperature. Slides were then washed with PBST and mounted with Vectashield (Vector Laboratories Ltd., Peterborough, UK).

Sections were examined on an Olympus BX51 upright microscope (Olympus UK Ltd., Watford, UK) and images were collected using a 10X or 20X objective with a Coolsnap ES camera (Photometrics UK, Marlow, UK) through MetaVue (Molecular Devices, Pennsylvania, USA).

**Data analysis**

Immunofluorescent images were processed and analysed using ImageJ (v1.47b; [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). 5-6 islets with maximum diameter longer than 100 µm from at least 3 images were analysed per patient or murine tissue. Every selected islet was cropped out along the margin based on nuclear clustering. For each islet, 6 selection lines angled 0°, 30°, 60°, 90°, 120° and 150° were drawn across the centre of mass and grey values of the staining intensity of insulin or glucagon along each line were tabulated and exported. Data from each line were divided into quartiles and the mean of the central two quartiles was defined as core while the mean of the other two was classified as periphery. Statistical significance was determined by unpaired Student’s t-test or One-way ANOVA followed by Tukey’s multiple comparisons test using GraphPad Prism 6 (GraphPad Software, Inc., California, USA). A $P$-value < 0.05 or better was considered as statistically significant. All quantification data are presented as Mean ± SEM.
3.4 Results

*Similar islet architecture in normal adult human and young child*

To evaluate the influence of age increase upon islet architecture, we first examined α- and β-cell distribution in adult and 3-year-old human controls. No obvious difference in islet structure was observed (Fig. 3.1A-H). While insulin-expressing β-cells appeared to be the predominant cell type and distributed throughout the islets, glucagon-producing α-cells mainly localised at the periphery of β-cell clusters but a good amount of α-cells managed to infiltrate and embed in between β-cells. Distribution analysis showed that α- and β-cells localised evenly in the islets and no significant difference in the organisation of α- and β-cells could be detected among the 2 adults and 1 toddler examined (Fig. 3.2).
Fig. 3.1 Islet structure in normal human and CHI patients. Pancreatic samples from normal adult (A-D) and 3-year-old (E-H) human, diffuse CHI patient 5 (I-L), lesion (M-P) and non-lesion (Q-T) domain from focal patient 10 and atypical patient 15 (U-X) were co-stained for insulin (green), glucagon (red) and DAPI (blue). Scale bars: 100µm.
Fig. 3.2 Distribution analysis of α- and β-cells in normal human adult and young child.

Distribution of insulin (A) and glucagon (B) staining was analysed in pancreatic samples from 2 adult and 1 3-year-old human controls. Results were transformed into peripheral/core staining ratio. ns: non-significant; n=6 islets/donor.
**Differential islet structure in CHI**

We next investigated the distribution of α- and β-cells in CHI patients. Most of the diffuse patients exhibited a similar pattern as the controls although the structure appeared to be slightly less compact (Fig. 3.1II-L; Fig. 3.3). On the other hand, patients with focal and atypical forms of CHI showed distinctive features in islet cell organisation. In focal CHI patients, adenomatous hyperplasia of the β-cells was evident inside the focal lesion and this led to the extreme clustering of islets as well as peripheralisation and loss of α-cells (Fig. 3.1M-P). However, distribution analysis failed to identify significant change of the localisation of α-cells (Fig. 3.3). Meanwhile, outside the focal domain, collapse of the cytoplasm and remarkable level of centralisation of the β-cells resulted in the significant migration of α-cells towards the periphery of the islets (Fig. 3.1Q-T; Fig. 3.3). Also, a slight increase of α-/β- cell number ratio was noticed in the majority of these shrunk islets, perhaps as a result of counterregulatory response to elevated insulin level. Interestingly, in all three atypical patients, α-cells exhibited a similar localisation pattern to patients with focal form of CHI (Fig. 3.1U-X). Intensity ratio of glucagon staining between the mantle and core region of the islets was significantly higher than diffuse patients ($P<0.05$); however, the resulted tendency of centralisation of the β-cells was not advanced enough to make a statistic significance (Fig. 3.3). Moreover, a marginal increase of α-cell number was observed (Fig. 3.1U-X).

In addition to the differential arrangement of the islets cells, co-expression of hormones was noticed in CHI patients. It seems that, while cells which co-expressed insulin and glucagon were not spotted in the adult and young child controls (Fig. 3.1A-H), large amount of co-expression of insulin and glucagon were found in 3 of the 7 diffuse and 1 of the 5 focal CHI patients as well as in a subpopulation of islets in 2 of the 3 atypical CHI patients.
Fig. 3.3 Distribution analysis of α- and β-cells in human controls and CHI patients.

Distribution of insulin (A) and glucagon (B) staining was analysed and compared among pancreatic samples from diffuse CHI (n=13), focal CHI (n=5 in lesion group and n=3 in non-lesion group) and atypical CHI patients (n=3) and normal controls (n=3). Results were transformed into peripheral/core staining ratio. *P<0.05; **P<0.01.
**Fig. 3.4 Co-expression of insulin and glucagon in CHI patients.** Pancreatic samples from diffuse CHI patient 1 (A-D), focal patient 12 (lesion; E-H) and atypical patient 15 (I-L) were co-stained for insulin (green), glucagon (red) and DAPI (blue). Significant amount of insulin/glucagon co-expression cells were detected in all three forms of CHI. Scale bars: 100µm.

Patients (Fig. 3.4). For the focal patient, the phenomenon was limited to the focal domain without affecting the surrounding tissue. Of the 3 diffuse patients who exhibited hormone co-expression, 2 had 2 separate pancreatectomies. Interestingly, this peculiar co-expression of insulin and glucagon was not found in the tissue from the second surgery of these two patients (data not shown). However, in all three patients who had 2 operations, there was a slight tendency (although not significant possibly due to the small sample number) of both α- and β-cell centralisation in the tissue from the second pancreatectomy (Fig. 3.5).
Fig. 3.5 Distribution analysis of α- and β-cells between 1st and 2nd operations in diffuse CHI patients. Distribution of insulin (A) and glucagon (B) staining was analysed and compared between pancreatic samples harvested from diffuse CHI during 1st and 2nd operations. Results were transformed into peripheral/core staining ratio. ns: non-significant.
α-cell re-organisation in SUR1-KO mice

Like in normal human islets, β-cells in young adult WT mice mainly localised in the centre of the islets though the distribution was more compact than that in humans. α-cells, as a result, were predominantly confined to the edge of the islets (Fig. 3.6A-D). $K_{\text{ATP}}$ channel inactivation via SUR1-KO induced an elevation in α-cell number (Fig. 3.6E-H) as well as a significant reduction in periphery/core ratio of glucagon staining towards 1:1, indicating a more even distribution of α-cells throughout the islets in SUR1-KO mice than in their age-matched WT littermates (Fig. 3.6I&J).
Fig. 3.6 Modified islet architecture in SUR1-KO mice. Pancreatic samples from 6 WT (A-D) and 6 SUR1-KO (lesion; E-H) mice were co-stained for insulin (green), glucagon (red) and DAPI (blue). Distribution of insulin (I) and glucagon (J) staining was analysed and compared between the WT and SUR-KO. Results were transformed into peripheral/core staining ratio. ns: non-significant; **P<0.01. Scale bars: 100μm.
3.5 Discussion

During human pancreatic development, consensus is β-cells emerge first at approximately 7.5-9 weeks post-conception (w.p.c.), followed by α-cells (8-9 w.p.c.), δ-cells (8-9 w.p.c.), ε-cells (9 w.p.c.) and PP-cells (9-10 w.p.c.), although minute discrepancies exist among different studies regarding the time points at which expression of each hormone commences (Polak et al., 2000, Piper et al., 2004, Andralojc et al., 2009, Meier et al., 2010, Vignjevic et al., 2012). From 12-13 w.p.c., scattered α- and β-cells form a compact structure with β-cells being in the islet centre and α-cells locating at the mantle (Clark and Grant, 1983, Stefan et al., 1983, Piper et al., 2004, Jeon et al., 2009). Weeks later (from about 16-18 w.p.c.), the majority of α- and β-cells migrate towards the opposite poles of the islets and present as bipolar-islet organisation and separate clusters (Piper et al., 2004, Jeon et al., 2009). Subsequently (sometime between 21-29 w.p.c.), this segregation of the two cell types appears to be reversed and β-cells are surrounded by a layer of peripherally located α-cells (Stefan et al., 1983, Piper et al., 2004). This islet cell organisation pattern is markedly altered during infancy: from term foetus to young infants, α-cells rapidly (probably within days to weeks after birth) infiltrate into the centre of islets and localisation along capillaries becomes evident (Rahier et al., 1981). Our examination of an infant’s pancreas revealed mature yet a little loose assembly of the islet cells, indicating that postnatal pancreatic development in human is approaching completion by the age of 3 years. The determination of the exact postnatal sequence of events which leads to the formation of mature islet architecture would, however, require systematic examination of pancreata from children between neonate and puberty.

Interestingly, compared to a previous report (Rahier et al., 1981), some CHI patients exhibited abnormalities in the process of islet structure formation. Similar to adult and
young child controls, diffuse patients showed high level of α-cell infiltration into the centre of the islets with less firm structural compactness. In contrast, focal patients had reduced number of α-cells scattered at the periphery of highly concentrated clusters of β-cells in the focal domain, whereas, outside the lesion, islets appeared to be shrunken, possibly as a result of β-cell resting due to the hypersecretion of insulin from the focal lesion (Sempoux et al., 2003), and α-cells were almost exclusively confined to the mantle of islets. Likewise, in atypical cases, the organisation of the islet cells looked immature considering their age (≥ 12 months). We presume that K\textsubscript{ATP} channel mutation is irrelevant since atypical patients, who were negative for ABCC8 and KCNJ11 defects, shared the same islet organisation pattern as focal patients. Indeed, peripheral α-cell distribution appeared to be universal in all the islets in focal patients no matter inside or outside the focal lesion when genetic mutations were only confined to the focal domain. One possible explanation is that, in focal patients, β-cell adenomatous hyperplasia due to accelerated proliferation rate inside the lesion has somehow “pushed” α-cells towards the periphery, while, in the rest of the pancreas, β-cell silencing has triggered adaptive islet cell re-organisation to maximise the injection of glucagon (and maybe other hormones) into the bloodstream while minimising the release of insulin into the circulation. A similar situation is believed to exist in atypical form of CHI when these cases examined could well be a subgroup of focal CHI if hyperactive islets are considered as small focal lesions (Henquin et al., 2012). Another possibility is that this α-cell peripheral localisation pattern is a result of stoppage or deferment of the normal islet cell re-organisation process that naturally happens during infancy (Rahier et al., 1981). However, we cannot explain why this phenomenon was only found in focal and atypical form of CHI but not diffuse CHI. Furthermore, changes in islets’ microvasculature and blood flow under different physiological and pathological conditions in rodent models have been reported previously (Nakamura et al., 1995,
Carlsson et al., 1997, Carlsson et al., 1998, Mizuno et al., 1999, Duvillie et al., 2002, Li et al., 2006). Thus, we speculate that this process, although yet to be confirmed, is accompanied with modifications in vasculature and blood flow in islets to match and adapt to alterations in body hormonal levels.

Surgical intervention in CHI treatment usually involves subtotal to near total removal of the pancreas (Shilyansky et al., 1997, Lovvorn et al., 1999). If hypoglycaemic condition cannot be properly controlled postoperatively, a second operation is often required (Shilyansky et al., 1997, Lovvorn et al., 1999, Martinez-Ibanez et al., 2002, Adzick et al., 2004). Although still debatable, regeneration of the pancreas after the first operation during the second operation has been previously documented and discussed where imaging techniques or surgeons’ reports were employed (Gough, 1984, Schönau et al., 1991, Shilyansky et al., 1997). To date, it is still unclear if pancreatectomy and/or subsequent regeneration process leads to any alterations in the islet structure. Our findings on 3 diffuse CHI patients who had 2 surgeries indicated a small migration process of α- and β-cells towards the centre of the islets occurs after the first operation. Unfortunately, we cannot determine if this centralisation tendency of the two cell types was a part of the regeneration event or islets were undergoing natural islet structure formation.

α-cell hyperplasia and/or hypertrophy has been previously reported in diabetic humans (Orci et al., 1976, Rahier et al., 1983, Yoon, 2003, Deng et al., 2004), implying the significant role that insulin resistance and loss of insulin production have played in increasing α-cell mass. On the other hand, in agreement with previous studies, our distribution studies using SUR-KO mice clearly demonstrated that, in rodent models of hyperinsulinism, α-cell mass expansion happens in adulthood and exacerbates along with
age increase and animals develop hyperglycaemia later on in their lives (Miki et al., 1997, Miki et al., 1998, Seghers et al., 2000a, Winarto et al., 2001, Marhfour et al., 2009). These observations indicate a possible paradoxical role of insulin in modulating α-cell mass expansion – both loss and excess of insulin release promotes α-cell proliferation/neogenesis. Coincidentally, in transient CHI patients who lack genetic mutation and spontaneously recover from hyperinsulinaemia, diabetes is a very common complication in adulthood (unpublished observation). Unfortunately, it was not possible to examine these patients for α-cell hyperplasia/hypertrophy. In the current study, the increase of glucagon-producing cells observed in focal and atypical patients was presumed to be caused by excessive insulin secretion. However, since diffuse form of CHI has not shown obvious sign of α-cell hyperplasia during our examination, it is possible that, as islet cells in the other two disease forms were under limitedly intra-islet hyperinsulinaemic condition, insulin hypersecretion disorder could be responsible for hampering adaptive mechanisms via inhibiting α-cell proliferation/neogenesis in a paracrine fashion.

Another peculiar finding in the present study is that some CHI patients exhibited insulin and glucagon co-expression in a population of the islets. Previously, morphological changes of the islets, increased β-cell activity, proliferation and apoptosis, and altered expression of proteins related to β-cell function and insulin secretion have been discovered in histological examination of the pancreas in CHI and discussed in details (Sempoux et al., 1998, Kassem et al., 2000, Sempoux et al., 2003, Kassem et al., 2010, Lovisolo et al., 2010, Rahier et al., 2011, Sempoux et al., 2011, Henquin et al., 2012). Few early studies have described the existence of these multihormonal cells in normal neonates and CHI patients but results were inconclusive due to the more primitive techniques and reagents used (Gould et al., 1983, Newman et al., 1986). Our results support these early discoveries.
and indicate that CHI is a disease which not only associates with physiological and morphological abnormalities but also presents with delay of the normal developmental maturation of the pancreas, or, in particular, islet cells. Polyhormonal cells have been found in the very early stages of islet cell development and fades when specification of the cell lineage further proceeds, although the proportion of these multihormonal cells within the islet cell population is controversial (Clark and Grant, 1983, Lukinius et al., 1992, Bocian-Sobkowska et al., 1999, Portela-Gomes et al., 1999, Polak et al., 2000, Piper et al., 2004, Jeon et al., 2009). The role and the significance of these polyhormonal cells in islet development are not fully understood but it has been suggested that insulin/glucagon co-expressing cells are precursors to mature α-cells (Riedel et al., 2012). Therefore, in CHI, these polyhormonal cells could well be β-cells that were transdifferentiating into α-cells in an attempt to alleviate the hyperinsulinaemic condition.

In summary, the present study has proved that diffuse, focal and atypical CHI had differential expression profile of insulin and glucagon, and that focal and atypical CHI exhibited delay in postnatal islet cell clustering caused by either adaptive remodelling of the islet structure or deferment in islet formation. Focal and atypical CHI also displayed α-cell hyperplasia in a population of islets and the degree of intra-islet hyperinsulinaemia seemed to be determinant. Co-expression of insulin and glucagon was spotted in all three forms of CHI and we speculate that these polyhormonal cells represent β- to α-cell transdifferentiation to counterregulate hyperinsulinism. These findings have provided some useful data to further our understanding on postnatal pancreatic development and CHI pathogenesis, and also have offered some assistive information to the diagnosis of CHI.
3.6 Acknowledgement

We acknowledge the support from members of Northern Congenital Hyperinsulinism Service (NORCHI) to the study. We thank Prof. N. Hanley for provision of human control histological samples and Prof. G. Dews (University of Tübingen, Tübingen, Germany) for gifting murine pancreatic tissue.

3.7 Reference


Diabetes, 53, 624-632.


HENQUIN, J. C., SEMPOUX, C., MARCHANDISE, J., GODECHARLES, S., GUIOT, Y.,


NAKAMURA, M., KITAMURA, H., KONISHI, S., NISHIMURA, M., ONO, J., INA, K., SHIMADA, T. & TAKAKI, R. 1995. The endocrine pancreas of spontaneously


SEMPOUX, C., GUIOT, Y., DUBOIS, D., NOLLEVAUX, M. C., SAUDUBRAY, J. M.,
NIHOUL-FEKETE, C. & RAHIER, J. 1998. Pancreatic B-cell proliferation in
persistent hyperinsulinemic hypoglycemia of infancy: an immunohistochemical
study of 18 cases. Mod Pathol, 11, 444-449.

95% pancreatectomy the procedure of choice for treatment of persistent
hyperinsulinemic hypoglycemia of the neonate? Journal of Pediatric Surgery, 32,
342-346.

ATP-sensitive K+ channel mediates the zinc switch-off signal for glucagon
response during glucose deprivation. Diabetes, 59, 128-134.


2012. Novel presentations of congenital hyperinsulinism due to mutations in the
MODY genes: HNF1A and HNF4A. J Clin Endocrinol Metab, 97, E2026-2030.

immunofluorescent study of the endocrine cell populations in the developing
human pancreas. Diabetes, 32, 293-301.

VIGNJEVIC, S., TODOROVIC, V., DAMJANOVIC, S., BUDEC, M., MITROVIC, O.,
DJIKIC, D., DRNDAREVIC, N., MICIC, M., MISKOVIC-KRIVOKAPIC, J.,
DJURICIC, S. & NIKOLIC, I. 2012. Similar developmental patterns of ghrelin-
and glucagon-expressing cells in the human pancreas. Cells Tissues Organs, 196,
362-373.

pancreatic islets of KATP channel-deficient mice: the involvement of KATP channels in the survival of insulin cells and the maintenance of islet architecture.

Arch Histol Cytol, 64, 59-67.


Chapter 4: Novel Variants of Congenital Hyperinsulinism Detected by Measurements of Serum GLP-1 (7-36) / GIP

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4.1 Abstract

The \textit{ABCC8} and \textit{KCNJ11} genes encode subunits of ATP-sensitive potassium (K\textsubscript{ATP}) channels and loss-of-function defects cause congenital hyperinsulinism (CHI). In focal-CHI these defects are localised to a small lesion of islet β-cells, whereas in patients with diffuse
disease gene defects will occur in all \( \text{K}_{\text{ATP}} \) channel expressing cells. As \( \text{K}_{\text{ATP}} \) channels are found in the enteroendocrine L- and K-cells, we hypothesized that measurements of serum concentrations of GLP-1(7-36) and GIP may be clinically valuable in the differential diagnosis of diffuse vs. focal CHI. We recruited 12 patients with CHI who presented with either transient or persistent disease and measured basal and post-prandial GLP-1(7-36) and GIP concentrations. Our data revealed no differences in serum incretin concentrations between transient and persistent CHI patients suggesting that \( \text{K}_{\text{ATP}} \) channels do not play a functional role in nutrient sensing in enteroendocrine cells. However, we did find marked differences in non-typical CHI patients suggesting that measurements of serum GLP-1(7-36) and GIP may be important in the diagnosis of this novel sub-group of CHI patients.
4.2 Introduction

Congenital hyperinsulinism (CHI) is the most common cause of persistent hypoglycaemia in early childhood. CHI is characterised by inappropriate release of insulin from pancreatic β-cells regardless of the level of glycaemia and is associated with brain injury and adverse neurological outcomes in a significant number of patients (De León and Stanley, 2007). CHI comprises a spectrum of severity of hypoglycaemia and includes children with both transient and persistent disease. The transient form of CHI is thought to account for approximately 50% of all patients (Banerjee et al., 2011) and despite being associated with significant long term abnormal neurological outcomes (Avatapalle et al., 2013), little is known about the mechanisms of disease and its resolution. For patients with persistent disease, the known genetic basis of CHI varies between 55% and 88% of patients in several relatively large and well characterized cohorts (Banerjee et al., 2011, Kapoor et al., 2013, Snider et al., 2013). The most common causes of persistent, medically-unresponsive CHI are mutations in the genes encoding ATP-sensitive potassium (K\textsubscript{ATP}) channel subunits: \textit{ABCC8} (encoding sulfonylurea receptor 1, SUR1) and \textit{KCNJ11} (encoding the potassium ion channel Kir6.2). \textit{ABCC8} / \textit{KCNJ11} defects are expressed in all islet β-cells in patients with diffuse CHI, but are only localized to a region of focal β-cell hyperplasia in patients with focal CHI since this form of the disease is caused by somatic loss of maternally-imprinted genes (De León and Stanley, 2007, Banerjee et al., 2011, Mohamed et al., 2012). Other identified genetic abnormalities associated with persistent CHI include mutations in \textit{GLUD1}, \textit{SLC16A1}, \textit{HADH}, \textit{UCP2}, \textit{HNF1A}, \textit{GCK}, and \textit{HNF4A} (encoding glutamate dehydrogenase 1, monocarboxylate transporter 1, hydroxyacyl-coenzyme A dehydrogenase, uncoupling protein 2, hepatocyte nuclear factor 1α, glucokinase and hepatocyte nuclear factor 4α, respectively) (De León and Stanley, 2007, Banerjee et al., 2011, Mohamed et al., 2012, Stanescu et al., 2012). Patients with non-K\textsubscript{ATP} channel mutations are generally responsive to
medical intervention with diazoxide and/or somatostatin analogues and this has led to the common use of genotyping as a means of informing medical and surgical management and predicting long-term outcomes. However, there are two concerns with this approach. First, a high proportion (i.e. >70%) of all CHI patients (transient and persistent) currently have no genetic basis to their condition (Banerjee et al., 2011, Mohamed et al., 2012, Banerjee et al., 2013) and second approximately 10% of patients undergoing pancreatectomy also have unknown genetic cause of disease and may represent a novel anatomopathological variant of CHI (Sempoux et al., 2011, Mohamed et al., 2012). Patients with such non-focal types of CHI, with no identified gene mutations and without the histopathological hallmarks of typical diffuse disease, may be categorised as unclassified variants (or atypical). These patients often present with symptoms later in childhood (up to several months after birth), are initially responsive to medication but become unresponsive later, and require pancreatectomy to ameliorate hypoglycaemia (Sempoux et al., 2011). Current techniques of imaging, including gold-standard computed tomography (PET-CT) using 6-L-\(^{18}\)F-fluorodihydroxyphenylalanine (\(^{18}\)F-DOPA) are not able to identify atypical CHI pre-operatively; therefore, although atypical CHI may represent a histologically distinct variant of CHI, no pre-operative investigation is presently diagnostic.

Incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are secreted from the enteroendocrine cells and have a substantial regulatory influence upon glucose homeostasis, including insulin secretion (Campbell and Drucker, 2013). The mechanisms of stimulus-response coupling of GLP-1 and GIP secretion from L- and K-cells respectively have not been completely resolved, but they are thought to be controlled by K\(_{\text{ATP}}\) channel activity since both L- and K-cells express the \(ABCC8\) and \(KCNJ11\) genes (Reimann et al., 2008, Parker et al., 2009). Depending on the
distribution and extent of pathological $K_{ATP}$ channels in CHI, incretin secretion could vary between different forms of CHI, mainly focal CHI – in which gene defects are localised solely to islet $\beta$-cells, and diffuse CHI affecting all $K_{ATP}$ channel-expressing cells. This hypothesis has not been previously investigated in patients with CHI and in the present study we have compared fasting and postprandial plasma GLP-1 and GIP concentrations amongst patients with persistent forms of CHI (focal, diffuse and atypical CHI) and those patients who had transient disease. Our results indicate that patients with atypical disease have a markedly altered ratio of GLP-1 (7-36):GIP, and that this may be used as a potential diagnostic biomarker for novel variants of CHI. These findings also suggest that $K_{ATP}$ channels do not play a major regulatory role in the control of GLP-1 and GIP stimulus-response coupling in man.

4.3 Materials and methods

Subjects and specimen collection

Twelve patients with CHI median age (range) 33 (1; 190) months were recruited between 2010 and 2012 with Local Ethical Approvals and Parental Consent. All patients were treated by the Northern Congenital Hyperinsulinism Service UK at the Royal Manchester Children's Hospital. Table 4.1 summarizes their clinical profiles with the classification of CHI being based on serology, genetic mutation, $^{18}$F-DOPA PET-CT and post-operative histological examination, where available, according to established diagnostic criteria as described previously (Banerjee et al., 2011, Banerjee et al., 2013). For analysis of serum peptides, patients were fasted for four hours before blood sample collection into BD P700 blood collection tubes (Becton, Dickinson U.K. Limited, Oxford, UK) pre-feed and 20 minutes (30 minutes for patients subjected to an oral glucose tolerance test, OGTT) after the start of
feeding (breast milk, whole milk, Nutrini Peptisorb/ polycal, or polycal alone). Ordinary milk feeds, without carbohydrate supplements, were given at 20mL / kg body weight. Two patients (#2, #12) were subjected to an oral glucose tolerance test and were fed with 9.82 mM glucose / kg body weight. All samples were centrifuged at 1300g for 10 minutes at 4°C and the plasma was removed, aliquoted and stored at -80°C prior to analysis.

**Enzyme-linked immunosorbent assay (ELISA)**

Plasma GLP-1 (7-36) and total GLP-1 (7-36 and 9-36) and total GIP concentrations were assessed using GLP-1 (Active 7-36) ELISA kit (ALPCO Diagnostics, USA), GLP-1 (7-36 and 9-36) ELISA kit (ALPCO Diagnostics, USA) and Human GIP (Total) ELISA kit (EMD Millipore Corporation, USA) respectively according to manufacturers’ instructions. Basal and stimulated GLP-1 (7-36) and GIP, concentrations and ratios of the incretins were analysed for difference between the different patient groups, i.e. transient, focal CHI, diffuse CHI and atypical CHI.

**Statistical analysis**

Data was analysed using GraphPad Prism 5 (GraphPad Software, Inc., California, USA). Statistical significance was determined One-way ANOVA and a *P*-value <0.05 was considered as significant. All data are presented as mean ± SEM.
<table>
<thead>
<tr>
<th>#</th>
<th>Classification of CHI</th>
<th>Age at diagnosis</th>
<th>Age at sample collection (months)</th>
<th>Birth Weight (Kg)</th>
<th>Gestation (weeks)</th>
<th>Mutation Status</th>
<th>PET-CT Diagnosis</th>
<th>Medical Treatment</th>
<th>Surgical Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Transient</td>
<td>6 days</td>
<td>1</td>
<td>2.08</td>
<td>38</td>
<td>Unknown</td>
<td>-</td>
<td>Diazoxide</td>
<td>No</td>
<td>Normoglycaemic</td>
</tr>
<tr>
<td>#2</td>
<td>Transient</td>
<td>1yr</td>
<td>190</td>
<td>3.45</td>
<td>40 +2</td>
<td>Unknown</td>
<td>-</td>
<td>Diazoxide</td>
<td>No</td>
<td>Normoglycaemic</td>
</tr>
<tr>
<td>#3</td>
<td>Transient</td>
<td>4 days</td>
<td>16</td>
<td>2.86</td>
<td>40</td>
<td>Unknown</td>
<td>-</td>
<td>Diazoxide</td>
<td>No</td>
<td>Normoglycaemic</td>
</tr>
<tr>
<td>#4</td>
<td>Transient</td>
<td>2 days</td>
<td>24</td>
<td>3.5</td>
<td>40</td>
<td>Unknown</td>
<td>-</td>
<td>Diazoxide</td>
<td>No</td>
<td>Normoglycaemic</td>
</tr>
<tr>
<td>#5</td>
<td>Transient</td>
<td>4 days</td>
<td>1</td>
<td>4.6</td>
<td>41</td>
<td>Unknown</td>
<td>-</td>
<td>Diazoxide</td>
<td>No</td>
<td>Normoglycaemic</td>
</tr>
<tr>
<td>#6</td>
<td>Diffuse</td>
<td>2 days</td>
<td>27</td>
<td>4.4</td>
<td>40</td>
<td>ABCC8</td>
<td>-</td>
<td>Octreotide</td>
<td>No</td>
<td>Continuous Therapy</td>
</tr>
<tr>
<td>#7</td>
<td>Diffuse</td>
<td>3 days</td>
<td>26</td>
<td>3.25</td>
<td>40</td>
<td>ABCC8</td>
<td>-</td>
<td>Diazoxide</td>
<td>No</td>
<td>Continuous Therapy</td>
</tr>
<tr>
<td>#8</td>
<td>Diffuse</td>
<td>6 days</td>
<td>33</td>
<td>4.73</td>
<td>40</td>
<td>ABCC8</td>
<td>-</td>
<td>Diazoxide / Octreotide</td>
<td>Near Total Pancreatectomy</td>
<td>Cured</td>
</tr>
<tr>
<td>#9</td>
<td>Focal</td>
<td>3 months</td>
<td>49</td>
<td>3.62</td>
<td>40</td>
<td>ABCC8</td>
<td>Focal</td>
<td>Diazoxide</td>
<td>Sub-Total Pancreatectomy</td>
<td>Cured</td>
</tr>
<tr>
<td>#10</td>
<td>Focal</td>
<td>7 days</td>
<td>48</td>
<td>4.9</td>
<td>38 +5</td>
<td>ABCC8</td>
<td>Focal</td>
<td>Diazoxide / Octreotide</td>
<td>Sub-Total Pancreatectomy</td>
<td>Cured</td>
</tr>
<tr>
<td>#11</td>
<td>Non-Typical</td>
<td>2 yr, 7 mo</td>
<td>36</td>
<td>2.72</td>
<td>34</td>
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<td>Diffuse</td>
<td>Diazoxide / Octreotide</td>
<td>Near Total Pancreatectomy</td>
<td>Cured</td>
</tr>
<tr>
<td>#12</td>
<td>Non-Typical</td>
<td>21 months</td>
<td>37</td>
<td>3.4</td>
<td>40</td>
<td>Unknown</td>
<td>Diffuse</td>
<td>Diazoxide</td>
<td>No</td>
<td>Continuous Therapy</td>
</tr>
</tbody>
</table>

Table 4.1 Clinical characteristics of the CHI patient cohort. All patients were treated for hypoglycaemia and classified as transient, diffuse, focal or atypical based upon either their clinical characteristics, genotyping, PET-CT diagnosis or pancreatic histology following surgery. Four patients with persistent disease (#8-11) underwent surgery to alleviate hyperinsulinism; samples for this study were taken after recovery from surgery. Patients #1-2 and #8-11 were sampled in the absence of drug treatment. Three patients with persistent disease are currently receiving medical interventions (#6, #7, #12) and were sampled during ongoing treatment. *Patient #12 was positive for a non-pathogenetic SNP variation in *UCP2*.
4.4 Results

Clinical, Genetic, PET-CT and Histolopathological Investigations

Five patients were diagnosed with transient CHI (Patients #1 to #5) on the basis that their condition spontaneously resolved following diazoxide therapy, with no further requirement for medication or surgery. No defects in \( ABCC8/KCNJ11 \) were found in these patients.

Seven patients were diagnosed with persistent CHI (Patients #6 to #12) on the basis that their condition failed to spontaneously resolve and glycaemic control could only be maintained using medical or surgical intervention. Diffuse CHI was anticipated on the basis of positive genotyping for defects in \( ABCC8/KCNJ11 \) in three patients with persistent CHI (Patients #6-#8) and subsequently confirmed by histology in one patient following pancreatectomy (Patient #8). Two of the three diffuse CHI patients remain on medical therapy, Table 4.1. Two patients with persistent CHI were diagnosed as focal CHI (Patients #9, #10) on the basis of paternally-inherited \( ABCC8 \) mutations and PET-CT scanning and confirmed by histological analysis following partial pancreatectomy. Two patients (Patients #11, #12) had late-onset presentation of persistent CHI, Table 4.1. Patients #11 and #12 were genotype negative for defects in \( ABCC8, KCNJ11, HADH, GCK, \) and \( HNF4A \). Patient #12 has a non-pathogenic missense variant c.167C>T in \( UCP2, \) p.A55V/N and is currently receiving diazoxide therapy (9mg/kg/day). Patient #11 was initially responsive to diazoxide/Octreotide therapy but failed to maintain adequate levels of glycaemia. During PET-CT a homogenous uptake of \(^{18}\text{F-DOPA}\) was suggestive, but not conclusive, of diffuse CHI. Following 95% pancreatectomy, examination of the resected pancreas revealed a heterogeneous pattern of pancreatic histopathology (diffuse distribution of abnormal islets with relatively large nuclei suggesting hyperfunction but many islets demonstrating normal resting pattern, on haematoxylin and eosin staining) and expression of hexokinase 1,
consistent with atypical CHI using established criteria (Sempoux et al., 2011, Henquin et al., 2012).

Enhanced post-prandial GLP-1 (7-36):GIP in unclassified variants of CHI

Basal and carbohydrate stimulated plasma incretin concentrations were measured and showed an incremental increase for all patients, summarised in Figure 4.1. The average basal GIP concentrations were not significantly different across each of the patient groups; 5.8 ± 0.7 pmol/L (n=5) vs. 6.2 ± 2.4 pmol/L (n=6) vs. 5.7 ± 1 pmol/L (n=2) for transient CHI vs. focal/diffuse CHI vs. atypical CHI, respectively. On average, post-prandial GLP-1 (7-36) concentrations increased to 3.9 ± 0.9 pmol/L (n=4) and 3.8 ± 1.1 pmol/L (n=5) in transient and focal/diffuse CHI patients, respectively, but was found to be markedly higher in atypical CHI; 82.2 pmol/L and 16.5 pmol/L for patients #11 and #12, respectively (Fig. 4.1A). In addition, patients with atypical disease were found to have higher fold changes in their post-prandial GIP concentrations – increasing by 14 fold vs. 8 and 6 fold increases for transient and focal/diffuse CHI groups, respectively (Figure 4.1B). The ratio of stimulated GLP-1 (7-36)/GIP was used to differentiate CHI patient groups (Figure 4.1C); the post-prandial GLP-1 (7-36):GIP ratio in patients with transient- and persistent CHI were found not to be significantly different: 0.020 ± 0.005 (n=4, Patients #1-#4) vs. 0.023 ± 0.005 (n=5, Patients #6-10), but was markedly elevated in patients with atypical CHI, 0.1 ± 0.01 (n=2, p=0.001, Patients #11, #12) (Fig. 4.1C). There were no distinguishable differences between basal GLP-1 (7-36):GIP values in the transient vs. focal/diffuse CHI groups and #11 (0.5 ± 0.05 vs. 0.5 ± 0.03 vs. 0.04, respectively), although this value was markedly elevated in patient #12 (0.3).
Fig. 4.1 Incretin hormone secretion in CHI patients. Panels A and B illustrate basal and post-prandial (“stimulated”) concentrations of GLP-1 (7-36) and GIP in CHI patients. White circles = transient CHI, black circles = focal/diffuse CHI (CHI-F-D) and black squares = atypical CHI (CHI-UV). Panel C summarises the GLP-1 (7-36):GIP ratio in the three patient groups. The integrated incretin ratio values showed no differences in transient and focal/diffuse CHI but was significantly larger in atypical CHI patients, p<0.001. White bars = transient CHI, hashed bars = focal/diffuse CHI and black bars = atypical CHI.
4.5 Discussion

GLP-1 and GIP have potent regulatory effects on hormone secretion, endocrine development and maintenance and incretin-based therapies have shown promise in the treatment of both diabetes (Campbell and Drucker, 2013) and CHI (Calabria et al., 2012). The precise mechanisms that control incretin secretion in response to physiological stimuli have not been fully elucidated, but ATP-sensitive K⁺-channels have been implicated in stimulus-response coupling mechanism in both L cells (Reimann et al., 2008, Gorboulev et al., 2012) and K cells (Nielson et al., 2007, Parker et al., 2009). As the loss of K<sub>ATP</sub> channels in focal CHI patients is restricted to the pancreas, our rationale for undertaking these studies was that the profile of stimulated GLP-1 (7-36):GIP could be used to differentiate CHI-F from CHI-D where ABCC8/KCNJ11 defects are also expressed in enteroendocrine cells. However, our observations, suggest that there are no major differences in the basal or stimulated GLP-1 (7-36) (Fig. 4.1A) or GIP concentrations (Fig. 4.1B) in patients with either focal or diffuse disease and that this patient group were also indistinguishable from transient CHI patients (Fig. 4.1). Whilst there are no established normal ranges for incretin hormones in the paediatric population our data are in general agreement with previous studies conducted on infants, children and young adults (Schou et al., 2005, Amin et al., 2008, Palladino et al., 2009, Kaas et al., 2012). GLP1 (7-36):GIP ratios were, however, found to be strikingly different for children with atypical CHI compared to patients with either transient - or persistent CHI disease. In atypical CHI patients the genetic basis of disease has not been described, although it has been suggested that heterogeneous expression of hexokinase 1 in β-cells may be associated with inappropriate insulin release (Henquin et al., 2012). In our cohort, we found a similar profile of hexokinase 1 expression in β-cells in the tissue of Patient #11 but this cannot be
evaluated in Patient #12 since the patient is currently receiving medical therapy for CHI. Interestingly, studies on rat insulinoma cell lines demonstrated upregulation of hexokinase 1 gene expression in response to GLP1 and GIP in vitro suggesting a possible link between our findings for patient #11 (Wang et al., 1995, Wang et al., 1996). We speculate that both atypical CHI patients would potentially have benefitted from GLP-1 receptor antagonist-based therapy described by Calabria et al. (2012).

Although numbers of patients were small for this pilot study, for CHI this is a significant sample size of very well-characterised cases. In our cohort the genetic basis of atypical CHI was not determined, which is consistent with the emerging profile of this novel group of CHI patients which constitute approximately 10% of cases requiring pancreatectomy. We did find that Patient #12 carried a p.A55V/N single nucleotide polymorphism in the UCP2 gene. This mutation does not have a direct pathological action on β-cell function, but it has previously been reported to have a positive linkage association with type 2 diabetes and obesity in individuals of Asian descent (Wang et al., 2004, Xu et al., 2011). UCP2 has recently been reported to be a negative regulator of glucose-induced GLP-1 secretion (Chen et al., 2012) and whilst p.A55V/N UCP2 is not associated with CHI, the possibility exists that the p.A55V/N UCP2 polymorphism may contribute to elevated serum concentrations of GLP-1 in this patient, which could lead to an inappropriate stimulation of insulin release in this case.

The rationale for our studies was based upon the premise that KATP channels have a functional role in the regulation of incretin hormone secretion. Although ion channel currents have not been shown to couple to physiological stimuli, there is strong support for their existence based upon gene and protein expression studies and also through the actions
of pharmacological modulators of $K_{ATP}$ channels on hormone secretion (Nielsen et al., 2007, Reimann et al., 2008, Parker et al., 2009, Gorboulev et al., 2012). However, our data which has been obtained from patients with defects in $K_{ATP}$ channels fails to support a prominent role for these channels in GLP-1 and GIP release following glucose ingestion. These findings are consistent with others who have also failed to link $K_{ATP}$ channel closure in enteroendocrine cells to secretion, including the actions of sulphonylureas which promote insulin release but do not stimulate GLP-1 secretion, for further discussion see (Tolhurst et al., 2012).

In summary, we have shown that CHI patients with defective $K_{ATP}$ channels do not have abnormal plasma incretin profiles while atypical patients with no identified disease-causing mutations presented with elevated basal and/or post-prandial GIP and/or GLP-1 (7-36) concentrations. Based upon these observations we propose that investigations of serum incretin concentrations in patients with CHI may identify a subset of patients with novel disease pathogenesis.

### 4.6 Acknowledgements

We thank Professor Sian Ellard, Dr Sarah Flanagan and Dr Meg Mashbat (Royal Devon and Exeter Foundation Trust, UK) for screening additional candidate gene mutations for this study. We acknowledge the invaluable support from the Northern Congenital Hyperinsulinism Service (NORCHI) team.
4.7 Reference


SCHONEMANN, M. D., RYAN, A. K., MCEVILLY, R. J., O'CONNELL, S. M., ARIAS,


Chapter 5: Differential Inhibitory Effects of GABAB Receptor Agonist Baclofen on Insulin Secretion in Isolated Islets from Mouse and Patients with Congenital Hyperinsulinism

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5.1 Abstract

Congenital hyperinsulinism (CHI) is a potentially lethal pancreatic disorder that is caused by genetic mutation related to β-cell function, including ATP-sensitive potassium (KATP) channel encoding genes ABCC8 and KCNJ11. Current treatment options for CHI include potassium channel activator (diazoxide), calcium channel antagonist nefidipine, glucagon, somatostatin analogues (octreotide and lanreotide) and partial to total pancreatectomy. These medical and surgical treatments all have their weaknesses and adverse effects.
Therefore, it is urgent to explore new medication for CHI treatment. \( \gamma \)-aminobutyric acid (GABA) operates as a neurotransmitter and endocrine function regulator in the pancreas. GABA modulates insulin secretion via \( \text{GABA}_B \) receptor. In order to determine if \( \text{GABA}_B \) receptor agonist baclofen is useful to the treatment of CHI, we applied baclofen to isolated islets from mouse and CHI patients. Our data suggested that 10 \( \mu \text{M} \) baclofen only suppressed glucose-induced insulin secretion but not at basal level or when glucose stimulation failed to increase insulin release. We also observed differences in the expression of \( \text{GABA}_B \) receptor subunits and subunits of calcineurin among mouse, adult human and CHI islets/whole pancreas. The cause(s) of these variations remains unclear but the involvement of glucose concentration in the culture environment was speculated.
5.2 Introduction

Congenital hyperinsulinism (CHI) is a heterogeneous pancreatic β-cell insulin secretion disorder that affects mostly neonates and infants. CHI is the most common cause of hypoglycaemia in children, and if not diagnosed or managed promptly, CHI could lead to severe brain injury and mental retardation (De León and Stanley, 2007). While the aetiology of approximately 50% of the CHI cases are still unclear, the rest are associated with mutations in genes encoding ATP-sensitive potassium (K\textsubscript{ATP}) channel (\textit{ABCC8} and \textit{KCNJ11}), glucokinase (\textit{GCK}), glutamate dehydrogenase 1 (\textit{GLUD1}), monocarboxylate transporter 1 (\textit{SLC16A1}), hydroxyacyl-coenzyme A dehydrogenase (\textit{HADH}), uncoupling protein 2 (\textit{UCP2}), hepatocyte nuclear factor 1α (\textit{HNF1A}) and hepatocyte nuclear factor 4α (\textit{HNF4A}) (Ashcroft, 2005, De León and Stanley, 2007, González-Barroso et al., 2008, James et al., 2009, Stanescu et al., 2012). There are two major histological forms of CHI: diffuse and focal. Diffuse CHI affects the entire pancreas while focal CHI is limited to a subpopulation of islet cells in a discrete region of the pancreas (De León and Stanley, 2007). Atypical CHI, as a novel form of CHI, has recently been discovered, and excessive expression of low-Km hexokinase 1 (HK1) or somatic mutation in the \textit{GCK} gene among abnormal islets, which are located in a few pancreatic lobules, are held responsible (Rahier et al., 2011, Sempoux et al., 2011, Henquin et al., 2012).

The immediate medical management of CHI following or concomitant to diagnosis starts with continuous feeding, glucose administration and/or glucagon infusion accompanied with frequent blood glucose monitoring to prevent the recurrence of hypoglycaemic episodes and consequently irreversible brain damage (Arnoux et al., 2010). As soon as normoglycaemia is accomplished and maintained, more specific treatments are initiated and aetiology has to be researched. Current common options for CHI treatment include
K\textsubscript{ATP} channel activator diazoxide (sometimes used in combination with a diuretic like chlorothiazide), calcium channels blocker nifedipine, glucagon, and somatostain analogue octreotide (Arnoux et al., 2010). All these pharmaceutical reagents have had some degree of success in managing CHI; however side effects, differential responsiveness and efficacy as well as difficulty in administration have challenged patients’ long-term tolerance to medical management (Aynsley-Green et al., 2000, Dunne et al., 2004, De León and Stanley, 2007, Arnoux et al., 2010). Therefore, it is important and urgently necessary to explore new options for CHI treatment.

\(\gamma\)-aminobutyric acid (GABA) is a major neurotransmitter in the central nervous system (CNS) as well as a regulator of hormone secretion and cell viability and function in the endocrine pancreas (Franklin and Wollheim, 2004, Ligon et al., 2007). GABA is synthesised from glutamate via enzyme glutamate decarboxylase (GAD) and interacts with ionotropic receptors to suppress neuron firing in the CNS (Franklin and Wollheim, 2004). In the pancreas, GABA is released from \(\beta\)-cells and acts in an autocrine as well as a paracrine fashion to modulate the secretion of insulin via metabotropic GABA\textsubscript{B} receptor (Brice et al., 2002, Braun et al., 2004, Braun et al., 2010) and glucagon through ionotropic GABA\textsubscript{A} receptor (Rorsman et al., 1989, Wendt et al., 2004, Xu et al., 2006). GABA\textsubscript{B} receptor agonist baclofen has been previously shown to inhibit glucose-stimulated insulin secretion (GSIS) in MIN6 cells (Brice et al., 2002), isolated rat islets (Gu et al., 1993, Brice et al., 2002, Braun et al., 2004) and isolated mouse islets (Bonaventura et al., 2012), while one other study claimed otherwise (Faraji et al., 2011). In vivo studies have raised further controversy regarding the effect of baclofen on insulin secretion regulation: the inhibitory effect of baclofen on insulin release in mice has been reported (Bonaventura et al., 2012); however, baclofen delayed the onset of diabetes in mice (Beales et al., 1995) as
well as improved glycaemic control in diabetic rats (Gomez et al., 1999), which indicates that baclofen also stimulates insulin release. Although further research is required, it has been demonstrated that mechanisms of baclofen action in regulating β-cell exocytosis is at least partially dependant on the activation of protein phosphatase calcineurin (Braun et al., 2004). In the current study, we aimed to determine the efficacy and mechanism(s) of baclofen in suppressing insulin secretion in CHI. We thus investigated the inhibitory effect of baclofen on GSIS and the expression of subunits of GABA<sub>B</sub> receptors and calcineurin in isolated islets from mice and patients with CHI.

5.3 Materials & methods

Human subjects

CHI patients involved in the current study were all diagnosed and treated at the Royal Manchester Children’s Hospital during 2008 to 2012. In all, the clinical diagnosis of CHI was made according to accepted criteria (Dunne et al., 2004, Hussain, 2008, Arnoux et al., 2010). A total of 5 diffuse CHI (including 1 suspected diffuse-atypical patients; age range from 2 to 36 months at the time of operation) were included in the study (Table 5.1). Tissue used from Patient 3 was harvested during his second pancreatectomy, 28 months after his first operation, due to uncontrolled hypoglycaemic condition. Cause of death of the adult pancreatic control sample donor was not related to the pancreas. No pancreatic disease was diagnosed prior to the death. Representative pancreatic tissue sections were examined by a certified histopathologist and normal morphology and good tissue preservation was confirmed. Information of the donor was anonymised due to ethical reasons.
<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age at surgery (months)</th>
<th>Diagnosis</th>
<th>Gene affected</th>
<th>Mutation (Paternal / Maternal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>13</td>
<td>Diffuse</td>
<td>ABCC8</td>
<td>c.4612-1G&gt;T / c.11C&gt;T</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>6</td>
<td>Diffuse</td>
<td>ABCC8</td>
<td>c.148+1G&gt;A / c.148+1G&gt;A</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>31*</td>
<td>Diffuse</td>
<td>ABCC8</td>
<td>c.512dupT / c.1818-1923+del</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>36</td>
<td>Atypical</td>
<td>None identified</td>
<td>None identified</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>2</td>
<td>Diffuse</td>
<td>ABCC8</td>
<td>c.1741T&gt;A / c.3992-9G&gt;A</td>
</tr>
</tbody>
</table>

Table 5.1 Clinical characteristics of CHI patients. *Patient 3 had his first pancreatectomy at the age of 3 month.

*Preparation of human and mouse islets*

Islets were prepared from pancreatic explants of human subjects (described above) and 10-12 week male C57/BL6 mice. For mouse islets, whole pancreas from 2 mice was used per isolation procedure. Pancreatic tissue was disassociated using liberase TL research grade (Roche Diagnostics Ltd., Burgess Hill, UK) digestion in Krebs ringer phosphate HEPES (KRH) buffer (129 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 10 mM HEPES, 2.5 mM CaCl₂ and 0.1% (w/v) BSA; pH 7.4) at 37°C. Islets were handpicked followed by a re-pick under a dissection microscope to minimise acinar cell contamination. Some islets from Patient 1 and 2 and mice as well as unprocessed tissue from Patient 3, 4 and 5 were stored in RNALater solution at -20°C immediately after the isolation procedure for RT-PCR. Prior to being used in insulin secretion assay, islets were cultured in tissue culture medium containing 88% (v/v) RPMI1640 medium (11mM D-glucose), 10% (v/v) foetal bovine serum and 2% (v/v) penicillin-streptomycin (final concentration - 200 U/ml and 200 µg/ml, respectively) in non-cell-culture-treated 6 well plates overnight in humidified atmosphere with 5% CO₂ supply at 37°C.
**Insulin secretion assay**

Islets isolated from mouse pancreata and pancreatic tissue from Patient 3, 4 and 5 were transferred into borosilicate tubes (5-9 islets/tube). Islets were allowed to equilibrate for 1 hour at 37°C and subsequently washed once in modified KRH buffer (136.9 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO₄, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.29 mM CaCl₂, 4.17 mM NaHCO₃, 10 mM HEPES and 0.1% (w/v) BSA; pH 7.5) containing 2mM D-glucose. After removing the KRH buffer, various treatments (all formulated upon modified KRH buffer), including 2mM D-glucose, 2mM D-glucose with 10 µM baclofen (Sigma-Aldrich Co. Ltd., Gillingham, UK), 18mM D-glucose and 18mM D-glucose with 10 µM baclofen, were applied to the batches of islets in replicates or triplicates. All incubations were done statically. After 1 hour incubation at 37°C, test solutions were removed and aliquoted while islets with leftover test medium were mixed with equal volumes of 2% (v/v) Triton-X100. All treatments were tested in duplicate or triplicates. All solutions were stored at -80°C until analysis.

**Determination of insulin concentration**

The concentration of insulin secretion in the test medium and insulin content of the islets were assessed using ultrasensitive insulin ELISA (ALPCO Diagnostics, New Hampshire, USA) for CHI islets or mouse insulin ELISA (ALPCO Diagnostics, New Hampshire, USA) for mouse islets.

**RT-PCR**

Total RNA was extracted from isolated islets or tissue pieces using RNeasy Mini Kit (QIAGEN Ltd. - UK, West Sussex, UK). Additional DNAse treatment was applied to remove genomic DNA contamination using RNase-Free DNase Set (QIAGEN Ltd. - UK,
West Sussex, UK). For each sample, 1µg of RNA was converted to cDNA with Oligo-dT and random nonamer primers using Precision qScript Reverse Transcription Kit (Primer Design Ltd., Southampton, UK). In order to monitor genomic DNA (gDNA) contamination, an RT negative control was established for every sample by omitting qScript enzyme from the reaction. PCR amplification was performed using Taq DNA polymerase (Life Technologies Ltd., Paisley, UK). The PCR program was composed of: 5 minutes at 94°C, 33 to 35 cycles of 45 seconds at 94°C, 30 seconds at 62.6, 64 or 65°C, 30 seconds at 72°C, and 8 minutes at 72°C. The products were analysed on 2% (w/v) agarose gel. The following primers were used for the detection of mouse genes:

*Ins1*: 5’CTTCAGACCTTGCGTTGGA3’ and 5’AGTGGCATTTACACGGTTGC3’;
*Ins2*: 5’GCAGAAGCGTGGCATTGTAG3’ and 5’GCTGGGTAGTGGTGGGTCTA3’;
*Gabbr1*: 5’AGGGGCATCTCGTTTGCTCC3’ and 5’GTGTGTGTGTGTTGAGGTTCC3’;
*Gabbr2*: 5’ACGCCTACCTCCCGTCCATT3’ and 5’ATCCGTGCTGAGTCTCTG3’;
*Ppp3ca*: 5’AACGCCAACGACAGCAGATGG3’ and 5’GCTACCTTCAGAGGAAGGCATCG3’;
*Ppp3cb*: 5’TCATAGCACCAGGCAGCGGA3’ and 5’CCAACACAGAGATAGGAGCGA3’;
*Ppp3cc*: 5’TGTCCCCTTCTTCTCAACG3’ and 5’ACCTCCTCTTCCACCCGACCT3’;
*Ppp3r1*: 5’TCTGCCCCATGTCAACCGCCA3’ and 5’TTTTCCACACAGCCGACAAGA3’;
*Ppp3r2*: 5’AGTGTGGGAAAGGACCGTGGGGT3’ and 5’TGCGTGGTGAGGAGGGACTT3’. 

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The following primers were used for the detection of human genes:

**INS:** 5’ACCAGCATCTGCTCCCTCTA3’ and 5’GGTCAAGGGCTTTATTCA3’;

**GABBR1:** 5’TCACTGCTCACACGCTGCCT3’ and 5’ACCCAAACACGCTCAAGGGCA3’;

**GABBR2:** 5’TCCCCCGTGACAGAACCACACT3’ and 5’TGTCCACCTGAGTGCCATCCGA3’;

**PPP3CA:** 5’GAGACTAACGGCAGGACAG3’ and 5’CAGCAAGCCACAACCAAGAC3’;

**PPP3CB:** 5’GGGCATCCACACCTCCATTT3’ and 5’TGGGTTTTCTCGGCACATTTTGC3’;

**PPP3CC:** 5’ATCAGAGGGTCTCAGCTTCA3’ and 5’ATGCTATCTCTTTCGGGGTGG3’;

**PPP3R1:** 5’AGGGAAAGGGGTTGGGCTTTTT3’ and 5’TGTCTGCTTGCGCCTTTTTGT3’

**PPP3R2:** 5’CGGAGGAGGAGCAGTAGGT3’ and 5’TGGTATGAGGACAGGATGC3’.

Every primer pair detects all possible splicing variants of the targeted gene and produces the same product size. All primers were designed using Primer3 software (Rozen and Skaletsky, 2000) based on mRNA sequences obtained from the National Centre for Biotechnology Information (NCBI) GeneBank database (Maryland, USA).

**Statistical analysis**

The data are expressed as Mean±SEM. Differences in the means were determined via ratio paired Student’s t-test (used in Fig. 5.1A) or unpaired Student’s t-test (used in Fig. 5.1B-D) using GraphPad Prism 6 (GraphPad Software, Inc., California, USA). A P-value < 0.05 is considered as statistically significant.
5.4 Results

**Baclofen inhibits GSIS in isolated islets from mice and CHI patients**

To study the effect of GABA<sub>B</sub>R agonist baclofen on insulin secretion from in mice and CHI patients, pancreatic islets isolated from mice and CHI patients 3-5 were stimulated with 2 mM glucose, 2 mM glucose with 10 µM baclofen, 18 mM glucose and 18 mM glucose with 10 µM baclofen, and insulin secretion was measured. In mice, 18mM glucose triggered an approximately 7.1-fold increase of glucose release over 2 mM glucose condition (Fig. 5.1A). This effect was significantly inhibited by 85% in the presence of 10 µM baclofen, while basal level of insulin secretion was unaffected by the drug (Fig. 5.1A). Similarly, in diffuse CHI Patient 3, while increase of glucose concentration from 2 mM to 18 mM raised insulin release by about 6.1-fold, baclofen was able to suppress this stimulation by 90% (Fig. 5.1B). No significant effect of baclofen was seen at the basal level (Fig. 5.1B). In suspected atypical Patient 4 (Fig. 5.1C) and diffuse Patient 5 (Fig. 5.1D), when compared to basal 2 mM glucose condition, 18 mM glucose and baclofen-supplemented 2 mM glucose both failed to significantly influence insulin secretion. Interestingly, unlike what we observed in mice and in Patient 3, 10 µM baclofen did not significantly inhibit insulin release in the presence of 18 mM glucose (Fig. 5.1C and 5.1D). Note that insulin secretion data was normalised to total insulin content in mice due to the high variability in islet size.

**Expression of GABA<sub>B</sub>Rs and calcineurin in mice, normal adult human and CHI**

We next sought to investigate the expression of GABA<sub>B</sub>Rs and calcineurin in mice and CHI. We were able to confirm the constant presence of GABA<sub>B</sub>R2, all three catalytic subunit isoforms of calcineurin and alpha isoform of the regulatory unit of calcineurin in isolated mouse islets (Fig. 5.2). In intact pancreatic samples from normal adult
Fig. 5.1 Differential effect of GABA$_B$R agonist Baclofen in isolated islets from mice and CHI patients. Insulin release from isolated islets was measured in the presence of 2 mM glucose (open bars), 2 mM glucose with 10 µM Baclofen (open bars with diagonal stripes), 18 mM glucose (filled bars) and 18 mM glucose with 10 µM Baclofen (open bars with vertical stripes). A: mice; data are mean±SEM of 4 experiments. B: Patient 3. C: Patient 4. D: Patient 5. *$P < 0.05$; ns = non-significant.
Fig. 5.2 RT-PCR on isolated islets from mice with specific primers targeting GABA<sub>B</sub>R subunits and calcineurin subunits. Total RNA extracted from isolated mice islets were reverse transcribed into cDNA and subsequently amplified using gene-specific primers against mouse GABA<sub>B</sub>R subunits 1 (Gabbr1) and 2 (Gabbr2); mouse calcineurin catalytic subunit alpha isoform (Ppp3ca), beta isoform (Ppp3cb) and gamma isoform (Ppp3cc); and mouse calcineurin regulatory subunit alpha isoform (Ppp3r1) and beta isoform (Ppp3r2). In RT negative controls (-), reverse transcriptase was omitted to monitor genomic DNA contamination. Samples from 4 separate isolation procedures were tested. gDNA: mouse genomic DNA positive control. M: PCR product size marker. Neg Ctrl: negative control with Taq polymerase omitted.

human, unlike mouse islets, GABA<sub>B</sub>R1 was expressed instead of GABA<sub>B</sub>R2 while alpha isoform of the catalytic subunit of calcineurin was missing (Fig. 5.3). Interestingly, a
variation was observed among CHI patients (Fig. 5.3). While the expression patterns of the subunits of calcineurin were consistent with those in adult human, Patient 1, in contrast to all other patients as well as adult human, did not express GABA$_B$R1. Moreover, isolated islets from Patient 2 and intact tissue sample from Patient 5 unexpectedly exhibited GABA$_B$R2 expression whereas other patients were negative. The strong bands of insulin in all samples tested indicated a high quality of the total RNA (Fig. 5.2, 5.3).

Fig. 5.3 RT-PCR on isolated islets and intact tissue samples from adult human and CHI patients with specific primers against GABA$_B$R subunits and calcineurin subunits. Total RNA extracted from isolated islets (Patient 1 and 2) and intact tissue samples (adult and Patient 3-5) were reverse transcribed into cDNA and subsequently amplified using gene-specific primers against human GABA$_B$R subunits 1 (GABBR1) and 2 (GABBR2); human calcineurin catalytic subunit alpha isoform (PPP3CA), beta isoform (PPP3CB) and gamma isoform (PPP3CC); and human calcineurin regulatory subunit alpha isoform (PPP3R1) and beta isoform (PPP3R2). In RT negative controls (-), reverse transcriptase was omitted to monitor genomic DNA contamination. gDNA: human genomic DNA positive control. M: PCR product size marker. Neg Ctrl: negative control with Taq polymerase omitted.
5.5 Discussion

To date, all the available treatment options for CHI are limited in their usefulness to some extent because of either nasty side effects or poor responsiveness. As a result, a good percentage of the patients have to undertake surgery to maintain euglycaemia; however, except for focal form of CHI, patients with severe diffuse disease are far from safe after the operation due to various possible post-operative complications, including recurring hypoglycaemic episodes (which might lead to reoperation), pancreatic exocrine insufficiency and diabetes mellitus (Lovvorn et al., 1999, Meissner et al., 2003, Arnoux et al., 2010). Therefore, it is paramount to explore new medical treatment options for CHI in order to reduce the risks of adverse effects from medication and surgical intervention.

GABA, as a potent inhibitory neurotransmitter, plays important roles in the regulation of neuron firing in the CNS (Franklin and Wollheim, 2004). However, controversial results have been reported trying to uncover the mechanisms by which GABA modulates hormone secretion in the endocrine pancreas. In the present study, we investigated the effects of GABA_B receptor agonist baclofen in controlling insulin secretion in islets from mice and patients with CHI. Results suggest that, islets from mice (Fig. 5.1A) and 1 of the CHI patients (Fig. 5.1B) exhibited GSIS at 18 mM glucose concentration and baclofen significantly reduced insulin release during 1 hour incubation while no obvious inhibitory effect was observed at 2 mM glucose concentration. On the other hand, islets from the other 2 CHI patients failed to show any increase in insulin secretion at high glucose concentration and baclofen did not have significant inhibitory effect on insulin release. Although the basal level of insulin secretion of these 2 patients was about 10-fold that of the other patient, significant decrease of insulin release was not observed in the presence of 10 µM baclofen (Fig. 5.1B, C). These data suggest that the inhibitory effect of baclofen is
independent of the basal level of exocytosis and $K_{\text{ATP}}$ channel activity but is dependant of glucose-induced exocytosis. This supports a previous report claiming that baclofen does not affect $[\text{Ca}^{2+}]_i$ or electrical activity of rat $\beta$-cells but instead directly inhibits the more distal end of the exocytosis process (possibly through influencing the release of ‘readily releasable pool’ (RRP)) (Braun et al., 2004). Indeed, as basal electrical and RRP activity are expected to be constantly high in CHI $\beta$-cells due to excessive $\text{Ca}^{2+}$ influx or uncontrolled glucose metabolism (Dunne et al., 2004), we speculate that the mechanism(s) by which $\text{GABA}_B$ receptor activation suppresses insulin exocytosis is tightly related to signaling pathways that are activated only during hyperglycaemia and likely does not depend on intracellular electrical activity.

$\text{GABA}_B$ receptor is a heteromeric G-protein-coupled receptor that is composed of 2 subunits: $\text{GABA}_B$R1 and $\text{GABA}_B$R2 (Jones et al., 1998, Kaupmann et al., 1998, White et al., 1998). $\text{GABA}_B$R1 is responsible for ligand binding (Galvez et al., 2000a, Galvez et al., 2000b, Galvez et al., 2001) and $\text{GABA}_B$R2 is critical for $\text{GABA}_B$R1 trafficking and downstream intracellular signalling including G-protein activation (Couve et al., 1998, Margeta-Mitrovic et al., 2000, Robbins et al., 2001, Duthey et al., 2002, Havlickova et al., 2002). The expression patterns of subunits of $\text{GABA}_B$ receptor in islets have been previously studied. Both $\text{GABA}_B$R1 and $\text{GABA}_B$R2 have been found to be expressed in human islets (Brice et al., 2002) and rat islets (Braun et al., 2004), whereas Bonaventura et al. (Bonaventura et al., 2012) confirmed the existence of $\text{GABA}_B$R2 in mouse islets. However, via RT-PCR, our data showed that, when during hypoglycaemia (no glucose was used during islet isolation), mouse islets only expressed $\text{GABA}_B$R2 whereas, by contrast, adult human islets did not express $\text{GABA}_B$R2. Since it is generally believed that both subunits of $\text{GABA}_B$ receptor are essential for proper receptor assembly and function and
downstream signal transduction (Jones et al., 1998, White et al., 1998, Kuner et al., 1999, Gassmann et al., 2004), we speculate that the expression of GABA_B receptor subunits and the successful formation of the receptor require at least a non-hypoglycaemic environment. In CHI patients, the expression patterns of GABA_B receptor subunits varied (Fig. 5.3). However, the reason to explain this phenomenon is still unclear. Further research into the alteration of GABA_B receptor subunit expression at different glucose concentration is required.

Protein phosphatase calcineurin plays an important role in modulating β-cell differentiation, development, growth and function (Heit et al., 2006, Tashiro et al., 2006, Heit, 2007, Soleimanpour et al., 2010, Demozay et al., 2011, Goodyer et al., 2012, Kragl and Lammert, 2012), and, as a critical player in Ca^{2+}-dependant exocytosis, is the downstream target upon baclofen stimulation (Braun et al., 2004). The activity of calcineurin depends on the proper formation of heterodimer by its catalytic subunit and regulatory subunit (Rusnak and Mertz, 2000). Here our data demonstrate the presence of all 3 isoforms of calcineurin catalytic subunit and alpha isoform of calcineurin regulatory subunit in mouse islets (Fig. 5.2), whereas in adult human and CHI patients beta and gamma isoform calcineurin catalytic subunit and alpha isoform of calcineurin regulatory subunit were present (Fig. 5.3). These findings confirm the existence of calcineurin in mouse and human islets on mRNA level; however, it still requires further investigation to confirm the level of calcineurin protein in islets as well as the level of baclofen-induced calcineurin activation at difference glucose concentration in both mouse and CHI islets.

Taken together, in the present study, we have demonstrated that, in mice and 1 of the diffuse CHI patient, 10 µM baclofen significantly inhibited GSIS in the presence of 18 mM glucose but not 2 mM glucose. This inhibitory effect of baclofen was not seen in the other
2 CHI patients tested, who also failed to exhibit any obvious increase of insulin release upon elevation of glucose concentration. These data suggest that baclofen might not be a good candidate for CHI treatment as GSIS is usually missing in CHI due to constant excessive insulin secretion. We were also able to prove that there was differentiation in the expression of GABA<sub>B</sub> receptor subunits and subunits of calcineurin among mouse, adult human and CHI islets. Further research into the field is necessary to determine the cause of these variations.

5.6 Acknowledgement

We are thankful to the support from members of Northern Congenital Hyperinsulinism Service (NORCHI) to the study. We acknowledge Prof. N. Hanley for provision of human adult control histological sample.

5.7 Reference


AYNSLEY-GREEN, A., HUSSAIN, K., HALL, J., SAUDUBRAY, J. M., NIHOUL-FEKETE, C., DE LONLAY-DEBENEY, P., BRUNELLE, F.,


agonist-binding site of GABAB type 1 subunit sheds light on the activation process of GABAB receptors. *J Biol Chem*, 275, 41166-41174.


GU, X. H., KUROSE, T., KATO, S., MASUDA, K., TSUDA, K., ISHIDA, H. & SEINO, Y.


PANGALOS, M. N. 2001. GABA(B2) is essential for g-protein coupling of the GABA(B) receptor heterodimer. *J Neurosci*, 21, 8043-8052.


WENDT, A., BIRNIR, B., BUSCHARD, K., GROMADA, J., SALEHI, A., SEWING, S.,


Chapter 6: Expression of Somatostatin Receptors in Congenital Hyperinsulinism: SOM230 as a Potential Replacement for Octreotide

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6.1 Abstract

In most cases, loss of function defects in ATP-sensitive potassium (K_{ATP}) channel encoding genes – ABCC8 and KCNJ11 – cause congenital hyperinsulinism (CHI). In diffuse CHI, gene defects are found in all K_{ATP} channel expressing cells, while, in focal form of CHI, gene defects are localised to a small and discrete region of the pancreas. Somatostatin plays important roles in the generation of pancreas and subsequent hormone expression.
Therefore, research into somatostatin receptors (SSTRs) and SST analogues is highly valuable to the investigation of CHI etiology and advancement of CHI treatment. We investigated the expression patterns of SSTRs in 12 CHI patients and found that (1) SSTR2 expression was reduced in half of the diffuse CHI cohort but recovered after surgery and octreotide withdrawal, while in focal patients SSTR2 expression was properly maintained; (2) in diffuse CHI, expression patterns of SSTR1, 3 and 4 (but not SSTR5) were similar to those in adult controls; and (3) in focal CHI, loss of the expression of SSTR1, 4 and 5 was noticed in β-cells from non-lesion, β-cells from lesion and all lesion cells, respectively, while the rest remain comparatively normal. We also tested and compared the efficacy of SST analogues octreotide and SOM230 in inhibiting insulin release in islets from a suspected atypical patient. Results suggest that SOM230, similar to octreotide, was able to inhibit insulin release but at higher concentrations than octreotide. This indicates the potential application of SOM230 in future CHI treatment.
6.2 Introduction

Congenital Hyperinsulinism (CHI) is a rare and potentially lethal pancreatic β-cell disorder and is associated with inappropriate insulin secretion for the level of glycaemia (Dunne et al., 2004). CHI is the most common cause of persistent hypoglycaemia in neonates and infants (De León and Stanley, 2007). The estimated incidence of CHI is within 1:27,000 to 1:50,000 in the general western population while can reach up to 1/3,000 in highly consanguine societies (Glaser et al., 2000). Although the molecular cause of approximately 50% of the CHI cases are still unknown, mutations in ATP-sensitive potassium channel (K\textsubscript{ATP}) channel subunits encoding genes \textit{ABCC8} and \textit{KCNJ11} are responsible for the majority of the cases with known aetiology while other known genetic defects have also been found including mutations in \textit{GLUD1}, \textit{HADH}, \textit{HNF4a} and \textit{GCK} (Giurgea et al., 2006, De León and Stanley, 2007, James et al., 2009). Histologically, CHI is commonly classified into two major forms: diffuse CHI and focal CHI. The entire pancreas is affected and enlarged nuclei in islets of Langerhans are found in diffuse form of CHI, while in focal CHI only a small discrete region is abnormal (presents as adenomatous hyperplasia of the endocrine cells) as a result of loss of heterozygosity of chromosome region 11p15 leading to imbalanced expression of cell proliferation regulatory genes (De León and Stanley, 2007). A third type of CHI (atypical CHI) has recently been identified which presents as morphological mosaicism in the islets (Rahier et al., 2011, Sempoux et al., 2011). Excessive expression of low-Km hexokinase 1 (HK1) and somatic activating mutation in \textit{GCK} gene in a subpopulation of islets, which are confined to a few pancreatic lobules, are held responsible for this particular form of CHI (Henquin et al., 2012). Common medical treatment options for CHI include oral administration of K\textsubscript{ATP} channel agonist diazoxide and calcium channel antagonist nefidipine and subcutaneous or intravenous injection of glucagon and octreotide (Hussain, 2008). When medical therapies fail to maintain
normoglycaemia, partial to total pancreatectomy is required (Arnoux et al., 2010).

Somatostatin receptors (SSTRs) belong to seven transmembrane G protein coupled receptor superfamily and 5 isoforms have been identified so far: SSTR1 to SSTR5 with SSTR2 being the only member that has 2 splicing variants- SSTR2a and SSTR2b (Patel and Srikant, 1997, Kumar and Grant, 2010). The expression of SSTRs in the pancreas in human has been previously reported: SSTR1-3 and 5 are highly expressed in the islets while SSTR4 is almost exclusively found in the acini (Kumar et al., 1999, Taniyama et al., 2005, Schmid et al., 2012), whereas one group claimed a strong SSTR4 positivity in the endocrine cells (Portela-Gomes et al., 2000, Portela-Gomes et al., 2010). Although controversial, it appears that β-cells strongly express SSTR1, 2 and 5 while SSTR2 is the predominant SSTR found in α-cells (Kumar et al., 1999). A recent study by Kailey et al. (Kailey et al., 2012) demonstrated dominant anti-secretory effect of SSTR2 over other isoforms in human α- and β-cells while SSTR3 and 5 activation also suppresses insulin exocytosis in β-cells and SSTR1 agonist inhibits exocytotic activity in α-cells.

Octreotide is a long-acting somatostatin analogue used in combination with frequent feeding in CHI management (De León and Stanley, 2007, Hussain, 2008). Octreotide suppresses hormone secretion mainly via SSTR2 and has low to no binding affinity to other somatostatin receptor subtypes (Patel and Srikant, 1997, Bruns et al., 2002, Brunicardi et al., 2003). However, while side effects, including tachyphylaxis, of short- to long-term octreotide treatment have always been concerning (Thornton et al., 1993, Laje et al., 2010, Avatapalle et al., 2012), the majority of the CHI patients fail to avoid surgery despite the use of octreotide (Thornton et al., 1993, Mohnike et al., 2008). On the other hand, SOM230 (also known as pasireotide), an alternative somatostatin analogue with
higher binding affinity to SSTR1, 3 and 5 compared to octreotide (Bruns et al., 2002, Schmid and Schoeffter, 2004), has shown promising efficacy in the treatment of acromegaly, neuroendocrine tumours and Cushing’s disease (Schmid, 2008).

The aim of this study was to immunohistochemically examine the expression of SSTRs in a panel of CHI samples and adult controls in order to address the question of whether any alterations occur in CHI, which could explain the irresponsiveness to octreotide administration. Five newly developed and reported monoclonal SSTR subtype selective antibodies were used (Schmid et al., 2012). Also, the current study aimed to investigate the possibility of using SOM230 as a potential replacement for octreotide in CHI medical management. Octreotide and SOM230 were applied to isolated islets which were extracted from a suspected atypical CHI patient, and results suggested that SOM230, in a smaller effective concentration range compared to octreotide, inhibits insulin release.

6.3 Materials & Methods

Subjects

CHI patients involved in the current study were all diagnosed and treated at the Royal Manchester Children’s Hospital during 1989 to 2012. In all, the clinical diagnosis of CHI was made according to accepted criteria (Dunne et al., 2004, Hussain, 2008, Arnoux et al., 2010). A total of 7 diffuse CHI (including 3 suspected diffuse-atypical patients; age range from 2 to 36 months at the time of operation) and 5 focal CHI (age range from 3 to 19 months at the time of operation) patients were included in the study (Table 6.1). Of the 7 diffuse patients, 3 have had 2 pancreatectomies and samples from both operations were analysed. In 5 focal CHI patients, tissue specimens from both inside and outside the lesion
removed during lesionectomy for 4 patients and therefore were examined comparatively. Of all the 12 patients, 8 were male and 4 were female.

Adult pancreatic control samples were obtained from 2 donors whose causes of death were not related to the pancreas. No pancreatic disease was diagnosed prior to the death of either donor. Representative pancreatic tissue sections from both donors were examined by a certified histopathologist and normal morphology and good tissue preservation was confirmed in both cases. Information of the donors was anonymised due to ethical reasons.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age at surgery (months)</th>
<th>Diagnosis</th>
<th>Gene affected</th>
<th>Mutation (Paternal / Maternal)</th>
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<td>F</td>
<td>2</td>
<td></td>
<td>ABCC8</td>
<td>c.1741T&gt;A / c.3992-9G&gt;A</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>4</td>
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<td>ABCC8</td>
<td>c.107A&gt;G / c.1630+1G&gt;T</td>
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<tr>
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<td>F</td>
<td>13</td>
<td></td>
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<tr>
<td>4</td>
<td>M</td>
<td>3</td>
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<td>ABCC8</td>
<td>T172fs / c.1818-? to 1923+?del</td>
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<td>M</td>
<td>12</td>
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<td>None identified</td>
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</tbody>
</table>

Table 6.1 Clinical characteristics of CHI patients.

_Tissue processing_
Pancreatic tissues were harvested during operations, and archived for subsequent analysis. Representative specimens were fixed with 4% (w/v) buffered formaldehyde for 24 hours at 4°C before being dehydrated, paraffinised and sectioned at 5µm thickness.

**Immunohistochemistry**

Pancreatic sections were deparaffinised in xylene and rehydrated through an ethanol gradient followed by multiple PBS/H$_2$O washes. Antigen retrieval was performed by microwave boiling in 10 mM citrate buffer (pH 6.0; supplemented with 0.05% (v/v) Tween-20) for 10 minutes. Sections were then permeabilised with 0.1% (v/v) Triton-X100 and blocked by 5% (w/v) fish skin gelatin for 1 hour each at room temperature. Subsequently, combinations of insulin (guinea pig anti-pig, 7.5 µg/mL; Life Technologies Ltd, Paisley, UK) or glucagon (mouse anti-pig; 1:1000 dilution; Sigma-Aldrich Co. Ltd., Dorset, UK) antibodies with human SSTR (hSSTR) antibodies (all hSSTR antibodies have been evaluated previously (Schmid et al., 2012); anti-hSSTR1-4 were all raised in mouse and were gifted from Novartis International AG, Basal, Switzerland; anti-hSSTR2 was raised in rabbit and was purchased from Epitomics, Inc., California, USA; anti-hSSTR1: 2.5 µg/mL; anti-hSSTR2: 1:400; anti-hSSTR3: 35.5 µg/mL; anti-hSSTR4: 4.17 µg/mL; anti-hSSTR5: 88 µg/mL) were applied to the sections for overnight at 4°C followed by multiple washes in 0.05% (v/v) Tween-20 supplemented PBS (PBST). Along with 2.5 µg/mL DAPI (Sigma-Aldrich Co. Ltd., Dorset, UK) for nucleus counterstaining, appropriate secondary antibodies conjugated with DyLight 488, Cy3 or DyLight 649 (all purchased from Stratech Scientific Ltd. (Suffolk, UK)) were applied to the sections at the concentration of 7.5 µg/mL for 1 hour at room temperature. Finally, slides were washed with PBST and mounted with Vectashield (Vector Laboratories Ltd., Peterborough, UK). Sections were examined on an Olympus BX51 upright microscope (Olympus UK Ltd.,
Watford, UK) and images were collected using a 20X objective with a Coolsnap ES camera (Photometrics UK, Marlow, UK) through MetaVue (Molecular Devices, Pennsylvania, USA). Images were processed and analysed using ImageJ (v1.47b; http://rsb.info.nih.gov/ij/).

Pancreatic islet isolation

Pancreatic tissue was disassociated using liberase TL research grade (Roche Diagnostics Ltd., Burgess Hill, UK) digestion in Krebs ringer phosphate HEPES (KRH) buffer (129 mM NaCl, 5 mM NaHCO3, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgCl2, 10 mM HEPES, 2.5 mM CaCl2 and 0.1% (w/v) BSA; pH 7.4) at 37°C. The final concentration of liberase was 0.73 mg/mL. Islets were handpicked under a dissection microscope and, before being used in insulin secretion assay, cultured in tissue culture medium containing 88% (v/v) RPMI1640 medium (with 11mM D-glucose), 10% (v/v) foetal bovine serum and 2% (v/v) penicillin-streptomycin (10000 U/ml and 10000 µg/ml, respectively) in non-cell-culture-treated 6 well plates overnight in humidified atmosphere with 5% CO2 supply at 37°C.

Insulin secretion assay

Islets were transferred into borosilicate tubes (5 islets/tube) and were allowed to equilibrate for 1 hour at 37°C and subsequently washed once in modified KRH buffer (136.9 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO4, 0.34 mM Na2HPO4, 0.44 mM KH2PO4, 1.29 mM CaCl2, 4.17 mM NaHCO3, 10 mM HEPES and 0.1% (w/v) BSA; pH 7.5) containing 2mM D-glucose. After removing the KRH buffer, various treatments (all formulated upon modified KRH buffer), including 2mM D-glucose, 18mM and 18mM D-glucose with 10^-6 to 10^-9 M Octreotide or SOM230 (both somatostatin analogues were gifted from Novartis
International AG, Basal, Switzerland), were applied to the islets for 2 hours at 37°C in triplicates. All incubations were done statically. Test solutions were removed and aliquoted while islets with leftover test medium were mixed with equal volumes of 2% (v/v) Triton-X100. All solutions were stored at -80°C until analysis.

**Determination of insulin concentration**

The concentration of insulin secretion in the test medium and insulin content of the islets were assessed using ultrasensitive insulin ELISA (ALPCO Diagnostics, New Hampshire, USA).

**Statistical analysis**

The data are expressed as Mean±SEM. Differences in the means were determined via one-way ANOVA followed by Dunnett’s *post hoc* test using GraphPad Prism 6 (GraphPad Software, Inc., California, USA). A *P*-value < 0.05 is considered as statistically significant.

**6.4 Results**

A summary of the immunohistochemical data can be found in Table 6.2. All data were categorised based on the form of CHI and the subtype of SSTRs, and were semi-quantified based on the coverage and intensity of the antibody staining using a single criterion. This criterion is that the assessment of the coverage of the antibody staining is based on the proportion of positive cells in the total cell count and the assessment of the intensity of the antibody staining is based on a subjective evaluation and comparison of the staining intensity across all the samples from a single person.
Expression of SSTR2 in diffuse CHI

Since SSTR2 is the most important SSTR isoform in controlling insulin and glucagon secretion (Kailey et al., 2012), we first examined SSTR2 co-localisation with insulin and glucagon. Dual immunostaining results suggested that, in normal adult, SSTR2 was strongly expressed in all β- (Fig. 6.1A) and α-cells (Fig. 6.1D). In diffuse CHI, half of the patients exhibited similar level of SSTR2 expression to normal adults in β-cells (Fig. 6.1B), while the rest of the patients had dramatically reduced SSTR2 expression (Fig. 6.1C). In α-cells, all diffuse CHI patients suffered from moderate (Fig. 6.1F) to complete (Fig. 6.1E) loss of SSTR2 staining.

Post-operative recovery of SSTR2 in diffuse CHI

By comparing the intensity and coverage of the staining of SSTR2 observed in islet cells between the pancreatic tissue harvested from 1st and 2nd operations, a recovery of the receptor was noticed in all diffuse patients. An obvious increase of SSTR2 expression was found in islet cells in Patient 4 after the surgery (Fig. 6.2). Further investigation, by co-localisation study, revealed that, SSTR2 expression was elevated post-operatively in β-cells (Fig. 6.3A vs. Fig. 6.3B) as well as in α-cells (Fig. 6.3C vs. Fig. 6.3D). A similar trend was seen in all 3 diffuse patients who had 2 operations, except for β-cells in Patient 2 (Table 6.2). SSTR2 expression in β-cells in Patient 2 during his first operation reaching a moderate level could explain the lack of further elevation after the surgery (Table 6.2). It is worth noticing that all 3 diffuse patients who underwent 2 operations were taken off octreotide for various period after their 1st pancreatectomy while their blood glucose levels were being monitored, which could suggest a possible connection between SSTR2 post-operative elevation and the withdrawal of octreotide treatment.
Table 6.2 Summary of expression of SSTRs in adult control and CHI. ND: Not determined; +: weak; ++: moderate; +++: strong; *< 50% of the cells; **≥50% but <100% of the cells.
Fig. 6.1 SSTR2 expression is modified in some diffuse CHI patients. Pancreatic tissue sections from adult control 2 (A and D) and diffuse CHI patients (B, C, E and F) were co-labelled with SSTR2 (red in all) and insulin (green in A-C) or glucagon (green in D-F). While Patient 1 (1st operation) (B) exhibited strong SSTR2 positivity in all β-cells, in Patient 4 (1st operation) (C), loss of SSTR2 expression was evident in the majority of insulin positive cells. SSTR2 expression in α-cells was completely lost in Patient 3 (1st operation) (E) and was remarkably reduced in Patient 4 (1st operation) (F). DAPI is displayed as blue in all panels. Scale bars = 50 µm.
Fig. 6.2 Recovery of SSTR2 expression in Patient 4 after pancreatectomy. Pancreatic tissue sections from diffuse CHI Patient 4 were collected after 1\textsuperscript{st} and 2\textsuperscript{nd} pancreatectomy and labelled with SSTR2 (red) and DAPI (blue). While SSTR2 expression was almost completely missing in pre-operative tissue (A), a general restoration of SSTR2 expression was noticed in the pancreas from the 2\textsuperscript{nd} surgery (B). Scale bars = 50 µm.
Fig. 6.3 Recovery of SSTR2 expression in α- and β-cells in diffuse CHI. Pancreatic tissue sections from Patient 2 (A and B) and Patient 4 (C and D) were co-labelled with SSTR2 (red in all) and insulin (green in A and C) or glucagon (green in B and D). Elevation of SSTR2 expression overlapped with insulin in Patient 2 (A vs. B) and glucagon in Patient 4 (C vs. D). DAPI is displayed as blue in all panels. Scale bars = 50 µm.
Expression of SSTR1 and 3-5 in diffuse CHI

SSTR1 was found to be strongly expressed in β-cells in all adult controls (Fig. 6.4A) and the vast majority of the diffuse CHI patients (Fig. 6.4B, Table 6.2). Unfortunately, we weren’t able to perform SSTR1/glucagon co-staining to further investigate the expression pattern of SSTR1 in α-cells because the primary antibodies were raised from the same species and were in the same subclass of IgGs.

SSTR3 appeared to have an α-cell-predominant expression pattern. It was moderately to strongly produced in α-cells of adult controls (Fig. 6.5A) and diffuse CHI patients (Fig. 6.5B, Table 6.2). On the other hand, SSTR3 was only weakly positive in the majority of β-cells in both adult controls (Fig. 6.4C) and in 50% the diffuse CHI patients (Fig. 6.4D, Table 6.2).

Similar to adult controls (Fig. 6.4E), weak to moderate level of SSTR4 expression was confined to β-cells in diffuse cases (Fig. 6.4F, Table 6.2), although moderate to high level of expression in the acinar cells could be frequently observed.

SSTR5, as the last member of the SSTR family, exhibited a β-cell-predominant expression profile. It was moderately produced in insulin-positive cells in both controls and in 2 of the 4 patients with diffuse CHI (Fig. 6.4G, Table 6.2). However, in the other 2 diffuse CHI patients, the level of SSTR5 expression in insulin-producing cells was remarkably weakened (Fig. 6.4H, Table 6.2). While co-localisation with glucagon could be rarely spotted in adult controls (Fig. 6.5C), SSTR5 was almost completely absent in diffuse CHI (Fig. 6.5D, Table 6.2).
Fig. 6.4 Differential expression of SSTR1, SSTR3-5 in β-cells in adult human and diffuse CHI patients. In all panels, insulin is labelled as green, SSTRs are red and DAPI is displayed as blue. High level of SSTR1 expression was found in β-cells in adult control 1 (A) and Patient 3 (1\textsuperscript{st} operation) (B). The vast majority of insulin producing cells showed weak SSTR3 positivity in adult control 2 (C) and patient 2 (1\textsuperscript{st} operation) (D). Low to moderate level of SSTR4 staining expression was observed in insulin positive cells in adult control 1 (E) and Patient 2 (2\textsuperscript{nd} operation) (F). SSTR5 predominantly resided in β-cells in adult control 1 (G) while Patient 4 (1\textsuperscript{st} operation) lost SSTR5 expression completely (H). Scale bars = 50 μm.
Fig. 6.5 Expression of SSTR3 and SSTR5 in α-cells in adult human and diffuse CHI patients. In all panels, glucagon is labelled green and DAPI is blue while red represents staining of SSTR3 (A and B) or SSTR5 (C and D). Co-staining of glucagon and SSTR3 indicated that all α-cells in adult control 2 (A) and Patient 3 (1st operation) (B) strongly expressed SSTR3. SSTR5 staining could only be found in a very small population of α-cells (as indicated by white arrows) in adult control 1 (C) and was completely absent in Patient 3 (1st operation) (D). Scale bars = 50 µm.
Focal CHI is associated with reduced expression of SSTR1 and 5 in resting islets as well as SSTR4 in the focal lesion

Expression profiles of SSTR1-5 were examined in α- and β-cells in focal CHI patients. Compared to adult controls, SSTR1 expression level in β-cells was maintained or only mildly reduced in the most of the focal lesions (Fig. 6.6A, Table 6.2) but the most of the surrounding tissue exhibited a more profound loss of SSTR1 staining (Fig. 6.6B). Similar to the case of diffuse CHI, we were unable to perform SSTR1/glucagon co-staining. SSTR2 positivity in α- and β-cells was properly preserved or only mildly reduced in the vast majority of the patients (Table 6.2), both inside (Fig. 6.6C and Fig. 6.7A) and outside the focal domain (Fig. 6.6D and Fig. 6.7B). Similarly, the expression profiles of SSTR3 in insulin- (Fig. 6.6E, F) and glucagon-producing cells (Fig. 6.7C, D) as well as SSTR4 in α-cells from the lesion (Fig. 6.7E) and α- (Fig. 6.7F) and β-cells (Fig. 6.6H) residing in the surrounding tissue were comparable to adult controls in most of the focal cases (Table 6.2). However, notably, colocalisation of SSTR4 and insulin was completely lost in 4 of the 5 lesion samples examined (Fig. 6.6G, Table 6.2). As to SSTR5, while focal domains maintained a similar expression pattern as adult controls (Fig. 6.6I and Fig. 6.7G), in non-lesion islets, staining intensity was mildly to moderately lessened in β-cells (Fig. 6.6J) and colocalisation with glucagon was missing (Fig. 6.7H).
**Fig. 6.6 Expression of SSTR1-5 in β-cells of focal CHI.** Co-stainings of SSTR1-5 with insulin were performed on pancreatic tissue from focal CHI patients. In all, insulin is green while SSTRs are labelled red and DAPI is blue. Panels on the left are from the inside the lesion while panels on the right are from outside the lesion. SSTR1 and SSTR5 were highly expressed in β-cells in the focal domain in Patient 9 (A) and Patient 6 (B) respectively but were heavily reduced in surrounding tissue from Patient 8 (I) and Patient 5 (J) respectively. In β-cells of Patient 9, SSTR2 was consistently strongly produced (C and D) and SSTR3 was feebly expressed (E and F) whereas SSTR4 expression was absent in the focal lesion (G) while feebly to moderately present in non-lesion tissue (H). Scale bars = 50 µm.
Fig. 6.7 Expression of SSTR2, 3 and 5 in α-cells of focal CHI. To determine the expression patterns of SSTRs in α-cells, pancreatic tissue sections from Patient 9 were co-labelled with glucagon and SSTR2, 3 and 5. In all panels, SSTRs, glucagon and DAPI are respectively labelled as red, green and blue. Panels on the left are from the focal domain while panels on the right are from the surrounding tissue. SSTR2 (A and B) and SSTR3 (C and D) were both intensely expressed in glucagon-producing cells. On the other hand, SSTR5 staining was only feebly present in α-cells in the focal lesion (E) but not outside the lesion (F). Scale bars = 50 µm.
Octreotide and SOM230 inhibit insulin release in isolated islets from atypical CHI

In order to evaluate and compare the power of octreotide and SOM230 in suppressing insulin release in CHI, islets extracted from a suspected diffuse-atypical CHI patient (Patient 12) were either maintained in 2 mM glucose or stimulated with 18mM glucose and various concentrations of octreotide and SOM230 were applied. The patient had low to moderate level of expression of SSTR1-5 in β-cells and SSTR2-4 in α-cells (Table 6.2). Results suggested that 18 mM glucose, instead of stimulating, significantly reduced insulin secretion compared to 2 mM glucose. In the presence of 18 mM glucose, 1 nM, 10 nM, 100 nM and 1 µM of octreotide significantly suppressed insulin release ($P<0.01$ to $P<0.05$) while only 10 nM, 100 nM and 1 µM of SOM230 significantly reduced insulin secretion ($P<0.01$ to $P<0.05$) (Fig. 6.8).
Fig. 6.8 Octreotide and SOM230 inhibited insulin secretion in suspected diffuse-atypical CHI. Islets isolated from suspected diffuse-atypical CHI patient 12 were stimulated with 2 mM and 18 mM glucose as well as $10^{-9}$ to $10^{-6}$ M of octreotide or SOM230 in the presence of 18 mM glucose. 18 mM glucose (n=3) significantly reduced insulin secretion compared to 2 mM glucose (n=2). $10^{-6}$ M (n=2), $10^{-7}$ M (n=2), $10^{-8}$ M (n=2) and $10^{-9}$ M (n=3) octreotide significantly inhibited insulin release in the presence of 18 mM glucose. $10^{-6}$ M (n=3), $10^{-7}$ (n=3) and $10^{-8}$ (n=2) but not $10^{-9}$ (n=2) SOM230 suppressed insulin secretion to a significant level. *$P<0.05$; **$P<0.01$; ns = non-significant.

6.5 Discussion

Somatostatin is expressed in various vital organs in the human body and exerts important inhibitory functions in regulating hormone secretion. Somatostatin functions through the
activation of SSTRs that are expressed in various combinations of the five subtypes-SSTR1 to SSTR5 at different densities (Kumar and Grant, 2010). In the pancreas, the distribution of SSTRs has been reported previously by a few groups although discrepancies exist presumably due to different antibodies used (Kumar et al., 1999, Portela-Gomes et al., 2000, Taniyama et al., 2005, Portela-Gomes et al., 2010, Schmid et al., 2012). In agreement with the study conducted by Schmid et al. (Schmid et al., 2012), which utilised the same antibodies as in the current study, we confirmed, in normal adult human, the predominant expression of SSTR1, 2 and 5 in β-cells and SSTR2 and 3 in α-cells, although SSTR4 expression was not found in the islets in their study but demonstrated by us.

To our knowledge, the current study is the first investigation of SSTR expression in CHI patients. Our results indicate that expression of SSTRs was altered in a subpopulation of diffuse and diffuse-atypical CHI patients. In these patients, SSTR2 expression was reduced in β-cells, which could significantly lower the effectiveness of SSTR2-specific somatostatin analogues like octreotide (Patel and Srikant, 1997, Bruns et al., 2002, Brunicardi et al., 2003) and lanreotide (Colao et al., 2010). Since these patients received pancreatectomy due to either their poor response to octreotide or the development of tachyphylaxis, reduction in SSTR2 expression could explain the clinical manifestation. In our study, diffuse patients who received more than one pancreatectomy showed elevation of SSTR2 expression while their octreotide treatment was cancelled, which indicates that tachyphylaxis (presented as loss of SSTR2 expression) could possibly be relieved by alteration of medication. However, it is also possible that the increase of SSTR2 expression was related to the regeneration of pancreas.

Octreotide, as an important anti-secretory reagent for glycaemic control, is widely used in
CHI management, especially in those diffuse diazoxide-irresponsive patients. Recently, long-acting release forms of octreotide and lanreotide have been reported to have satisfactory effect in CHI management (Modan-Moses et al., 2011, Kuhnen et al., 2012, Le Quan Sang et al., 2012). However, given the reduced or complete loss of SSTR2 expression in β-cell population and that insulin release can be inhibited by SSTR3 and 5 activation (Kailey et al., 2012), one would speculate that alternative somatostatin analogues like SOM230 which targets SSTR subtypes other than SSTR2 could be beneficial (Bruns et al., 2002). Indeed, according to our observation, almost all the diffuse and suspected diffuse-atypical CHI patients had SSTR5 expression in β-cells while SSTR3 was found in all these patients (Table 6.2). Moreover, since SSTR2 was present in α-cells in almost all of these patients (Table 6.2) and SSTR2 is the functionally dominant SSTR in human α-cells (Kailey et al., 2012), replacing octreotide and lanreotide with SOM230 can also minimise the influence of the medication to the natural counter-regulatory response in the patients.

In the present study, we were able to prove that SOM230 was capable of suppressing insulin release in the presence of high glucose concentration in a suspected diffuse-atypical patient although, compared to octreotide, the inhibitory effect was only observed in a higher concentration range (Fig. 6.5). Surprisingly, 18 mM glucose did not increase but significantly reduced insulin release. This peculiar phenomenon has been previously reported in both focal and diffuse CHI patients with $K_{\text{ATP}}$ channel mutations although no definitive cause could be identified (Henquin et al., 2011). We acknowledge that we cannot confirm that the inhibitory effect that we have observed from SOM230 was only caused by SSTR3 and SSTR5 activation as SOM230 still exhibits moderate level of binding affinity to SSTR2 (Schmid, 2008). However, in murine corticotroph cells, SOM230 exerts its
higher potency than octreotide in suppressing forskolin-induced cAMP accumulation and calcium oscillation primarily via SSTR5 while the ligand effect on SSTR2 is negligible (Ben-Shlomo et al., 2009). Furthermore, *in vitro* study on HEK293 cells expressing human SSTRs have revealed that, compared to octreotide, SOM230 is more potent in SSTR3 and SSTR5 activation but not SSTR2 (Lesche et al., 2009). Therefore, it is safe to assume that SOM230 inhibits insulin secretion mainly via SSTR3 and/or SSTR5.

In summary, we have demonstrated high variation in the expression of SSTRs in diffuse and suspected diffuse-atypical CHI patients while the expression was more consistent in focal CHI patients. Notably, a subpopulation of the diffuse and suspected diffuse-atypical CHI patients lost SSTR2 expression in β-cells while SSTR2 expression was present in α-cells in almost all the patients. Therefore, because octreotide predominantly targets SSTR2, an alternative somatostatin analogue is needed to resolve irresponsiveness to octreotide while minimising the inhibitory effect on glucagon secretion. Also, we have shown that, on isolated islets from a suspected diffuse-atypical CHI patient who had weak to moderate levels of expression of all 5 SSTRs in β-cells, SOM230 was capable of inhibiting insulin release at a higher concentration range than octreotide, suggesting its potential application in CHI treatment. Further research is needed to determine if SOM230 outperforms octreotide in patients with very weak or no SSTR2 and in a bigger patient cohort. Moreover, more studies are required to further understand the pharmacodynamics of SOM230 in order to evaluate its clinical potentials.

6.6 Acknowledgement

We thank Novartis Novartis International AG for gifting us the SSTR antibodies and
somatostatin analogues. We also acknowledge the members of Northern Congenital Hyperinsulinism Service (NORCHI) for their support to the study.

6.7 References


DIFFERENTIAL EFFECTS OF OCTREOTIDE AND PASIREOTIDE ON SOMATOSTATIN RECEPTOR INTERNALIZATION AND TRAFFICKING IN VITRO. *J Clin Endocrinol Metab*, 94, 654-661.


Chapter 7 Summary

The purpose of this thesis was to explore new diagnostic and therapeutic options for the treatment of CHI as well as to expand our knowledge on islet development and maintenance and CHI pathogenesis. A number of hypotheses in the relevant field have been evaluated and examined in a series of four “papers” (Chapters 3 to 6). The following is a summary of the original objectives and of the outcomes of each paper. The additional experiments required to take each paper to “publication-quality” are also described.

7.1 Chapter 3: Differential Presentation of Pancreatic Islet Architecture in Congenital Hyperinsulinism

The objectives of Chapter 3 were to investigate the morphological changes of the islet structure in CHI and their significance to disease pathogenesis and progression by examining the distribution patterns of α- and β-cells in CHI patients and SUR1-KO mice.

The following outcomes were achieved:

(1) Even distribution of α-cells and β-cells throughout the islets were found in normal adult and young child;

(2) There was no significant structural difference between islets from normal adult and 3-year-old infant which indicates the completion of postnatal islet maturation around the age of 3 years;

(3) Compared to adult and young child controls, diffuse CHI patients exhibited similar islet architecture, while focal and atypical CHI patients showed significant α-cell peripheralisation and/or β-cell centralisation;
(4) Delayed postnatal islet development was found in all three forms of CHI as indicated by the appearance of cells that co-express insulin and glucagon;

(5) A different islet cell organisation pattern to human was seen in mice: α-cells localised at the periphery of the islets and β-cells resided in the core area; however, this structure was altered in SUR-1 KO mice whose α-cells were significantly more evenly distributed throughout the islets.

Additional work required includes:

(1) It will be interesting to research the normal process of postnatal islet formation by determining the alterations in islet cell distribution from birth to puberty so that the comparison between controls and CHI patients could be more conclusive;

(2) When quantifying the staining intensity of hormones across the islets, it will be better to divide the data into 10 or more parts rather than 4 parts so that the periphery and the core of the islets can be more clearly defined; also, a figure to demographically explain how this quantification method works is needed;

(3) The sample number was 5-6 islets from at least 3 images in this Chapter for quantification. It would be better to test sample tissues from more areas of the pancreas for each patient/mice so that the difference of islet structure across the whole pancreas could be taken into account;

(4) There are vague expressions in this Chapter about the “compactness” of the islets; however, there is no data but only observation. Therefore, in order to quantitatively compare the degree of islet maturation, it is important to measure the density of islet cells (e.g. islet cell count/10000µm²).
7.2 Chapter 4: Novel Variants of Congenital Hyperinsulinism Detected by Measurements of Serum GLP-1 (7-36) / GIP

The objectives of Chapter 4 were to test the hypothesis that serum levels of enteroendocrine incretins GLP-1 and GIP vary among different forms of CHI and to see if this can be used as a biomarker for the diagnosis of CHI.

The following outcomes were achieved:

(1) No significant differences in serum GLP-1 and GIP concentrations were observed in serum from patients with transient, diffuse and focal CHI. These data suggest that $K_{\text{ATP}}$ channels may not play a major role in nutrient-sensing by enteroendocrine cells;

(2) Patients with atypical CHI exhibited markedly elevated post-prandial concentrations of serum GLP-1 and GIP and GLP-1 (7-36)/GIP ratio suggesting that measurements of serum GLP-1(7-36) and GIP may be important in the diagnosis of this novel sub-group of CHI patients.

Additional work required includes:

(1) The diet of the patients during tests was not consistent. This inconsistency has significant impact on the accuracy of the results. Thus, a more carefully monitored and executed study should be conducted to avoid this problem;

(2) The ages of the patients were markedly different, which may lead to different degrees of digestive system development. Since oral feeding was used, inconsistent levels of digestive system development could lead to uncontrolled variability in the data from these patients. Hence, if more patients can be recruited, a sub-division of the patients
based on their ages could be useful to enhance the accuracy and creditability of the results;

(3) There were only 2 atypical CHI patients recruited in this Chapter, which made it impossible to conduct statistical analysis on this sub-group of patients. Hence, it will be pivotal to recruit more atypical patients so that statistical analysis could be performed and a more persuasive conclusion could be drawn;

(4) It will be helpful to establish the normal ranges of the serum concentrations of GLP-1 and GIP so that the results could be more clinically applicable.

### 7.3 Chapter 5: Differential Inhibitory Effects of GABAB Receptor Agonist Baclofen on Insulin Secretion in Isolated Islets from Mouse and Patients with Congenital Hyperinsulinism

The objectives of Chapter 5 were to study the potential use of the GABA<sub>B</sub> receptor agonist baclofen in suppressing insulin secretion in CHI.

The following outcomes were achieved:

(1) 10 µM baclofen significantly reduced GSIS in mouse islets without affecting basal insulin secretion;

(2) 10 µM baclofen significantly inhibited insulin secretion in CHI islets where GSIS occurred; however, when glucose failed to influence insulin release, baclofen did not suppress insulin secretion. 10 µM baclofen did not significantly affect basal insulin secretion in CHI islets.
(3) Mouse islets expressed: GABA\textsubscript{B}R2, all 3 isoforms of calcineurin catalytic subunit and the alpha isoform of calcineurin regulatory subunit. In human tissue, expression of the following was determined: the beta and gamma isoform calcineurin catalytic subunit and the alpha isoform of calcineurin regulatory subunit. Expression of GABA\textsubscript{B} receptor subunits was variable.

Additional work required includes:

(1) As GSIS rarely occurs in CHI, a study that investigates the effect of baclofen on suppressing insulin secretion at different glucose concentrations in CHI islets would be more useful in determining baclofen suitability for CHI treatment;

(2) The expression patterns of GABA\textsubscript{B} receptor subunits and calcineurin subunits should be determined by quantitative RT-PCR. Western blot should also be used to determine GABA\textsubscript{B} receptor expression and its downstream signals;

(3) It would be interesting to see if the expression of GABA\textsubscript{B} receptor subunits and calcineurin subunits vary under different physiological conditions (e.g. different glucose levels) and with or without baclofen treatment.

7.4 Chapter 6: Expression of Somatostatin Receptors in Congenital Hyperinsulinism: SOM230 as a Potential Replacement for Octreotide

The objectives of Chapter 6 were to examine the pancreatic expression of SSTRs in CHI and to evaluate and compare the efficacy of SST analogues octreotide and SOM230 in inhibiting insulin release.
The following outcomes were achieved:

(1) SSTR2 expression was reduced in half of the diffuse CHI cohort but recovered after surgery and octreotide withdrawal, while in focal patients SSTR2 expression was properly maintained;

(2) In diffuse CHI, expression patterns of SSTR1, 3 and 4 (but not SSTR5) were similar to those in adult controls;

(3) In focal CHI, loss of the expression of SSTR1, 4 and 5 was noticed in β-cells from non-lesion, β-cells from lesion and all lesion cells, respectively, while the rest remain comparatively normal;

(4) SOM230, similar to octreotide, was able to inhibit GSIS but at higher concentrations than octreotide.

Additional work required includes:

(1) To make the results clearer, the Table 2 in this Chapter should be made simpler by only showing the data that represent the majority of the patients in each category;

(2) An insulin secretion assay comparing the efficacy of SOM230 and octreotide should be conducted at basal glucose level instead of the high glucose concentration currently employed;

(3) More CHI patients, especially typical diffuse CHI patients, should be recruited for insulin secretion assay to determine the reproducibility of the results;

(4) Highly SSTR-subtype-selective SST analogues should be tested for their ability to suppress insulin secretion in CHI islets to see if there are also candidates to replace octreotide.
References (Chapter 2 and 7)


BONNER-WEIR, S., INADA, A., YATO H, S., LI, W. C., AYE, T., TOSCHI, E. &


BRICE, N. L., VARADI, A., ASHCROFT, S. J. & MOLNAR, E. 2002. Metabotropic glutamate and GABA(B) receptors contribute to the modulation of


DATE, Y., NAKAZATO, M., HASHIGUCHI, S., DEZAKI, K., MONDAL, M. S.,


*Curr Opin Nephrol Hypertens*, 18, 495-500.


putative role as an islet cell paracrine-signalling molecule. *J Gen Physiol*, 123, 185-190.


GUIOT, Y., SEMPOUX, C., MOULIN, P. & RAHIER, J. 2001. No decrease of the
beta-cell mass in type 2 diabetic patients. Diabetes, 50 Suppl 1, S188.


MAZOR-ARONOVITCH, K., GILLIS, D., LOBEL, D., HIRSCH, H. J.


SAKURABA, H., MIZUKAMI, H., YAGIHASHI, N., WADA, R., HANYU, C. &
YAGIHASHI, S. 2002. Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. 
*Diabetologia*, 45, 85-96.


SNIDER, K. E., BECKER, S., BOYAJIAN, L., SHYNG, S. L., MACMULLEN, C., HUGHES, N., GANAPATHY, K., BHATTI, T., STANLEY, C. A. & GANGULY, A.


WENDT, A., BIRNIR, B., BUSCHARD, K., GROMADA, J., SALEHI, A., SEWING, S.,
from rat alpha-cells is mediated by GABA released from neighboring beta-cells.
*Diabetes*, 53, 1038-1045.

novel developmentally regulated islet cell in the human pancreas. *Regul Pept*, 107,
63-69.

diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, 27,
1047-1053.

WITHERS, D. J., GUTIERREZ, J. S., TOWERY, H., BURKS, D. J., REN, J. M., PREVIS,
S., ZHANG, Y., BERNAL, D., PONS, S., SHULMAN, G. I., BONNER-WEIR, S.
391, 900-904.

CASTEELE, M., MELLITZER, G., LING, Z., PIPELEERS, D., BOUWENS, L.,
SCHARFMANN, R., GRADWOHL, G. & HEIMBERG, H. 2008. Beta cells can be
generated from endogenous progenitors in injured adult mouse pancreas. *Cell*, 132,
197-207.

XU, E., KUMAR, M., ZHANG, Y., JU, W., OBATA, T., ZHANG, N., LIU, S., WENDT, A.,
Intra-islet insulin suppresses glucagon release via GABA-GABAA receptor system.
*Cell Metab*, 3, 47-58.

YOOON, K. H., KO, S. H., CHO, J. H., LEE, J. M., AHN, Y. B., SONG, K. H., YOO, S. J.,
KANG, M. I., CHA, B. Y., LEE, K. W., SON, H. Y., KANG, S. K., KIM, H. S., LEE,

Appendix 1 Materials and Methods

A1.1 Materials

A1.1.1 Chemical Reagents

Unless otherwise stated, all general laboratory reagents used for standard solutions and buffers preparation are analytical reagent grade and supplied by BDH Chemicals Ltd. (Poole, UK), Sigma-Aldrich Co. Ltd. (Gillingham, UK) or Fisher Scientific UK Ltd. (Loughborough, UK).

A1.1.2 Murine Pancreatic Tissue

Mouse pancreatic tissues used in immunohistochemical examination were either kindly gifted from Professor Gisela Drews (University of Tübingen, Tübingen, Germany) or purchased from the Biological Services Facility at the University of Manchester. The generation and characteristics of these SUR1-KO mouse have been described previously (Seghers et al., 2000). Pancreata were fixed through 24h incubation in 4% (w/v) paraformaldehyde and washed twice with PBS, 10 min each, and finally stored in PBS for transportation. Upon reception, tissues were sent to the Histology Facility within the Faculty of Life Sciences for dehydration, paraffinisation and sectioning (performed by Anne Warhurst or Peter Walker, FLS Histology Facility, University of Manchester). Each slide contained 1 piece of 5 µm of tissue.
A1.1.3 Human Pancreatic Tissue

Pancreatic tissue from normal human and CHI patients were either gifted from Professor Neil Hanley (normal human pancreata; University of Manchester, Manchester, UK) or gifted/loaned from Royal Manchester Children’s Hospital (Manchester, UK). Two normal adult human donor pancreata used in the studies were considered unfit for transplantation but otherwise normal. The information of the donors was anonymised for confidentiality. Disease tissue samples were obtained at the time of surgery and fixed with 4% (w/v) PFA for 24h followed by paraffinisation. Tissue sectioning of all tissue samples was accomplished by the Histology Facility within the Faculty of Life Sciences (University of Manchester, Manchester, UK) and all sections were 5 µm thick. Details of CHI patients’ age (at which time the tissue samples were harvested), pathology and identified gene mutations are listed in each relevant ‘Results’ chapters.

A1.1.4 Blood sample collection

CHI patients and patients with transient hypoglycaemia were diagnosed and treated by the Northern Congenital Hyperinsulinism Service at the Royal Manchester Children's Hospital (Manchester, UK). Patients were recruited prospectively between 2010 and 2012 and retrospectively between 2003 and 2010. Local ethical approvals and consents were obtained. Blood samples were collected into BD P700 blood collection tubes (Becton, Dickinson U.K. Limited, Oxford, UK) pre-feed and 20 or 30 minutes (only for patients subjected to OGTT) after the start of the feeding. Samples were centrifuged at 1300g for 10 minutes at 4°C and the plasma was removed, aliquoted and stored at -80°C for future analysis.
**A1.2 Methods**

**A1.2.1 Immunohistochemistry**

*1.2.1.1 Immunofluorescent microscopy*

Tissue sections were deparaffinised and rehydrated through 5 minutes of xylene wash twice, 3 minutes of 99% ethanol wash twice, 3 minutes of 90% (v/v) ethanol, 3 minutes of 70% (v/v) ethanol, 3 minutes of PBS (pH7.2) wash twice and finally brief distilled water (dH₂O) wash. Subsequently, heat induced antigen retrieval was performed by immersing the slides in citrate buffer (pH6.0) and heating in microwave for 2.5 minutes using high power until buffer started to boil (i.e. 100°C) followed by 20 minutes of heating using medium power (i.e. 99-100°C). dH₂O was used to top up the buffer volume to prevent dehydration of the tissue. Then the slides were allowed to cool down at room temperature for 20-30 minutes followed by brief dH₂O rinse. The tissue sections were then circled by a hydrophobic barrier pen and permeabilised for 1 hour by 0.1% (v/v) Triton-X following 1 hour blocking with 5% (w/v) FSG at room temperature in humidified box. The sections were then incubated with primary antibodies (diluted in 1% (w/v) FSG) listed in Table A1 at 4°C overnight in humidified chamber followed by 10 minutes of wash with PBST for 3 times. Subsequently, the sections were incubated with secondary antibodies listed in Table A2 for 1 hour at room temperature in humidified box in dark followed by 10 minutes of wash with PBST for 3 times in dark. Finally, the sections were mounted with coverslips with Vectashield mounting medium (Vector Laboratories, Peterborough, UK) and left overnight at room temperature in dark to dry. In order to monitor autofluorescence and non-specific binding of secondary antibodies, each test slide was accompanied by 1 negative slide where primary antibody was omitted. Images were collected on an Olympus BX51 upright microscope (Olympus UK Ltd, Watford, UK) using a 10X, 20X, 40X or 60X
objective and captured using a Coolsnap ES camera (Photometrics UK, Marlow, UK) through MetaVue Software (Molecular Devices, Pennsylvania, USA). Specific band pass filter sets for DAPI, FITC, Cy3 and Cy5 were used to prevent bleed through from one channel to the next.

<table>
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<tr>
<th>Antibody</th>
<th>Host</th>
<th>Reactivity</th>
<th>Working Concentration</th>
<th>Manufacturer</th>
</tr>
</thead>
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<tr>
<td>Anti-glucagon (clone K79bB10; monoclonal)</td>
<td>Mouse</td>
<td>Human</td>
<td>1:1000 dilution</td>
<td>Sigma-Aldrich Co. Ltd. (Gillingham, UK)</td>
</tr>
<tr>
<td>Anti-insulin (polyclonal)</td>
<td>Guinea pig</td>
<td>Pig</td>
<td>7.5 μg/mL</td>
<td>Life Technologies Ltd. (Paisley, UK)</td>
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<tr>
<td>Anti-hSSTR1 (monoclonal)</td>
<td>Mouse</td>
<td>Human</td>
<td>2.5 μg/mL</td>
<td>Gifted from Novartis International AG (Basel, Switzerland)</td>
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<td>Anti-hSSTR3 (monoclonal)</td>
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<td>Human</td>
<td>35.5 μg/mL</td>
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<td>Anti-hSSTR4 (monoclonal)</td>
<td>Mouse</td>
<td>Human</td>
<td>4.17 μg/mL</td>
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</tr>
<tr>
<td>Anti-hSSTR5 (monoclonal)</td>
<td>Mouse</td>
<td>Human</td>
<td>88 μg/mL</td>
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<tr>
<td>Anti-hSSTR2 (polyclonal)</td>
<td>Rabbit</td>
<td>Human</td>
<td>1:400 dilution</td>
<td>Epitomics, Inc. (California, USA)</td>
</tr>
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Table A1 Primary antibodies used in immunohistochemical studies.
<table>
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<th>Antibody</th>
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<th>Distributor</th>
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<td>Guinea pig</td>
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<td></td>
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<tr>
<td>DyLight 488-conjugated IgG (IgG1 specific)</td>
<td>Goat</td>
<td>Mouse</td>
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<tr>
<td>Cy3-conjugated IgG</td>
<td>Donkey</td>
<td>Mouse</td>
<td>7-7.5 µg/ml</td>
<td>Stratech Scientific Ltd. (Suffolk, UK)</td>
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<td>Cy3-conjugated IgG (IgG2a specific)</td>
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<td>Mouse</td>
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<td></td>
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<tr>
<td>Cy3-conjugated IgG (IgG2b specific)</td>
<td>Goat</td>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DyLight 649-conjugated IgG</td>
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<td>Rabbit</td>
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</table>

Table A2 Secondary antibodies used in immunohistochemical studies.

A1.2.1.2 Image processing, analysis and quantification

Immunofluorescent images were processed and analysed using ImageJ (v1.47b; [http://rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij)).

For the analysis of endocrine cell distribution, 5-6 islets with maximum diameter longer than 100 µm from at least 3 images were analysed per patient or murine tissue sample. Every selected islet was isolated along the margin based on nuclear clustering. For each islet, 6 selection lines angled 0°, 30°, 60°, 90°, 120° and 150° were drawn across the centre of mass and grey values of the staining intensity of insulin or glucagon along each line were tabulated and exported. Data from each selection line were divided into quartiles and the mean of the central two quartiles was defined as core while the mean of the other two was classified as periphery.
A1.2.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

All RT conversion and PCR experiments were conducted using a Techne TC-512 thermal cycler (Bibby-Scientific Ltd., Stone, UK).

A1.2.2.1 RNA Extraction and Conversion

All RNA extractions were performed using QIAGEN RNeasy Mini Kit (QIAGEN Ltd. - UK, West Sussex, UK) according to manufacturer’s handbook. For RNA extraction from tissue samples, approximately 25 mg of tissue was processed. In brief, tissue pieces were lysed in RLT buffer and thoroughly homogenised using a homogenizer. The lysates were then mixed with equal volumes of 70% (v/v) ethanol and centrifuged in RNeasy spin columns. Subsequently, RNA that has bound to the RNeasy spin columns was washed with RW1 buffer and treated with DNase I solution (prepared from RNase-Free DNase Set (QIAGEN Ltd. - UK, West Sussex, UK)) for 15 minutes at room temperature to eliminate DNA carryovers. The columns were then washed with RW1 buffer followed by RPE buffer wash twice. Finally, RNA was collected by adding 30 or 50 µL of RNase-free water and thorough centrifugation. RNA concentration in each sample was then determined by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA).

RT conversion from RNA to complementary DNA (cDNA) was performed using Precision qScript Reverse Transcription Kit (Primer Design Ltd., Southampton, UK). In short, for each RNA extract, 1 µg of template RNA, 1 µL of Oligo-dT primer and appropriate amount of Rnase/Dnase free water were used to constitute a total of 10 µL of reaction mixture and heated to 65°C for 5 minutes. Then the reaction mixture was immediately transferred to ice bath followed by addition of a 10 µL mixture that is composed of 2 µL qScript 10X buffer,
1 µL dNTP mix (10 mM of each), 2 µL DTT (100 mM), 4 µL Rnase/Dnase free water and 1 µL qScript enzyme. Then the mixture was incubated at 55°C for 20 minutes and subsequently inactivated by 75°C incubation for 15 minutes. In addition, in order to monitor genomic DNA (gDNA) contamination in the RNA extracts, each RNA sample has an RT negative control (RT-). During RT conversions of these negative controls, qScript enzyme was replaced by Rnase/Dnase free water.

**A1.2.2.2 PCR**

All primer pairs (Table A3) that have been utilised in the experiments were designed using Primer3 software (Rozen and Skaletsky, 2000) according to mRNA sequences which were obtained from human GeneBank database (NCBI, Maryland, USA).

The ingredients of each PCR reaction mixture (50 µL) are listed in Table A4. For each primer pair, a negative control reaction was performed to indicate that there was no contamination from foreign DNA by replacing gDNA or cDNA with ddH$_2$O. The PCR programme was composed of: initial denaturation for 5 min at 94°C; cycles of 30 seconds annealing at appropriate temperature (see Table A3 for details), 30 seconds elongation at 72°C and 30 seconds denaturation at 94°C; and 8 minutes final elongation at 72°C.
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<th>Gene</th>
<th>Forward (F) / Reverse (R)</th>
<th>Primer Sequence</th>
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<th>Annealing Temperature (°C)</th>
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<tr>
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<td>35</td>
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</table>

Table A3 Gene specific primer pairs used for mouse and human gene expression detection.
A1.2.2.3 Agarose Gel Electrophoresis

PCR products were analysed using agarose gel electrophoresis. Briefly, for each sample, 2 µL of 6X blue/orange loading dye (Promega Corporation, Madison, USA) was mixed thoroughly with 10 µL of PCR product. The mixtures, together with DNA ladder (New England Biolabs (UK) Ltd., Hitchin, UK), were loaded into 2% (w/v) agarose (Lonza, Rockland, USA) gel prepared with 1X Tris base, acetic acid and EDTA (Daoudi et al.) buffer (Promega Corporation, Madison, USA) containing 1 µg/mL ethidium bromide (Promega Corporation, Madison, USA). Loaded gel was then immersed in 1X TAE buffer and electrified at 110 volts. The electrophoresis was accomplished using a HU-13 Midi horizontal gel unit with a PSU 400/600 power supply (Scie-Plas Ltd., Cambridge, UK). The bands were visualised and photographed under Ultra-Violet light using a gel documentation system and a camera (Uvitec Cambridge, Cambridge, UK).

A1.2.3 Glucose induced insulin secretion assay

A1.2.3.1 Pancreatic islet isolation

Islets were prepared from pancreatic explants of human subjects or 10-12 week male
C57/BL6 mice. For mouse islets, whole pancreas from 2 mice was used per isolation procedure. Pancreatic tissue was disassociated using liberase TL research grade (Roche Diagnostics Ltd., Burgess Hill, UK) digestion in Krebs ringer phosphate HEPES (KRH) buffer (129 mM NaCl, 5 mM NaHCO$_3$, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgCl$_2$, 10 mM HEPES, 2.5 mM CaCl$_2$ and 0.1% (w/v) BSA; pH 7.4) by gentle swirling and vigorous shaking at 37°C. When the digestion was complete, enzymatic reaction was terminated by repeatedly washing the digestion mixture with ice cold KRH buffer and centrifugation. Islets were handpicked followed by a re-pick under a dissection microscope to minimise acinar cell contamination. Islets as well as unprocessed tissue from some CHI patients were stored in RNALater solution at -20°C for future detection of gene expression. Prior to being used in insulin secretion assay, islets were cultured in tissue culture medium containing 88% (v/v) RPMI1640 medium (11 mM D-glucose), 10% (v/v) foetal bovine serum and 2% (v/v) penicillin-streptomycin (final concentration - 200 U/mL and 200 µg/mL, respectively) in non-cell-culture-treated 6 well plates overnight in humidified atmosphere with 5% CO$_2$ supply at 37°C.

A1.2.3.2 Insulin secretion assay

Isolated islets that have been cultured overnight were transferred into borosilicate tubes (5-9 islets/tube). Islets were allowed to equilibriise for 1 hour at 37°C and subsequently washed once in modified KRH buffer (136.9 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO$_4$, 0.34 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 1.29 mM CaCl$_2$, 4.17 mM NaHCO$_3$, 10 mM HEPES and 0.1% (w/v) BSA; pH 7.5) containing 2 mM D-glucose. After removing the KRH buffer, various treatments (all formulated upon modified KRH buffer) were applied to the batches of islets. All incubations were done statically. After 1 or 2 hour incubation at 37°C, test solutions were removed and aliquoted while islets with leftover test medium
were mixed with equal volumes of 2% (v/v) Triton-X100. All treatments were tested in duplicate or triplicates. All solutions were stored at -80°C until analysis.

### A1.2.3.3 Determination of insulin concentration

The concentration of insulin secretion in the test solution and insulin content of the islets were assessed using ultrasensitive insulin ELISA (ALPCO Diagnostics, New Hampshire, USA) for CHI islets or mouse insulin ELISA (ALPCO Diagnostics, New Hampshire, USA) for mouse islets. Ultrasensitive insulin ELISA has <11.1% inter- and intra-assay variation and has 100% cross-reactivity to human insulin. The assay does not cross-react with human C-peptide or proinsulin (intact) and has <0.05% cross-reactivity to human IGF-1 and IGF-2. The sensitivity of the assay is 0.135 µIU/mL. Mouse insulin ELISA has <0.01% cross-reactivity to mouse C-peptide 1, C-peptide 2, IGF-1 and IGF-2. Both inter- and intra-assay variation are <5%. The sensitivity of the assay is 0.06 ng/mL.

### A1.2.3.4 Determination of incretin concentration

Plasma active GLP-1 and total GIP levels were assessed using GLP-1 (Active 7-36) ELISA kit (ALPCO Diagnostics, NH, USA) and Human GIP (Total) ELISA kit (EMD Millipore Corporation, MA, USA), respectively, according to manufacturers’ instructions. GLP-1 (Active 7-36) ELISA has <10% inter- and intra-assay variation and has 100% cross-reactivity to GLP-1 (7-36) amide but <0.1% cross-reactivity to GLP-1 (9-36) amide, GLP-1 (9-37), GLP-1 (7-37), GLP-1 (1-36) amide, GLP-2 and glucagon. The sensitivity of the assay is 0.05 pM. Human GIP (Total) ELISA has 100% cross-reactivity to GIP (1-42) and GIP (3-42) but do not cross-react with glucagon, oxyntomodulin, GLP-1 and GLP-2. Both inter- and intra-assay variation are <10%. The sensitivity of the assay is 4.2 pg/mL.
A1.2.4 Statistical Analysis

All data was analysed using GraphPad Prism 6 (GraphPad Software, Inc., California, USA). Statistical significance was determined by unpaired or paired student’s t-test or One-way ANOVA followed by Tukey’s multiple comparisons test using GraphPad Prism 6 (GraphPad Software, Inc., California, USA) as appropriate. All results are presented as Mean±SEM.

A1.3 Reference
