Metabolite profiling of a human pancreatic ductal cell line for cell culture medium optimisation: enhancing trans-differentiation to endocrine cells

A thesis submitted to The University of Manchester for the degree of
MPhil Physiology
in the Faculty of Biology, Medicine and Health

2016

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Contents

Abstract

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Degree Title: MPhil Physiology
Date: 2016
Thesis Title: Metabolite profiling of a human pancreatic ductal cell line for cell culture medium optimisation: enhancing trans-differentiation to endocrine cells

Although pancreatic islet transplantation has been largely successful there are a number of caveats for this treatment method, mainly concerning the limited availability of donor islets. There has therefore been an advance in the field of regenerative medicine for the generation of functional β-cells for transplantation for the treatment of diabetes. Pancreatic development is tightly regulated by a cascade of transcription factors and regulatory signalling pathways. Extensive research has been carried out into mapping the transcription factors onto specific stages of pancreatic development. Understanding these regulatory networks has been critical in the establishment of *in-vitro* differentiation protocols. There is a myriad of data supporting pancreatic plasticity and the ability of pancreatic exocrine cells to trans-differentiate towards an endocrine lineage. Previously in our laboratory, using the human pancreatic ductal cell line PANC-1, a small compound molecule (*N*-cyclopropyl-5-(2-thienyl)-3-isoxazole-carboxamide) referred to in this study as isoxazole, was shown to reprogramme PANC-1 cells towards an endocrine phenotype. The aim of this current project was to generate a metabolic profile of isoxazole treated PANC-1 cells and optimise the *in-vitro* cell culture environment to enhance trans-differentiation, with a hypothesis that metabolism may be driving some aspects of endocrine differentiation. Following a 48 hr treatment of PANC-1 cells with isoxazole, cells showed distinct changes in cell morphology with the formation of islet-like clusters and 'pseudo-ductal' structures. Morphometric data analysis using the CellProfiler software showed that isoxazole treatment induced enhanced cellular elongation, whilst decreasing cell symmetry and compactness, phenotypic of a migratory cell. Isoxazole up-regulated key genes involved in pancreatic development like *Ngn3* and *NeuroD1* and also mature β-cell markers such as *insulin, SLC30A8* and the GLUT2 glucose transporter *SLC2A2* measured by qRT-PCR. In addition to changes in gene expression, a metabolic profile of PANC-1 cells following isoxazole treatment was also generated using LC and GC mass spectrometry. This profile highlighted significantly altered metabolites from isoxazole treated cells and used cell culture medium, suggesting active utilisation of these metabolites during the trans-differentiation process. Many of the metabolites altered by isoxazole treatment were involved in the tricarboxylic acid (TCA) cycle. Interestingly, two metabolites (arginine and aspartate) were significantly lowered in the cell culture medium following isoxazole treatment when compared to the DMSO vehicle control. Supplementation of the cell culture medium with arginine and aspartate led to the enhanced expression of β-cell associated genes and transcription factors such as *Ngn3, NeuroD1, insulin*, and *SLC30A8*. Changes were also observed at a protein level, with an up-regulation in the levels of SUSD2, a novel endocrine progenitor cell surface marker, following isoxazole treatment. Collectively, this data suggests a key role for amino acid metabolism in endocrine differentiation, which could be used as a potential tool for enhancing the generation of pancreatic β-cells for transplantation.

DECLARATION

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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Acknowledgements

This project was done in parallel to the BBSRC/BRIC funded project entitled 'Application of single cell metabolite profiling for the optimisation of stem cell bioprocessing'. I am appreciative for the support and feedback from supervisors on this project especially to Dr Karen Cosgrove and Prof Alan Dickson. Also, to my colleague Dr Katherine Hollywood for her support over the course of this project and for her contribution to metabolomics and mass spectrometry data analysis. I'd like to thank my laboratory peers for their support and encouragement, especially to Dr Alexander Ryan for his helpful comments and feedback. And finally to my family, especially to my older brothers Qasim and Kabir, for their endless support, understanding and their simple presence during difficult times which has helped me immensely throughout this MPhil.

List of abbreviations

ADI	arginine deiminase
AICAR	5-aminoimidazole-4- carboxamide ribonucleotide
AMA	Aminomalonic acid
AMP	adenosine monophosphate
Arx	Aristaless-related homeobox protein
ASNS	asparagine synthetase
ASS1	argininosuccinate synthetase 1
AST	aspartate amino transferase
AZA	5' Azadeoxycytidine
CAII	Carbonic anhydrase II
CK19	Cytokeratin-19
DMSO	Dimethyl sulfoxide
FACS	Fluorescence-activated cell sorting
FBS	foetal bovine serum
FFA	Free fatty acid
GC-MS	Gas chromatography mass spectrometry
GFP	green fluorescent protein
GLP-1	Glucagon-like peptide 1
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GPR50	G-protein coupled receptor-50
GSIS	glucose stimulated insulin secretion
HAT	histone acetyl transferase
HDAC	histone de-acetyltransferase
hESC	human embryonic stem cell
HK I	Hexokinase 1
HK II	Hexokinase 2
HN3b	Hepatic Nuclear Factor
HNF 1b	Hepatic Nuclear Factor
HNF6	Hepatic Nuclear Factor
hTERT	human telomerase reverse transcriptase
IBMX	3-isobutyl-1-methylxanthine
ICA	Islet-like cell aggregates

IPC	Insulin Producing Cell
ISOXAZOLE	*N*-Cyclopropyl-5-(2-thienyl)-3-isoxazole-carboxamide
LC-MS	Liquid chromatography mass spectrometry
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescent intensity
miRNA	micro RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NeuroD1	Neurogenic differentiation 1
Ngn3	Neurogenin 3
Nkx2.2	Nkx2 homeobox 2
Nkx6.1	Kkx6 homeobox 1
Pax4	Paired box gene 4
PDAC	Pancreatic ductal adenocarcinoma
PDL	Pancreatic Duct Ligation
PDX1	Pancreatic and duodenal Homeobox 1
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative real-time polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SFM	Serum free medium
STMN2	Stathmin 2
STZ	Streptozotocin
SUSD2	Sushi-domain containing-2
TCA	Tricarboxylic acid
TROP2	Tumour-associated calcium signal transducer
TSA	Trichostatin A
VPA	Valproic acid

CHAPTER 1 General Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterised by hyperglycaemia due to an imbalance in insulin hormone levels (reviewed in Atkinson & Gianani 2009). DM can be classified into two main groups; type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D or insulin - dependent diabetes is caused by autoimmune destruction of insulin secreting β-cells whereas T2D or non-insulin dependent diabetes is caused by decreased insulin sensitivity and impaired β-cell function (Godfrey et al., 2012). DM encompasses a spectra of conditions which also include gestational diabetes, maturity onset diabetes of the young (MODY) and neonatal diabetes. DM is a global epidemic and a major health concern due to its increasing prevalence and serious health complications, such as cardiovascular disease, retinopathy, and neuropathy (reviewed in J. C. and J. O. Manilay 2012). Currently, there is no cure for diabetes and insulin injections are the gold standard for the treatment of T1D. However, this is not without its drawbacks, as the application of exogenous insulin does not recapitulate physiological glucose homeostasis and patients with T1D can often suffer hypoglycaemic episodes (reviewed in Godfrey et al. 2012; Tan et al. 2014).

Following the discovery of insulin in the early 1920's there has been a concerted effort in the understanding of the human pancreas and normal pancreatic development (Lakey et al. 2006). Whole pancreatic organ transplantation was first performed in 1966, however with little success due to complications with immune rejection (Miranda et al. 2013). The next major advance was with the transplantation of healthy pancreatic islets into diabetic patients following the Edmonton protocol in 2000. Although successful there were a number of drawbacks for this method of treatment, as this method requires large numbers of islets from multiple donors and there is a limitation in the availability of cadaveric donor islets for transplantation (Shapiro et al. 2000; Manning Fox et al. 2015). There has thus been a drive towards the *in-vitro* generation of functional pancreatic β-cells for regenerative medicine and the treatment of diabetes.

1.1 The pancreas

The adult pancreas is a highly structured organ with extensive vascularisation (Slack 1995). The pancreas has dual functions with both endocrine and digestive roles essential for glucose homeostasis and physiology (Pieler & Chen 2006). The human pancreas is composed of both endocrine and exocrine tissue. The exocrine portion consists of acinar and ductal cells which make up 85% of the whole pancreas (Gittes, 2009). Acinar cells cluster at the end of ducts and secrete digestive enzymes for the breakdown of macromolecules such as carbohydrates, fats and proteins (Slack 1995). The endocrine portion consists of clusters of cells known as the Islets of Langerhans which are made up of 5 different cell types; α, β, δ, ε and PP cells (Collombat et al. 2010). Each is responsible for the secretion of specific hormones: glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively (Slack, 1995).

1.2 Pancreatic embryology and development

There has been extensive research into pancreatic development over the past two decades characterising the morphometric events occurring in a stage wise manner and mapping the associated signalling networks (Pan & Wright 2011). Understanding the regulatory pathways essential for normal pancreatic development has been instrumental in establishing *in-vitro* differentiation protocols. The majority of information on pancreatic development is based on rodent models due to the ethical restrictions and limited access to human foetal pancreatic tissue, especially during the first trimester (Piper et al. 2004). A series of signalling cascades control exocrine and endocrine cell fate during embryogenesis. The pancreas arises from the endodermal germ layer and is formed from the gut tube by fusion of dorsal and ventral pancreatic ducts. Pancreatic development can be divided into three main stages; primary, secondary and final transition phases (Pan & Wright 2011). During the primary transition phase, pancreatic budding occurs in the mouse at embryonic day 9.5 (E9.5), after which extensive branching of the epithelium into the surrounding mesenchyme leads to a highly branched structure occurring at E11.5 (Jørgensen et al. 2007; Pandol 2010). The secondary transition phase is marked by extensive remodelling, cell proliferation and cyto-differentiation into endocrine and exocrine cells at around E15.5 (Slack 1995; Jørgensen et al. 2007). Hormone expressing cells become apparent at this stage and there is a significant amount of differentiation and morphogenetic

transformation towards all three pancreatic cell types (islets, ductal and acinar cells) from multipotent progenitor cells (MPC's) (Madsen et al. 1996; Pan & Wright 2011). The final stage of pancreatic development is expansion and maturation of all three pancreatic cell types. Islets of Langerhans form organised structures and maturation continues after birth where human β-cells secrete insulin in response to dietary intake (Madsen et al. 1996; Mastracci & Sussel 2012; O'Dowd & Stocker 2013).

1.3 Transcriptional regulation of the pancreas

There are a number of extrinsic and intrinsic factors involved in the development of the pancreas. Molecular control involves a cascade of transcription factors essential for the development of mature pancreatic cells (Slack, 1995).

Pancreas and duodenal homeobox factor 1 (*PDX1*) is referred to as the 'master regulator' of pancreatic development and β-cell differentiation (Slack, 1995). *PDX1* expression levels have been reported to vary during pancreatic development. During early pancreatic development, in particular the primary transition phase the expression of the transcription factor *PDX1* is critical and highly expressed (see Figure 1.1). However, during the second transition phase of pancreatic development, bipotent progenitor cells express diminished levels of *PDX1*, and this transiently disappears in endocrine progenitor cells however re-emerges at high levels in mature β-cells (Offield et al. 1996). *PDX1* expression is significantly reduced in ductal and acinar cell lineages and expression is restricted to endocrine cells only (Deramaudt et al., 2006; Offield et al., 1996). Other transcription factors involved during the primary transition phase include PTF1α, SOX9 and HNF1β (Slack 1995; Jørgensen et al. 2007).

Neurogenin 3 (*Ngn3*) is a basic helix loop helix transcription factor and the only neurogenin gene member within its family involved in pancreatic development (Jørgensen et al., 2007; Madsen et al., 1996). *Ngn3* is involved in the secondary transition phase of pancreatic development, where it is transiently expressed and expression has been reported in mouse models to peak in endocrine progenitor cells, shown in diagram 1 (Slack 1995). Studies have shown *Ngn3* positive cells can give rise to all five endocrine cell types (α, β, δ, ε and PP cells), making it essential for endocrine differentiation and a marker of endocrine progenitor cells (Slack 1995). It is tightly regulated upstream by the transcription factors *HNF-1β, HNF-6*, and *HNF-3β*, but also regulates the expression of downstream transcription

factors such as *NeuroD1, Pax4, Arx, Nkx2.2* and *Nkx6.1* (Gittes, 2009; Jørgensen et al., 2007).

Other transcription factors involved in the secondary transition phase of pancreatic development are *NeuroD1*, another basic helix loop helix transcription factor that is involved in the regulation of insulin gene transcription in pancreatic β-cells (Madsen et al., 1996; O'Dowd & Stocker, 2013). *Pax4* is a member of the paired box homeoprotein family and is involved in pancreatic development; after birth its expression is restricted to insulin positive mature β-cells. *Nkx2.2* and *Nkx6.1* are both members of the Nk homeoprotein transcription factor family and involved in the regulation and differentiation of pancreatic endocrine cells.

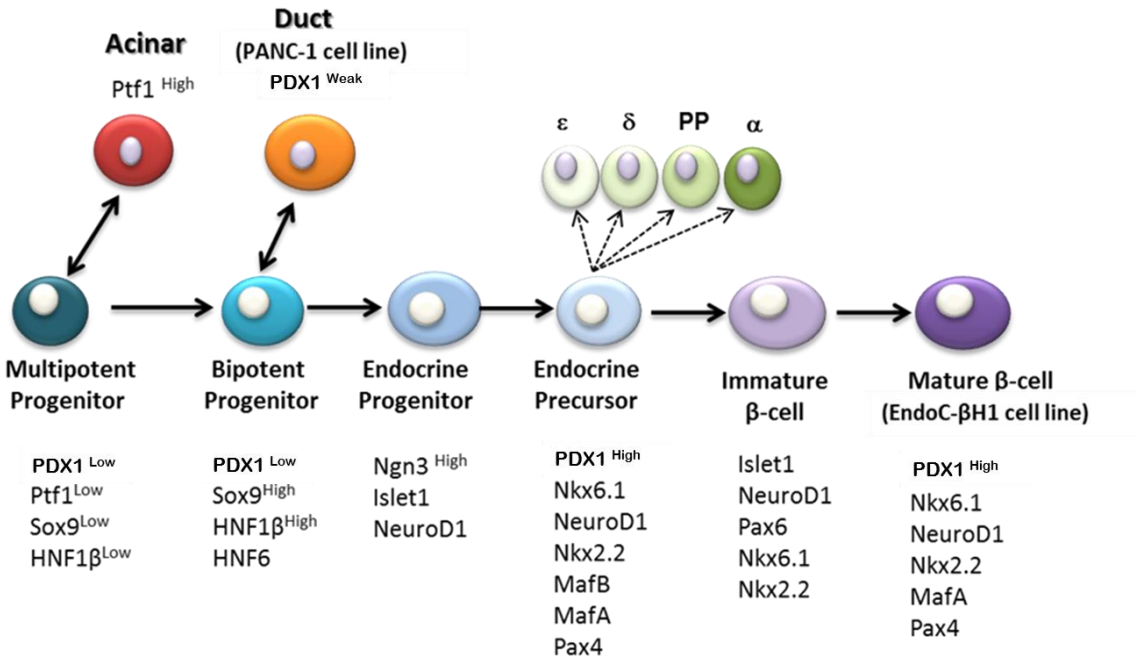


Figure 1.1 Transcriptional factors involved in pancreatic development. Schematic representation of the stages of pancreatic development towards a mature β-cell with associated stage-specific transcription factors (adapted from Pan & Wright 2011; Piper et al. 2004). Cell lines used in this study (PANC-1 and EndoC-βH1) are shown at the equivalent stages of development.

1.4 Insulin biosynthesis and secretion

Insulin biosynthesis and secretion in pancreatic β-cells is tightly regulated from gene transcription to translation and exocytosis of insulin. Messenger RNA (mRNA) transcripts of insulin are translated into an inactive protein called pre-proinsulin on the rough endoplasmic reticulum. Pre-proinsulin is enzymatically cleaved to form proinsulin consisting of chains A, B and C (Steiner & Philipson 2009). At the Golgi apparatus proinsulin is packaged into immature secretory granules, which become acidic in pH and leads to the proteolytic cleavage of proinsulin into insulin and C-peptide inside mature secretory granules (Hou et al. 2009; Fu et al. 2013). Although little is known about the function of C-peptide, insulin and C-peptide are released in equimolar amounts, and C-peptide is often used experimentally as a marker of insulin synthesis and release (Troitza K. Bratanova-Tochkova et al. 2002). Inside these mature granules, insulin is crystallised with calcium and zinc to form dense granules (Hou et al. 2009). Pancreatic β-cells contain three distinct populations of intracellular insulin granule pools which are involved in the bi-phasic glucose stimulated insulin secretion (GSIS) (Butler 2000). There is a small readily releasable pool of insulin granules (approximately 50-100 granules in a mouse β-cell) and a morphologically docked pool of granules that are temporarily lodged to the plasma membrane (approx. 1000 granules in a mouse β-cell) (Steiner & Philipson 2009). These are involved in the acute phase of insulin secretion at a rate of 1 granule per 3 seconds (T. K. Bratanova-Tochkova et al. 2002). The second (chronic) phase of insulin secretion is dependent on the trafficking on reserve granules to the plasma membrane and can take up to several hours at a rate of 1 granule per 10 seconds (Butler 2000; Troitza K. Bratanova-Tochkova et al. 2002; Steiner & Philipson 2009).

1.5 Regenerative medicine

One of the key objectives in the field of diabetes and regenerative medicine is to identify methods of replenishing pancreatic insulin producing cells (IPCs) for cell based therapy. There are a number of approaches to source new β-cells. One such method is the induction of the proliferative capacity of existing pancreatic β-cells (Ackermann & Gannon, 2007; Tan, Elefanty, & Stanley, 2014). Terminally differentiated cells exhibit little if any ability to proliferate *in vitro*, however it is known that β-cell mass changes in response to physiological stresses such as pregnancy, obesity and aging *in vivo* (Ong & Ozanne 2015;

Ackermann & Gannon 2007). For example, during pregnancy and obesity, there is an increase in β-cell mass to meet changes in insulin demands suggesting a capacity for β-cell neogenesis and regeneration (Wu, Yang, Chen, & Xu, 2015). In other cases, the rate of pancreatic β-cell proliferation has been shown to decrease with increasing age; thus highlighting the finely tuned balance between β-cell neogenesis, proliferation, and apoptosis (Ackermann & Gannon 2007). There is much on-going research looking into the intrinsic factors that regulate pancreatic β-cell proliferation (Heit, Karnik, & Kim, 2006; Tan et al., 2014). Nevertheless, it is debatable whether these newly generated β-cells are derived from existing β-cells, or are derived from pancreatic stem and progenitor cells (Manilay, 2012; Tan et al., 2014).

1.6 Pancreatic plasticity

One emerging method for the regeneration of pancreatic β-cells utilises the plasticity of pancreatic cells to 'trans-differentiate' from one terminally differentiated cell type to another, often occurring under physiological stress or pancreatic injury (Ackermann & Gannon, 2007). In rat pancreatic duct ligation (PDL) experiments (which mimic pancreatic injury) there is a considerable mass of new islets formed, composed of "β-cell like" cells. Note, that use of the term "β-cell like" implies that the cells demonstrate some key aspects of functional β-cells, albeit not all the features of a fully mature adult human β-cell. Key characteristics include; gene and protein expression profiles, cell morphology that mimics embryological development and ultimately the cells ability to synthesise and secrete insulin in a glucose-responsive manner (Zhu et al. 2016). In this study, proliferation of existing β-cells alone could not account for this expansion (Godfrey et al., 2012). In other studies, *Ngn3*$^{+ve}$ cells have been harvested following PDL in mice showing that *in vivo* there is reactivation of *Ngn3* expression in a small subset of *PDX1*$^{+ve}$ ductal cells, indicating the presence of multipotent endocrine progenitor cells in the adult mouse pancreas. More recently, Valdez et al. (2016) showed that pro-inflammatory cytokines that are associated with Type 1 and Type 2 diabetes (i.e. TNFα, IL-1β, IFNγ) could stimulate the expression of the endocrine genes *Ngn3* and *insulin* in the human ductal cell line PANC-1. They also showed that inflammatory cytokines caused epithelial-to-mesenchymal transition as ductal cells differentiated towards an endocrine lineage (Valdez et al. 2016). There is a growing amount of compelling data to suggest there is a high degree of cellular plasticity within the pancreas in response to various stress and stimuli (Pandian et al. 2014; Lefebvre et al.

2010). In one proposed model of β-cell regeneration, it is hypothesised that under normal physiological conditions there is no β-cell replication. However during pancreatic injury (e.g. partial pancreatectomy and duct ligation) when β-cell mass decreases to 30% of its total mass, β-cells are regenerated from trans-differentiation of α-cells and differentiation from pancreatic stem cells and progenitor cells (J. C. and J. Manilay 2012).

1.7 Reprogramming acinar cells

Acinar cells which are the most abundant cell type of the pancreas have been reported to trans-differentiate into IPCs under certain conditions *in vitro*. Studies using the acinar cell line AR42J have shown that treatment with both factors activin A and hepatocyte growth factor (HGF) lead to the production of insulin positive cells (Mashima et al. 1996). Furthermore, studies show that acinar cells, (which are usually discarded during the purification step of preparing islets for transplantation) can be reprogrammed into 'β-cell like' cells. By re-expressing the combination of transcription factors *PDX1, Ngn3* and *MafA in vivo*, differentiated acinar cells were reprogrammed into insulin producing and secreting cells capable of normalising blood glucose levels in mice (Zhou et al. 2008). Such data supports the concept of pancreatic plasticity and the ability to produce functional IPCs from exocrine sources.

1.8 Ductal to endocrine differentiation

During embryonic development, islets, ductal and acinar cells arise from a common precursor epithelium (O'Dowd & Stocker, 2013). Endocrine cells have been thought to delaminate from the epithelium to form clusters (Pan & Wright 2011; Bonner-Weir & Weir 2005). The formation of the Islets of Langerhans during pancreatic development involves epithelial to mesenchymal transition (EMT) and these islets are still closely associated with the parental ducts (Pan & Wright 2011). Physiologically the ductal epithelium is a site where pancreatic stem cells and progenitor cells can reside (Bonner-Weir & Weir 2005; Bonner-Weir & Sharma 2002). This has been observed in a number of studies using rodent models in partial pancreatectomy and PDL studies as previously stated. Cell lineage studies using the ductal marker carbonic anhydrase 2 (CA2) showed that following PDL the progeny of the ductal cells gave rise to both exocrine and endocrine cells suggesting a common progenitor (Godfrey et al. 2012). There is a growing amount of literature on pancreatic

plasticity and the ability of pancreatic exocrine tissue to trans-differentiate into endocrine lineages (Pandian et al. 2014; Yuan et al. 2013; Mashima et al. 1996; Zhou et al. 2008). Endocrine differentiation is a complex process involving a cascade of transcription factors and regulatory signalling networks.

1.9 Background work leading to current project

PANC-1 cells are a human ductal epithelial cell line, routinely used as a model for *in vitro* differentiation protocols (derivation of the cell line described in Lieber et al. 1975). The Cosgrove laboratory has previously shown the ability of the isoxazole derivative (*N*-cyclopropyl-5-(2-thienyl)-3-isoxazolecarboxamide) to reprogramme PANC-1 cells towards an endocrine lineage. Based on unpublished data from within the laboratory, gene expression profiles were determined for isoxazole treated PANC-1 cells and proteomic profiling revealed significant alterations in 257 proteins following isoxazole treatment (Gsour 2015).

1.10 Aims and hypothesis

The aim of this current project was to quantify changes in cell morphology and generate a metabolic profile of PANC-1 cells before and after 48hr isoxazole treatment. Based on the generation of the metabolic profile, the aim was to manipulate the *in-vitro* environment and optimise the cell culture medium composition with the hypothesis that metabolism may drive some aspects of differentiation.

CHAPTER 2 Materials and Methods

Cell Culture

PANC-1 (pancreatic epithelial ductal carcinoma) cell line was obtained from American Type Culture Collection (ATCC) and cultured in Dulbeccos Modified Eagles Medium (DMEM D6429) (Gibco, Life Technologies Ltd, Paisley UK) supplemented with 10% (v/v) foetal calf serum (FCS). Once cells were 70-80% confluent, they were sub-cultured enzymatically using trypsin EDTA (Sigma Alrich, UK). Cells were routinely, passaged at a 1:3-1:5 ratio and grown in a humidified incubator at 37^{o}C in air with 5% CO_2. STR profiling was carried out to confirm cell line authenticity of the cell lines used in this study; PANC-1 and EndoC-βH1 cell lines (Supplementary Table 1). Routine mycoplasma testing was performed every 6 months to confirm cell cultures were mycoplasma free (Supplementary Figure 1).

Proliferation and Cell Viability

Cells were seeded at 30,000 cells/well in a 6-well plate for 2, 4, and 6 days in quadruplet and cell counts were performed using a Neubauer haemocytometer. Trypan blue exclusion was used to determine cell viability. Medium was changed every 48hr.

MTT assay

MTT (3-(4,5 dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide) (Sigma M2128) was used to determine cell viability of cell culture medium supplements on PANC-1 cells. The MTT assay was performed in 96-well plates as described in (Mosmann 1983). A colorimetric assay used to determine cell proliferation, viability and toxicity by assessment of the metabolic activity of cells. Growth curves were used to determine the optimum seeding density for 6 day experiments. Cells were subsequently seeded at 6,250 cells/well for 2, 4, and 6 days with the addition of test conditions. MTT stock was prepared at 5mg/ml in PBS, sterile filtered, and 10µl/well was added. Plates were incubated for 4-6hr at 37^{o}C. Cells were lysed with 100µl/well of lysis solution (isopropanol/10% (v/v) triton X-100/0.04N HCL) and incubated on a plate shaker for 10-20mins at room temperature. Optical density was measured spectrophotometrically at 595nm using a 96-well plate reader (Synergy, HT, BioTek Instruments, Inc.). The development of deep purple/blue formazan product is directly proportional to the number of viable and metabolically active cells.

Cell Culture

EndoC-βH1 cells were gifted to us from EndoCells at passage 50, Paris, France. Cells were grown on pre-coated flasks containing Matrigel (100µg/ml) and Fibronectin (2µg/ml). Cells were maintained in DMEM containing 5.6mM glucose and supplemented with the reagents listed in Table 2.1

Table 2.1 EndoC-βH1 growth medium reagents

Compound	**Final Concentration**	**Supplier**	**Catalogue Code**
DMEM (low glucose)	5.6mM	Invitrogen	31885-023
Beta-mercaptoethanol	50µM	Sigma Aldrich	M3148
Nicotinamide	10mM	VWR	481907
Pen/Strep	100µg/ml	Sigma Aldrich	P0781
BSA Fraction V FA free	2% (w/v)	Roche Diagnostics	1077583001
Human transferrin	5.5µg/ml	Sigma Aldrich	T8158
Sodium Selenite	6.7ng/ml	Sigma Aldrich	214485

Glucose Stimulated Insulin Secretion Assay

Glucose stimulated insulin secretion (GSIS) was carried out on the EndoC-βH1 cells. Cells were seeded at 70,000 cells/cm^2 in 100µl growth medium in a 96-well plate. After 3 days incubation at 37^{o}C, growth medium was aspirated and replaced with 100µl starvation medium containing 2.8mM glucose overnight. EndoC-βH1 cells were incubated in Krebs Ringer Buffer (KRB; 116mM NaCl, 5mM KCL, 24mM $NaHCO_3$, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES and 0.2% (w/v) BSA) containing 0.5mM glucose for 1hr. Finally, cells were incubated in test conditions; 0.5, 5, 11, 15mM glucose, with and without addition of IBMX (final concentration at 0.5mM) (Sigma Aldrich, UK). Supernatant was carefully removed and assayed for insulin secretion, cells were lysed using TETG lysis buffer (1M Tris, 1% Triton X-100, 10% (v/v) glycerol, 5MNaCl, 0.2M EGTA) containing protease inhibitor cocktail (CompleteOne, Roche Diagnostics). Insulin was measured in cell culture medium and cell lysates by ELISA (Stratech, USA) and presented as percentage insulin secretion normalised to total insulin content.

Medium Manipulation Experiments

PANC-1 cell culture medium was optimised with the addition of amino acid supplements shown in Table 2.2. Supplementation with arginine, aspartate and asparagine (Sigma Aldrich, UK) was based on the concentrations in the media formulations listed in the supplementary information Table 2. The pH of optimised medium was also measured and not significantly altered from pH 7.2.

Table 2.2 PANC-1 amino acid supplementations

Metabolite	Basal Conc. (mM)	Supplementation Final conc. (mM)		
		2x	4x	6x
Arginine	1.2	2.40	4.80	7.20
Aspartate	0.05	0.10	0.20	0.30
Asparagine	0.06	0.12	0.24	0.36

Differentiation Protocol

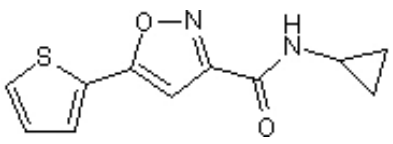
A differentiation protocol was optimised within the laboratory. For cell adherence PANC-1 cells were first seeded in normal growth medium containing 10% (v/v) FCS. After 24h cells were washed in PBS and medium was switched to serum free medium. The compositions of reagents are listed in Table 2.4. Cells were incubated in serum free DMEM/F12 for 48h before being treated with Isoxazole. Isoxazole (Tocris, 4439) was dissolved in DMSO (Sigma, UK) and used at a final concentration of 20µM as previously reported (Dioum et al. 2011). A DMSO vehicle control was used in all experiments at a final concentration of 0.03% (v/v).

<u>Isoxazole Properties</u>

Molecular formula: $C_{11}H_{10}N_2O_2S$

Molecular weight: 234.27

Structure:



Differentiation Protocol

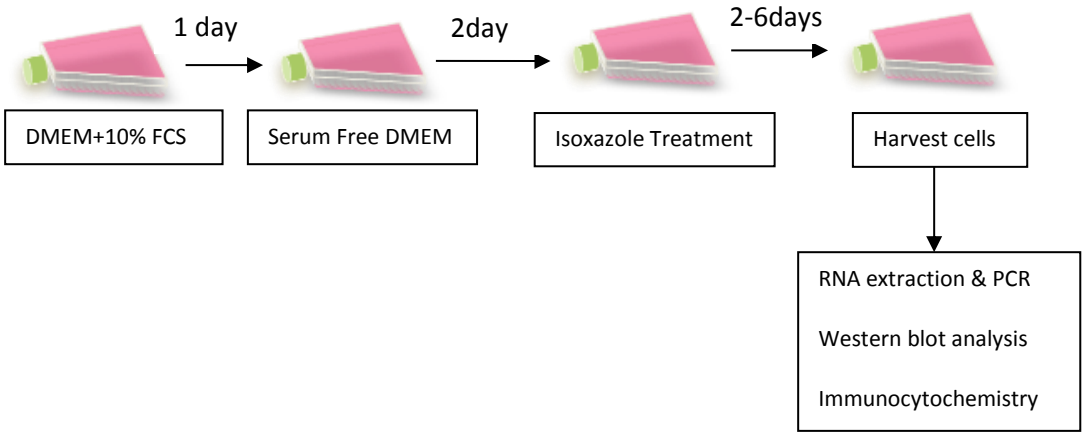


Diagram 2.3 Schematic representation of the differentiation protocol. PANC-1 cells were first seeded into growth medium containing 10% (v/v) serum for 24hr, medium was replaced with serum free DMEM/HAMS F12 for 48hr after which drug treatment was added for 2-6 days.

Table 2.4. Optimised Serum Free Medium

Reagent	**Final Concentration**	**Supplier**	**Catalogue Number**
DMEM	49.5% (v/v)	Gibco	11960-044
HAMS F12	49.5% (v/v)	Sigma	N6658
Insulin Transferrin Selenium (ITS)	1% (v/v)	Gibco	41400-045
L-Glutamine	2mM	Sigma	G7513
L-Ascorbic Acid	128µg/ml	Sigma	A4544
L-Glutathione	20µg/ml	Sigma	G4251
Epidermal Growth Factor (EGF)	0.75ng/ml	Sigma	824831

Note: Final glucose concentration 11 mM

Gene Expression Analysis

RNA extraction

Total RNA was isolated from PANC-1 cells using Qiagen RNAeasy Mini Kit (Qiagen, UK) according to manufacturer's instructions. To summarise, cells were trypsinised and resuspended in RLT buffer containing 1% (v/v) β-mercaptoethanol. Cells were lysed using a QIAshredder (Qiagen, UK) according to manual instruction RNA was extracted and eluted using the RNAeasy columns. To eliminate genomic DNA, DNase digest was performed using DNA Free Kit (Ambion, Life Tech, UK). RNA was incubated with DNase enzyme at 37^{o}C for 30mins and inactivated with beads for 2mins at room temperature. RNA was centrifuged at 10,000g for 2mins and purified RNA was collected. The concentration of RNA was measured using the Nanodrop^{TM} 8000 spectrophotometer (Thermo Scientific) and the integrity of RNA was assessed by the A_{260}/A_{280} ratio, a ratio of >1.95 was used as a cut-off for all samples. A 260:280 ratio of ~2.0 indicates 'pure' RNA, a ratio less than 1.80 indicates protein/phenol contamination.

Complementary DNA conversion

cDNA was synthesised using the Nanoscript Reverse Transcription (RT) kit (Primer Design, Southampton, UK) according to the manufacturer's instructions. For all samples purified RNA was adjusted to 200ng/µl. Annealing and extension steps using random hexamers were carried out on the Veriti 96-well Thermal Cycler (Applied Biosystems, UK). For each sample a negative control was included that omitted the addition on the RT enzyme. cDNA was stored at -20^{o}c until required.

Reverse Transcription PCR

Primer sequences were checked using the National Centre for Biotechnology Information (NCBI) database and primers were obtained from Eurofins MWG Operon (Germany). Primers were reconstituted to 100µM stock, and diluted to 10µM working stock. The reagents used for PCR are listed in Table 2.5. A list of primers and relevant conditions are referenced in Table 2.6 and the PCR cycling conditions in Table 2.7. PCR products were run a 2% agarose gel and visualised on the Bio-Rad ChemiDoc MP system (Hemel Hempstead, UK)

Table 2.5 PCR reagents

Reagents	Volume (µl) /reaction	Final Concentration
10x Buffer	2.5	-
dNTP's	0.5	0.2mM
Forward Primer	0.625	0.25µM
Reverse Primer	0.625	0.25µM
Template	2	-
Taq Polymerase	0.2	1-2.5 U/µl
RNase free water	18.55	
Final volume	25µl	

Table 2.6 Primer list

Gene	Primer Sequence (5' - 3')	Annealing Temp (^{o}c)	No. of cycles	Product Size (bp)	NCBI reference
PDX1	F:AACAACTATTCACGAGCCAGTA R:CGATTTCCCACAAACATAAC	59	34	139	NM_000209
Ngn3	F:CGAATGCACGACCTCAAC R:AGTCAGCGCCCAGATGTAGT	59	34	126	NM_020999.3
NeuroD1	F:CTCGGACTTTTCTGCCTGAG R:GAAGTTGCCATTGATGCTGA	61	34	275	NM_002500.4
Pax4	F:TTGCCTTCCTTGCCCTTCCTCC R:TCTCCTTCCCACTCCTGCCTCA	58	34	119	NM_006193.2
Islet1	F: TATTTTGCCACAAGCGTCTC R: TTCAAAGACCACCGTACAACC	56	34	281	NM_002202
Nkx6.1	F: AAAGACGGGAAGAGAAAACA R: CCAGAGGCTTATTGTAGTCG	59	34	298	NM_006168
MafA	F: GGCTTCAGCAAGGAGGAGGTCA R: GCCCGCCAGCTTCTCGTATTTC	59	34	222	NM_201589.3
MafB	F: TCTTTTTCCTCTCTGGCTTT R:CTGAGGGTGATTCTGGTTAC	52	34	237	NM_005461.3
INSULIN	F: ACCAGCATCTGCTCCCTCTA R: GGTTCAAGGGCTTTATTCCA	58	35	115	NM_000207
SLC2A2 (GLUT2)	F: CACCTCAACAGAATCAAAGCC R: AGGCCTGAAATTAGCCCACA	58	34	152	NM_001278658.1
GCK	F: CAGTCCTGGCCATTTTCTTG R: ATGCTGCTTGGGGTTTCTT	60	34	278	NM_000162
SLC30A8	F: CTGTGGTTTTGCACCAGAGA R:TGATACTCTGAAATAGATCTCCAAGG	58	34	111	NM_001172815.2
18S	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	60	24	151	NM_003286.2

Table 2.7 PCR cycling conditions

Step	Temp (^{o}C)	Time	No. of cycles
Initial Denature	94	5min	
Denature	94	45sec	See Table 2.6
Anneal	See Table 2.6	30sec	
Extension	72	45sec	
Final Extension	72	2min	

Quantitative Real-time PCR

Quantitative gene expression was determined using TaqMan gene expression assays (Life Technologies, UK) listed in Table 2.8. A total of 64ng of cDNA was loaded per well. The plate was assayed on the StepOne Plus^{TM} Real Time PCR Systems (Applied Biosystems, UK) using standard thermal cycling conditions (Hold 95^{o}C 10mins, 40x cycles at 95^{o}C 15sec, 60^{o}C 1min). Data was normalised to 18S housekeeping gene and is represented as a fold change of isoxazole against DMSO vehicle control. Relative gene expression was calculated based on the $2^{-\Delta\Delta Ct}$ method. 3 independent experiments presented as mean ± SD.

Table 2.8 List of TaqMan Gene Expression Assays used

Primer	Accession No.	Amplicon size	Probe exon location	Catalogue No.
PDX1	NM_000209.3	73	1-2	Hs00236830_m1
Ngn3	NM_020999.3	127	2	Hs01875204_s1
NeuroD1	NM_002500.4	110	2	Hs00159598_m1
SLC30A8	NM_001172811.1	73	5-6	Hs00545183_m1
Insulin	NM_000207.2	139	2-3	Hs02741908_m1
GPR50	NM_004224.3	73	1-2	Hs00173684_m1
TROP2	X77753.1	115	1 - 1	Hs01922976_s1
SUSD2	NM_019601.3	84	10-11	Hs00995026_m1

Immunocytochemistry

PANC-1 cells were grown on 15mm sterilised glass coverslips in 12-well plates and fixed in 4% paraformaldehyde (PFA) for 20mins for internal proteins or ice-cold acetone for 5 mins for cell surface antigens. For internal protein detection, cells were permeabilised with 0.5% (v/v) Triton X-100 for 15mins at room temperature, blocked in 10% (v/v) normal goat serum (NGS) for 1hr at room temperature. The primary antibody was diluted in 3% (v/v) NGS overnight at 4^{o}C, (list of antibodies in Table 2.9). Cells were subsequently washed 3x

5mins in PBS-Tween20, and incubated with the secondary antibody for 1hr at room temperature (Table 2.10). Slides were mounted with Prolong Gold Diamond Anti-fade containing DAPI (Life Technologies, UK). Images were taken on the Olympus BX51 upright microscope using MetaVue Software (CA, USA) and analysed on ImageJ.

Table 2.9 List of Primary Antibodies

Primary Antibody	Host	Specificity	Dilution	Supplier	Catalogue Code
Anti-Insulin	Rabbit	Polyclonal	1:200	Abcam	ab63820
Anti-SUSD2	Mouse	Polyclonal	1:50	BioLegend	327401
Anti-CK19	Rabbit	Monoclonal	1:250	Abcam	ab76539

Table 2.10 List of Secondary Antibodies

Secondary Antibody	Host	Reactivity	Dilution	Supplier
Alexa Flour488	Goat	Rabbit	1:1,000	Life Technologies, UK
Alexa Flour488	Goat	Mouse	1:1,000	Life Technologies, UK

Cell Morphology Assessment

For assessment of changes in cell morphology, wheat germ agglutinin (WGA) stain was carried out on PANC-1 cells grown on glass coverslips in 12-well plates. Cells were fixed with 4% (w/v) PFA for 15mins at 37^{o}C, washed 3x in PBS and incubated with WGA (Life Technologies, UK) at 5µg/ml for 10mins at RT. Slides were then mounted in Prolong Gold Diamond Anti-fade containing DAPI (Life Technologies, UK) and visualised on the Olympus BX51 upright microscope using MetaVue Software (CA, USA). Images were further analysed using CellProfiler Software.

Morphometric measurements; the minimum and maximum Feret's diameter ($Feret_{min}$ and $Feret_{max}$) is a measure of cell length between any two parallel tangents at a perpendicular angle (Olson 2011).

Aspect ratio is defined as: $AR = \frac{Feret\ min}{Feret\ max}$ where a perfect circle has the aspect ratio of 1 and an oval has an aspect ratio of less than 1. As both the $Feret_{min}$ and $Feret_{max}$ approach the same value, the aspect ratio equals to 1; therefore this assessment can also be used as a measure of symmetry (Olson 2011).

Eccentricity is a measure of cellular elongation. A perfect circle has the eccentricity value of zero, whereas an oval shape would have a greater eccentricity value (Olson 2011).

Compactness is defined as: $C = \frac{\sqrt{\frac{4}{\pi} \times Area}}{Max\ Diameter}$ and is a measure of solidity; as cells become more irregular in shape this leads to increased concavity making the cell less solid or compact. A perfect circle or square has a compactness value of 1, whereas a star shape will have a lower value (Olson 2011).

Form factor is defined as: $FF = \frac{4\pi \times Area}{(Perimeter)^2}$ and although similar to compactness, it takes into consideration the complexity of the cell perimeter as well as the cell area. For example, a cell with a smooth edge would have a form factor of 1 whereas a cell with a rough or jagged edge would have a form factor of zero. As this calculation takes into account the smoothness of the perimeter, it is also used as a measure of sphericity and circularity (Olson 2011).

Statistical Analysis

GraphPad Prism (GraphPad Software Inc, USA) was used for the graphical representation of data and statistical analysis. Analysis of variance one-way ANOVA was used to analyse qRT-PCR fold changes between multiple treatment groups with post-hoc analysis performed using Dunnetts's multiple comparisons test. The specific statistical analysis is stated for each graph, respectively. Where stated, NS $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ for an average of 3 independent experiments $\pm$ SEM.

CHAPTER 3 Characterisation of endocrine differentiation in a human pancreatic ductal cell line

3.1 Introduction

3.1.1 Beta-cell regeneration

There are a number of different sources for generating new β-cells for cell based therapy in the treatment of diabetes where there is a loss of functional insulin producing and secreting β-cells. These alternative routes include; expansion of existing pancreatic β-cells by increased proliferation; differentiation from embryonic stem (ES) cells, differentiation from pancreatic progenitor cells (PPC's) and finally trans-differentiation from exocrine sources such as acinar and ductal cells (Bonner-Weir & Weir 2005a) (summarised in Figure 3.1).

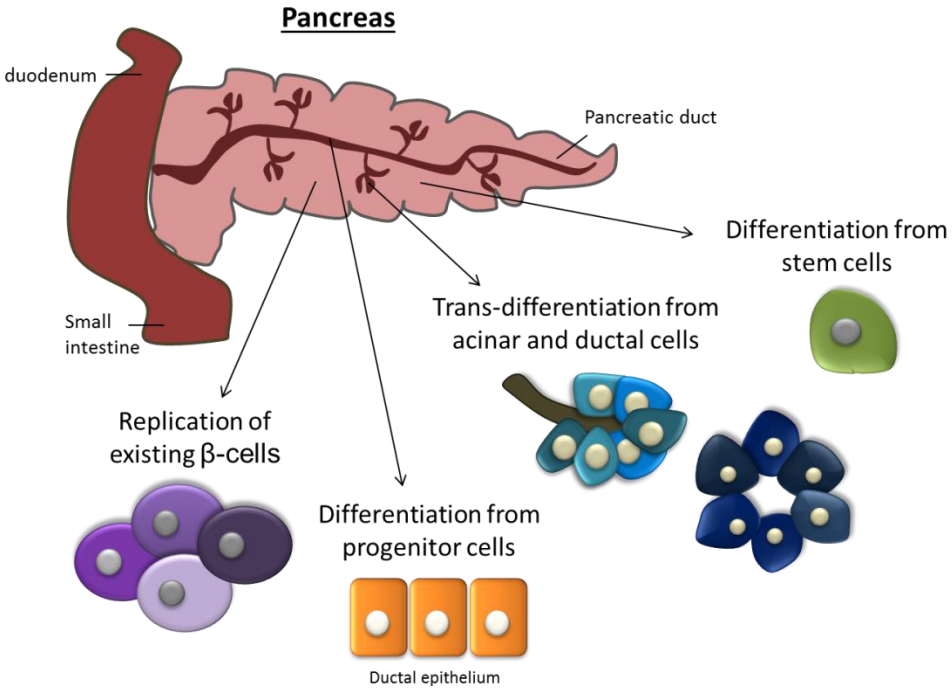


Figure 3.1 Sources for β-cell regeneration; Potential sources of new β-cells include replication of existing β-cells, differentiation from pancreatic progenitor cells residing in the ductal epithelium, trans-differentiation from exocrine cells (acinar and ductal cells) and differentiation from stem/progenitor cells (non- islet and non- exocrine origin) (Adapted from Bonner-Weir & Weir 2005)

3.1.2 Cellular reprogramming of PANC-1 cells

PANC-1 is a ductal epithelial carcinoma cell line widely used in studies of pancreatic cancer but has also been utilised in endocrine differentiation studies. PANC-1 cells were first derived from a 56 year old Caucasian male and established in culture in 1975 (Lieber et al. 1975). Studies have shown that the addition of exogenous factors can cause cellular reprogramming of PANC-1 ductal cells toward an endocrine lineage *in vitro*. One such compound is stem cell factor (SCF) which is reported to be present in the pancreatic mesenchyme during development, and its interaction with the receptor tyrosine kinase c-Kit is reported to promote islet differentiation and proliferation (Linnekin 1999). Wu et al. showed that exogenous SCF treatment in PANC-1 cells, led to increased expression of the transcription factors *PDX1* and *Pax6*, as well as *insulin* and *glucagon*. Immunostaining also showed C-peptide and insulin expression indicating the synthesis of newly formed insulin granules. This correlated with islet like cluster formation and a reduction in CK19, a ductal cell marker (Wu et al. 2010).

3.1.3 Small compounds which trigger ductal to endocrine trans-differentiation

In addition to studies using naturally occurring signalling factors such as SCF, there have been a -number of studies using small molecules to induce ductal to endocrine differentiation. Yuan et al. screened over 60,000 compounds in a high throughput qPCR gene expression assay using the pancreatic ductal cell line PANC-1 to identify compounds that induced the endogenous expression of *PDX1*, *Ngn3* and *MafA* (Yuan et al. 2013). A novel molecule (BRD7552) was identified as increasing *PDX1* mRNA levels in a dose dependent and time dependent manner. Exposure of PANC-1 cells to BRD7552 also induced the expression of *insulin* mRNA and caused insulin biosynthesis, suggesting that small compounds could be useful tools for transcriptional regulation and cellular reprogramming (Yuan et al. 2013). There has been a growing amount of research into the role of epigenetic changes in the control of pancreatic development and endocrine cell fate. A study using the de-methylating agent 5' Azadeoxycytidine (AZA) and the HDAC inhibitor Trichostatin A (TSA) in PANC-1 cells, showed that AZA induced the expression of key endocrine genes *Ngn3*, *NeuroD1* and *Nkx6.1* in a time dependent manner (Lefebvre et al. 2010). Although no insulin was detectable at the mRNA level, the expression of the hormones somatostatin and glucagon increased in cells cultured with AZA, suggesting that this compound may induce ductal to endocrine differentiation (Lefebvre et al. 2010).

3.1.4 Epigenetic regulation of gene transcription

There are a number of post-translational modifications of histones that regulate gene transcription including; acetylation, methylation, phosphorylation and ubiquitination. More importantly, histone acetylation regulates gene transcription by promoting an open chromatin structure, leading to gene activation (Marks et al. 2001). Histone methylation however promotes a closed chromatin structure leading to gene silencing (Haumaitre et al. 2009; Marks et al. 2001). Histone acetylation is regulated by two families of enzymes: histone acetyl transferases (HAT's) and histone de-acetyltransferases (HDAC's). HAT's cause acetylation of lysine residues, adopting a 'relaxed' chromatin structure that leads to the recruitment of transcriptional machinery (Delcuve et al. 2012; Marks et al. 2001). Conversely, HDAC's oppose the effect of HAT's by reversing acetylation of lysine resides and thereby stabilising chromatin structure (Yang & Seto 2007). A number of studies have shown the use of pharmacological small molecules with HAT and HDAC inhibitor (HDACi) activity involved in chromatin re-modelling play a role in directing pancreatic cell fate. The HDACi molecules valproic acid (VPA) and trichostatin A (TSA) have been previously reported to repress acinar cell differentiation whilst increasing a ductal cell phenotype and also increasing $Ngn3^{+ve}$ endocrine progenitor cells (Haumaitre et al. 2009).

3.1.5 Isoxazole; a novel potential transducer of ductal trans-differentiation?

The isoxazole derivative (*N*-Cyclopropyl-5-(2-thienyl)-3-isoxazole-carboxamide) has been previously reported to enhance GSIS and up-regulate the expression of β-cell associated transcription factors in human islets. Six-month-old human islets showing diminished insulin secretion following glucose stimulation when treated with 40μM isoxazole for up to 48hr displayed over a 50-fold increase in *insulin* mRNA and a significant increase in intracellular insulin (Dioum et al. 2011).

The MAPKs (mitogen activated protein kinases) ERK1/2 play a role in the control of insulin gene transcription in pancreatic β-cells (Dioum et al. 2011). Isoxazole has been shown to increase insulin secretion in human islets through biphasic activation of ERK1/2 and increasing histone acetyl transferase (HAT) activity (Dioum et al. 2011).

The insulin promoter region contains DNA binding domains upstream from the transcriptional start site for the transcription factors PDX1, NeuroD1 and MafA. Under high glucose conditions (10-30mM glucose) PDX1, NeuroD1 and MafA can activate *insulin* gene

transcription in a synergistic and co-ordinated manner (Sreenath S Andrali et al. 2008). PDX1 regulates insulin gene transcription by recruiting the HAT p300 as a co-activator to the insulin promoter region in high glucose conditions, ultimately leading to the acetylation of histones and insulin gene transcription. NeuroD1 can be phosphorylated by ERK1/2 which causes its translocation into the nucleus (shown in Figure 3.2). As NeuroD1 cannot bind directly to DNA, it forms a heterodimer with the DNA binding protein E47, within the insulin promoter region. This complex recruits the HAT p300 which causes acetylation of NeuroD1 and in turn induces insulin gene transcription (Dioum et al. 2011).

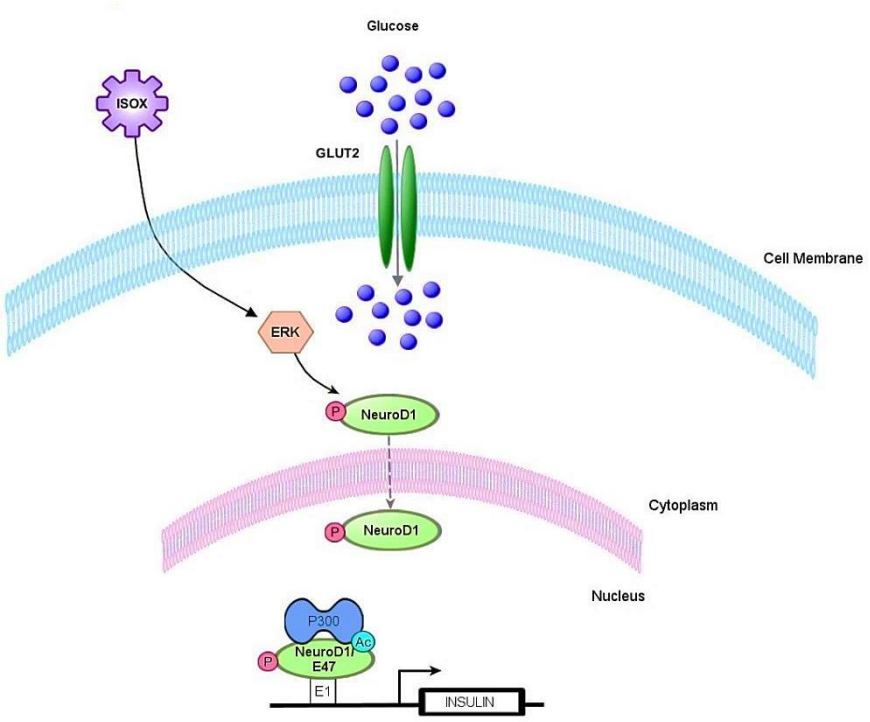


Figure 3.2 Mechanism of insulin gene transcription by isoxazole. Isoxazole (and high glucose concentrations) causes biphasic activation of ERK1/2 leading to phosphorylation of NeuroD1 and subsequent translocation into the nucleus, where it forms a complex with the HAT molecule p300 and induces insulin gene transcription (adapted from Sreenath S. Andrali et al. 2008).

3.1.6 EndoC-βH1 as a model β-cell line

From studies aiming to produce functional β-cells via differentiation of stem/progenitor cells, or trans-differentiation from different cell types, it is vital to have a readily available source of human β-cells for comparison. There are very few human pancreatic β-cell lines commercially available for this purpose due to a number of caveats. Firstly, access to tissue is restricted. Secondly, although β-cells make up the majority of cell types within the Islets of Langerhans, they actually account for approximately 1-2% of all pancreatic cell types. Given that the pancreas consists of both endocrine and exocrine regions it is therefore challenging to isolate a homogenous β-cell population for expansion *ex vivo* (Weir & Bonner-Weir 2011). Thirdly, and more importantly, human β-cells are susceptible to de-differentiation *in vitro* losing their glucose responsiveness and mature markers such as insulin, PDX1, and GLUT2 (Gershengorn et al. 2004). Furthermore, insulin secreting cell lines need to be assessed for their β-cell phenotype over long term cell culture to ensure a mature phenotype is maintained and that other islet-cell phenotypes don't arise (Weir & Bonner-Weir 2011). Finally, the proliferative capacity of β-cells is limited as somatic cells are affected by telomere shortening and therefore subjected to replicative senescence (Lee et al. 2004). One approach to tackle replicative senescence is to immortalise cell lines using human telomerase reverse transcriptase (hTERT) (Lee et al. 2004).

The human β-cell line EndoC-βH1 cell was gifted to our laboratory from Endocells (Paris, France). The EndoC-βH1 cell line was established by transduction of human foetal pancreatic buds with the simian virus 40 (SV40) within the insulin promoter region (Ravassard et al. 2011). The genetically altered rudiments of pancreatic tissue were then grafted into immuno-compromised SCID mice until mature pancreatic tissue was developed. The newly generated and differentiated β-cells were then transduced with hTERT. The subsequent transformed and immortalised cells were then expanded *in vitro* (Ravassard et al. 2011).

The EndoC-βH1 cells have been characterised and compared to human adult pancreatic islets; it was found that they express β-cell specific markers and secrete insulin in response to glucose stimulation (Ravassard et al. 2011). They have also been reported to contain 0.48 μg of insulin per million cells- less than 10% insulin content when compared to normal β-cells. Furthermore, these cells have been shown to restore normal blood glucose levels in chemically induced diabetic mouse models (Ravassard et al. 2011; Weir & Bonner-Weir 2011).

3.1.7 Aims and Hypothesis

Isoxazole has been previously reported to induce insulin gene transcription and enhance GSIS in human pancreatic islets as discussed above. The aims for this chapter were to investigate the ability of isoxazole to initiate endocrine trans-differentiation in a human pancreatic ductal cell line, PANC-1. Following treatment of the PANC-1 cells for 48 hr with isoxazole we aimed to characterise the changes observed in cell morphology, proliferation rate, gene expression and protein expression. The EndoC-βH1 cells express an array of mature β-cell markers. We used the model β-cell line-EndoC-βH1 cells comparatively to determine whether differentiated PANC-1 cells following isoxazole treatment were reaching a mature β-cell phenotype.

3.2 Results

3.2.1 EndoC-βH1 cells as a model β-cell line

EndoC-βH1 is a human pancreatic β-cell line and was used as a model cell line for insulin detection and secretion. The EndoC-βH1 cells have been described in the literature as being stable for up to 80 passages with no significant changes in cell morphology (Ravassard et al. 2011). The EndoC-βH1 cells are an adherent cell line that grew in a monolayer as shown in Figure3.3A. The seeding density required for maintaining these cells was high at 70,000 cells per $cm^{2.}$ The growth rate of the EndoC-βH1 cells was slow, and although the doubling rate in the literature was described as 5 days (Ravassard et al. 2011), within our laboratory the proliferation rate was lower with a 1.5 fold increase at day 7 as shown in Figure3.3B.

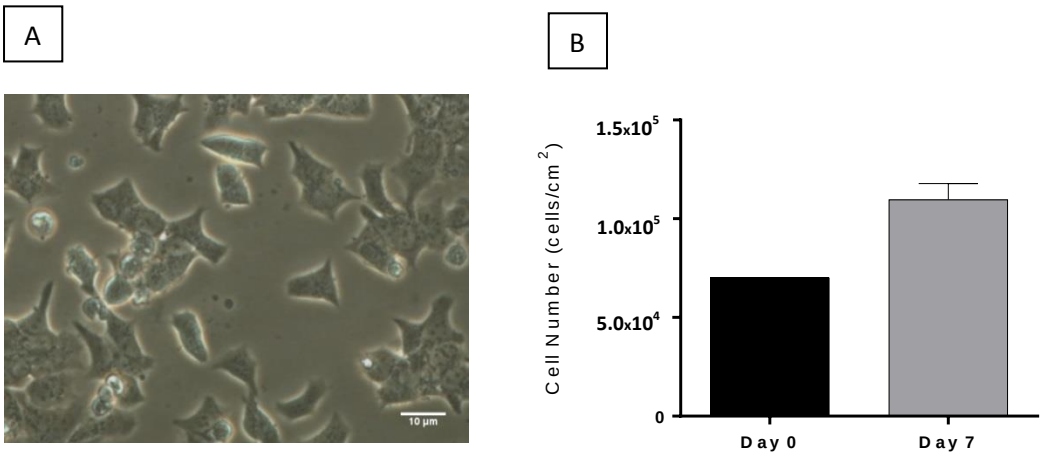


Figure 3.3 Growth of EndoC-βH1 cells. Panel A. Phase contrast micrograph of EndoC-βH1 cells at passage 52. Scale bar=10µm. Panel B. Cell number of EndoC-βH1 cells after 7 days in culture. Mean ± SEM n=8 from passage 52-65.

Immunocytochemical staining showed that insulin was detected in untreated EndoC-βH1 cells as shown in Figure 3.4.

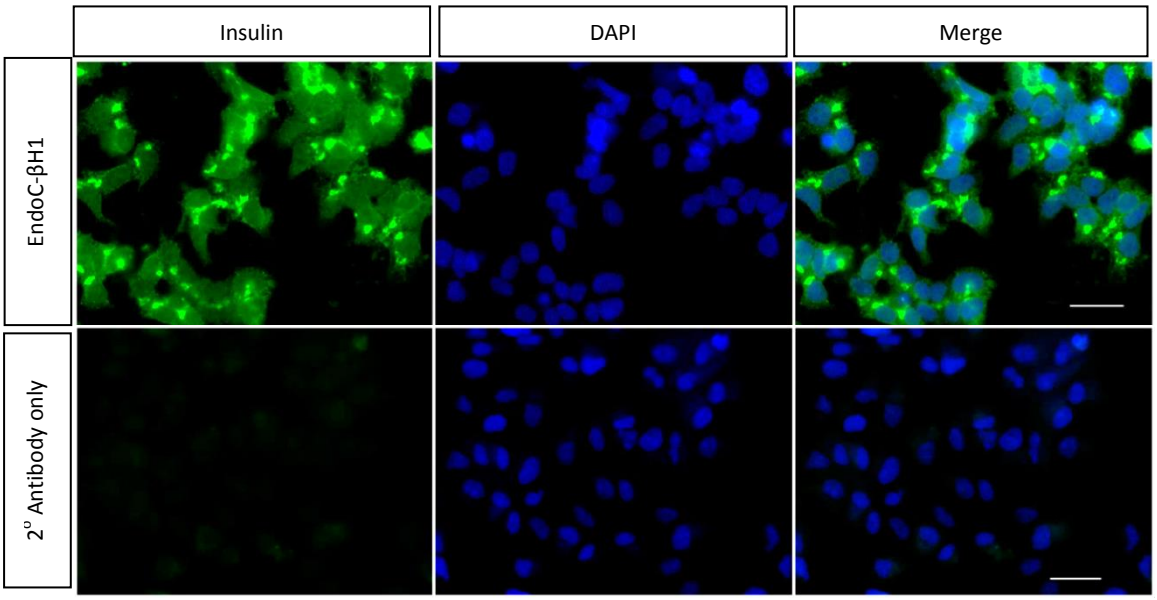


Figure 3.4 Detection of insulin in model β-cell line EndoC-βH1. Immunocytochemical staining of insulin (green) and DAPI nuclear staining (blue) in untreated EndoC-βH1 at passage 60. Secondary antibody only negative control. Scale bar = 20µm. Data typical of n=3 experiments from passages 52-65.

Glucose stimulated insulin secretion (GSIS) assay in the EndoC-βH1 cells was measured across 0.5-15mM glucose range as shown in Figure 3.5. Increasing the glucose concentration did not significantly increase insulin secretion in the EndoC-βH1 cells, except at 5 mM glucose ($p<0.05$ when compared to basal 0.5 mM glucose). This may be due to low experimental replicates and high variability between treatment groups. Insulin secretion was increased due to elevated cyclic AMP levels when treated with the phosphodiesterase inhibitor IBMX at a final concentration of 0.5 mM ($p<0.05$ when compared to the relevant glucose treatment). Although the addition of IBMX enhanced basal insulin secretion, it did not however improve the glucose responsiveness of the cells.

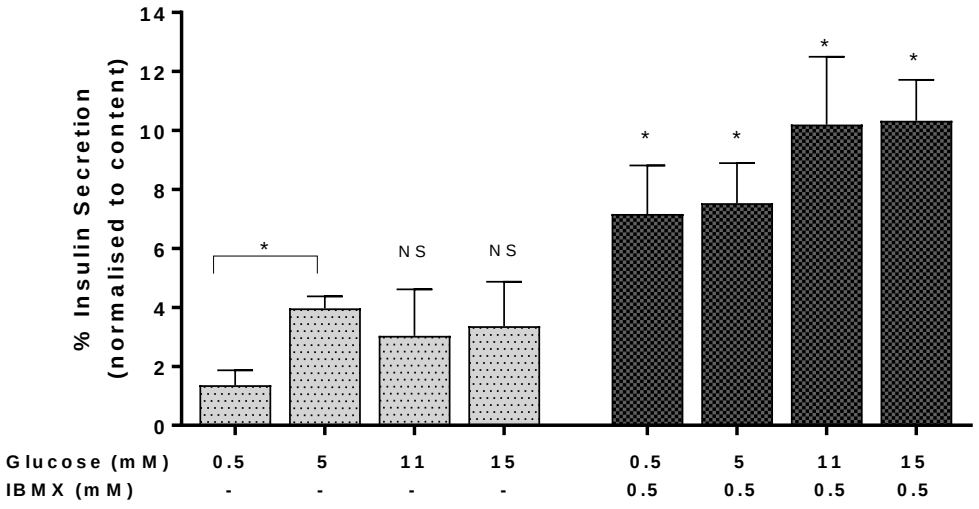


Figure 3.5 Glucose stimulated insulin secretion in EndoC-βH1 cell. EndoC-βH1 cells challenged with glucose at 0.5, 5, 11 and 15mM glucose with and without IBMX (0.5 mM). Insulin secretion presented as percentage of total insulin content. Mean ± SEM of 3 independent experiments. (*$p<0.05$, NS $p>0.05$ 0.5 vs 11 and 15mM glucose, t-test)

3.2.2 PANC-1 heterogeneity

Due to the difficulties with the EndoC-βH1 cells, focus was shifted to the PANC-1 cells, which have been shown to transdifferentiate towards an endocrine lineage (Hui et al. 2001; Lefebvre et al. 2010; Yuan et al. 2013; Wu et al. 2010). As shown in Figure 3.6A PANC-1 cells show a degree of morphological variability at a single cell level. Using haemotoxylin and eosin staining, microscopic cytological analysis of PANC-1 cells show distinct cellular morphologies (as shown in Figure 3.6B). The presence of large cells, intermediate stellate cells and small morula shaped cells indicate a heterogeneous cell population as highlighted by the arrows.

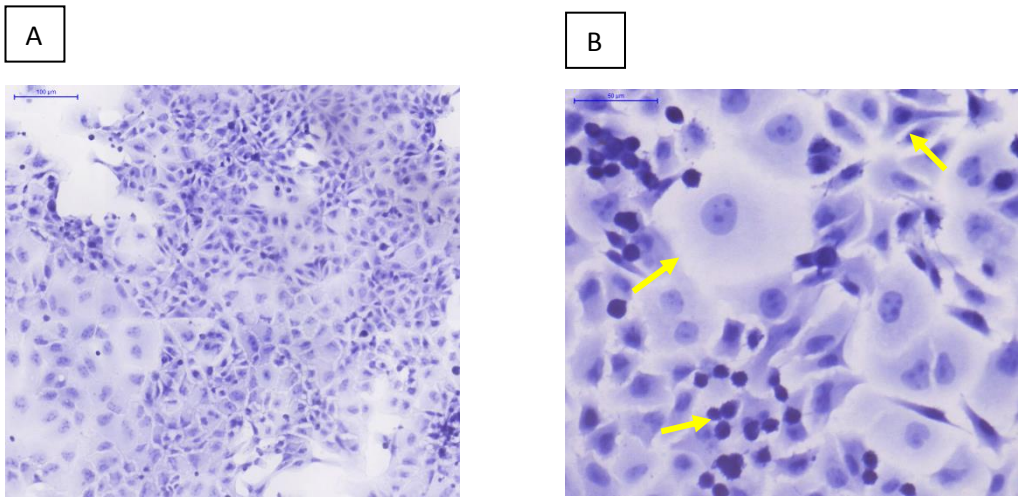


Figure 3.6 PANC-1 morphological heterogeneity. Immuno-histochemical staining and microscopic observations of PANC-1 show distinct cellular morphologies indicating a heterogeneous cell population. Arrows pointing to different cell types (large, intermediate stellate and small morula shaped cells). Scale bar A= 100µm, B=50µm; typical data shown from n=2 experiments from passages 23-35.

3.2.3 PANC-1 Cell Proliferation and Viability

To investigate the actions of isoxazole on pancreatic endocrine differentiation, the effect of isoxazole on PANC-1 cell proliferation and viability was initially determined using both the MTT assay and trypan blue cell counts. PANC-1 cells have been reported to have mean doubling time of 52hrs (Lieber et al. 1975). Data from this study show that PANC-1 cells grown in the presence of 10% FBS to have a population doubling time of 54.3 hrs. From the proliferation curves in Figure 3.7 it was evident that the biggest difference observed in cell proliferation was due to the removal of serum from the cell culture medium. As cells were seeded at the same seeding density, increased absorbance (at 595nm) is indicative of increased cell proliferation (van de Loosdrecht et al. 1994). PANC-1 cells grown in serum free conditions had a significantly lower proliferation rate. There was no significant difference in proliferation rate between isoxazole, DMSO vehicle control and untreated PANC-1 cells in serum free medium.

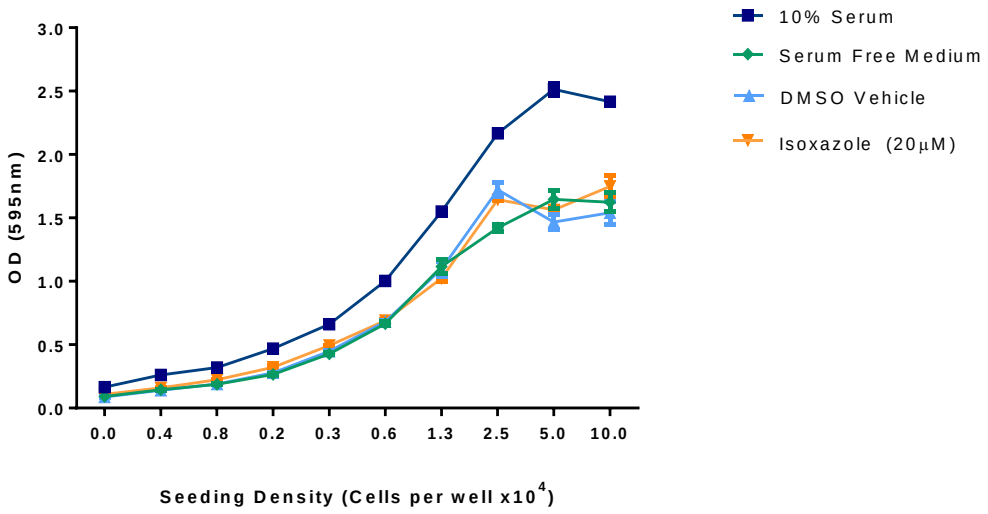


Figure 3.7 Proliferation rate of PANC-1 cells after 48 hr. PANC-1 cells in 10% serum and in a defined serum free medium, with the addition of (20µM) isoxazole, and DMSO vehicle control, measured by MTT cell proliferation/viability assay. Mean ± SEM of 3 independent experiments. ($p<0.01$ for 10% FCS and other treatment groups for all cell concentrations, using one way ANOVA and Dunnet's post-hoc test).

To further explore the effect of isoxazole on cell growth, cell counts and viability were determined using trypan blue cell counts over 6 days of treatment with isoxazole and the DMSO vehicle control. Cell proliferation was not significantly affected following a 6 day treatment with isoxazole compared to the DMSO control (shown in Figure 3.8 Panel A). Isoxazole treatment did however significantly decrease the cell viability following 6 days of treatment when compared to day 0 ($p<0.001$), although this was not significantly different from the DMSO vehicle control at that time point (Figure 3.8 Panel B).

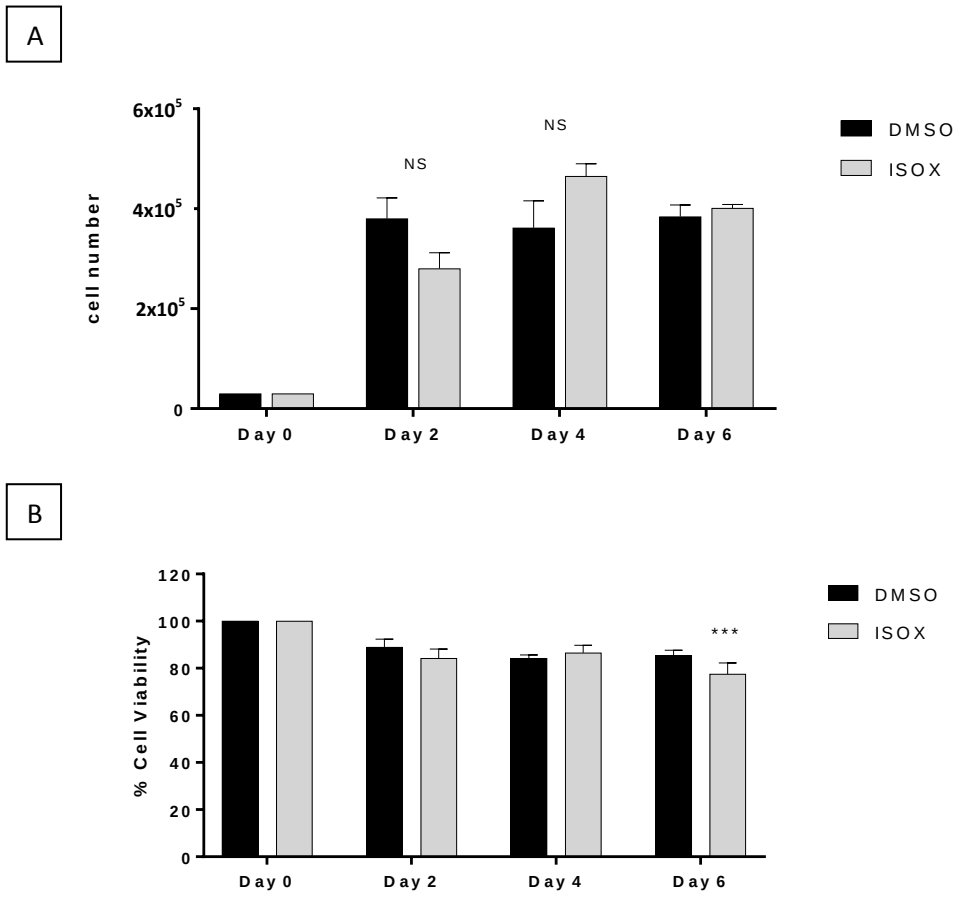


Figure 3.8 Proliferation and cell viability of PANC-1 cells treated with isoxazole and DMSO vehicle control 2, 4, and 6 days. Panel A. Cell proliferation measured by trypan blue cell counts over 6 days treatment with isoxazole and DMSO vehicle control. Panel B. Percentage cell viability calculated from trypan blue exclusion. Mean ±SEM of 3 independent experiments, statistical analysis Panel A- NS $p>0.05$ student t-test, Panel B- one-way ANOVA (***$p<0.001$).

3.2.4 Effect of isoxazole on cell morphology

We observed distinct morphological changes at a population level in PANC-1 cells following a 48hr treatment with isoxazole when compared to the DMSO vehicle control (Figure 3.9). Single cells became elongated and more mesenchymal in appearance; cells appeared to collectively migrate and begin forming aggregates or clusters at day 2 of isoxazole treatment.

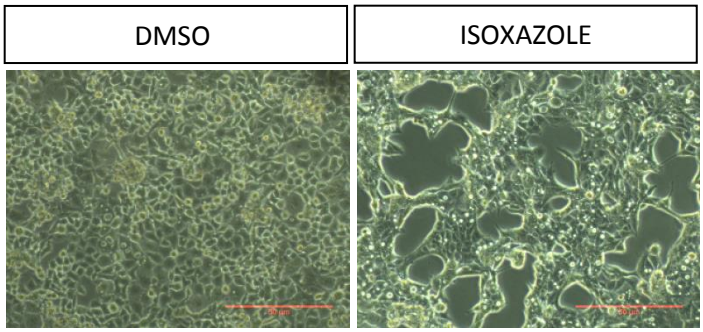


Figure 3.9 Isoxazole induces changes in cell morphology. Phase contrast micrographs of PANC-1 cells with 48 hr isoxazole treatment show distinct morphological changes when compared to DMSO control. Scale bar= 100µm (x10 magnification). Data typical of n=6 independent experiments between passages 23-35.

Following 6 days of isoxazole treatment, cluster formation was evident, and furthermore the formation of 'pseudo-ductal' structures forming on the periphery of these cell aggregates was clear as shown in Figure 3.10. The number of 'pseudo–ductal' structures in a given area (1mm^2) was quantified and was 10-fold higher with isoxazole treatment, significantly higher when compared to the DMSO vehicle control (Figure 3.11 A). 'Pseudo-ductal' structures were quantified by measurement of total free area (µm^2) formed as a result of the cellular organisation observed with isoxazole treatment (Figure 3.11 B). The area formed by these 'pseudo-ductal' structures was significantly higher in the isoxazole treated cells compared to the DMSO control.

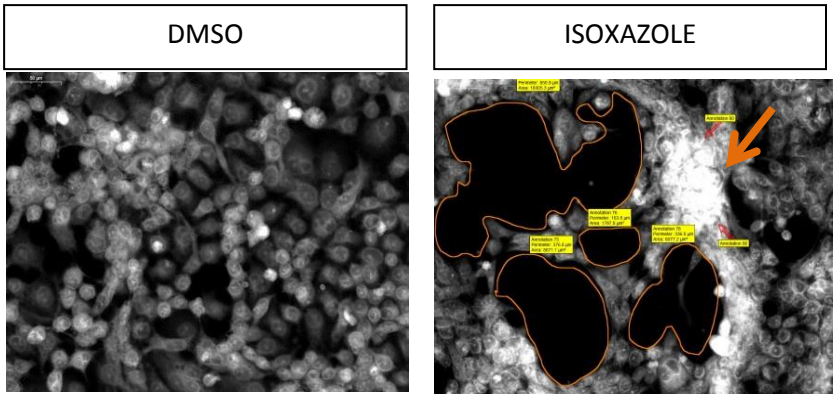


Figure 3.10 Isoxazole induces formation of 'pseudo-ductal' structures and cell aggregates. Representative images of DMSO and isoxazole treated PANC-1 cells following 6 day treatment showing ductal structures and cluster formation.

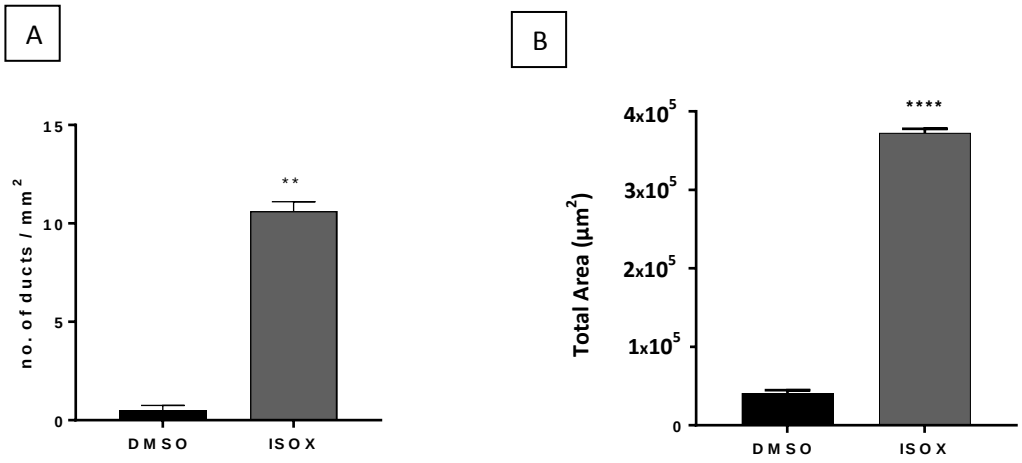


Figure 3.11 Quantification of 'pseudo-ductal' structures using CaseViewer Software. Quantification of the number of pseudo-ducts per 1mm^2 of cell area shown in Panel A and the total area formed (µm^2) by the ductal structures in Panel B. Mean ± SEM of 9 coverslips across 3 independent experiments between passages 23-35. Statistical analysis 2 tailed t-test (**p<0.01 compared against DMSO control).

To further explore the interesting changes observed in cell morphology, additional analysis was done to quantify the morphometric changes in single cells induced by isoxazole treatment. Plasma membrane staining with wheat germ agglutinin was carried out on PANC-1 cells. Nine representative images of untreated, DMSO and isoxazole treated PANC-1 cells were taken at 40x magnification across 3 independent experiments and assessed for cell shape. Figure 3.12 highlights important stages in the processing of images using the CellProfiler software. Key parameters included the identification of 'primary objects' which were the nuclei and 'secondary objects' which were the outer cell membranes.

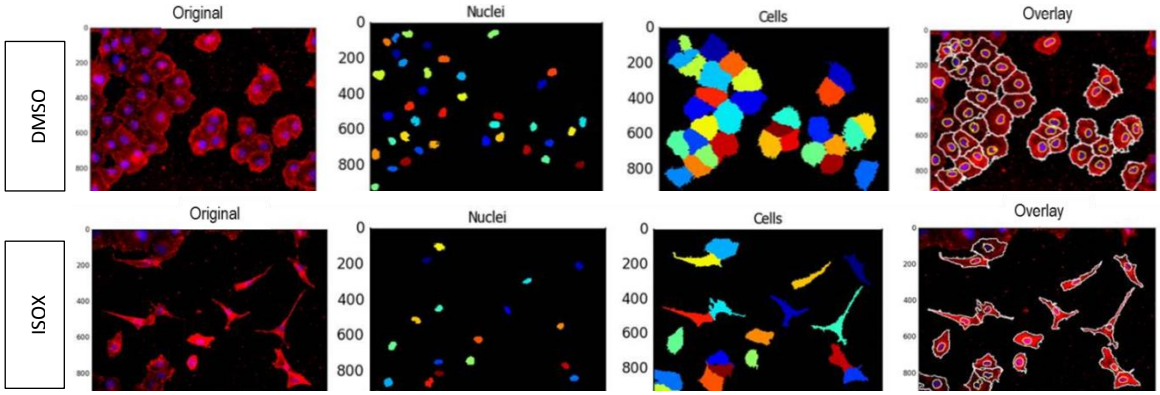


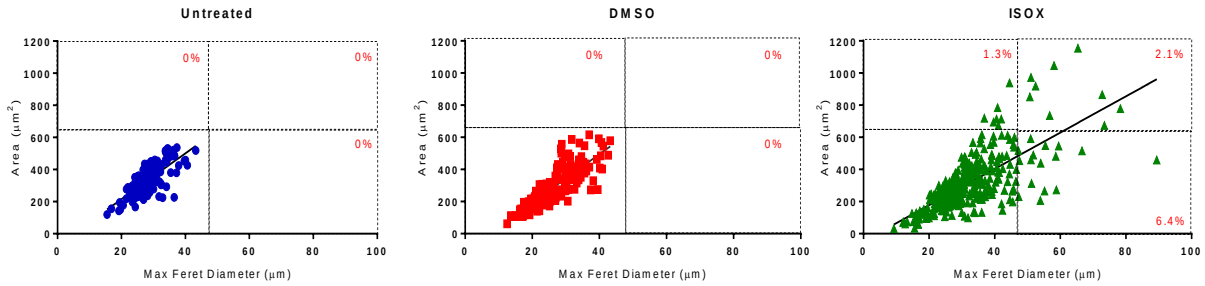
Figure 3.12 Assessment of isoxazole induced changes in cellular morphology using CellProfiler. Representative images taken at x40 magnification (n=3) assessed for cell shape.

The maximum Feret's diameter is a measure of the maximum distance between any two parallel tangents and is used as a measure of cell length (Moore et al. 1987). Figure 3.13 Panel A, shows the area of cells (μm^2) plotted against the maximum Feret's diameter (µm) for each treatment group. Although 90% of isoxazole treated cells appeared similar in length and size to the controls, nearly 10% of cells showed a unique profile following isoxazole treatment. Quadrant cut-off levels revealed that 6.4% of cells had increased length while 2.1% of cells had both increased length and area while 1.3% of cells had increased in area only.

Statistical analysis showed that although isoxazole treatment had a significant effect on cell length and area, DMSO to a lesser extent also had a significant effect. Despite the low DMSO concentration (0.03% v/v) the vehicle control also induced changes in cell morphology minimising the effect observed by isoxazole. Subsequently, there was no

significant difference in cell area, maximum feret diameter and also perimeter (data not shown) between DMSO and isoxazole. However, from descriptive statistical analysis (shown in Table 3.13 Panel B), percentiles revealed that 95% of isoxazole treated cells have an area greater than 98µm^2 and less than 609µm^2, while untreated PANC-1 cells have an area between 187µm^2 and 513µm^2. Thus illustrating that isoxazole treatment gave the largest spread in the distribution of cell area (Δ 511 µm^2 in cell area) while untreated PANC-1 cells had the smallest spread in the distribution (Δ 326 µm^2 in cell area).

A



B

	Area (µm^2)		
	Untreated	**DMSO**	**ISOX**
5% Percentile	187	113	98
95% Percentile	513	547	609
Δ cell area (µm^2)	326	434	511

Figure 3.13 Quantification of isoxazole induced changes in cellular morphology. Cell area (µm^2) versus maximum Feret's diameter (µm) is compared in isoxazole treated PANC-1 cells (A). Descriptive statistical analysis showing percentile ranges for cell area in isoxazole treated PANC-1 cells (B).

Other morphometric measurements were also analysed which revealed a more descriptive profile of isoxazole treated cells (descriptions and formulae for the morphometric measurements are included in the Materials and Methods chapter of this thesis). The aspect ratio is calculated as the minimum Feret's diameter divided by the maximum Feret's diameter (Olson 2011). If these values are the same (for example a circle), then the aspect ratio is equal to 1 (Moore et al. 1987). Therefore, this parameter can be used as measure of symmetry. Isoxazole treatment significantly lowered the aspect ratio in PANC-1 cells indicating a less symmetrical phenotype (shown in Figure 3.14 A). Eccentricity is a measure of elongation and this was significantly higher in the isoxazole treatment group suggesting that isoxazole caused cellular elongation (Figure 3.14 B). Compactness takes into account the cell area and maximum diameter (Moore et al. 1987). Isoxazole decreased cellular compactness when compared to the untreated and vehicle controls (Figure 3.14 C). Form factor is similar to compactness; however it also takes into consideration the complexity of the perimeter (Olson 2011). A cell with a smooth edge will have a form factor of 1 whereas a cell that has micro-villi or a jagged edge will have lower form factor. Whilst DMSO also affected the form factor, isoxazole significantly decreased the form factor further; suggesting isoxazole increased cellular protrusions or membrane 'roughness' (Figure 3.14 D). This data suggests that whilst the majority of isoxazole treated cells appeared similar in morphology to the controls, there was a small, but significant, percentage of cells with a distinctive cellular profile.

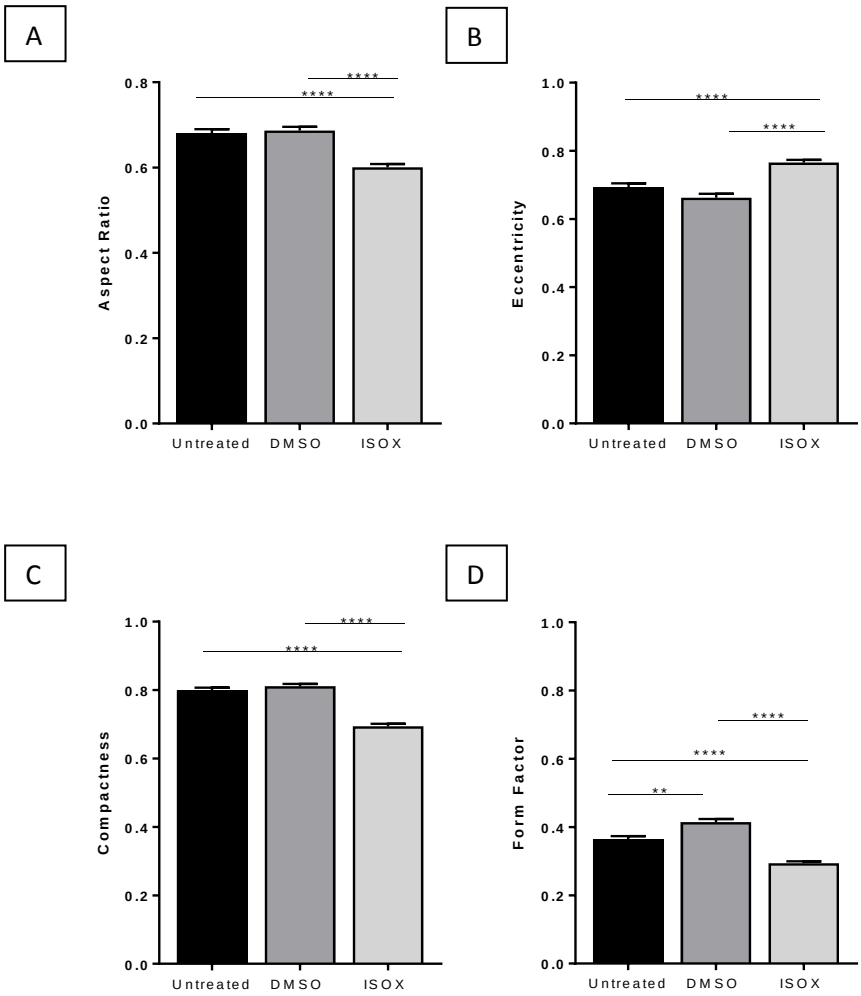


Figure 3.14 Morphometric analysis of PANC-1 cells treated with Isoxazole. Isoxazole treatment was compared with DMSO vehicle control and untreated PANC-1 cells. Aspect ratio (A), eccentricity (B), compactness (C) and form factor (D) were altered with isoxazole treatment. Mean ±SEM represented from 3 independent experiments. Statistical analysis one-way ANOVA (**p<0.01, ****p<0.0001).

3.2.5 Changes in gene expression

During pancreatic development, the transcription factor *Ngn3* is expressed in all pancreatic progenitor cells and gives rise to all endocrine cell lineages including β-cells. *Ngn3* initiates a signalling cascade of transcription factors that leads to the differentiation towards mature β-cells. Isoxazole treatment induced the expression of *Ngn3* with 48hr treatment (as shown in Figure 3.15) and further quantitative analysis showed a significant up-regulation with a 150-fold increase in *Ngn3* mRNA levels compared to DMSO vehicle control as shown in Figure 3.16B. Low levels of *Ngn3* mRNA were detected in the EndoC-βH1 cell line as expected (Figure 3.16B), since *Ngn3* is expressed at high levels at the endocrine progenitor stage and is later down regulated in more mature β-cells (Jørgensen et al. 2007). Isoxazole treatment had little effect on PDX1 mRNA expression (Figure 3.15 and 3.16A).

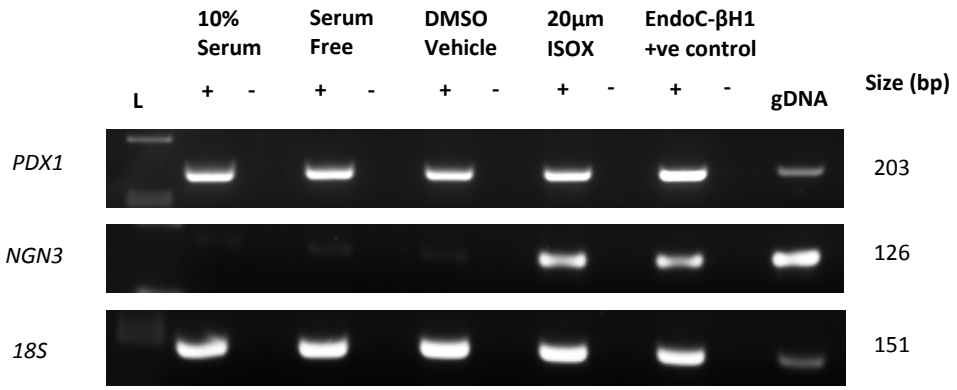


Figure 3.15 Isoxazole induces the expression of *Ngn3* in PANC-1 cells. Reverse transcription-PCR shows no change in the expression of *PDX1* and the induction of *Ngn3* expression following a 48hr isoxazole treatment. 18S house-keeping gene. Data typical of n=3 independent experiments.

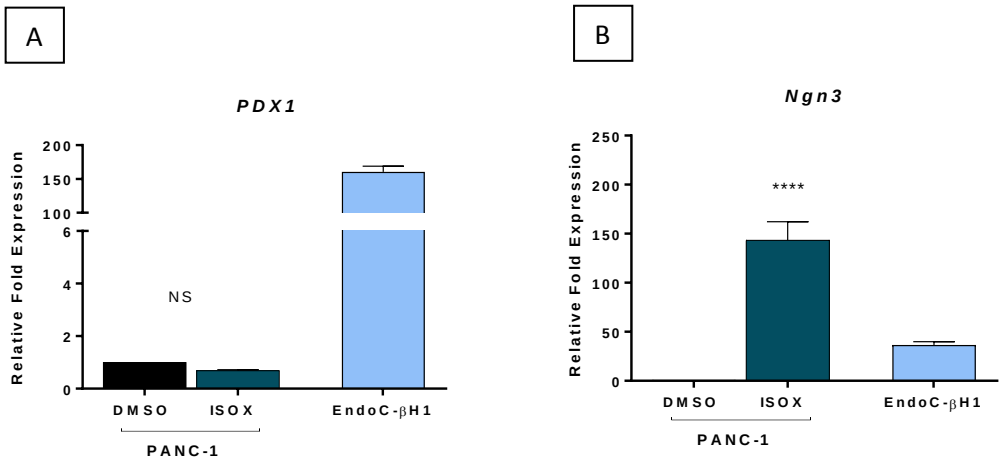


Figure 3.16 Quantitative RT-PCR of genes *PDX1* (A) and *Ngn3* (B). qRT-PCR shows no change in *PDX1* expression and an up-regulation of *Ngn3* after 48 hr isoxazole treatment when compared to the DMSO control. Normalised to 18s housekeeping gene. Model β cell line EndoC-βH1 gene expression fold change also compared to DMSO control. Mean ± SEM of 3 independent experiments. Unpaired t-test (****p<0.0001 compared to DMSO control).

Isoxazole treatment also induced the expression of transcription factors *NeuroD1, Pax4* and increased the expression of *Islet1*. Expression of transcription factors *Nkx6.1, MafA* and *MafB* were also observed in untreated controls and isoxazole treated PANC-1 cells, although not quantified, shown in Figure 3.17.

NeuroD1 is a helix-loop-helix transcription factor that works downstream of *Ngn3* and plays a critical role in insulin gene transcription in β-cells and islet formation (Jørgensen et al. 2007). *NeuroD1* was significantly up-regulated with isoxazole treatment with nearly a 1,500-fold increase compared to DMSO control (Figure 3.18A). *Pax4* is a paired box homeoprotein transcription factor, and is detected in predominantly insulin positive cells. It plays a role in the terminal differentiation stage of pancreatic development and maturation of β-cells (Madsen et al. 1996). *Pax4* expression was significantly up-regulated with isoxazole treatment with nearly a 600-fold increase in mRNA levels (Figure 3.18B). *Islet1* is a LIM homeodomain transcription factor and is expressed in all endocrine cell types including mature β-cells (Madsen et al. 1996). *Islet1* expression was significantly up-regulated with isoxazole treatment with over a 10-fold increase in mRNA levels compared to DMSO control (Figure 3.18C).

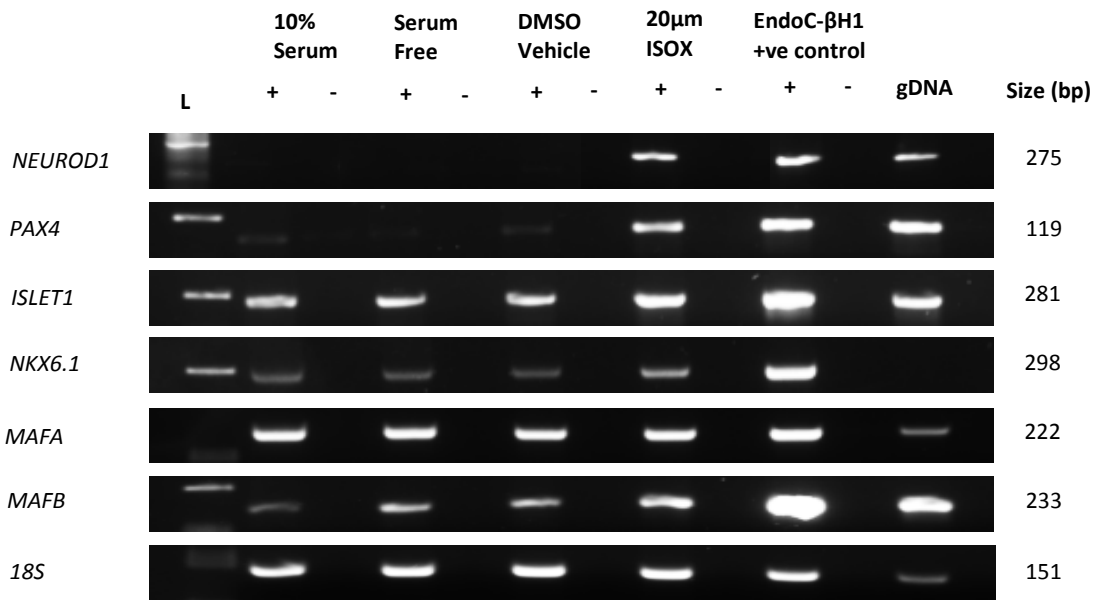


Figure 3.17 Isoxazole induces expression of *NeuroD1* and *Pax4*. Reverse transcription PCR shows the induction of *NeuroD1, Pax4* and up-regulation of *Islet1* following 48 hr isoxazole treatment. Transcription factors *Nkx6.1, MafA* and *MafB* also detected in untreated PANC-1 cells. 18S house-keeping gene. Data typical of n=3 independent experiments.

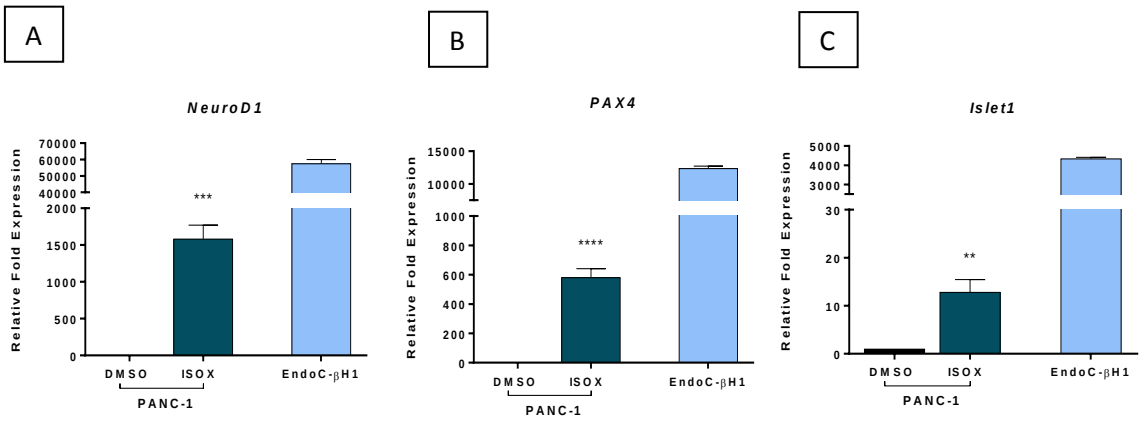


Figure 3.18 Quantitative RT-PCR of genes *NeuroD1* (A*), Pax4* (B), *Islet1* (C). qRT-PCR shows up-regulation in *NeuroD1, Pax4* and *Islet1* after 48 hr isoxazole treatment compared to DMSO control. Normalised to 18s housekeeping gene. Model β cell line EndoC-βH1 gene expression fold change also compared to DMSO control. Mean ± SEM of 3 independent experiments. Un-paired t-test (**p<0.01, ***p<0.001, ****p<0.0001 compared to DMSO control).

Furthermore, the expression of mature β-cell markers was also assessed. *SLC30A8* expression was induced with isoxazole treatment (Figure 3.19) and further analysis shows a significant up regulation with isoxazole treatment with nearly a 150-fold increase compared to the DMSO control (Figure 3.20A). Isoxazole treatment also induced the expression of the *insulin* gene. Although this was barely detectable by basic RT-PCR, qRT-PCR however showed nearly a 20-fold increase in *insulin* mRNA levels with isoxazole treatment compared to the DMSO control (Figure 3.20B). *SLC2A2* gene encodes for the GLUT2 glucose transporter and is necessary for glucose sensing and GSIS. *SCL2A2* expression was significantly up-regulated with a 30-fold increase with isoxazole treatment when compared to the DMSO control (Figure 3.20C). Levels of *SLC30A8, Insulin* and *SLC2A2* were also quantified in the EndoC-βH1 cell line and were considerably greater.

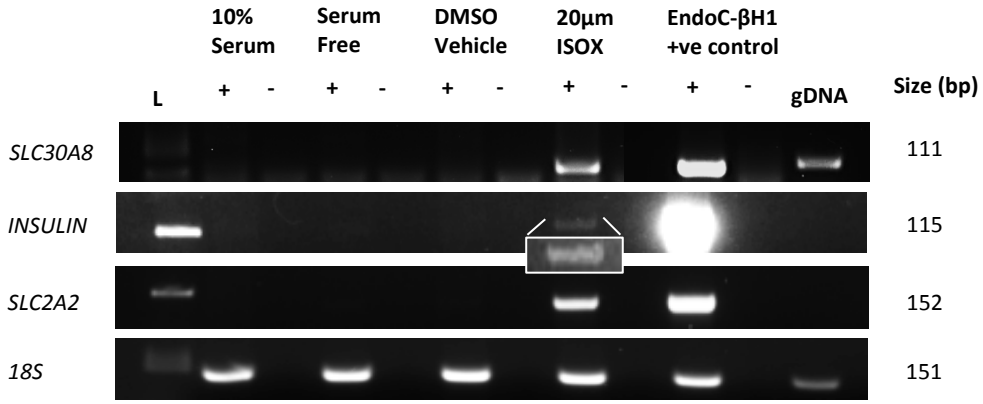


Figure 3.19 Isoxazole induces the expression of mature β-cell markers. Induction in the expression of mature markers *SLC30A8, Insulin* and *SCL2A2* following 48hr isoxazole treatment, measured by reverse transcription PCR. 18S house-keeping gene. Data representative of n=3 independent experiments from passages 23-35.

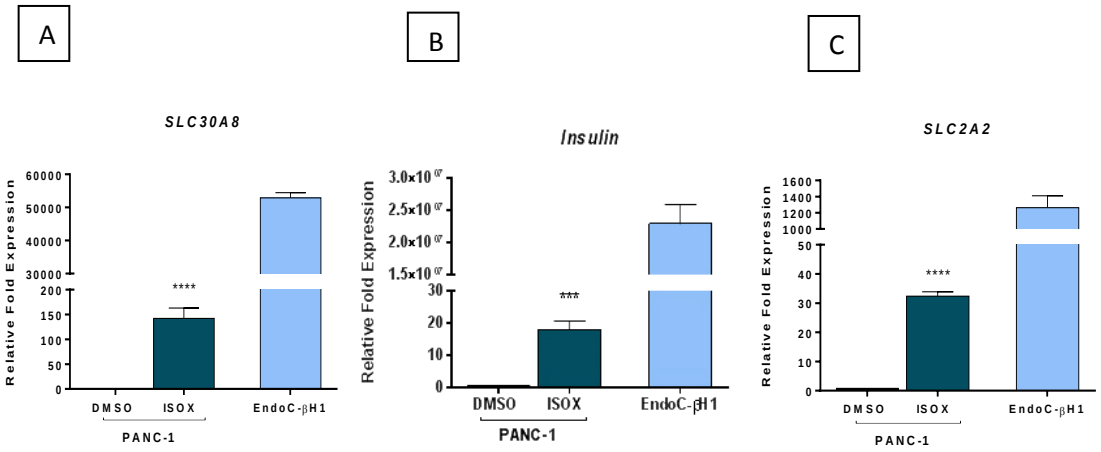


Figure 3.20 Quantitative RT-PCR of mature β-cell associated genes *SLC30A8* (A), *Insulin* (B), and *SLC2A2* (C) Isoxazole induces up-regulation of *SLC30A8, insulin* and *SLC2A2* after 48 hr isoxazole treatment compared to DMSO control. Normalised to 18s housekeeping gene. Model β cell line EndoC-βH1 gene expression fold change also compared to DMSO control. Mean ± SD of 3 independent experiments. Un-paired t-test (***p<0.001, ****p<0.0001 compared to DMSO control).

3.2.6 Ductal marker CK19 expression in PANC-1

Having established the up-regulation of pro-endocrine genes in PANC-1 cells following isoxazole treatment, this study aimed to explore if changes were also occurring at a protein level. CK19 is a pancreatic ductal cell specific epithelial marker (Bouwens 1998). Given the heterogeneity observed by microscopic analysis discussed at the beginning of this chapter, the PANC-1 cell population was subsequently further investigated. As shown in Figure 3.21, ICC staining of untreated PANC-1 cells showed a differential expression of CK19 with regions of $CK19^{+ve}$ and $CK19^{-ve}$ cells.

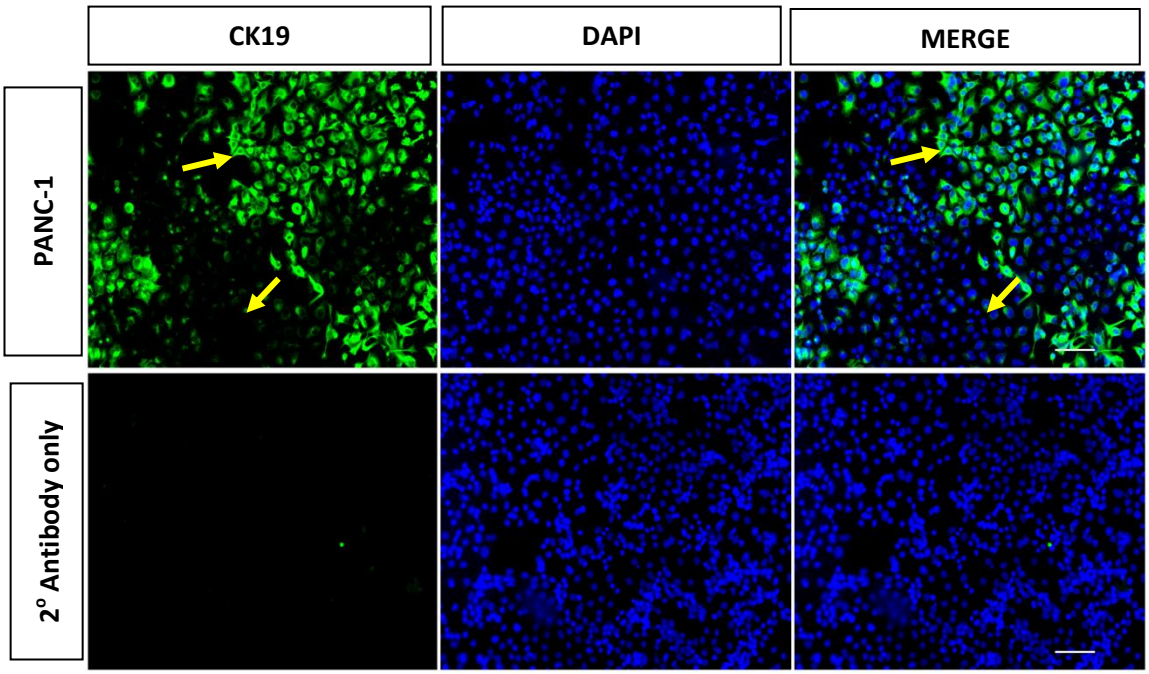


Figure 3.21 Differential expression of CK19 in PANC-1. Immunocytochemical staining of untreated PANC-1 cells with ductal cell marker cytokeratin 19 (CK19) reveal $CK19^{+ve}$ and $CK19^{-ve}$ cell populations indicated by arrows. Secondary antibody only control. Scale bar= 50µm (X10 magnification). Data representative of 3 independent experiments from passages 23-35.

Given the differential CK19 ductal marker expression in untreated PANC-1 cells, CK19 staining was quantified using CellProfiler Software. Quantification revealed that approximately 80% of PANC-1 cells were $CK19^{+ve}$ while nearly 20% are $CK19^{-ve}$ (Figure 3.22 A). While the vast majority of cells are significantly positive for CK19, within this population there are variable levels of expression. Figure 3.22 B shows the variations in fluorescent intensity (MFI) of CK19 staining. This ranges from 0 (undetectable) to 1 which is highly fluorescent. Analysis of the descriptive statistics of percentiles show that 8% of cells are within the lower 25% (MFI<0.5), 45% of cells are in the median range (MFI range 0.1-0.3) while 28% of cells are within the upper 75% percentile (MFI range 0.3-1).

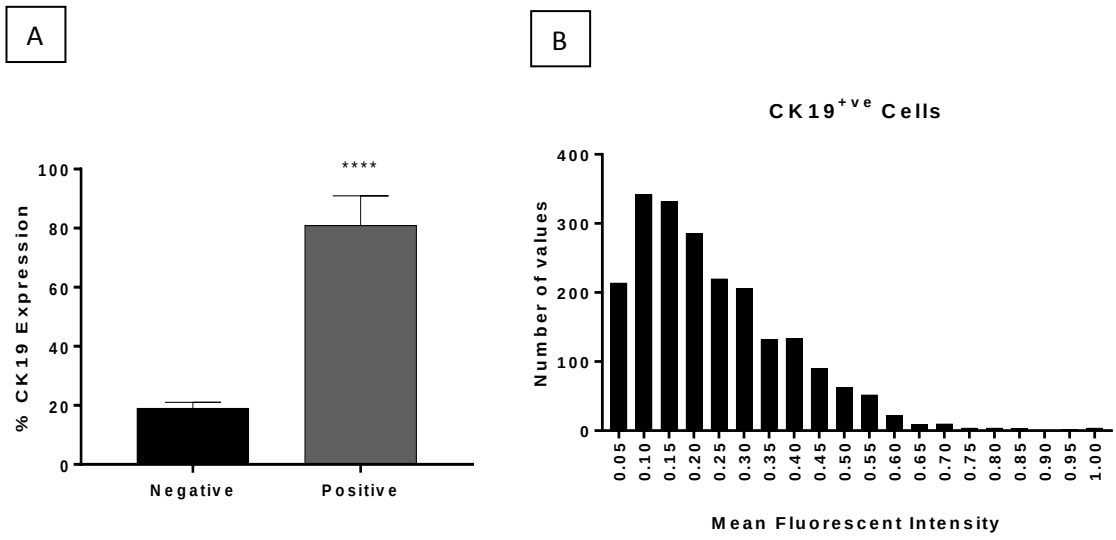


Figure 3.22 Quantification of CK19 in untreated PANC-1 cells. Percentage $CK19^{+ve}$ cells calculated based on mean fluorescent intensity (MFI) (A). Histogram of $CK19^{+ve}$ cells in PANC-1 (B). (****p<0.0001 t-test)

Previous data has revealed heterogeneity in PANC-1 cell morphology, with three distinct cell types detected in untreated PANC-1 cells and in addtion to this the differential expression of CK19 staining. This led to the question of whether PANC-1 cells highly expressing CK19 were associated to a particular cell type i.e large cells. To determine if there was a relationship between CK19 staining and cell morpholgy, the MFI of CK19 was compared against cell area (μm^2) shown in Figure 3.23. Pearson correlation test showed the R^2 value of 0.25, indicating no correlation between CK19 staining and cell area in untreated PANC-1 cells.

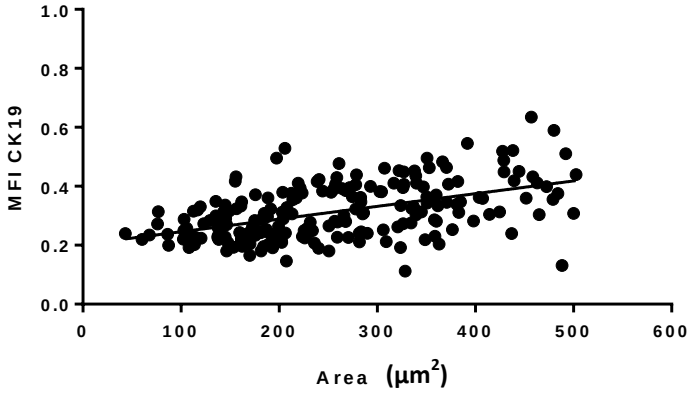


Figure 3.23 No correlation between PANC-1 cell morphology and CK19^{+ve} cells. Mean fluorescent intensity (MFI) of CK19 staining compared with cell area (μm^2) in untreated PANC-1 cells shows no correlation (Pearson correlation test, R^2=0.25).

Following on from this, having already established the up-regulation of pro-endocrine genes with isoxazole treatment, CK19 expression in response to isoxazole treatment was further investigated. ICC staining of CK19 was carried out on untreated, DMSO vehicle control and isoxazole treated PANC-1 cells with 48 hr treatment (Figure 3.24). By observation there appeared to be no distinct differences in CK19 expression between the treatment groups.

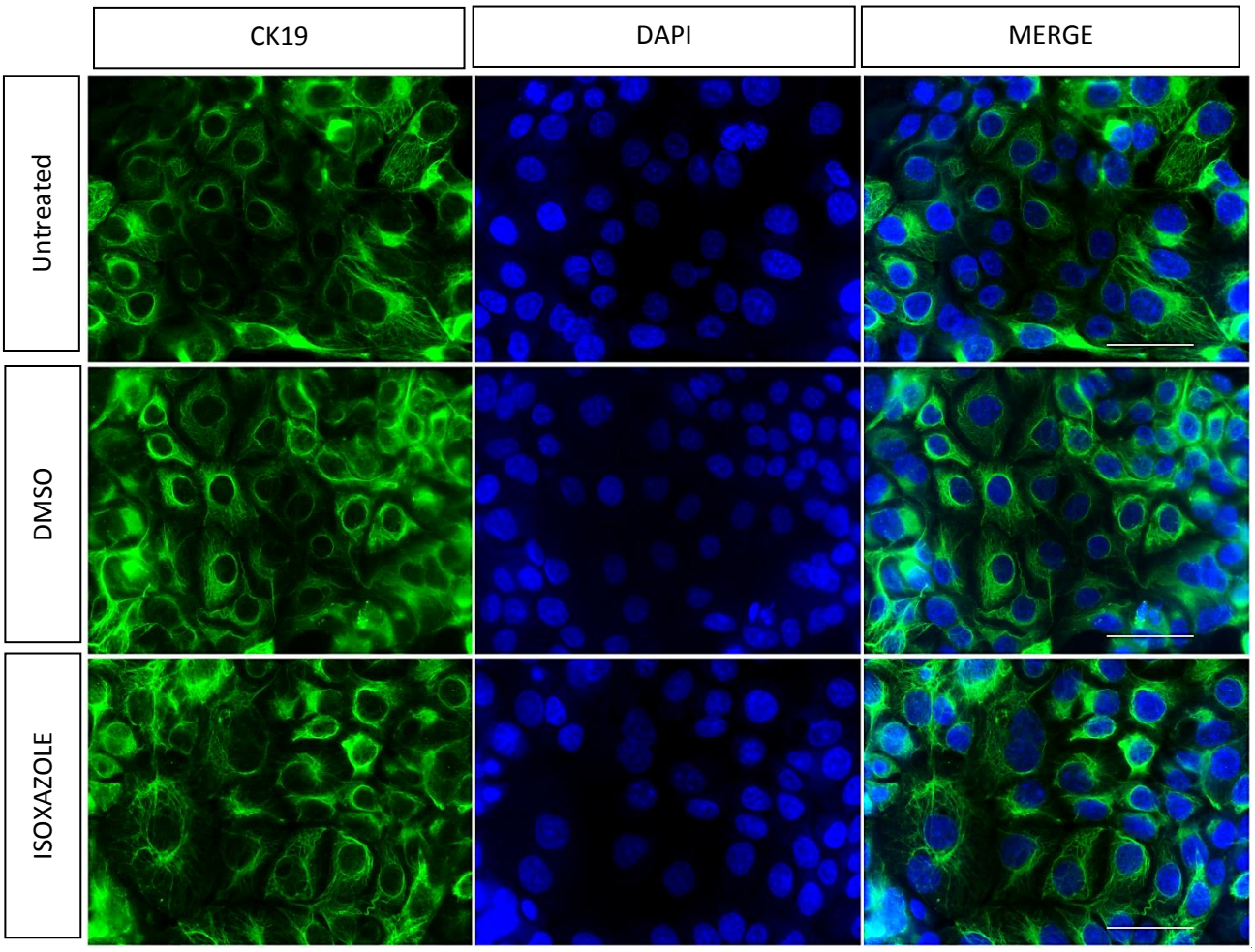


Figure 3.24 Isoxazole doesn't affect CK19 expression in PANC-1 cells. Immunocytochemical staining of Cytokeratin-19 ductal cell specific marker in untreated, DMSO vehicle control and isoxazole treated PANC-1 cells following 48hr treatment. Scale bar = 50µm (x40 magnification)

Quantification of CK19 staining in PANC-1 cells was done using CellProfiler Software. Quantification of CK19 staining showed that isoxazole treatment had no significant effect on CK19 expression (Figure 3.25).

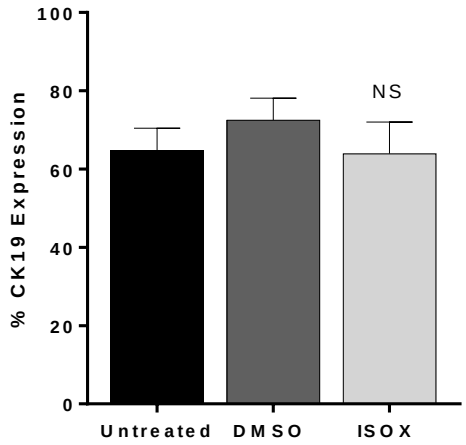


Figure 3.25 Quantification of CK19 staining in isoxazole treated PANC-1 cells. Percentage of CK19 positive cells in untreated, DMSO vehicle control and isoxazole treated PANC-1 cells following a 48hr exposure. Mean± SEM of 3 independent experiments from passages 23-35.

3.3 Discussion

3.3.1 Insulin secretion in a model β-cell line

Although the EndoC-βH1 cells have been a useful tool in the detection of insulin and insulin secretion assays they are not without their drawbacks. One such caveat is the high seeding density and slow proliferation rate which make this cell line experimentally difficult to work with. The EndoC-βH1 cells had a high insulin content of 0.65 μg ± 0.02 (mean ± SEM) per 10^6 cells, which was comparable to that reported in the literature of 0.48 μg/10^6 cells (Ravassard et al. 2011). However, the levels of GSIS observed experimentally was lower than what has been reported previously (Ravassard et al. 2011). The EndoC-βH1 cells have been previously reported to have a 3-fold increase in insulin secretion in a dose dependent manner across 0.5-20mM glucose range (Ravassard et al. 2011). Furthermore, Ravassard *et al* (2011) also reported stable GSIS at 15mM glucose from passage 25-75. However, GSIS assays performed on the EndoC-βH1 cells within this study (which were gifted to the Dunne/Cosgrove laboratory at passage 50) showed a consistent lack of glucose responsiveness with no correlation between insulin secretion and cell age between passages 55-87. The capacity of cells to secrete insulin is dependent on a multitude of factors and discrepancies can be related to batch-to-batch variation in reagents, the investigator and techniques used (Wallace & Matthews 2002). Furthermore, studies have shown that inter-laboratory variation in the measurement of insulin can vary up to 3-fold (Robbins et al. 1996). This is partly owing to assay bias; as insulin measurements quantified using ELISA vary considerably from those taken on radio-immuno assay (RIA) (Wallace & Matthews 2002). Thus there is a great need for the standardisation and use of calibration references for insulin assays across laboratories (Robbins et al. 1996).

There are many potential applications for the use of insulin secreting cell lines in research; most importantly they provide a model cell line for β-cell function in health and disease (Skelin 2010; Poitout et al. 1996). However a study comparing a range of commercially available insulin secreting cell lines (RIN, HIT, β-TC, MIN6 and INS-1) showed that some cell lines and cell line clones exhibited a loss in the functional characteristics of primary β-cells (Poitout et al. 1996). This included a complete loss or diminished glucose responsiveness, decreased insulin content, alterations in glucose sensing, and co-expression of insulin with glucagon and somatostatin (Poitout et al. 1996). Such characteristics are representative of a de-differentiating β-cell, which is another caveat of growing and culturing insulin secreting cell lines *in vitro* (Skelin 2010; Poitout et al. 1996). As a result, the EndoC-βH1

cells were subsequently used where possible as a positive control in the re-programming and differentiation of PANC-1 ductal cells towards a β-cell lineage.

Although the EndoC-βH1 cells were a useful comparative tool, the detection of insulin protein in isoxazole treated PANC-1 cells was far more challenging. It is widely reported that cells take up exogenous insulin from the cell culture medium or is released from dying cells *in-vitro* (Rajagopal et al. 2003). As the defined cell culture medium used for the differentiation protocol contained a high concentration of insulin, insulin detection in PANC-1 cells using antibody based methods was therefore problematic.

3.3.2. PANC-1 heterogeneity

MIA-PaCa-2 is another widely used human pancreatic epithelial cell line for the study of pancreatic cancer and endocrine differentiation studies (Neureiter et al. 2005). The heterogeneity of pancreatic ductal cell lines PANC-1 and MIA-PaCa-2 have been previously reported in the literature (Gradiz et al. 2016). Although PANC-1 cells appear to have three distinct cellular morphologies, by flow cytometry this has been revealed as one viable cell population indicating pleomorphism (Gradiz et al. 2016). This is where cells from the same parent can assume different morphologies at different stages of the cell cycle and can also change between these forms. In comparison, the MIA-PaCa-2 cells have been reported to have 2 distinct populations based on microscopic analysis; this has been confirmed by flow cytometry revealing two distinct viable cell populations indicating polymorphism; whereby cells can exist in multiple forms and shapes but cannot interchange between these, but rather can only assume the one form (Neureiter et al. 2005; Gradiz et al. 2016).

3.3.3. Growth and viability

For potential cell based therapy it is essential to have a chemically defined, serum free and xeno-free cell culture medium. Foetal calf/bovine serum contains a number of macromolecules including proteins, hormones and growth factors (Usta et al. 2014). Such native serum derived proteins can contaminate and interfere with experimental protocols. With the additional complication of batch variability it is therefore essential to strive towards a serum free cell culture system. A serum free and chemically defined cell culture medium was developed within the Dunne/Cosgrove laboratory (listed in Table 2.4 Materials and Methods chapter). Forty-eight hour PANC-1 proliferation curves in 10% serum and serum free cell culture medium showed that the removal of serum had the biggest effect ($p<0.01$) at all cell concentrations (Figure 3.7). However there was no

significant difference between untreated, DMSO vehicle control and isoxazole treated PANC-1 cells in serum free conditions. It has been previously reported that isoxazole significantly decreased cell proliferation in the rat insulinoma cell line INS1E without affecting cell viability, measured by lactate dehydrogenase activity (Kalwat et al. 2016). Isoxazole caused no significant reduction in cell proliferation in PANC-1 cells following 6 days treatment (Figure 3.8, Panel A). Isoxazole did however affect cell viability following 6 days of treatment when compared to the start of treatment (Figure 3.8 Panel B). A decrease in cell viability to over 70% suggests cellular toxicity of this compound over extended periods of time. Thus all experiments in this study were performed following two days of isoxazole treatment.

3.3.4 Isoxazole changes cell morphology

Isoxazole caused changes in cell morphology with cell cluster formation and 'pseudo-ductal' structures in PANC-1 cells (Figure 3.9-3.10). Isoxazole treatment caused a significant increase in the number of 'pseudo-ductal' structures formed in any $1mm^2$ area (Figure 3.11A) and a significant increase in the free area formed as a result of these structures (Figure 3.11B) when compared to the DMSO control. Cluster formation in PANC-1 cells during ductal to endocrine differentiation protocols have been previously reported. PANC-1 cells have been widely reported to trans-differentiate into 'islet-like' clusters often under serum free conditions and with additional stimuli such as glucagon-like peptide-1 (GLP-1) (Hui et al. 2001), fibroblast growth factor 2 (FGF-2) (Hardikar et al. 2003) or stem cell factor (SCF) (Wu et al. 2010). Wu *et al.* showed that culturing PANC-1 cells in serum free medium supplemented with 1% BSA, transferrin and insulin like growth factor 1 (IGF-1) for 7 days resulted in cell cluster formation. These 'islet-like' clusters displayed an endocrine phenotype, showing a significant decrease in the ductal marker CK19 and an up-regulation in the expression of insulin, glucagon, C-peptide, PDX1 and Pax6 protein (Wu et al. 2010). This transformation into clusters has been reported elsewhere, where PANC-1 cells have been reported to form 'islet-like cell aggregates' (ICA's) (Hardikar et al. 2003). Following brief trypsin exposure to detach cells from the substratum, cells were grown in serum free medium and formed 3D ICA's that resembled human islets of Langerhans and expressed the hormones insulin, glucagon and somatostatin. PANC-1 cells exposed to trypsin and grown in 10% FCS did not form ICA's (Hardikar et al. 2003).

Such cluster structures have been reported in 3D cell culture systems, whereby PANC-1 cells were grown in hydrogels and 'cyst-like cell clusters' that closely resemble the 'pseudo–

ductal' structures were observed (Raza et al. 2013). In the present study, clusters and ductal formation were rarely observed in untreated PANC-1 cells (data not shown). Although cluster formation and 'pseudo-ductal' structures were observed at a range of seeding densities, PANC-1 cells seeded at a very low seeding density (5,000cells/cm^2) for single cell mass spectrometry techniques (data not shown) did not form these structures, suggesting that extensive cell to cell contact is required.

Further analysis was done to investigate the changes in cell morphology (Figure 3.12). A comparison of cell area (μm^2) against maximum Feret's diameter (μm) in untreated, DMSO control and isoxazole treated PANC-1 cells revealed that nearly 10% of isoxazole treated cells had a unique phenotype (Figure 3.13A). Descriptive statistical analysis showed that isoxazole treatment gave the largest spread in the cell area (μm^2) (Figure 3.13B). Furthermore, as shown in Figure 3.14, the aspect ratio, eccentricity and compactness measurements show that isoxazole treatment resulted in PANC-1 cells becoming significantly less symmetrical, more elongated and less compact indicative of cell migration (Driscoll et al. 2014). It is difficult to determine the proportion of cells undergoing differentiation from gene expression data as these are arbitrary values displaying relative fold changes. It could be speculated that the sub-population of elongated/migratory cells which constituted less than 10% of isoxazole treated PANC-1 cells were possibly under-going ductal to endocrine trans-differentiation.

Dimethyl sulfoxide (DMSO) is a small amphiphilic molecule with a number of chemical properties. It has a primary role in cryopreservation and is routinely used at a final concentration of 10% (v/v) in the freezing down of cells (Notman et al. 2006). It is widely accepted that higher doses of DMSO have toxic effects on cell growth and metabolism (Yu & Quinn 1998). Although cell line dependent, studies report that a DMSO concentration of 0.1-0.5% (v/v) have little or no effect on cellular toxicity (Jamalzadeh et al. 2016). Despite the low DMSO concentration used in this study (0.03% v/v), DMSO did affect cell morphology and had a significant effect on cell area, length, and perimeter which subsequently impacted the form factor which measures the complexity of the cellular perimeter (Figure 3.14D). However, the chemical properties of DMSO allow for its direct interaction with lipids, where it readily incorporates into the lipid-water interface of cell membranes thus affecting the area and permeability of cellular membranes (de Ménorval et al. 2012; Yu & Quinn 1998; Notman et al. 2006). Given the amphiphilic nature of DMSO

and its ability to integrate into plasma membranes the affects observed on cell form in this study are therefore not surprising (Notman et al. 2006).

Collectively, this data suggests that isoxazole induced changes in cellular morphology with the formation of clusters and 'pseudo- ductal' structures. Although no migration assays were performed, the significant increase in eccentricity observed with isoxazole treatment and cluster formation is indicative of cellular migration (Driscoll et al. 2014). It has been proposed that pancreatic progenitor cells (PPCs) reside within the ductal epithelium and during pancreatic injury and β-cell loss, these PPCs undergo epithelial to mesenchymal transition and migrate from the duct into the surrounding mesenchyme to form islets of Langerhans (Bonner-Weir et al. 2000; Inada et al. 2008). To observe changes in cell morphology and possible cell migration with isoxazole treatment is interesting as it could potentially suggest that a small population of cells may be differentiating and replicating the events that would normally occur during pancreatic development.

3.3.5 Gene Expression

On a population level, isoxazole induced the expression of β-cell associated genes suggesting differentiation toward a β-cell lineage. Transcriptional regulation and therefore assessment of gene expression levels is central to directed differentiation protocols as it recapitulates normal pancreatic development in a stage-wise manner. Therefore a number of transcription factors involved in endocrine differentiation and β-cell development were measured using reverse transcription PCR and quantified using quantitative real time PCR (qRT-PCR). *PDX1* was expressed in both untreated controls and isoxazole treated PANC-1 cells and further analysis by qRT-PCR showed no fold change in the expression of *PDX1*, despite the relatively high abundance in mRNA levels (average Ct values of 20) as shown in Figure 3.15-3.16A. This is consistent with other reports of *PDX1* expression in PANC-1 cells showing an average Ct value of 28 (Yuan et al. 2013). *PDX1* expression during early embryonic pancreatic development is essential, and is later important for endocrine differentiation, however levels of *PDX1* are down regulated during ductal and acinar development (Deramaudt et al. 2006; Offield et al. 1996). It has been proposed that PANC-1 cells can assume a less differentiated status of being $PDX1^{+ve}$ in order to begin endocrine development (Bonner-Weir et al. 2000). Even a moderate and transient induction in *PDX1* expression has been shown to be sufficient to cause cellular reprogramming in PANC-1 cells (Yuan et al. 2013). Isoxazole also induced the expression of the endocrine progenitor stage transcription factor *Ngn3* following 48 hr exposure (Figure 3.15) and qRT-PCR showed a

significant nearly 150-fold induction in *Ngn3* expression with isoxazole treatment (Figure 3.16B). Levels of *Ngn3* were comparatively lower in the model β-cell line EndoC-βH1, which is characteristic of a mature human β-cell line. Levels of *PDX1* were also compared to the model β-cell line EndoC-βH1, which had considerably increased mRNA levels compared to both DMSO and isoxazole treated PANC-1 cells. *PDX1* mRNA levels were approximately 150-fold higher in the EndoC-βH1 cell line (Figure 3.16A). Statistical analysis was not carried out between isoxazole treated PANC-1 cells and the EndoC-βH1 cells as they are two different cell lines and treated under different conditions. Graphical representation of gene expression levels of both cell lines was used simply for approximation.

Isoxazole induced the expression of the endocrine associated transcription factors; *NeuroD1, Pax4, Islet 1*, which were further quantified by qRT-PCR and significantly up-regulated with isoxazole treatment when compared to the DMSO control (Figure 3.17-3.18). Other transcription factors such as *Nkx6.1, MafA* and *MafB* were constitutively expressed in PANC-1 cells (Figure 16).

NeuroD1, PDX1 and *MafA*, which were induced by isoxazole treatment or already expressed in PANC-1 cells have been shown to synergistically activate insulin gene transcription by binding to elements within the insulin promoter region and are critical for the synthesis of new insulin transcripts and aiding GSIS (Aramata et al. 2005). mRNA levels of *NeuroD1, Pax4* and *Islet1* was also quantified in the EndoC-βH1 cell line and were considerably greater compared to isoxazole treated PANC-1 cells as shown in Figure 3.18 A-C. It is noteworthy that the gene expression levels of the β-cell transcription factors *PDX1, NeuroD1, MafA, Pax6* and *Nkx6.1* were reported in the EndoC-βH1 cell line as being equivalent to that of human islets (Ravassard et al. 2011). Although not directly comparable to isoxazole treated PANC-1 cells, it does place into context the complexity of transcription factor expression and progression of differentiation towards a β-cell phenotype.

Furthermore, the mRNA expression of mature β-cell markers such as; *SLC30A8, insulin* and *SLC2A2* was also assessed. *SLC30A8* encodes for the zinc transporter ZnT8 which plays a role in transporting zinc ions into insulin containing granules which is necessary for insulin secretion (Tamaki et al. 2013). Isoxazole significantly up-regulated *SLC30A8, insulin* and *SLC2A2* (encoding for the GLUT2 glucose transporter) mRNA levels in PANC-1 cells (Figure 3.19-3.20).

Isoxazole significantly up-regulated *insulin* mRNA levels nearly 20-fold when compared to the DMSO control in PANC-1 cells (Figure 3.20B). If the levels of *insulin* mRNA were to be compared between the functional human β-cell line EndoC-βH1 and isoxazole treated PANC-1 cells, the EndoC-βH1 cells would have 1 million times (or 6 orders of magnitude) greater *insulin* mRNA levels than PANC-1 treated cells. It is interesting to note, that the EndoC-βH1 cells have been reported to contain *insulin* mRNA one order of magnitude lower than that expressed in adult human pancreatic islets (Ravassard et al. 2011). Although not directly comparable, it does put into perspective the amount of *insulin* mRNA physiologically required for functional and transplantable β-cells for cell based therapy. Therefore, a greater drive is needed to understand the molecular mechanisms involved in the signal transduction mechanisms and transcriptional activation of genes essential for pancreatic β-cell development.

3.3.6 CK19 Ductal Marker expression in PANC-1

Having established that the isoxazole derivative up-regulates key β-cell associated genes involved in pancreatic development, the aim was to investigate this further and determine whether during this process, ductal associated proteins were down-regulated. Intermediate filaments (IFs) are important for the cellular architecture of the epithelium as well as the subcellular compartmentalisation of proteins (Schüssler et al. 1992). Cytokeratins (CK) which are keratin containing IFs, are commonly used in the in the examination of pancreatic cell differentiation (Schüssler et al. 1992). In normal pancreatic tissue acinar cells express CK8 and CK18, whereas ductal cells have been shown to express CK7, CK8, CK18 and CK19 (Neureiter et al. 2005). The study of cytokeratin expression is useful especially in elucidating the origin of pancreatic cells during pancreatic injury or trans-differentiation (Bouwens 1998; Bonner-Weir et al. 2000).

CK19 expression in untreated PANC-1 cells showed a differential expression pattern, with regions of cells staining negative for the ductal cell specific marker (Figure 3.21). Quantification of CK19 staining in PANC-1 cells showed that the significant majority of cells, approximately 80% were positive for CK19, whilst nearly 20% were negative (Figure 3.22A). Wu *et al* reported similar levels of $CK19^{+ve}$ cells with approximately 70% of PANC-1 cells positive for this marker at day 0 of their differentiation protocol (Wu et al. 2010). The mean fluorescent intensity of CK19 was investigated showing varying degrees of CK19 expression across the PANC-1 population (Figure 3.22B). To elucidate if there was a correlation between CK19 expression and cell type, the mean fluorescent intensity of CK19 expression

was compared against the cell area (μm^2) in Figure 3.23. Three populations of cells (large, intermediate and small) have been reported in the literature for the PANC-1 cell population (Gradiz et al. 2016). No correlation was observed between CK19 expression and the cell area as indicated by the low R^2 value (0.25). This indicates that the expression of CK19 is homogenously expressed, which supports other findings where cytokeratin 5.6 was found to be expressed in all three PANC-1 cell morphology types (Gradiz et al. 2016). However, PANC-1 cells have been previously reported to have a heterogeneous expression of cellular makers. The expression of epithelial, mesenchymal, neuroendocrine and hormonal receptor markers were assessed in PANC-1 and MIA-PaCa-2 cells and the expression profiles varied depending on the cell morphology (Gradiz et al. 2016). Larger PANC-1 cells have been reported to have increased expression of the neuroendocrine marker chromogranin A and also the neuronal glycoprotein CD56, whilst the small morula cells have been shown to have increased expression of the mesenchymal marker vimentin (Gradiz et al. 2016).

Following on, CK19 expression was investigated in untreated, DMSO vehicle control and isoxazole treated PANC-1 cells to determine if isoxazole treatment altered the levels of CK19. Isoxazole had no effect on CK19 expression in PANC-1 cells following 48hr exposure (Figure 3.24). Quantification of CK19 staining in all the treatment groups showed no significant changes in CK19 expression (Figure 3.25). There is a discrepancy the literature with regards to CK19 expression in pancreatic tissue and cells following isoxazole treatment. In a recent study, human islets in long-term culture for 8-14 months were treated with isoxazole for 7 days. Microarray data showed that isoxazole upregulated mRNAs that encode β-cell specific proteins, essential for insulin transcription and GSIS. Furthermore, comprehensive analysis into the microarray data also showed a significant 2.69-fold increase in CK19 expression in isoxazole treated human islets (Kalwat et al. 2016). However, based on unpublished proteomics data from within the Dunne/Cosgrove laboratory 6 day isoxazole treated PANC-1 cells showed a 3.74-fold significant decrease in CK19 expression. Similarly, Wu et al. showed a significant decrease in the percentage of $CK19^{+ve}$ PANC-1 cells when grown in serum free medium for 7 days after the formation of 'islet-like' clusters (Wu et al. 2010).

However, given the heterogeneity within the PANC-1 population based on the cellular assessment and variable CK19 levels, it is difficult to draw any conclusions between cell morphology (large, intermediate stellate and small morula shaped cells) and differentiation

status. Isoxazole did not affect CK19 ductal marker expression in PANC-1 cells. Twenty percent of untreated PANC-1 cells were $CK19^{-ve}$, the identity of these non-ductal cells is interesting and yet to be determined. Despite being a ductal epithelial cell line, other phenotypes have been reported within PANC-1 cells displaying mesenchymal, neuroendocrine, and hormonal receptor markers (Gradiz et al. 2016).

In summary, results reported in this chapter demonstrate the heterogeneity of the PANC-1 cell population with three distinct cell types and differential CK19 ductal marker expression. However, given this, isoxazole treatment induced the expression of key β-cell associated genes essential for pancreatic endocrine development at an mRNA level. Isoxazole also induced the formation of islet-like clusters and 'pseudo-ductal' structures which is interesting in the context of pancreatic organogenesis and formation of the Islets of Langerhans. Assessment of the changes in cell morphology showed that isoxazole treatment induced greater cellular elongation with a reduction in symmetry and compactness which is phenotypic of a migratory cell.

With the number of factors contributing to the complexity in determining the differentiation status of PANC-1 cells post isoxazole treatment it is evident that a greater understanding and characterisation of the cells is needed. The next step was to understand cellular metabolism and processes that drive β-cell development, focussing on stage-specific cell surface markers to elucidate the population of cells potentially undergoing endocrine differentiation.

CHAPTER 4 Metabolite profiling and cell culture medium manipulation in PANC-1 cells

4.1 Introduction

4.1.1 Transcription to translation

From the numerous human and rodent studies, there is a myriad of data characterising the transcription factors, forming a rigorous gene expression profile depicting the stages of β-cell development (Slack 1995). Epigenetic regulation also contributes to pancreatic cell fate and pharmacological factors such as HAT's and HDAC inhibitors can be used to direct endocrine differentiation (Haumaitre et al. 2009). However, it is difficult to relate gene expression profiles to protein expression data as mRNA transcripts are not necessarily translated into fully functional proteins (Cenik et al. 2015; Vogel & Marcotte 2012). There are a number of stages following on from mRNA synthesis including; mRNA stabilisation, degradation, translation into protein, and post-translational modifications to consider (Vogel & Marcotte 2012). In fact a study in 2004 by Tian *et al*, investigated the steady state of proteins and mRNA expression in a multipotent mouse cell line and its differentiated progeny, identified changes in 150 key genes and corresponding proteins. The study showed that 45% of changes observed at an mRNA level showed no change at a protein level; 35% showed changes in protein but no change in mRNA with only 20% of the key genes showing a good correlation between mRNA and protein (Tian 2004). Therefore, mRNA levels cannot be equated with protein abundance (Tian 2004). Therefore to determine if PANC-1 cells in this study are becoming progressively more 'β-cell like' following isoxazole treatment, the expression profile of proteins is also required in addition to observable changes in gene expression.

4.1.2 Pancreatic cell surface markers

Classic differentiation protocols are long and complex, with a poor efficiency often yielding low amounts of mature β- cells (D'Amour et al. 2006). In order to improve the efficiency of differentiation protocols, it is essential that we identify and isolate markers for stage specific cells that are progressively becoming 'β-cell like'. Thus, there is increasing amounts of data emerging on the stage specific cell surface markers in the development of pancreatic β-cells.

One such marker that has recently been identified is sushi-domain containing-2 (SUSD2) transmembrane protein. A putative novel cell surface marker, SUSD2 has been reported to

be enriched in pancreatic endocrine progenitor cells and early endocrine cells in both human ESCs and in the developing human pancreas (H. Liu et al. 2014). SUSD2 enriched cells were placed under the kidney capsule of SCID mice and after 19 weeks immunostaining of the engraftments showed SUSD2^{+ve} cells had differentiated into all five hormone producing cell types; α-, β-, δ-, ε-, and PP cells (H. Liu et al. 2014). A comparison of 11 and 18 week human foetal pancreatic tissue showed increased SUSD2^{+ve} cells at 18 weeks and high SUSD2 expression was co-localised with Nkx2.2 (H. Liu et al. 2014). Expression levels of SUSD2 have also been shown to be altered with isoxazole treatment in a number of different models. In a transcriptomics microarray analysis, carried out on long-term cultured human islets which were treated for 7 days with isoxazole, increased mRNA levels of *SUSD2* were also reported. *SUSD2* was significantly up-regulated 2.37-fold in isoxazole treated human islets when compared to the DMSO control (Kalwat et al. 2016). In addition, gene array data on a pancreatic mesenchymal stem cell line that was derived from patient tissue from within the laboratory showed a significant increase in *SUSD2* mRNA when treated with isoxazole (unpublished data). Furthermore, based on unpublished data from within the Dunne/Cosgrove laboratory, proteomics data analysis in 6 day isoxazole treated PANC-1 cells, showed a staggering 57.2-fold increase in SUSD2 protein when compared to the DMSO control (Gsour 2015).

Other cell surface markers for mature β-cells have been recently reported. G-protein coupled receptor-50 (GPR50) and tumour-associated calcium signal transducer (TROP2) were identified as novel mature pancreatic β-cell markers (Fishman et al. 2012). A study that directed differentiation of transfected human embryonic stem cells (hESC's) showed that a population of double positive GPR50 and TROP2^{+ve} cells, expressed the mature transcription factors *insulin, Ngn3, Pax4, Nkx6.1* and *Sox9* (Fishman et al. 2012).

4.1.3 Understanding metabolomics

It is important to consider cellular processes such as survival, proliferation, apoptosis and differentiation in a more 'holistic' manner, using proteomic and metabolite profiling to look into the systems biology of a cell (Zhang et al. 2013). Metabolomics is defined as the metabolic equivalent to functional genomic activity and phenotype (Yanes et al. 2010). It can be described as the downstream product of biochemical reactions taking place within living cells, exposing the biological pathways and networks necessary for cell biology (Yanes et al. 2010; Zhang et al. 2013).

4.1.4 Role of amino acid metabolism in cell biology and endocrine differentiation

It has been long established that amino acid metabolism, under the appropriate conditions in conjunction with glucose, affects insulin secretion (Newsholme et al. 2007; Patel et al. 2016; Maechler & Wollheim 2000). A number of metabolites such glutamine, arginine and alanine have been shown to synergistically promote insulin secretion in pancreatic β-cells (Patel et al. 2016; Smith et al. 1997; Newsholme et al. 2007). Whereas arginine causes direct depolarisation of the plasma membrane, alanine and glutamine are associated with mitochondrial metabolism and the generation of TCA cycle intermediates, culminating in the increase in intracellular ATP levels, leading to closure of K_{ATP} channels and depolarisation of the cell membrane (Newsholme et al. 2007; Zhang & Li 2013). This leads to the opening of voltage-dependent Ca^{2+} channels and the influx of calcium ions ultimately resulting in the exocytosis and secretion of insulin granules (Patel et al. 2016; Komatsu et al. 2013; Zhang & Li 2013).

Until recently, very little was known about the relationship between amino acid metabolism and developmental stem cell biology (Kilberg et al. 2016). A number of recent studies have shown that amino acid metabolism is essential for self-renewal and differentiation particularly in ESC's (Kilberg et al. 2016). A recent study using metabolomics, nutrition and genetics, revealed that threonine is an essential amino acid for the maintenance of pluripotency and self-renewal in mouse ESC's (Chen & Wang 2013). Likewise human ESC's have been reported to have a high dependency on methionine, where deprivation of this amino acid caused cells to differentiation into all three germ layers, and prolonged exposure led to apoptosis (Shiraki et al. 2014). Furthermore, the non-essential amino acid L-proline has been shown to act as a signalling molecule to promote mouse ESC to mesenchymal transition. Interestingly, this was reversed with the removal of L-proline from culture conditions or by the addition of ascorbic acid (Vitamin C) (Zhang et al. 2012).

ESC's and iPSC's have been shown to have a unique metabolic phenotype regarding energy consumption, macromolecule (protein, DNA and lipid) synthesis and metabolite flux (Kilberg et al. 2016). L-leucine, a branched chain amino acid, has been reported to impair β-cell development by inhibiting $PDX1^{+ve}$ pancreatic progenitor cells from differentiating into $Ngn3^{+ve}$ endocrine progenitor cells (Rachdi et al. 2012). In this particular study, the addition L-leucine (at 10mmol/L) following 1, 3, 5 and 7 days in culture caused a significant reduction in $Ngn3^{+ve}$ and $insulin^{+ve}$ cells in rat pancreatic explants (Rachdi et al. 2012).

Furthermore, L-leucine was shown to increase intracellular levels of hypoxia inducible factor-1α (HIF-1α), a known repressor of endocrine cell fate by activation of the mTOR signalling pathway (Rachdi et al. 2012). Collectively, these studies highlight the impact of the components in the *in-vitro* cell culture environment on determining cell fate, whether to maintain a pluripotent status or induce differentiation.

4.1.5 Effect of isoxazole on metabolites

Isoxazole has recently been reported to alter metabolites in the mouse insulin-secreting cell line MIN6 (Kalwat et al. 2016). Metabolites were compared in cells treated with isoxazole in low (5mM) and high (25mM) glucose concentrations for a 48hr period. In the study over 130 metabolites were significantly altered with changes in glucose concentration having the most profound effect (Kalwat et al. 2016). Increased levels of malate and 5-aminoimidazole-4- carboxamide ribonucleotide (AICAR) were observed in the high glucose isoxazole treatment group (Kalwat et al. 2016). Also intermediates of glycolysis and the TCA cycle were significantly up-regulated in the high glucose group as expected.

4.1.6 Aims and hypothesis

The studies reviewed here have investigated the effect of amino acid metabolism on the maintenance of pluripotency in ESC's and iPSC's. One study by Scharfmann *et al,* 2012 has shown the impact of *in-vitro* amino acid supplementation on arresting pancreatic β-cell development (Rachdi et al. 2012). With the need for transplantable β-cells in the treatment of diabetes so paramount; this study attempts to elucidate amino acid metabolism involved in pancreatic endocrine differentiation using metabolomics, gene expression and *in-vitro* metabolite manipulation.

The aim for this chapter was to elicit the effect of isoxazole on metabolites in PANC-1 cells during ductal to endocrine trans-differentiation. With a metabolic profile, the aim was to manipulate the cell culture environment in order to drive *in-vitro* pancreatic endocrine differentiation further, with a hypothesis that altering metabolism can enhance endocrine differentiation.

4.2 Results

4.2.1 Isoxazole alters metabolites

In order to generate a metabolic profile of PANC-1 cells pre- and post- isoxazole treatment metabolomics was performed. Liquid chromatography (LC-) and gas chromatography (GC-) mass spectrometry (MS) was carried out by Dr Katherine Hollywood (University of Manchester) on PANC-1 cell culture medium (footprint) and lysates (fingerprint) following 48hr isoxazole treatment. Several metabolites were significantly altered by isoxazole compared to the DMSO control (Figure 4.1). Most of the amino acids listed in Figure 4.1 were glucogenic and significantly increased intracellularly. Alanine, leucine, serine and threonine were significantly increased in fingerprint samples.

Metabolite	Matrix	Fold Change	Instrument	Property
Arginine	Footprint	- 1.3	LC-MS	Glucogenic
Aspartate	Footprint	- 1.3	GC-MS	Glucogenic
Aspartate	Fingerprint	+ 1.3	GC-MS	Glucogenic
Alanine	Fingerprint	+ 1.7	GC-MS	Glucogenic
Leucine	Fingerprint	+ 1.4	GC-MS	Ketogenic
Serine	Fingerprint	+ 1.5	GC-MS	Glucogenic
Threonine	Fingerprint	+ 1.2	GC-MS	Gluco/Keto

Figure 4.1 Isoxazole alters metabolites in PANC-1 cells. Table of metabolites significantly altered by isoxazole treatment compared to DMSO control after normalisation ($p<0.05$). Analysed in cell lysates (fingerprint) samples and used cell culture medium (footprint) samples. Measured by LC- and GC- mass spectrometry[1].

[1] LC- and GC- Mass Spectrometry analysis was carried out by Dr Katherine Hollywood.

Interestingly, 2 amino acids, arginine and aspartate, were significantly decreased in the cell culture medium (Figure 4.2 A-B). Aspartate concentrations were also significantly increased inside isoxazole treated PANC-1 cells shown in Figure 4.2 C.

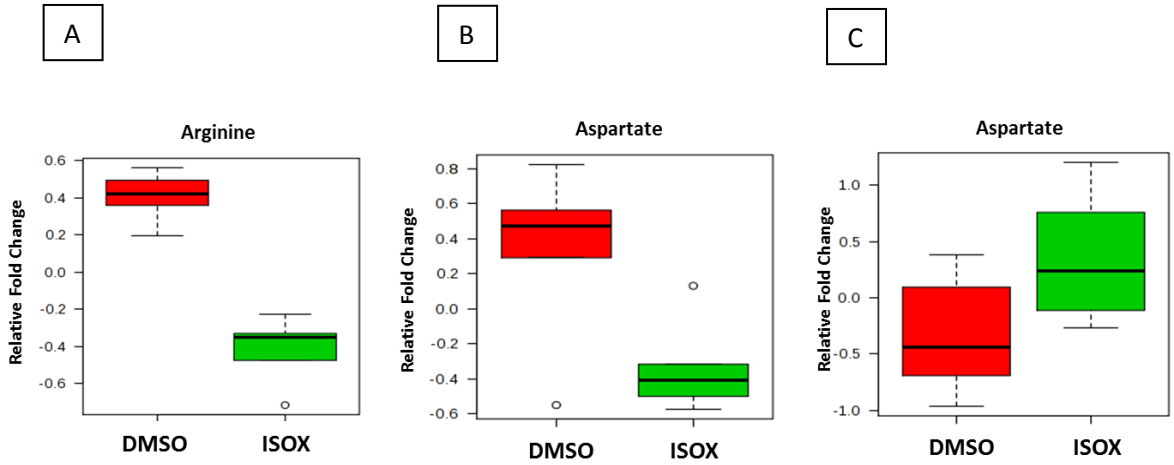


Figure 4.2 Isoxazole changes arginine and aspartate concentrations. Panel A-B. Box and whisker plots showing significant decreases in arginine and aspartate concentrations in used cell culture medium treated with isoxazole (ISOX) 48hr compared to DMSO control. Panel C. Aspartate concentrations are significantly increased inside isoxazole treated cells following 48hr isoxazole treatment.

Analysis of the metabolomics data set revealed that other metabolites were significantly altered with isoxazole treatment, such as fructose, galactose and fatty acids (Figure 4.3). Interestingly the compound called undecanedioic acid was increased nearly 40-fold in the cell culture medium exposed to isoxazole treated PANC-1 cells. C-6 sugars such as galactose were found to be significantly decreased in cell culture medium and fructose significantly decreased endogenously in isoxazole treated PANC-1 cells. Fatty acids, collectively as a group were significantly increased inside isoxazole treated PANC-1 cells.

Metabolite	Matrix	Fold Change	Instrument
C6 sugar- Galactose	Footprint	- 1.2	GC-MS
Undecanedioic Acid	Footprint	+ 39.9	LC-MS
HomoCitrate	Footprint	+ 1.4	LC-MS
Hexadecatrienoic Acid	Footprint	+ 1.4	LC-MS
Fatty Acids	Fingerprint	> 1.0	LC-MS
C6 sugar- Fructose	Fingerprint	- 1.6	GC-MS
Aminomalonic Acid	Fingerprint	- 1.4	GC-MS
Phosphoric Acid	Fingerprint	- 3.1	GC-MS

Figure 4.3 Isoxazole alters sugars and fatty acids. Table of metabolites significantly altered by isoxazole treatment compared to DMSO control after normalisation ($p<0.05$). Analysed in cell lysates (fingerprint) samples and used cell culture medium (footprint) samples. Measured by LC- and GC- mass spectrometry[2].

[2] LC- and GC- Mass Spectrometry analysis was carried out by Dr Katherine Hollywood.

4.2.2 Medium Manipulation: Arginine supplementation

4.2.2a. Effects of arginine supplementation on cell proliferation

Having generated a metabolic profile of PANC-1 cells exposed to isoxazole treatment, experiments were devised which involved supplementing the cell culture medium with 2x, 4x and 6x the basal concentration of arginine, aspartate and the aspartate precursor-asparagine to isoxazole treated PANC-1 cells for 2, 4, and 6 days. The final concentrations are listed in the Materials and Methods chapter, Table 2.2.

Initially, cell proliferation and viability with the modified cell culture medium was determined using the MTT assay. Proliferation rate over 6 days with 2x, 4x and 6x arginine concentration in DMSO vehicle control and isoxazole treated PANC-1 cells compared to basal (1x) arginine levels are shown in Figure 4.4 Panel A. Arginine supplementation alone did not affect the proliferation rate of cells after 2, 4 or 6 days (data not shown). In this present study there was no significant difference between DMSO and isoxazole-treated cells after 2 and 4 days of treatment (Figure 4.4, Panel A). However, after 6 days of isoxazole treatment cell proliferation was significantly decreased in 2x and 6x arginine supplementation, with a trend for 1x and 4x ($p<0.05$ and $p<0.001$ respectively). No significant decrease in cell viability was observed with arginine supplementation (Figure 4.4, Panel B).

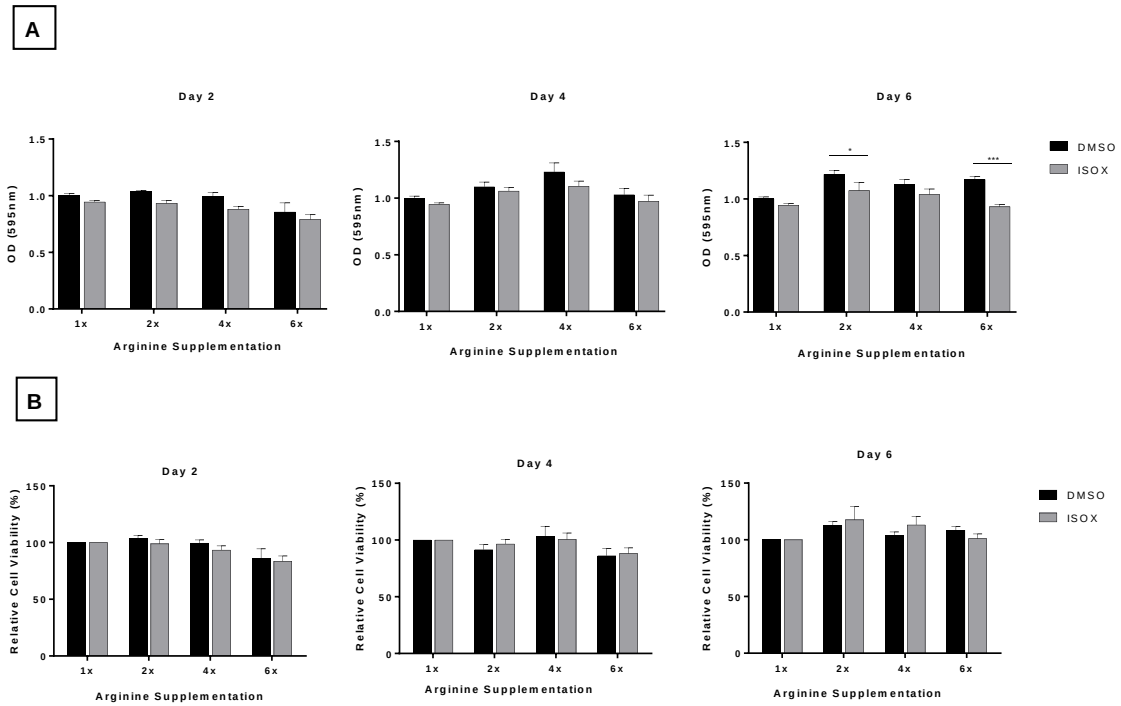


Figure 4.4 Proliferation and viability with arginine amino acid additions. Panel A. Cell proliferation of PANC-1 cells treated with 2, 4, and 6x arginine supplementation over 2, 4, and 6 days measured by MTT assay. Panel B. Relative cell viability calculated as a percentage of the control (untreated, DMSO and Isoxazole without the addition of amino acids). Mean $\pm$ SEM of 3 independent experiments. Statistical analysis using one-way ANOVA and Bonferroni's post-hoc test (*p<0.05, **p<0.01, ***p<0.001).

4.2.2b.Effect of arginine supplementation on gene expression

PANC-1 cells treated with isoxazole and relevant controls (DMSO plus arginine supplementation at 2, 4 and 6x basal concentration) for 2 days were assessed. Quantitative RT-PCR (qRT-PCR) was carried out for endocrine specific transcription factors (*PDX1, Ngn3* and *NeuroD1*) mature markers (*SLC30A8, insulin*) and the novel cell surface markers (*SUSD2, GPR50, TROP2*) as shown in Figure 4.5 Panel A-C respectively. As previously shown, isoxazole had no significant effect on the levels of *PDX1*, despite being abundantly expressed at an mRNA level (average Ct value of 20). The addition of arginine alone at increasing concentrations in the DMSO control group also elicited no effect. In comparison to the DMSO vehicle control, gene inductions in isoxazole treated groups were all significantly higher (with the exception of *PDX1*). Therefore, further statistical analysis was carried out in isoxazole treated groups, with a focus on the impact of increasing amino acid supplementation against basal conditions (1x).

Arginine supplementation did significantly up-regulate the transcription factors *Ngn3* and *NeuroD1* in dose-dependent manner, with a trend of 6x arginine supplementation to increase *Ngn3* and *NeuroD1* mRNA levels in isoxazole treated PANC-1 cells ($p<0.0001$) (Figure 4.5, Panel A). Although isoxazole significantly up-regulated *SLC30A8* levels when compared to the DMSO control, the addition of arginine in the cell culture medium did not further increase expression. There was a trend for 4x arginine supplementation to increase the expression *insulin* mRNA levels in isoxazole treated PANC-1 cells ($p<0.001$) (Figure 4.5, Panel B).

qRT-PCR data showed that isoxazole significantly up-regulated SUSD2 expression in PANC-1 cells when compared to the DMSO control. Increased arginine concentrations (at 4x and 6x basal concentrations) further increased the amount of SUSD2 mRNA (Figure 4.5, Panel C). Both *GPR50* and *TROP2* were significantly up-regulated with isoxazole treatment in PANC-1 cells following a 48 hr exposure (Figure 4.5, Panel C). Increasing arginine concentrations with isoxazole treatment caused significant increase in *GPR50* mRNA levels when compared to the basal (1x) arginine concentration. However, increasing the arginine concentration did not elicit the same effect with *TROP2* mRNA. With variable levels, only 6x arginine concentration significantly increased *TROP2* mRNA levels when compared to isoxazole treated PANC-1 cells under basal conditions ($p<0.05$) (Figure 4.5, Panel C).

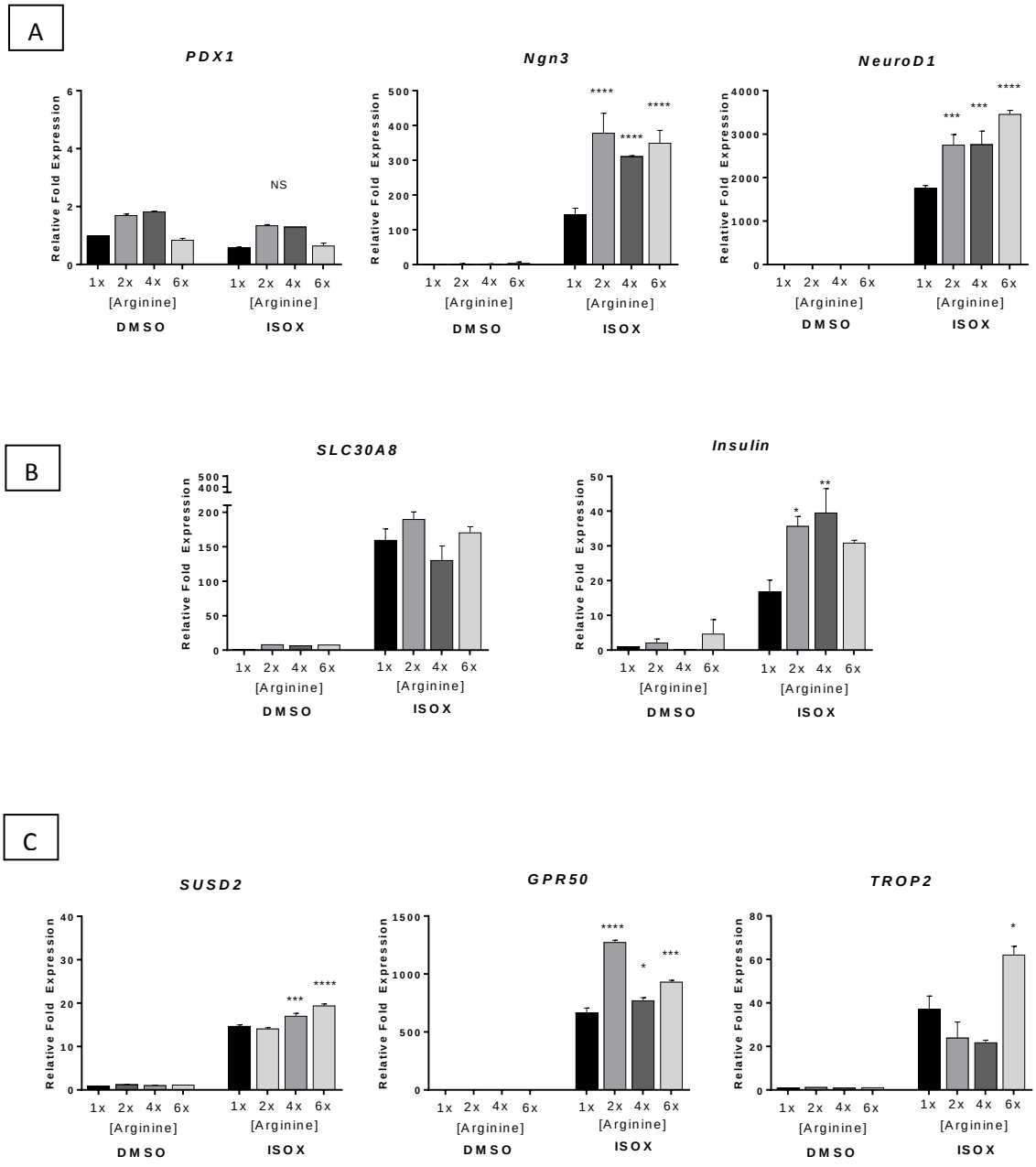


Figure 4.5 Arginine supplementation enhances β-cell associated genes in isoxazole treated PANC-1 cells. Gene expression of transcription factors PDX1, Ngn3 and NeuroD1 (A), mature markers SLC30A8 and insulin (B), and novel developmental cell surface markers SUSD2, GPR50 and TROP2 (C), measured by qRT-PCR isoxazole (48hr) compared to relevant DMSO control. Mean ± SEM of 3 independent experiments. Statistical analysis was done using one-way ANOVA and Dunnett's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared against isoxazole 1x arginine concentration).

4.2.3 Medium manipulation: Aspartate supplementation

4.2.3a Effects of aspartate supplementation on cell proliferation

Proliferation rates over 6 days with 2x, 4x and 6x aspartate concentration in untreated, DMSO vehicle control and isoxazole treated PANC-1 cells are shown in Figure 4.6 A. The proliferation rate with isoxazole and increasing aspartate concentrations were varied showing an unusual trend over 6 days. Aspartate supplementation alone did not affect the proliferation rate in PANC-1 cells (data not shown). At day 2, isoxazole significantly decreased proliferation and cell viability (Figure 4.6 B) with 6x aspartate supplementation. However, cell proliferation and viability at 6x aspartate supplementation has recovered by day 6 of treatment. At day 4 of treatment, 2x and 4x aspartate supplementation with isoxazole significantly decreased proliferation and cell viability. By day 6, isoxazole significantly reduced the proliferation rate of 4x aspartate supplementation with no effect on cell viability ($p<0.0001$). Again, cell proliferation and viability for 2x aspartate supplementation had recovered 2 days later by day 6 of treatment.

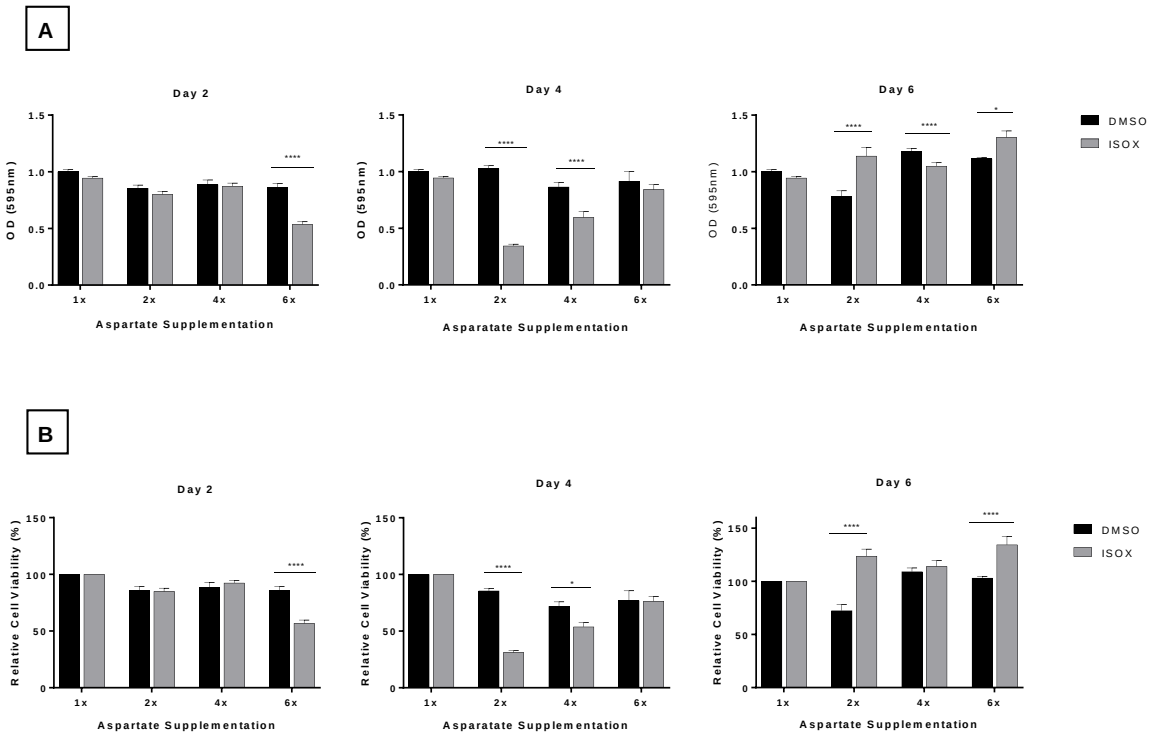


Figure 4.6 Cell proliferation and viability with isoxazole and aspartate additions. Cell proliferation of PANC-1 cells treated with 2, 4, and 6x aspartate supplementation over 2, 4, and 6 days measured by MTT assay. Relative cell viability calculated as a percentage of the control (untreated, DMSO and Isoxazole without the addition of amino acids). Mean ± SEM of 3 independent experiments. Statistical analysis using one-way ANOVA and Bonferroni's post-hoc test (*p<0.05, **p<0.01, ***p<0.001 ****p<0.0001).

4.2.3b Effect of aspartate supplementation on gene expression

qRT-PCR was carried out at day 2 of isoxazole treatment with aspartate medium supplementation. *PDX1* mRNA levels were unaffected by isoxazole and amino acid supplementation. With the exception of *PDX1*, fold changes in gene expression with isoxazole treated groups were significantly up-regulated when compared to the DMSO control; however focus was on the effect of amino acid supplementation within the isoxazole treated group. Medium manipulation experiments and statistical analysis compared the addition of amino acids with isoxazole to basal conditions (1x). Aspartate supplementation significantly increased the expression of the transcription factors *Ngn3* and *NeuroD1*, (Figure 4.7, Panel A), insulin (Figure 4.7, Panel B), and the endocrine progenitor cell surface marker *SUSD2* in a dose dependent manner (Figure 4. 7, Panel C). There was a trend for increasing aspartate concentrations with isoxazole treatment to significantly decrease mRNA levels of the mature marker *GPR50* (6x arginine supplementation compared to 1x arginine $p<0.0001$). Arginine supplementation had little effect on *TROP2* and *SLC30A8* mRNA levels in isoxazole treated PANC-1 cells (Figure 4.7, Panel C).

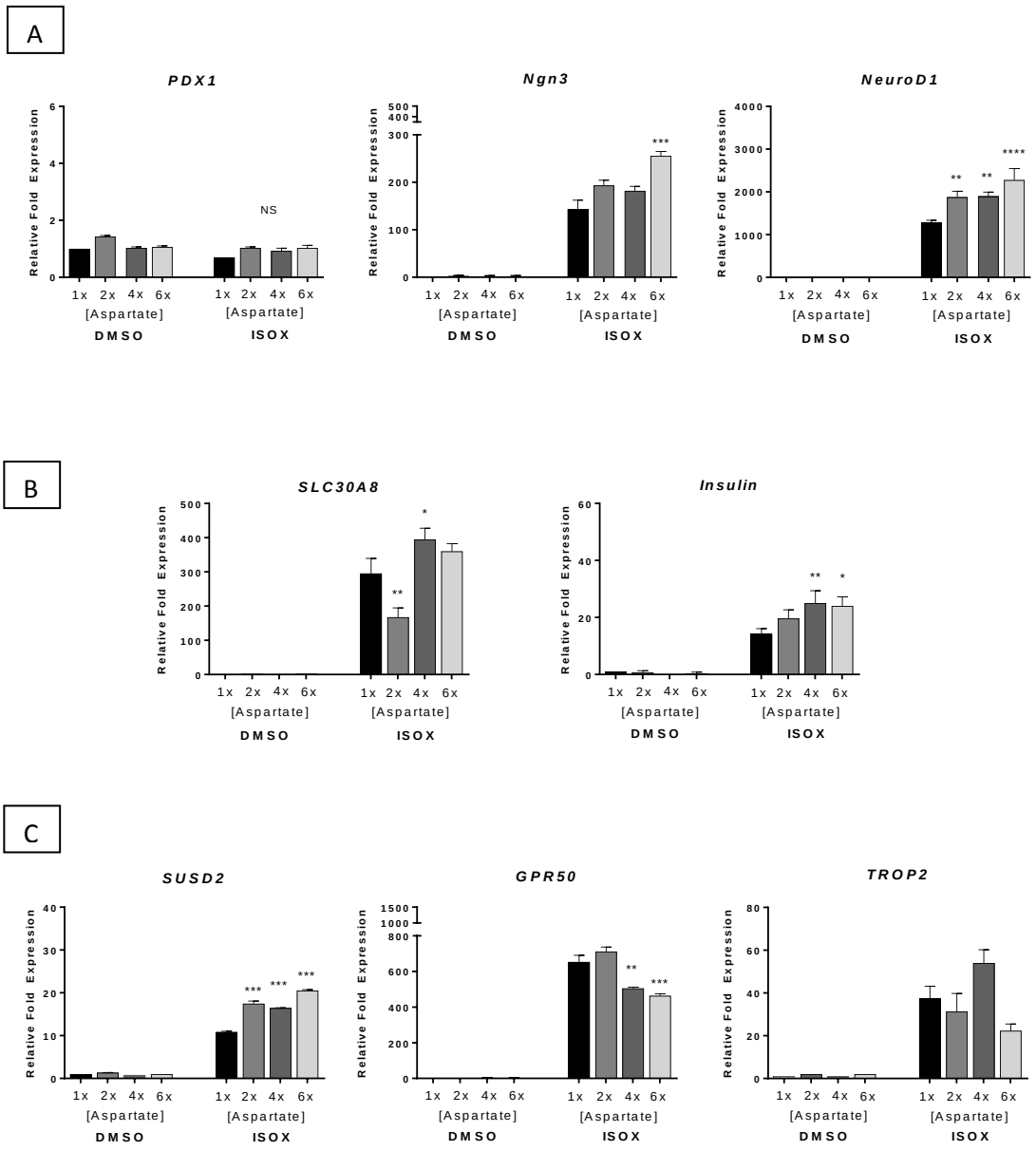


Figure 4.7 Aspartate supplementation alters gene expression and enhances β-cell associated genes in isoxazole treated PANC-1 cells. Panel A. Gene expression of transcription factors *PDX1, Ngn3* and *NeuroD1*. **Panel B**. expression of mature markers *SLC30A8* and *insulin*. **Panel C**. expression of novel developmental cell surface markers *SUSD2, GPR50* and *TROP2*, measured by qRT-PCR, normalised to 18S housekeeping gene and compared to relevant DMSO control. Mean ± SEM of 3 independent experiments. Statistical analysis was done using one-way ANOVA and Dunnett's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared against isoxazole 1x aspartate concentration).

4.2.4 Medium manipulation: Asparagine supplementation

4.2.4a. Effects of Asparagine supplementation on cell proliferation

Proliferation rates over 6 days with 2x, 4x and 6x asparagine concentration in DMSO vehicle control and isoxazole treated PANC-1 cells are shown in Figure 4.8, Panel A. At day 2, isoxazole significantly lowered the proliferation rate with asparagine supplementation (4x, 6x the basal concentration) ($p<0.0001$ and $p<0.01$, respectively). At day 6, 2x and 4x basal asparagine concentrations with isoxazole treatment significantly lowered proliferation rate compared to the controls with no effect on cell viability (Figure 4. 8, Panel B).

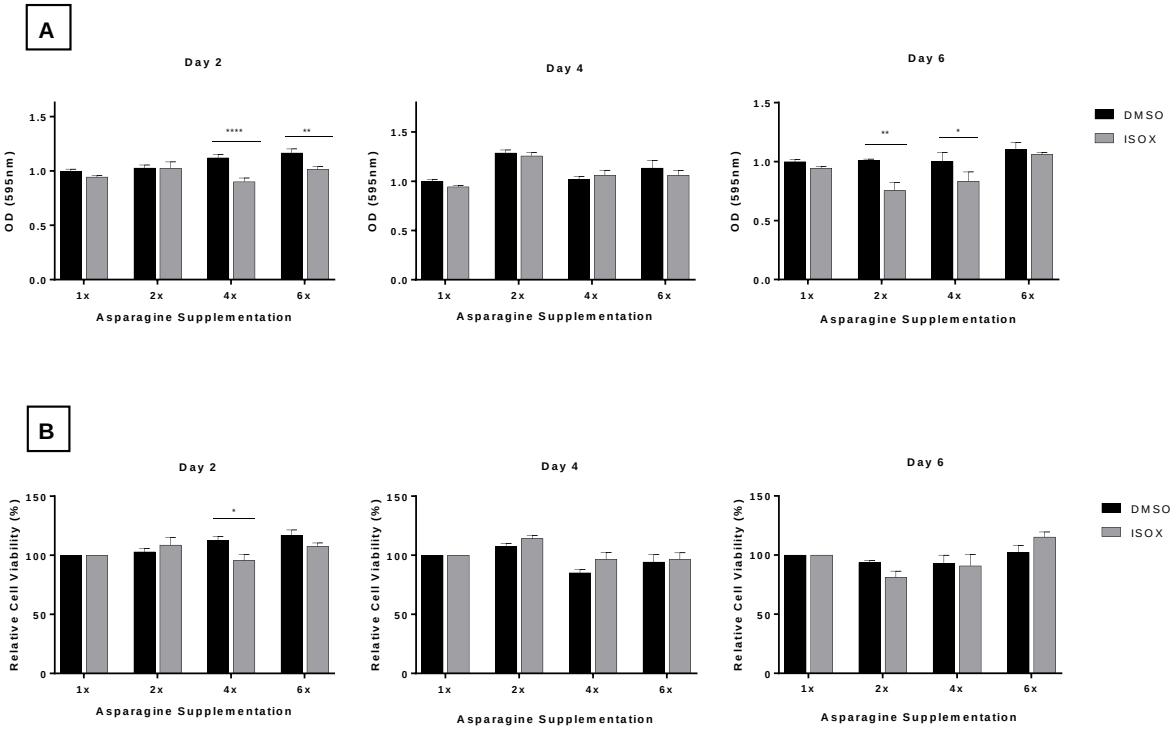


Figure 4.8 Cell proliferation and viability with isoxazole and asparagine supplementation. Cell proliferation of PANC-1 cells treated with 2, 4, and 6x asparagine supplementation over 2, 4, and 6 days measured by MTT assay. Relative cell viability calculated as a percentage of the control (untreated, DMSO and Isoxazole without the addition of amino acids). Mean ± SEM of 3 independent experiments. Statistical analysis using one-way ANOVA and Bonferroni's post-hoc test (*$p<0.05$, **$p<0.01$, ***$p<0.001$ ****$p<0.0001$).

4.2.4b Effect of Asparagine Supplementation on gene expression

Similarly to the other amino acids (arginine and aspartate), qRT-PCR showed that increasing asparagine concentrations had no effect on *PDX1* expression in isoxazole treated PANC-1 cells. With the exception of *PDX1*, fold changes in gene expression with isoxazole treated groups were significantly up-regulated when compared to the DMSO control. Medium manipulation experiments and statistical analysis shown here compare the addition of amino acid supplementation within the isoxazole treated group to basal conditions (1x).

In contrast to arginine and aspartate, the addition of asparagine attenuated the 'isoxazole effect' in PANC-1 cells often in a dose-dependent manner. Increased asparagine supplementation was associated with a significant decrease in the β-cell related genes *Ngn3, NeuroD1* (Figure 4.9, Panel A), *SCL30A8, Insulin* (Figure 4.9, Panel B), and *GPR50* (Figure 4.9, Panel C) expression. Asparagine supplementation did however increase the levels of the putative endocrine progenitor marker *SUSD2* and had little effect on the mature β-cell marker *TROP2* (Figure 4.9, Panel C).

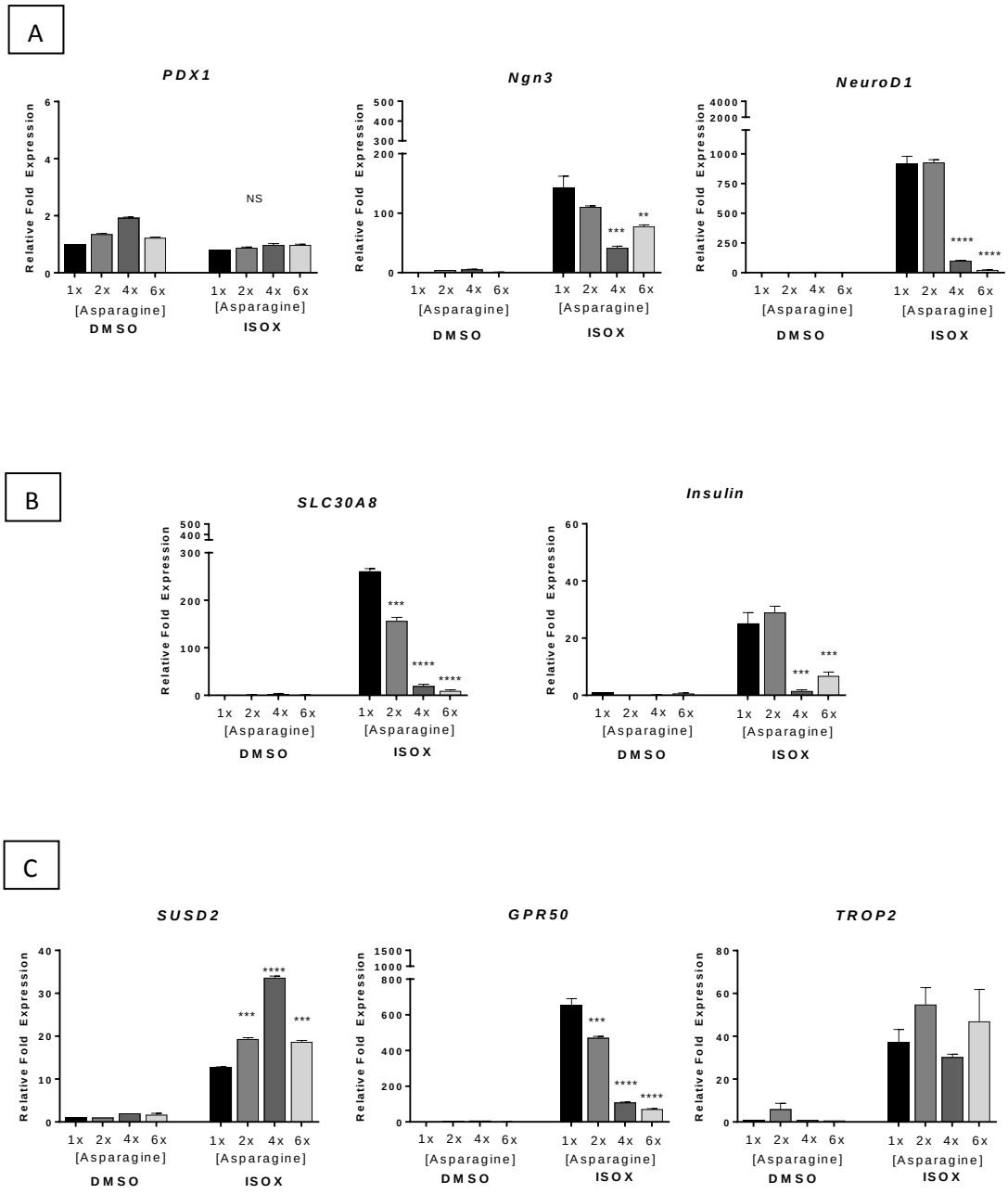


Figure 4.9 Asparagine supplementation alters gene expression in isoxazole treated PANC-1 cells. Gene expression of transcription factors *PDX1, Ngn3* and *NeuroD1*, mature markers *SLC30A8* and *insulin*, and novel developmental cell surface markers *SUSD2, GPR50* and *TROP2*, measured by qRT-PCR normalised to 18S housekeeping gene and compared to DMSO control. Mean ± SEM of 3 independent experiments. Statistical analysis was done using one-way ANOVA and Dunnett's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared against isoxazole 1x asparagine concentration).

4.2.5 Changes in protein expression following cell culture medium manipulation

4.2.5a. CK19 expression in PANC-1 cells

Having established the impact of altering metabolites in the cell culture medium on the expression β-cell associated genes, the focus shifted towards the ductal protein marker CK19. As described in Chapter 3, CK19 expression varied in untreated PANC-1 cells, however the addition of isoxazole had no effect on its expression. The aim of this present study was to investigate whether the optimised cell culture medium would down-regulate CK19 expression in isoxazole treated PANC-1 cells.

PANC-1 cells were treated for 6 days with isoxazole alone and with the addition of amino acids; arginine, aspartate and asparagine at 6x the basal conditions. Immunostaining with the pancreatic ductal marker CK19 showed generally consistent expression of CK19 across the supplemented media (Figure 4.10). Isoxazole treatment showed 'pseudo-ductal' structures which were described earlier in Chapter 3 and were also observed in isoxazole treated PANC-1 cells with the addition of arginine. The addition of aspartate and asparagine to isoxazole treated PANC-1 cells showed ductal formations albeit to a lesser degree.

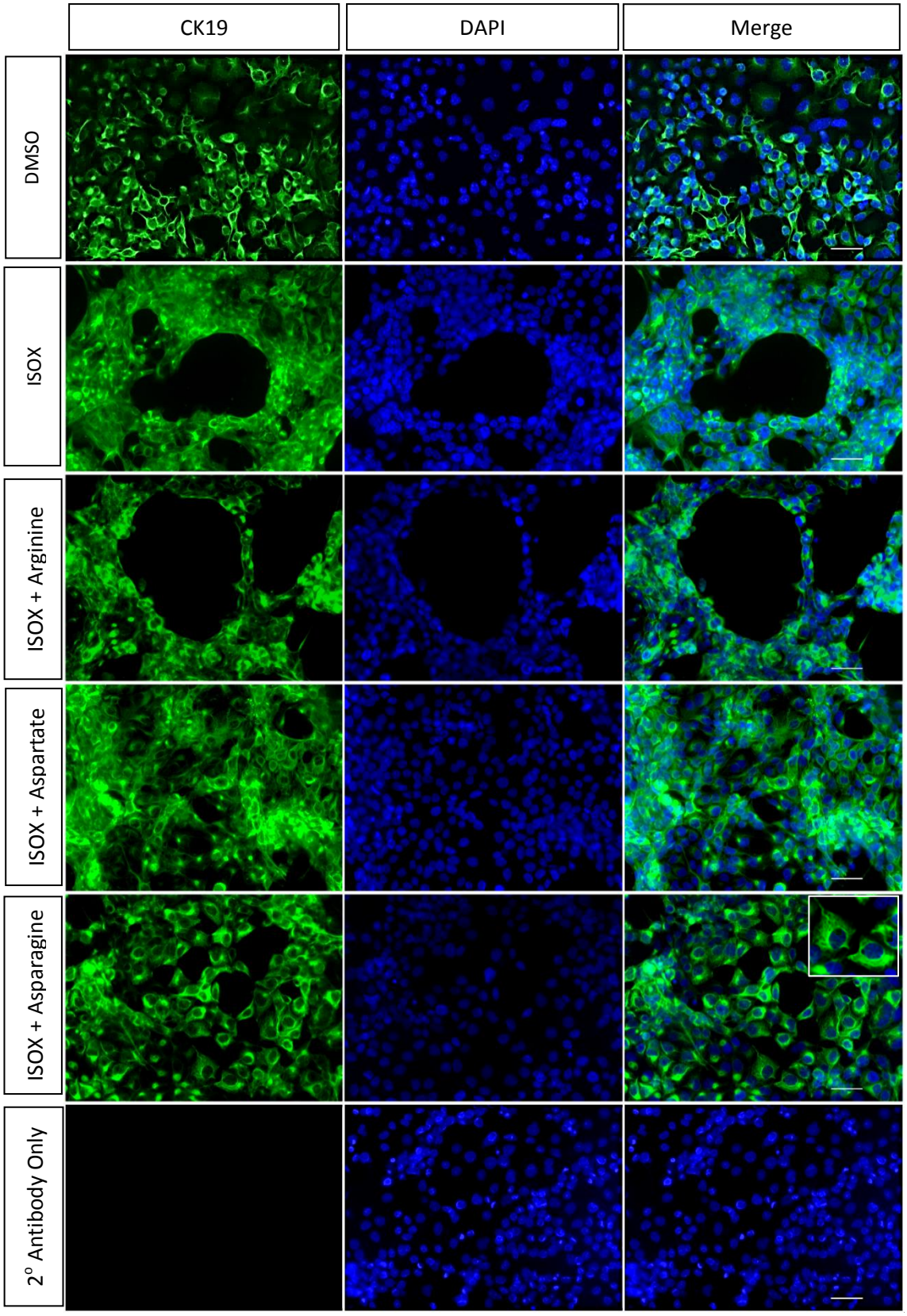


Figure 4.10 Medium manipulations alter CK19 ductal maker expression in PANC-1 cells. Immunocytochemical staining of PANC-1 cells with ductal marker CK19 (green). Treatment for 6 day with isoxazole alone and isoxazole with amino acid additions (arginine, aspartate and asparagine) 6x basal conditions. Secondary antibody only control. Scale bar= 50µm. Data representative of 3 independent experiments between passage 23-35.

The levels of the pancreatic ductal marker CK19 in the modified cell culture medium was quantified using the mean fluorescent intensity (MFI) as shown in Figure 4.11. The number of $CK19^{+ve}$ cells significantly increased with isoxazole/arginine supplementation compared to isoxazole treatment alone. Whilst asparagine supplementation significantly decreased the number $CK19^{+ve}$ cells compared to isoxazole treatment alone.

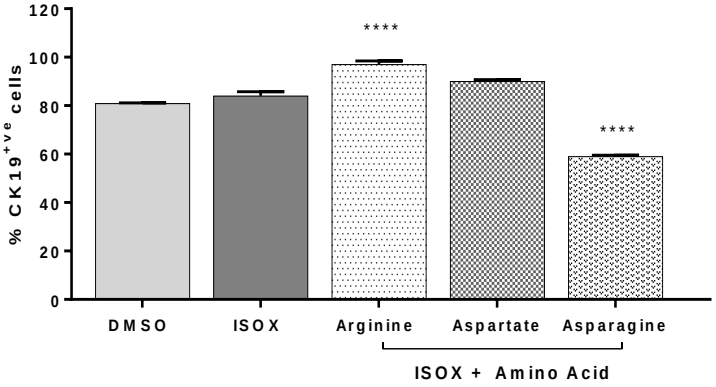


Figure 4.11 Isoxazole and medium manipulation alter percentage $CK19^{+ve}$ cells. Mean fluorescent intensity (MFI) of CK19 staining in isoxazole treated PANC-1 cells and medium supplementation with arginine, aspartate and asparagine, compared to DMSO vehicle control. Six day treatment. Mean $\pm$ SEM of 3 independent experiments. Statistical analysis was done using one-way AVOVA comparing isoxazole treatment to arginine, aspartate and asparagine (****$p<0.0001$). ISOX= isoxazole.

4.2.5b. SUSD2 expression in PANC-1 cells

SUSD2 is a putative and novel pancreatic endocrine progenitor marker (H. Liu et al. 2014). Following on from CK19 detection and quantification with the optimised cell culture medium, the objective was to firstly investigate whether isoxazole treatment in PANC-1 cells would induce the expression of SUSD2 protein. And secondly to determine whether manipulating the cell culture medium with metabolites actively being utilised by isoxazole treated PANC-1 cells, would enhance SUSD2 protein levels. As previously discussed, qRT-PCR showed that the addition of arginine, aspartate and asparagine to the cell culture medium significantly up-regulated the mRNA levels of SUSD2 in isoxazole treated PANC-1 cells.

Immunostaining with the putative endocrine progenitor cell surface marker SUSD2 in isoxazole treated PANC-1 cells showed a clear up regulation when compared to the DMSO control (Figure 4.12). SUSD2 expression was measured in the modified medium containing amino acid supplementation (6x the basal levels) following a 6 day treatment and showed variable expression levels in the PANC-1 population. A minority of cells in all the isoxazole/amino acid treatment groups exhibited a unique cell morphology; appearing more mesenchymal with an example highlighted in isoxazole/asparagine treated PANC-1 cells (Figure 4.12).

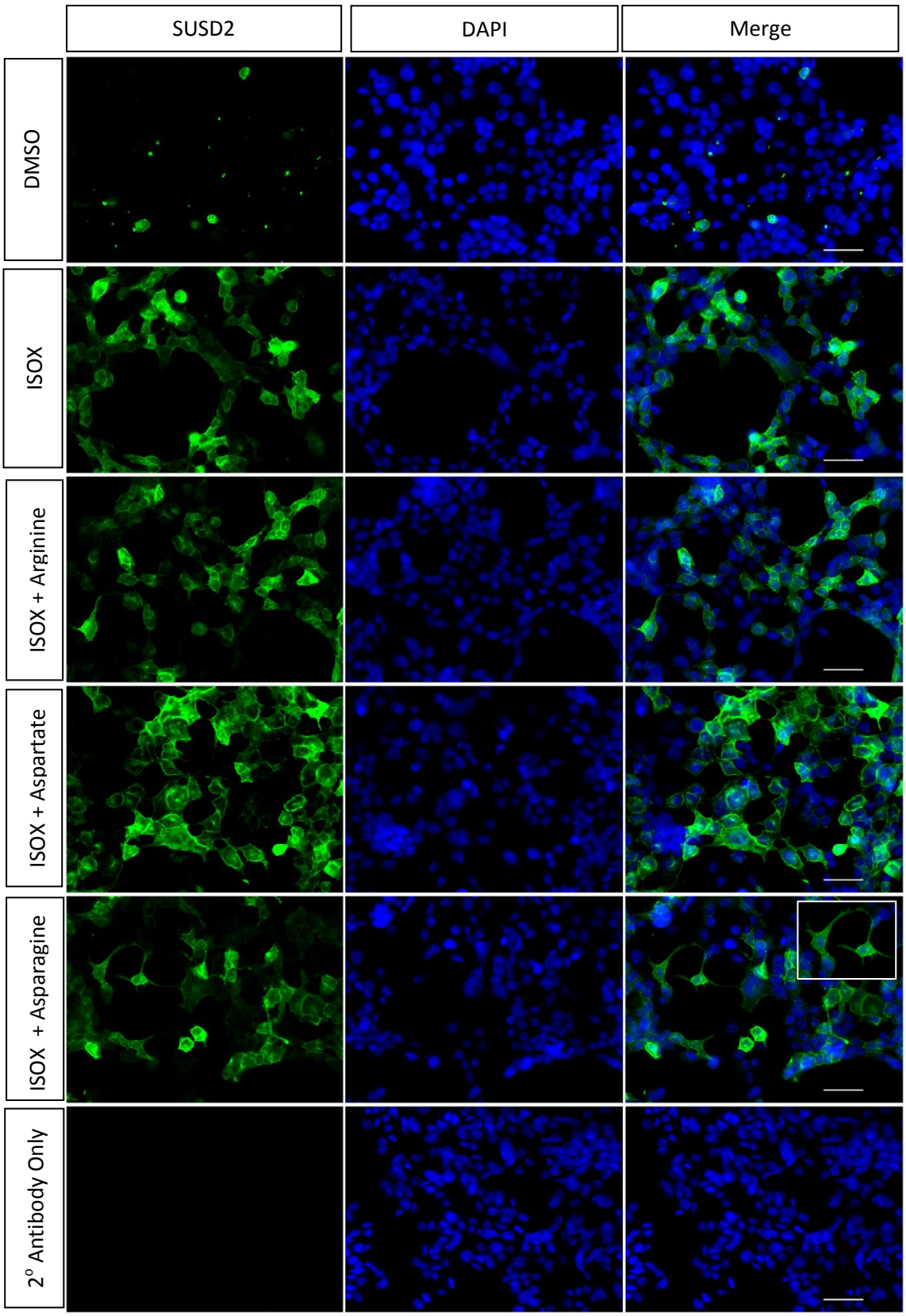


Figure 4.12 Isoxazole treatment up-regulates SUSD2 expression. Immunocytochemical staining of PANC-1 cells with the putative and novel endocrine progenitor marker SUSD2 (green). Treatment for 6 days with isoxazole (ISOX) alone and isoxazole with amino acid additions (arginine, aspartate and asparagine) 6x basal conditions. Scale bar= 50µm x20 magnification. Data typical of 3 independent experiments from passage 23-35.

Quantification of SUSD2 expression in PANC-1 cells treated with isoxazole and amino acid supplementations was measured using the MFI. Isoxazole significantly up-regulated the percentage of $SUSD2^{+VE}$ cells in the PANC-1 population when compared to the DMSO control (Figure 4.13). The addition of arginine, aspartate and asparagine also significantly up-regulated the expression of SUSD2 in comparison to the DMSO controls. However, the addition of amino acids did not significantly affect SUSD2 expression from basal isoxazole levels.

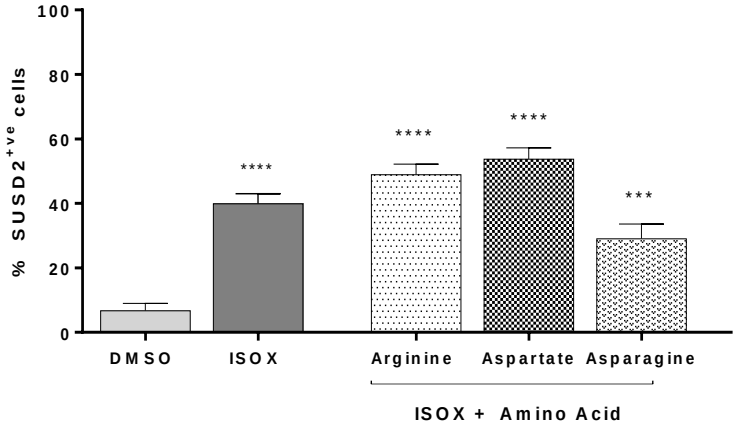


Figure 4.13. Isoxazole increases the percentage of $SUSD2^{+ve}$ cells in PANC-1 cells. Percentage $SUSD2^{+ve}$ calculated from mean fluorescent intensity (MFI) of staining using CellProfiler Software. Mean ± SEM of 3 independent experiments. Statistical analysis was done using one-way ANOVA (***P<0.001, **** P<0.0001 against DMSO control). No significant difference between isoxazole and isoxazole/amino acid supplementation.

Analysis of both CK19 and SUSD2 data showed varying levels of CK19 positive PANC-1 cells and an up-regulation in the expression of SUSD2 protein in isoxazole treated PANC-1 cells. To determine whether the cells highly positive for SUSD2 were CK19 negative and vice versa, led to dual- immunostaining of PANC-1 cells with the pancreatic ductal marker CK19 and cell surface marker SUSD2. PANC-1 cells were treated with isoxazole and isoxazole in the modified medium for 6 days and stained accordingly as shown in Figure 4.14. Levels of SUSD2 expression were variable across the different treatment groups. Interestingly, by observation it was apparent that cells highly positive for SUSD2 co-stained weakly positive for CK19.

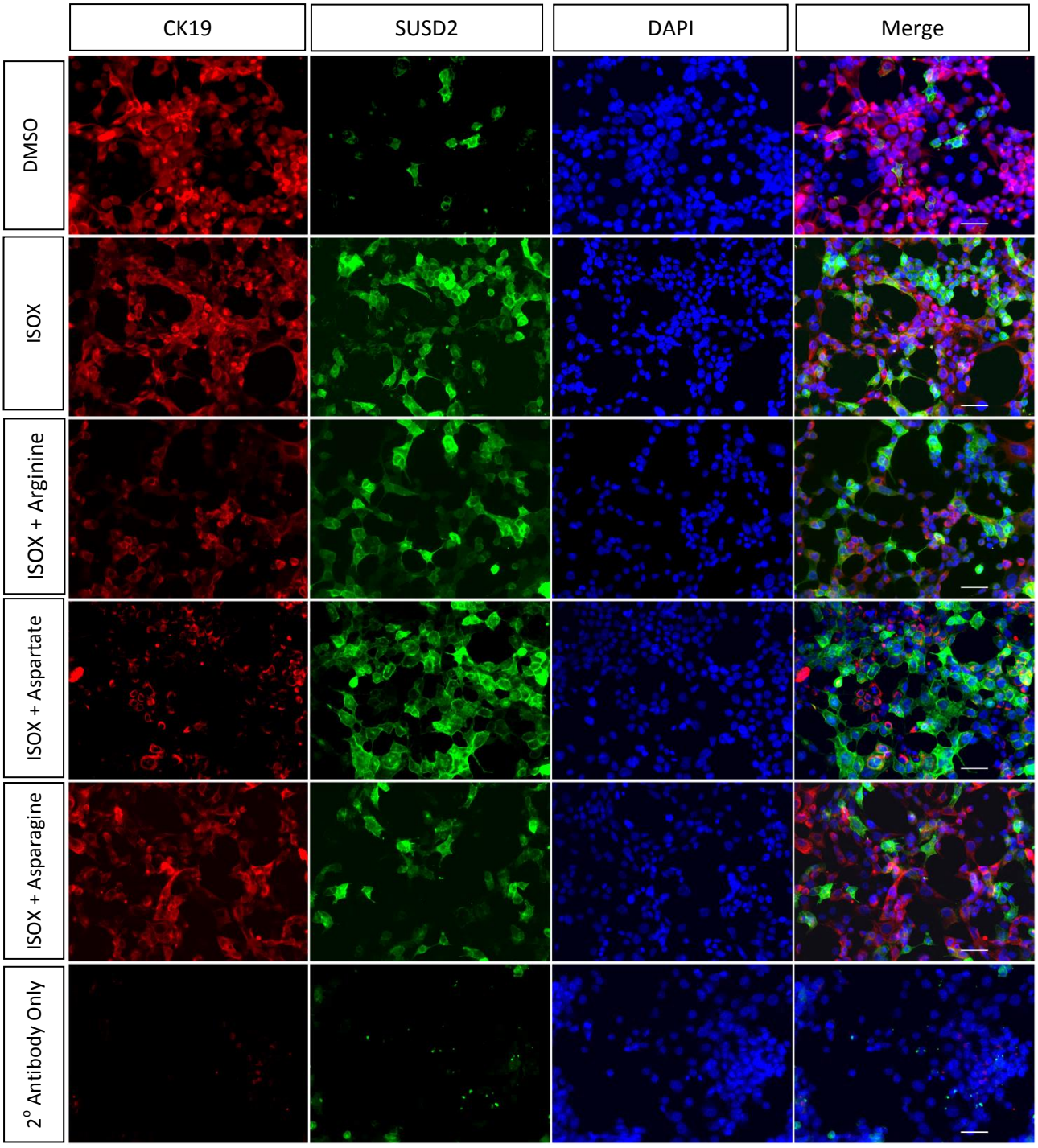


Figure 4.14 Amino acid supplementation with isoxazole alters CK19 and SUSD2 expression. Dual immunocytochemical staining of PANC-1 cells with CK19 (red) and SUSD2 (green). Treatment for 6 day with isoxazole (ISOX) alone and isoxazole with amino acid additions (arginine, aspartate and asparagine) 6x basal conditions. Scale bar= 50µm x20 magnification. Data representative of 3 independent experiments between passage 23-35.

Upon further analysis, dual immunostaining revealed 4 populations of cells; $CK19^{+ve}$ cells, $SUSD2^{+ve}$ cells, $SUSD2/CK19^{+ve}$ co-expressing cells and cells negative for both markers (indicated by arrows in Figure 4.15). Co-stained PANC-1 cells ($SUSD2/CK19^{+ve}$) were less frequently observed.

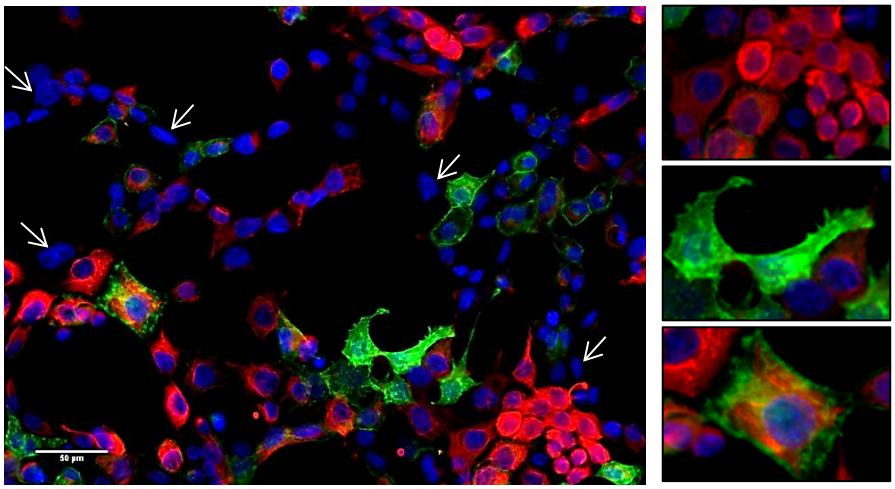


Figure 4.15 Dual immunocytochemical staining of CK19 and SUSD2 reveal 4 populations of cells. Isoxazole treated PANC-1 cells stained with CK19 (red) and SUSD2 (green) following 6 day treatment. Regions selected showing cells $CK19^{+ve}$ cells (top panel), $SUSD2^{+ve}$ cells (middle panel) and $CK19^{+ve}/SUSD2^{+ve}$ co-expressing cells (lower panel). Arrows indicating $SUSD2^{-ve}/CK19^{-ve}$ cells. Scale bar= 50µm. Data representative of 3 independent experiments between passage 23-35.

4.2.5c. SUSD2 expression and cell morphology

Isoxazole treatment has been previously shown to cause changes in cell morphology. Morphometric analysis revealed that isoxazole treatment caused PANC-1 cells to become distinctively more elongated and less symmetrical (discussed in Chapter 3). From microscopic observation of immunocytochemical staining, it seemed that cells highly positive for SUSD2 appeared more elongated. Therefore, to investigate the relationship between SUSD2 expression and cell morphology, the mean fluorescent intensity (MFI) of $SUSD2^{+ve}$ cells was compared against the maximum Feret's diameter (μm) shown in Figure 4.16. There was a moderately positive correlation, showing that cells highly positive for SUSD2 marker expression also had the longest cell length.

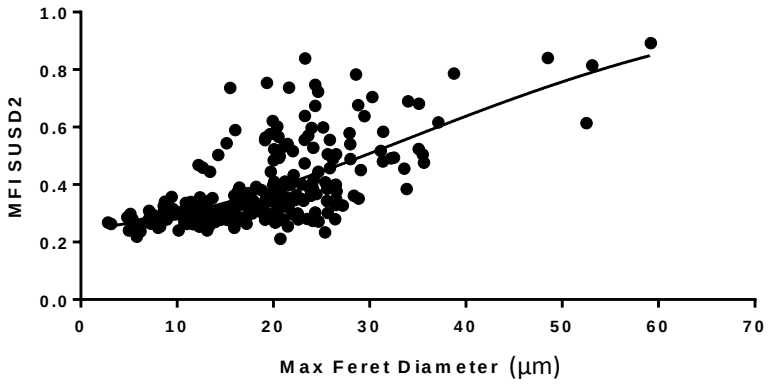


Figure 4.16 Assessment of cell morphology and SUSD2 expression in isoxazole treated PANC-1 cells. Mean fluorescent intensity (MFI) of $SUSD2^{+ve}$ cells compared against maximum Feret's diameter (μm) shows a moderately positive correlation (R^2=0.49 Pearson correlation test).

4.3 Discussion

4.3.1 Isoxazole alters metabolites

Isoxazole treatment in PANC-1 cells altered metabolites endogenously and also in the cell culture medium; selectively affecting amino acids that were involved in the TCA cycle. Depending on the metabolic cell fate, amino acids are defined as being glucogenic or ketogenic (Berg et al. 2002a). Glucogenic amino acids are broken down into pyruvate or one of the intermediates of the TCA cycle; α-ketoglutarate, succinyl CoA, fumarate or oxaloacetate as show in Figure 4.3.1. (Berg JM, Tymoczko JL 2002). These can be oxidised into carbon dioxide and water to generate ATP via the TCA cycle and oxidative phosphorylation (Berg et al. 2002b). Furthermore, the carbon skeleton can be used to synthesise glucose via gluconeogenesis. In contrast, ketogenic amino acids are broken down into acetyl CoA or acetoacetate, the precursors to fatty acids and ketone bodies and cause no net change in glucose production (Berg et al. 2002b). Most of the amino acids significantly altered by isoxazole treatment in PANC-1 cells were glucogenic (results Figure 4.1) such as alanine, serine, threonine, arginine and aspartate. Leucine was the only ketogenic amino acid significantly altered by isoxazole treatment.

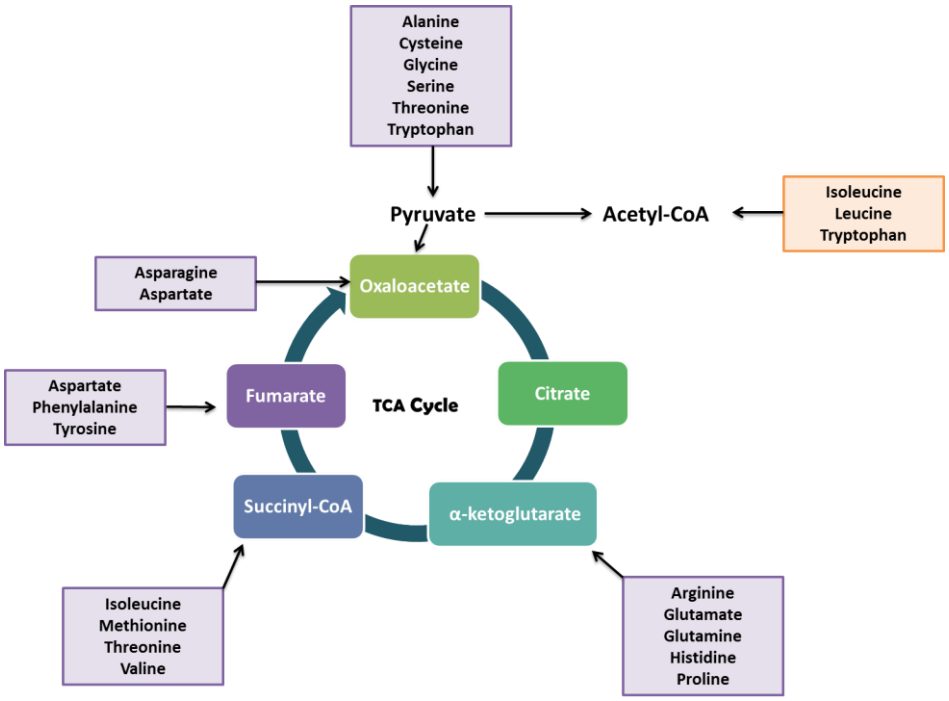


Figure 4.3.1 Amino acids involved in the TCA cycle. Amino acids are classified into glucogenic and ketogenic amino acids depending on cell fate. Glucogenic amino acids (purple) are metabolically converted into pyruvate, α-ketoglutarate, succinyl-CoA, fumarate, oxaloacetate. Ketogenic amino acids (yellow) are converted into acetyl-CoA (Adapted from Berg et al. 2002).

4.3.2 Changes in arginine and aspartate levels

Interestingly, LC- and GC- MS data showed that 2 amino acids; arginine and aspartate, were significantly decreased 1.3-fold in the cell culture medium (results Figure 4.2A-B) suggesting cells were actively taking up and using these particular amino acids during the differentiation protocol.

Based on unpublished proteomics data from within the laboratory, isoxazole treated PANC-1 cells were selectively up-regulating enzymes involved in the biosynthesis and metabolism of arginine, aspartate and asparagine.

4.3.3 Biosynthesis and metabolism of arginine, aspartate and asparagine

Arginine is synthesised from citrulline and ornithine in the urea cycle as shown in Figure 4.3.2 Pancreatic cancers are described as being auxotrophic for arginine, which is defined as the inability to synthesise nutrients essential for growth and metabolism (Lodish et al. 2000). Most cancers, including PANC-1 cells, express very low levels of the enzyme argininosuccinate synthetase 1 (ASS1) (Delage et al. 2010). ASS1 catalyses the reaction between citrulline to argininosuccinate in the urea cycle (shown in Figure 4.3.2). This is a rate limiting step in the biosynthesis of arginine, resulting in the intrinsic dependence of PANC-1 cells on exogenous arginine (Delage et al. 2010). A study found that arginine deprivation with the use of arginine deiminase (ADI) which degrades arginine into citrulline resulted in inhibition of tumour growth (J. Liu et al. 2014). Based on unpublished proteomics data from within the laboratory, the enzyme ASS1 was found to be significantly up-regulated 3.6-fold in 6 day isoxazole treated PANC-1 cells compared to DMSO control (Gsour 2015).

Aspartate can be synthesised by 2 routes; firstly transamination from glutamate and oxaloacetate to form aspartate and α-ketoglutarate by the enzyme aspartate amino transferase (AST); secondly, derivation from asparagine with the enzyme L-asparaginase as shown in Figure 4.3.2 (Berg et al. 2002b). The reverse of this reaction (aspartate to asparagine) is catalysed by the enzyme asparagine synthetase (ASNS) which interestingly was also significantly up-regulated by 2.9-fold in 6 day isoxazole treated PANC-1 cells (based on unpublished proteomics data analysis). Fifty-two percent of pancreatic adenocarcinoma's express very low or no ASNS enzyme and studies show that treatment of patients with the enzyme L-asparaginase resulted in inhibition of tumour growth, via depletion of plasma asparagine (Boroughs & DeBerardinis 2015; Dufour et al. 2012). Similar

studies in PANC-1 and MIA PaCa-2 cells have shown that treatment with L-asparaginase resulted in inhibition of protein synthesis and cell growth (Milman, Cooney, et al. 1979).

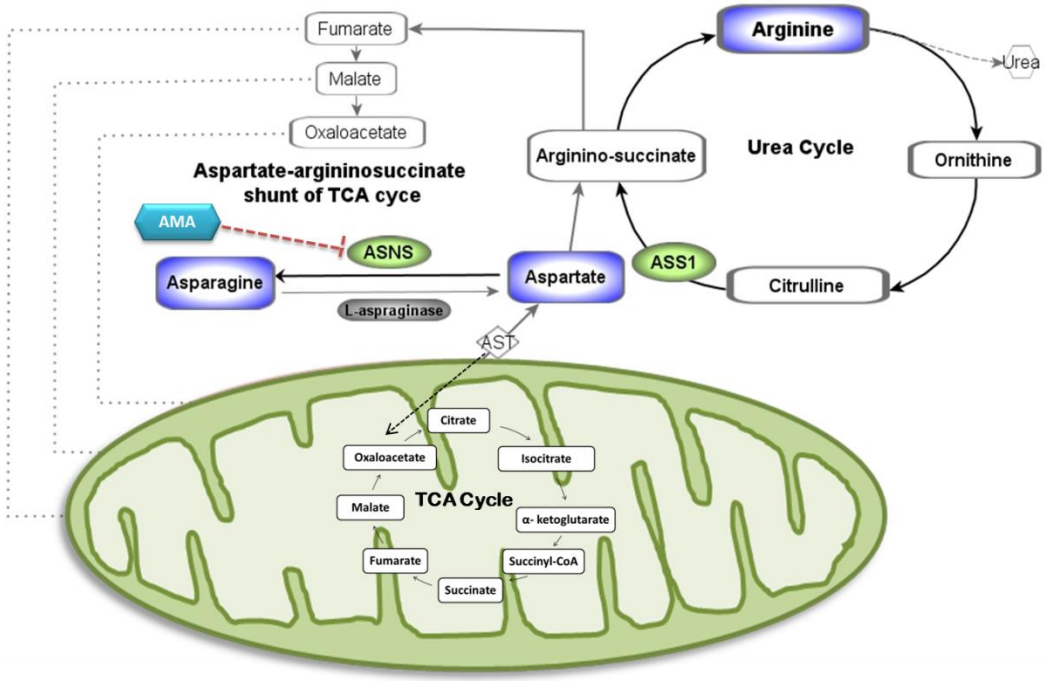


Figure 4.3.2 Metabolism and biosynthetic pathways for asparagine, aspartate and arginine. Metabolites and enzymes altered by isoxazole treatment in PANC-1 cells assessed by proteomics and metabolomics. Pathways highlight metabolism and catabolism of asparagine, aspartate and arginine in the urea cycle and tricarboxylic acid (TCA) cycle. ASNS=asparagine synthetase, ASS1= argininosuccinate synthetase 1, AST= aspartate amino transferase, AMA= aminomalonic acid (Adapted from Berg et al. 2002).

Aspartate metabolism and catabolism can occur from a number of different routes as shown in Figure 4.3.2. It can form argininosuccinate, which can either be used in the synthesis of arginine, or form fumarate and other intermediates of the TCA cycle. These intermediates can replenish and maintain the TCA cycle by the 'aspartate-argininosuccinate shunt' (Berg et al. 2002b). Aspartate can also be transaminated to form asparagine, and can also form oxaloacetate to 'feed' directly back into the TCA cycle.

4.3.4 Isoxazole and glycolysis

Collectively from metabolomics and proteomics data sets, glycolytic intermediates and enzymes were generally unaltered. Pancreatic ductal adenocarcinoma (PDAC) cell lines such as PANC-1 have a distinct metabolic phenotype with an enhanced glycolytic rate (described as the Warburg effect), increased glucose consumption and over-expression of glycolytic enzymes (Finley et al. 2013). In addition, cells display increased pyruvate to lactate conversion, reduced mitochondrial activity and increased cell proliferation and tumour invasiveness (Kennedy & Dewhirst 2010). In highly proliferative cancer cells it is believed that cells take up large amounts of nutrients such as glucose, whilst the intermediates of glycolysis and the TCA cycle are 'shuttled off' to produce the building blocks for nucleic acids, amino acids and fatty acids (Perera & Bardeesy 2015). Exogenous glutamine from extracellular sources is taken up and converted to glutamate and then to α-ketoglutarate which maintains the TCA cycle (Finley et al. 2013). The growth of PANC-1 and MIA PaCa-2 cells has been shown to be absolutely dependent on L-glutamine (Finley et al. 2013; Boroughs & DeBerardinis 2015).

Glucose, fructose and galactose are simple sugars and structural isomers that share the same molecular formula ($C_6H_{12}O_6$) (Gropper Sareen, Smith Jack 2009). As shown in Figure 4.3, glucose concentrations were unaltered by isoxazole treatment; fructose however was significantly decreased 1.6-fold inside isoxazole-treated PANC-1 cells, whilst galactose was found significantly decreased 1.2-fold in the cell culture medium. This suggests isoxazole treated PANC-1 cells were actively taking up and utilising C-6 sugars. Significant accumulation of lactate was also not observed intracellularly, which suggests that isoxazole did not affect glycolysis despite the highly proliferative and glycolytic nature of PANC-1 cells. Isoxazole did significantly up-regulate the gene *SLC2A2* encoding for the GLUT2 glucose transporter (discussed in Chapter 3). However, no change was observed in the glucose transporter GLUT1 and Hexokinase II in PANC-1 cells treated with isoxazole although were abundantly expressed at an mRNA level, as expected (n=1, data not shown). HK II and GLUT1 are overexpressed glycolytic enzymes in PDAC (Perera & Bardeesy 2015). The glycolytic enzyme glucokinase was not detected in PANC-1 cells (n=1, data not shown) consistent with what has been reported in the literature (Cohen et al. 2015; Perera & Bardeesy 2015).

4.3.5 Lipid metabolism and isoxazole

Collectively, fatty acids were significantly increased inside isoxazole treated PANC-1 cells when compared to the DMSO control (Figure 4.3). Free fatty acids (FFA's) are important for healthy pancreatic β-cell function and studies have shown that deprivation of FFA's leads to reduced GSIS (Nolan et al. 2006).

Undecanedioic acid, a medium chain fatty acid, was significantly increased nearly 40-fold in cell culture medium exposed to isoxazole, the highest fold change of all the metabolites (Figure 4.3). Given that both isoxazole ($C_{11}H_{10}N_2O_2S$) and undecanedioic acid ($C_{11}H_{20}O_4$) are both 11-carbon molecules, it was speculated that undecanedioic acid could be a by-product of isoxazole metabolism. However given the unique heterocyclic structure of the isoxazole compound this is unlikely and the role of undecanedioic acid in metabolism or differentiation is still unknown.

Hexadecatrienoic acid (HTA) was significantly increased 1.4-fold in the cell culture medium (Figure 4.3). HTA is an omega-3 polyunsaturated fatty acid (PUFA). PUFA's such as omega-3 and omega-6 play an important role in cell membrane integrity, growth and development (Nolan et al. 2006).

4.3.6 Other metabolites altered by isoxazole

Furthermore, aminomalonic acid (AMA) was significantly decreased 1.4-fold inside isoxazole treated PANC-1 cells (Figure 4.3). AMA is involved in protein synthesis and amino acid biosynthesis. Furthermore, AMA is a strong inhibitor of the enzyme ASNS (Milman, Muth, et al. 1979), which catalyses the conversion of aspartate to asparagine and this enzyme was found to be significantly up-regulated with isoxazole treatment (as previously discussed). It is interesting that metabolomics data shows that an inhibitor of ASNS was significantly decreased with isoxazole treatment; further supporting the concept of a bottleneck effect and increased aspartate catabolism.

Phosphoric acid (H_3PO_4) was significantly decreased 3.1-fold intracellularly following isoxazole treatment. Phosphoric acid is an inorganic acid which can dissociate to form phosphate ions. With a a wide range of cellular functions, it also provides a phosphorous source for DNA synthesis (Boer et al. 2010).

The metabolite AICAR was not found to be altered by isoxazole treatment in PANC-1 cells. AICAR an adenosine monophosphate (AMP) analogue involved in *de novo* purine synthesis

and the generation of nucleotides was found in both high and low glucose conditions to be significantly down regulated with isoxazole treatment when compared to the DMSO control in MIN6 cells (Kalwat et al. 2016). However this metabolite was not detected in the LC- and GC- MS analysis carried out in PANC-1 cells.

4.3.7 Impact of medium optimisation on cell proliferation

Having generated a metabolic profile of PANC-1 cells pre- and post- isoxazole treatment, this study aimed to manipulate the *in-vitro* cell culture environment in order to enhance trans-differentiation in PANC-1 ductal cells.

The study first determined the effect of amino acid supplementation on cell proliferation and viability in isoxazole treated PANC-1 cells. Amino acid supplementation on PANC-1 cells alone did not affect cell growth. Arginine supplementation with isoxazole treatment had no effect on cell proliferation and viability during the first 4 days of treatment. However, isoxazole treated PANC-1 cells supplemented with arginine following 6 days of treatment did significantly lower the proliferation rate with no effect on cell viability (Figure 4.4 A-B). It is believed that cellular processes such as proliferation and differentiation are mutually exclusive and isoxazole has been previously reported to decrease cell proliferation whilst inducing endocrine differentiation (Kalwat et al. 2016).

Asparagine supplementation on isoxazole treated PANC-1 cells also decreased the cell proliferation rate at day 2 and day 6 of treatment with little or no effect on cell viability (Figure 4.8). PANC-1 cells are described as being auxotrophic for arginine and asparagine and therefore depend heavily on extracellular sources for growth and proliferation (Delage et al. 2010). It would therefore be expected that increasing arginine and asparagine concentrations in the cell culture medium would correlate with increased proliferation rates in PANC-1 cells, however this was not observed. In fact, cell proliferation decreased with isoxazole treatment suggesting a possible role for those metabolites in endocrine differentiation rather than proliferation.

Aspartate supplementation on isoxazole treated PANC-1 cells resulted in a significant decrease in cell proliferation and viability (6x supplementation at day 2 and 2-4x supplementation at day 4). However by day 6, cell proliferation and viability had recovered (Figure 4.6). Metabolomics data on isoxazole treated PANC-1 cells revealed that aspartate was not only being selectively utilised from the cell culture environment, but also

significantly increased 1.3-fold intracellularly when compared to the DMSO control (Figure 4.2 B-C). Unlike the amino acids arginine and asparagine, PANC-1 cells are not auxotrophic for aspartate, as this can be biosynthesised from a number of metabolic routes (summarised in Figure 4.3.2). Therefore exogenously increasing the aspartate concentrations could lead to cellular toxicity, possibly accounting for the decreased cell viability observed with initial treatment (Figure 4.6).

Metabolic reprogramming is an intrinsic characteristic and hallmark of cancer; whereby cells can restructure their metabolism to compensate for a lack of nutrients such as glucose or L-glutamine in their microenvironment (Hanahan & Weinberg 2011; Boroughs & DeBerardinis 2015). PANC-1, like other PDAC cell lines, has been previously reported to have a degree of metabolic plasticity, with an inherent capacity for cells to sense nutrient availability and switch to alternative bio-energetic pathways (Daemen et al. 2015; Boroughs & DeBerardinis 2015). Given the recovery in cell growth of PANC-1 cells following 6 days of treatment with aspartate, it can be speculated that a selection process is occurring, whereby the remaining viable cells compensate by rapidly proliferating with prolonged exposure. This may be an adaptive metabolic mechanism utilised in the face of nutrient excessiveness, analogous to that observed in nutrient deprivation in order to promote cell survival and growth (Boroughs & DeBerardinis 2015).

4.3.8 Impact of medium optimisation on gene expression

After studying the changes in cell proliferation and viability with isoxazole in optimised cell culture medium, focus shifted towards assessing the genes involved in endocrine development. Very little has been done in the field of metabolomics for enhancing pancreatic endocrine differentiation with a greater focus being on amino acid metabolism in maintaining ESC pluripotency (Kilberg et al. 2016). A panel of genes and transcription factors involved in the different stages of pancreatic development was assessed, including the transcription factors *PDX1, Ngn3, NeuroD1*, mature β-cell markers *SLC30A8* and *insulin* and finally, the novel cell surface markers, *GPR50, TROP2* and *SUSD2*.

Although cell proliferation and viability was done over 6 days, qRT-PCR was carried out at day 2 of isoxazole treatment as it was not feasible to assay samples over a number of time points. Arginine supplementation had no effect on *PDX1* expression in isoxazole treated PANC-1 cells as shown in Figure 4.5A. This was also true for aspartate and asparagine supplementation (Figure 4.7A and 4.9A). However, increasing the arginine concentrations in the cell culture medium did significantly increase the mRNA levels of the β-cell

associated genes *Ngn3, NeuroD1* and *insulin.* Arginine supplementation also significantly increased the expression of the mature β-cell associated cell surface marker *GPR50*, in a dose-dependent manner. However, arginine supplementation had little effect on the expression of other genes associated with a mature β-cell phenotype such as the zinc transporter *SLC30A8*, and the novel cell surface marker *TROP2* (Figure 4.5B-C). Arginine supplementation did however significantly increase the expression of the novel endocrine progenitor associated cell surface marker *SUSD2* in a dose-dependent manner. This suggests a synergistic effect of arginine supplementation with isoxazole treatment, enhancing ductal to endocrine trans-differentiation to an endocrine progenitor stage of β-cell development.

Aspartate supplementation with isoxazole treated PANC-1 cells significantly increased β-cell associated genes including; *Ngn3, NeuroD1, SLC30A8* and *insulin* as shown in Figure 4.7. *SUSD2* signifies early endocrine and endocrine progenitor stage of β-cell development, and *SUSD2* mRNA levels were enhanced with increased aspartate supplementation, (Fishman et al. 2012; Watson et al. 2013). Aspartate supplementation significantly decreased *GPR50* mRNA levels and had no effect on *TROP2* expression, possibly suggesting the limitations of this differentiation protocol on pancreatic development. Collectively this data suggests a synergistic effect of aspartate supplementation to the cell culture medium of isoxazole treated PANC-1 cells resulting in enhanced endocrine differentiation.

Unlike arginine and aspartate, asparagine supplementation on isoxazole treated PANC-1 cells appeared to have attenuated the 'isoxazole effect' in relation to mature β-cell associated genes (Figure 4.9). Asparagine significantly decreased *Ngn3, NeuroD1, SLC30A8, insulin* and *GPR50* expression, in a dose-dependent manner when compared to basal (1x asparagine) levels. Conversely, asparagine additions did significantly up-regulate *SUSD2* expression. Collectively this data suggests that asparagine supplementation to the cell culture medium of isoxazole treated PANC-1 cells repressed endocrine differentiation.

To summarise the results so far, arginine and aspartate supplementation appeared to synergistically enhance endocrine differentiation to varying degrees, whilst asparagine had the reverse effect on pancreatic developmental associated genes, suggesting a link between specific amino acids, metabolism and differentiation status. In particular, the metabolites arginine and aspartate were decreased in used cell culture medium. Replenishing these amino acids by manipulation of the cell culture medium resulted in enhanced expression of β-cell associated genes, suggesting endocrine development.

However, supplementation of the medium with asparagine (the precursor to aspartate) had the reverse influence, attenuating the 'isoxazole effect'. The role of asparagine in cellular metabolism is protein synthesis, whereas aspartate and arginine are involved in nucleotide biosynthesis (Boroughs & DeBerardinis 2015). This difference in metabolites during endocrine differentiation supports the mechanism of action for isoxazole. Isoxazole causes epigenetic changes by increasing histone acetyl transferase activity, thus initiating gene transcription (Dioum et al. 2011), hence would require increased nucleotide synthesis for mRNA transcription. Therefore cells maybe preferentially utilising aspartate and arginine over asparagine. Also, studies have shown that PANC-1 cells in particular express low levels of the enzyme L-asparaginase (Milman, Cooney, et al. 1979). Therefore surplus asparagine would not be broken down into aspartate where it could enter the TCA cycle or the urea cycle. Thus potentially leading to an excessive accumulation of intracellular asparagine impeding metabolic flux or resulting in a 'bottleneck' effect (Berg et al. 2002). This may account for the lack of differentiation observed with asparagine supplementation. It is important to note that isoxazole did not affect asparagine levels in the metabolomics analysis and was assayed speculatively as a potential target for manipulating cellular metabolism in PANC-1 cells.

4.3.9 Impact of cell culture medium optimisation on CK19 expression

After determining the gene expression profiles of isoxazole treated PANC-1 cells in modified cell culture medium, focus shifted to the characterisation of protein markers. Firstly, this study aimed to investigate whether the manipulation of cell culture medium had any effect on the pancreatic ductal marker CK19, as this has been previously reported to decrease with induction of endocrine differentiation (Wu et al. 2010). Expression of the ductal marker CK19 was unaltered by isoxazole treatment in PANC-1 cells (reported in Chapter 3); however the addition of arginine supplementation significantly increased CK19 expression compared to isoxazole treatment alone (Figure 4.10). The addition of arginine to isoxazole treated PANC-1 cells also revealed similar 'pseudo-ductal' structures which were found to be significantly increased with isoxazole treatment, described in Chapter 3. Aspartate had no effect on CK19 expression, whereas asparagine showed to significantly decrease CK19 expression (Figure 4.11). Suggesting that arginine supplementation was making isoxazole treated PANC-1 cells more ductal in phenotype whilst asparagine was making the cells less ductal.

4.3.10 Impact of cell culture medium optimisation on SUSD2 expression

Following on from CK19 quantification in modified cell culture medium, the aim was to investigate whether SUSD2, a putative endocrine progenitor cell surface marker, would be induced with isoxazole treatment at a protein level and whether amino acid supplementation could enhance SUSD2 levels (H. Liu et al. 2014). SUSD2 was found to be significantly up-regulated nearly 30% in isoxazole treated PANC-1 cells when compared to the DMSO control (Figure 4.13). The addition of arginine, aspartate and asparagine to isoxazole at 6x the basal concentration for 6 days resulted in significantly higher SUSD2 levels compared to the DMSO control. Although the percentage of $SUSD2^{+ve}$ cells did appear lower in asparagine treated PANC-1 cells the addition of amino acids did not significantly alter the levels of SUSD2 expression when compared to isoxazole alone.

Reviewing data independently on the variable CK19 and SUSD2 percentage positive cells, this raised the question of whether the $SUSD2^{+ve}$ cells were $CK19^{-ve}$ and vice versa. This lead to dual immunostaining of CK19 and SUSD2 in isoxazole treated PANC-1 cells with the addition of arginine, aspartate and asparagine (Figure 4.14). Levels of SUSD2 expression increased with isoxazole treatment and the levels were variable across the different treatment groups. Furthermore, by observation it appeared that cells highly positive for SUSD2 were not highly positive for CK19. This was further explored in Figure 4.15, where isoxazole treated PANC-1 cells were dual stained for CK19 and SUSD2, and revealed a heterogeneous staining pattern. There appeared to be four distinct cell populations; $SUSD2^{+ve}$, $CK19^{+ve}$, $SUSD2/CK19^{+ve}$ and finally $SUSD2/CK19^{-ve}$ cells. It is interesting to note that the highly $CK19^{+ve}$ cells appeared spherical in cell morphology and formed clusters or aggregates. Whereas, the highly $SUSD2^{+ve}$ cells were fewer in number and interspersed with a distinct elongated cellular morphology suggestive of a migratory cell (Moore et al. 1987).

4.3.11 Correlation of SUSD2 expression and cell morphology

Although there is newly emerging data to support the presence of SUSD2 during endocrine differentiation, very little is known about the actual role SUSD2 plays in pancreatic development as it is a novel and putative cell surface marker (H. Liu et al. 2014). There is much more data that elucidates the role of SUSD2 in cancer with a role in cell migration and metastasis, especially in breast cancer cells (Watson et al. 2013). Increased expression of SUSD2 is often linked with lower survival rate and poor prognosis (Watson et al. 2013). PANC-1 cells are a ductal cell line, therefore migration and adhesion would be essential for

exocrine to endocrine trans-differentiation as this would recapitulate normal pancreatic development. Studies have shown epithelial to mesenchymal transition in PANC-1 cells during endocrine differentiation (Quint 2012).

To investigate whether there was any relationship between cell morphology and SUSD2 expression in isoxazole treated PANC-1 cells, the mean fluorescent intensity of SUSD2 staining was compared to the maximum Feret's diameter (a measure of cell length in μm). In general, there appeared to be a moderately positive correlation (R^2=0.49) confirming that the highly SUSD2 positive cells displayed a longer cell phenotype (Figure 4.16). It is worth noting that in the literature, SUSD2 expression in 18-week old human foetal pancreatic tissue was reported to be highly expressed in endocrine progenitor cells and early endocrine cells but excluded from 'islet-like' structures. Cells highly positive for SUSD2 were not found present in rounded clusters or 'islet-like' structures that displayed hormone expressing cells positive for insulin and glucagon (H. Liu et al. 2014). Metabolomics data from this current study also showed a significant increase in lipids inside isoxazole treated cells, which would further support changes observed in cell morphology. Isoxazole treatment in PANC-1 cells significantly increased cellular elongation (reported in Chapter 3), which would require increased lipid synthesis for the extension of the plasma membrane and increased cell length which has been correlated with high SUSD2 expression.

To summarise, results reported in this chapter demonstrate that isoxazole not only induces the expression of key pancreatic endocrine genes, but this is accompanied by alterations in metabolites both intrinsically and extrinsically. Supplementing the *in-vitro* cell culture environment back with metabolites such as arginine and aspartate, which were actively being utilised by isoxazole treated PANC-1 cells, led to enhanced expression of endocrine and β-cell associated genes (such as *NeuroD1, Ngn3* and *insulin*). Changes also occurred at a protein level, with an up-regulation in the levels of the endocrine cell surface marker SUSD2, where PANC-1 cells also displayed a distinctive elongated cellular morphology following isoxazole treatment. An overall summary of the changes observed in genes, metabolites and proteins following isoxazole treatment in PANC-1 cells are shown in Figure 4.3.3.

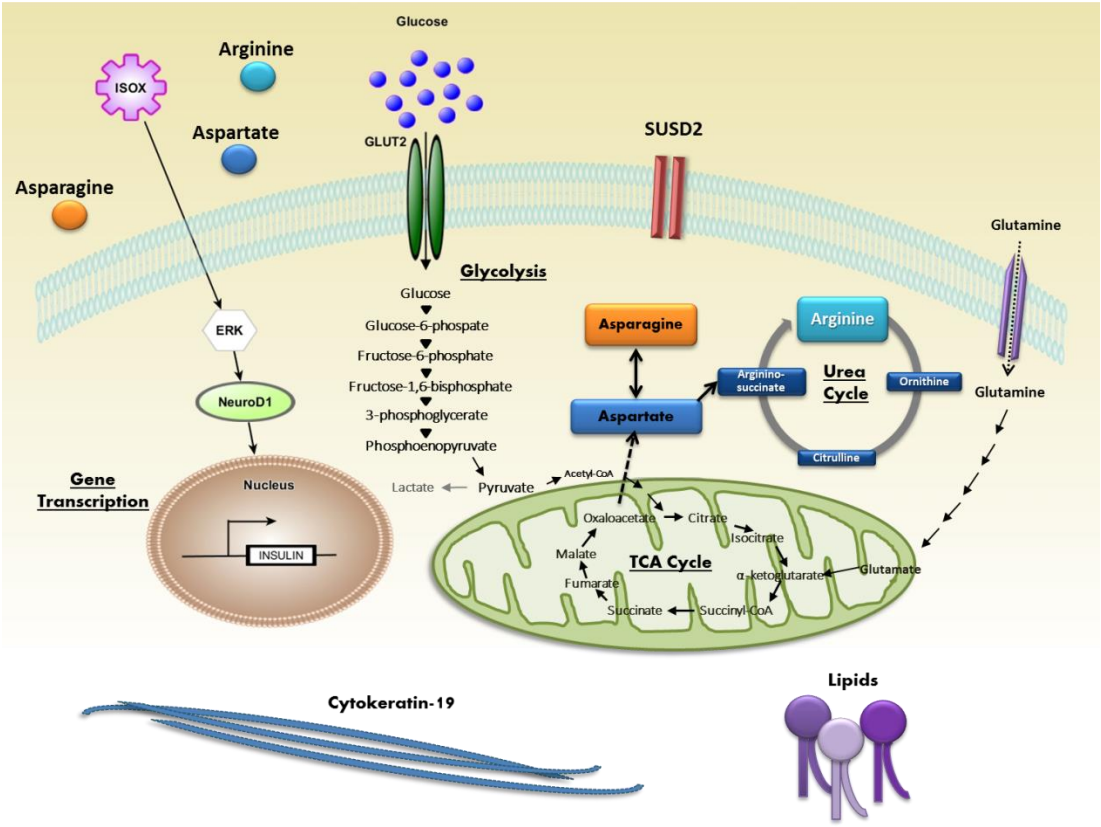


Figure 4.3.3 Summary of metabolic and transcriptional pathways altered by isoxazole treatment in PANC-1 cells. Metabolites and enzymes altered by isoxazole treatment in PANC-1 cells. Isoxazole affects ERK1/2 signalling and induces insulin gene transcription. Isoxazole affected pathways involved in metabolism and catabolism of asparagine, aspartate and arginine involved in the urea cycle and tricarboxylic acid (TCA) cycle. Isoxazole had little effect on glycolysis. The addition of exogenous arginine, aspartate and asparagine affected insulin gene transcription, the TCA cycle and lipid metabolism, altered cytokeratin-19 expression and SUSD2 cell surface marker expression. ASNS=asparagine synthetase, ASS1= argininosuccinate synthetase 1, AST= aspartate amino transferase.

CHAPTER 5 Final Discussion

In this study, it has been shown that treatment of a ductal cell line, PANC-1 with the isoxazole compound enhanced expression of β-cell associated genes. Albeit, mRNA levels were to a much lesser degree than what is observed in the model human β-cell line, EndoC-βH1. Isoxazole treatment also affected the metabolites endogenously and within the cell culture medium; suggesting that PANC-1 ductal cells were actively selecting components of the cell culture microenvironment in order to drive endocrine specific gene transcription. The supplementation arginine and aspartate back into the cell culture medium, synergistically enhanced endocrine and β-cell associated gene transcripts such as *insulin* and *Ngn3* that were up-regulated with isoxazole treatment. However, the addition of asparagine (precursor to aspartate) had the reverse effect on endocrine differentiation, decreasing the expression of β-cell associated genes. From collectively assessing all the data, it would broadly appear that the addition of arginine and aspartate to cell culture medium was enhancing endocrine differentiation from a ductal cell phenotype toward an endocrine progenitor stage of development.

Isoxazole treatment alone appeared to up-regulate the mature β-cell markers *GPR50* and *TROP2*, however, the addition of amino acids elicited little effect on *TROP2* mRNA expression. Arginine supplementation appeared to increase *GPR50* expression in a dose dependent manner, whereas aspartate and asparagine had the opposing effect. It is often difficult to identify and test novel cell surface markers, especially at the final stages of β-cell maturation as it is difficult to obtain the necessary tissue for a positive control. The mature β-cell markers *GPR50* and *TROP2* were not expressed in the positive control cell line EndoC-βH1 (data not shown). Without access to human pancreatic tissue or human islets it was difficult to ascertain if the later stages of pancreatic development were reached with the optimised cell culture medium and isoxazole treatment. However, preliminary western blot data suggests that isoxazole does not induce the expression of GPR50 and TROP2 protein (N=1 data not shown).

However data in this study identified SUSD2, a putative endocrine progenitor marker to be up-regulated in isoxazole treated PANC-1 cells. Furthermore, amino acid supplementation to cell culture medium showed a significant up-regulation of SUSD2 protein levels when compared to the DMSO control. One would expect to see an inverse relationship of CK19 expression compared to SUSD2 levels; however this was not the case. Levels of CK19 were high and remained high even with isoxazole treatment and the addition of amino acids

(except for asparagine). This could be attributed to the heterogeneity of PANC-1 cells. A caveat of interpreting gene expression data is that it doesn't take into account the number of cells in the population. It could be possible that a small sub-population of cells are undergoing ductal to endocrine trans-differentiation whilst the vast majority of cells remain unchanged.

Furthermore, it was difficult to determine the source of potentially differentiating SUSD2^{+ve} cells within the isoxazole treated PANC-1 population. PANC-1 cells are heterogeneous in nature; with approximately 80% of cells expressing the pancreatic ductal marker CK19 and three distinct cellular types. Isoxazole treatment yielded a 30% increase in SUSD2^{+ve} cells compared to the DMSO control. However this leads to the question of where the SUSD2^{+ve} cells have originated from. Are the SUSD2^{+ve} cells derived mainly from the 20% of non-ductal (CK19^{-ve}) cells including a smaller percentage of co-expressing SUSD2/CK19^{+ve} cells? Or have they originated from CK19^{+ve} cells that have lost expression of CK19 with isoxazole treatment to become SUSD2^{+ve}?

PANC-1 ductal cells consist of large, intermediate stellate and small morula shaped CK19^{+ve} cells. PANC-1 cells are also described as 'pleomorphic' and can assume different cell morphologies at different stages of the cell cycle (Gradiz et al. 2016). With the appropriate stimuli, such as isoxazole treatment under defined cell culture conditions, hypothetically, it could be possible that PANC-1 cells during endocrine differentiation lose expression of CK19, change cell morphology and migrate into islet-like clusters. This theory could be supported by proteomics data from within the laboratory showing that isoxazole up-regulated proteins involved in cytoskeletal re-organisation and cell adhesion mediated by the Rho GTPase signalling pathway in PANC-1 cells (unpublished data). Studies have shown that the Rho signalling pathway is important in regulating delamination of ductal epithelial cells in endocrine differentiation in which Ngn3^{+ve} cells form islets (Kesavan et al. 2014). In a gene array study that treated human islets with isoxazole for 7days, *stathmin 2* (STMN2) was significantly up-regulated 18-fold compared to the DMSO control, the highest fold induction with isoxazole treatment (Kalwat et al. 2016). Data from within the Dunne/Cosgrove laboratory showed that STMN2 was also significantly up-regulated 3.09-fold in isoxazole treated PANC-1 cells (unpublished data). STMN2 is crucial in microtubule stability, and although its preferentially expressed in neurons, its role is to stabilise microtubules and control cell length (Di Paolo et al. 1997). Collectively this data supports

the concept of cell migration and remodelling of the cell membrane during ductal to endocrine trans-differentiation with isoxazole treatment.

It is important to recognise cell line specific mutations that lead to auxotrophy and nutrient sensing. Thus a concerted effort is required to develop a chemically defined cell culture medium optimised to maintain cell growth whilst promoting differentiation and synthesis of macromolecules. The use of metabolomics and proteomics can help in the understanding of the systems biology and cellular stresses leading to bottle-neck effects thus hindering cellular processes such as differentiation. With a greater drive in understanding the metabolic profiles of stage specific pancreatic cells, we can enhance differentiation towards mature β-cell phenotypes, for potential large scale production of transplantable cells for therapeutic use.

Future work

The cumulative effects of arginine and aspartate were not investigated due to time constraints; however, it would be interesting to determine if the addition of both arginine and aspartate further enhanced endocrine differentiation or whether this reached a plateau. Although it broadly appears that isoxazole with the addition of amino acids such as arginine and aspartate enhanced endocrine differentiation from a ductal stage towards an endocrine progenitor stage, this would have to be confirmed by investigating whether SUSD2^{+ve} cells co-stained with a nuclear transcription factor present at that stage of development, such as *Ngn3* or *NeuroD1*. If positive, SUSD2 could ultimately be used as a marker for cell sorting. This would greatly reduce the complexity of working with a heterogeneous cell population. It would appear that isoxazole induces the onset of endocrine differentiation by 'switching on' genes such as *Ngn3*, which is essential for pancreatic development as it gives rise to all 5 hormone expressing cell types. Following isoxazole treatment and after harvesting SUSD2^{+ve} cells, these cells could be used for a more rigorous step-wise differentiation protocol to further differentiate cells toward a mature β-cell phenotype.

Supplementary Information

Table 1. Cell line authentication of PANC-1 and EndoC-βH1 cell lines showing Short Tandem Repeat (STR) results at specific loci.

Locus Name	PANC-1 (X,Y)	EndoC-βH1 (X,Y)
D5S818	11 ,13	11, 12
D13S317	11	11, 12
D7S820	8, 10	10, 12
D16S539	11	15, 21
vWA	15	28, 31
TH01	7,8	15, 17
TPOX	8, 11	11, 12
CSF1PO	10, 12	8
D18S51	12	14, 15
D21S11	28	18.2, 24
D3S1358	17	8,
D8S1179	14, 15	13, 14
FGA	21	6, 8
Penta_D	14	7, 11
Penta_E	7, 14	15, 18

Figure 1. Mycoplasma testing of PANC-1 and EndoC-βH1 cell lines

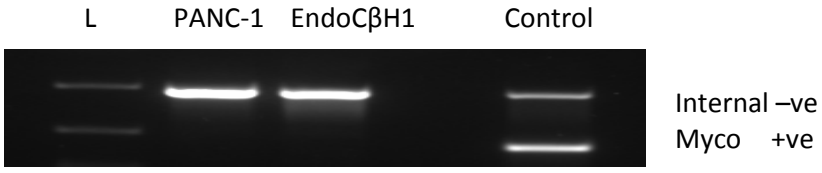


Table 2. PANC-1 medium formulations of basal growth medium (DMEM D6429) and serum free medium for differentiation protocol (DMEM/HAMS-F12)

Maintenance Growth Medium (+10% FCS)			
DMEM (D6429)			
Components	**g/L**	**mg/L**	**Final Conc (mM)**
Amino Acids			
L-Alanyl-L-Glutamine	none		
L -Arginine • HCl	0.08	84.00	0.40
L -Cysteine • 2HCl	0.06	62.60	0.52
L -Glutamine	0.58	584.00	4.00
Glycine	0.03	30.00	0.40
L-Histidine • HCl • H2O	0.04	42.00	2.80
L-Isoleucine	0.11	105.00	0.80
L-Leucine	0.11	105.00	0.80
L-Lysine • HCl	0.15	146.00	0.80
L-Methionine	0.03	30.00	0.20
L-Phenylalanine	0.07	66.00	0.40
L-Serine	0.04	42.00	0.40
L-Threonine	0.10	95.00	0.80
L-Tryptophan	0.02	16.00	0.08
L-Tyrosine • 2Na • 2H2O	0.10	103.79	0.40
L-Valine	0.09	94.00	0.80
Vitamins			
Choline Chloride	0.00	4.00	0.03
Folic Acid	0.00	4.00	0.01
myo -Inositol	0.01	7.20	0.04
Niacinamide	0.00	4.00	0.03
D-Pantothenic Acid • ½	0.00	4.00	0.02
Pyridoxal • HCl	none		
Pyridoxine • HCl	0.00	4.04	0.02
Riboflavin	0.00	0.40	0.00
Thiamine • HCl	0.00	4.00	0.01
Inorganic Salts			
CaCl2	0.20	200.00	1.80
Fe(NO 3) 3 • 9H 2O	0.00	0.10	0.00
MgSO4	0.10	97.67	0.81
KCl	0.40	400.00	5.33
NaHCO3	3.70	3700.00	44.05
NaCl	6.40	6400.00	110.34
Na H 2PO 4	0.11	109.00	0.79
Other			
D-Glucose	4.50	4500.00	25.00
HEPES	none		
Phenol Red • Na	0.02	15.90	0.04
Pyruvic Acid • Na	0.11	110.00	1.25

Serum Free Medium for Isox (50:50) + Supplements								
DMEM (11960-044)				HAMS F12 (N6658)				
Components	**Molecular Weight**	**mg/L**	**mM**	**Components**	**g/L**	**mg/L**	**mM**	**Final Conc (mM)**
Amino Acids				**Amino acids**				
L-Arginine hydrochloride	211	84	0.40	L-Alanine	0.009	9	0.101	0.051
L-Cystine 2HCl	313	63	0.20	L-Alanyl-L-Glutamine	—			0
Glycine	75	30	0.40	**L-Arginine.HCL**	**0.21**	**211.00**	**1.00**	**1.20**
L-Histidine hydrochl	210	42	0.20	**L-Asparagine.2H20**	**0.02**	**15.01**	**0.11**	**0.06**
L-Isoleucine	131	105	0.80	**L-Aspartic Acid**	**0.01**	**13.30**	**0.10**	**0.05**
L-Leucine	131	105	0.80	L-CysteineHCL .H20	0.04	35.00	0.29	0.39
L-Lysine hydrochloride	183	146	0.80	L-Glutamic Acid	0.01	14.70	0.10	0.05
L-Methionine	149	30	0.20	L-Glutamine	0.15	146.00	1.00	2.50
L-Phenylalanine	165	66	0.40	Glycine	0.01	7.51	0.10	0.30
L-Serine	105	42	0.40	L-Histidine HCL H20	0.02	20.96	0.14	0.24
L-Threonine	119	95	0.80	L-Isoleucine	0.00	3.94	0.03	0.43
L-Tryptophan	204	16	0.08	L-Leucine	0.01	13.10	0.10	0.50
L-Tyrosine disodium	261	104	0.40	L-Lysine HCL	0.04	36.50	0.20	0.60
L-Valine	117	94	0.80	L-Methionine	0.00	4.48	0.03	0.13
Vitamins				L-Phenylalanine	0.00	4.96	0.03	0.23
Choline chloride	140	4	0.03	L-Proline	0.03	34.50	0.30	0.15
D-Calcium pantothenate	477	4	0.01	L-Serine	0.01	10.50	0.10	0.30
Folic Acid	441	4	0.01	L-Threonine	0.01	11.90	0.10	0.50
Niacinamide	122	4	0.03	L-Tryptophan	0.00	2.04	0.01	0.05
Pyridoxine hydrochloride	206	4	0.02	L-Tyrosine.2Na.2H20	0.01	7.78	0.03	0.23
Riboflavin	376	0.4	0.00	L-Valine	0.01	11.70	0.10	0.50
Thiamine hydrochloride	337	4	0.01	**Vitamins**				
i-Inositol	180	7.2	0.04	D-Biotin	0.00	0.01	0.00	0.00
Inorganic Salts				Choline chloride	0.01	13.96	0.10	0.11
Calcium Chloride (Ca	111	200	1.80	Folic Acid	0.00	1.32	0.00	0.01
Ferric Nitrate (Fe(NO	404	0.1	0.00	myo-Inositol	0.02	18.00	0.10	3.70
Magnesium Sulfate (	120	97.67	0.81	Niacinamide	0.00	0.04	0.00	0.02
Potassium Chloride (	75	400	5.33	D-Pantothenic Acid 1/2C	0.00	0.48	0.00	0.01
Sodium Bicarbonate	84	3700	44.05	Pyridoxine HCL	0.00	0.06	0.00	0.01
Sodium Chloride (NaCl)	58	6400	110.34	Riboflavin	0.00	0.04	0.00	0.00
Sodium Phosphate n	138	125	0.91	Thiamine HCL	0.00	0.34	0.00	0.01
Other Components				Vitamin B12	0.00	1.36	0.00	0.00
D-Glucose (Dextrose)	180	4500	25.00	**Inorganic Salts**				
Phenol Red	376.4	15	0.04	CaCl2 2H20	0.04	44.10	0.40	1.30
				CaCl2	—			
				CuSO4. 5H20	0.00	0.00	0.00	0.00
				FeSO4.7H20	0.00	0.83	0.01	0.00
				MgCl2	0.06	57.60	0.61	0.30
				KCl	0.22	224.00	2.99	5.65
				NaCl	7.60	7599.00	131.02	186.19
				NaHCO3	1.18	1176.00	14.00	36.02
				Na2HPO4	0.14	142.04	1.03	1.48
				ZnSO4.7H20	0.00	0.86	0.01	0.00
				Other				
				D-Glucose	1.80	1802.00	10.01	22.5
				Hypoxanthine	0.00	4.73	0.03	0.02
				Hypoxanthine Sodium S	—			
				Linoleic Acid	0.00	0.08	0.00	0.00
				Phenol Red Na	0.00	1.30	0.00	0.02
				Putrescine.2HCL	0.00	0.16	0.00	0.00
				Pyruvic Acid Na	0.11	110.00	1.25	0.63
				Thioctic Acid	0.00	0.21	0.00	0.00
				Thymidine	0.00	0.73	0.00	0.00
				Supplements				
				ITS (insulin, transferrin, selenium)		500ul per 50ml		1%
				Ascorbic Acid		128ug/ml		0.727
				Growth factor-EGF		0.75ng/ml		117pmol/L
				Glutathione		20ug/ml		0.065

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