Cell lineage tracing in the human pancreas

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Veterinärprogrammet

Examensarbete 2008:24
ISSN 1652-8697
Uppsala 2008

Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Anmial Sciences
Veterinary Medicine Programme

Degree project 2008:24
ISSN 1652-8697
Uppsala 2008

ACKNOWLEDGEMENTS

Mairi Brittan
Malcolm Alison
Stina Ekman
Ronny Fransson-Steen

All members of the department for diabetes and metabolic diseases at QMUL
Dilsa, for always keeping me smiling as well as my “mtDNA-team”
Staff at Histopathology Unit, Cancer Research UK
Staff at the Immunohistochemistry lab at Hammersmith Hospital, London
Prof. Doug Turnbull and Dr. Iryna Ziabrevain, the mitochondrial research group at the University of Newcastle
Astra Zeneca, Sweden
Royal Veterinary College
Wellcome Trust
Swedish University of Agricultural Sciences, SLU

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SAMMANFATTNING

Cellerna i bukspottkörteln hos människa är normalt inte proliferativa, om dessa celler skadas är regenerationen snabb. Den bakomliggande mekanismen är inte känd men det innebär att pankreas funktion bibehålls. Forskning har ännu inte kunnat identifiera en definitiv stamcell i pankreas. I djurförsök är det möjligt att använda radioaktiva eller genetiska markörer för att följa cellinjer, vilket inte är möjligt i humanforskning utan där används istället stamcellsmarkörer. Det finns därför ett behov att utveckla bättre teknik för att kunna följa, isolera och identifiera celler av samma ursprung (stamceller). Kvalitativ teknik skulle kunna möjliggöra kartläggning av ursprunget till celler, vilka härstammar från stamceller med mutationer, eftersom mutationer av mitokondriell-DNA ackumuleras natruligt i våra celler allt eftersom vi åldras.

Eftersom proteinet cytokrom c oxidas kodas från mitokondriellt-DNA uppstår ofta defekter i cytokrom c oxidas om DNA:t är muterat. I den redovisade studien undersöktes muterade områden i normal pankreas där klonala grupper med celler kunde påvisas med hjälp av immunohistokemi avseende förekomsten av cytokrom c oxidas. Dessa grupper av celler har troligtvis samma ursprung. I studien försökte vi visa att dessa celler även uppvisade normal funktion, även vad gäller proliferationskapacitet. Undersökningen visade dock att celler med muterat mitokondriellt-DNA inte har normal funktion.

Tekniken att spåra cellers delning och rörelse i human vävnad är användbar för att följa hur celler prolifererar och förflyttar sig i pankreas. Fortsatta studier inom området skulle kunna identifiera markörer för att påvisa förändringar i pankreasceller, som exempelvis förstadier till cancer.

ABSTRACT

Background and aims: The human pancreas is normally proliferatively quiescent; however loss of cells gives a rapid regenerative response to restore a functionally healthy tissue mass. The mechanism for this is unclear and to date research has failed to identify a definitive pancreatic stem cell. Cell lineage analyses are possible in animals by labelling cells with radioactive tags or genetic markers, but this is not feasible in humans. Current human studies rely on stem cell markers, thus there is a need to develop a better technique to track, isolate and identify cells of related origin. Mitochondrial DNA mutations amass naturally within our cells as we age. Therefore, cells formed by division of a stem cell carrying mutated mitochondrial DNA should, given a qualitative assay, permit the progeny of this cell to be traced.

Methods and results: Mitochondrial DNA mutations often result in a defect in the mitochondrial DNA-encoded protein cytochrome c oxidase, and can be identified using a histochemical stain. Individual mutant patches were studied by staining serial sections of normal pancreas. This revealed the presence of clonal units of cells, strong evidence that pancreatic cells within each patch are derived from a common progenitor. We tried to show that these cells are normal in synthetic and proliferative function, although this showed that the mitochondrial DNA mutated cells didn't have normal synthetic function. However this did not negate our evidence of clonal proliferation.

Conclusion: This novel means of tracing patterns of cell division and migration in human tissues is suitable for finding how cells proliferate and move in the human pancreas, and with continued work it might be useful as a marker for other changes e.g. pre-cancerous lesions.

ABBREVIATIONS

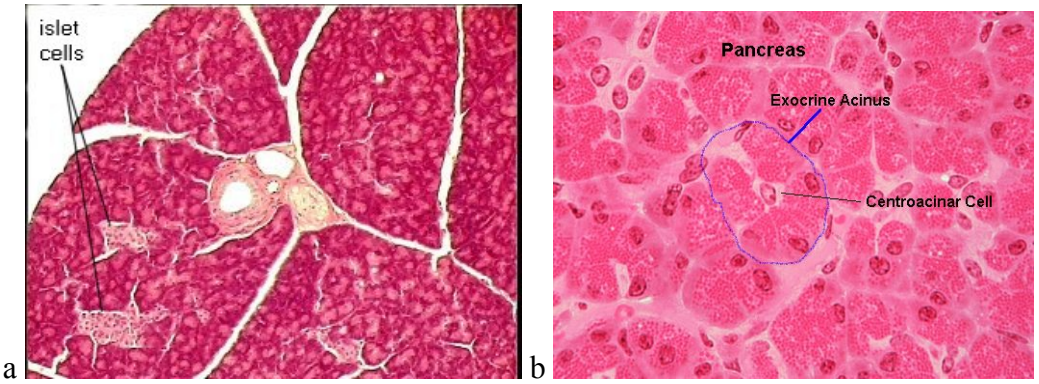
COX	cytochrome c oxidase
CSC	cancer stem cells
DAB	diaminobenzidine
g	gram
H_2O_2	hydrogen peroxide
m	milli- (suffix)
mtDNA	mitochondrial DNA
μ	micro- (suffix)
μl	microlitre
PBS	phosphate buffered saline
pH	$-\log[H^+]$
SC	stem cell
SDH	succinate dehydrogenase

1. INTRODUCTION

The aims of this project were to try to locate adult stem cells (SCs) in the human pancreas using mitochondrial DNA (mtDNA) mutations as a marker and to see if we could elucidate the clonal nature of normal pancreatic cells. In order to do this, we performed histochemical staining for cytochrome c oxidase (COX) expression, which is encoded by mtDNA. We firstly screened for the presence of COX-negative (COX-ve) patches of cells, and after that we serially sectioned tissues to elucidate the patch architecture and patterns of cell migration. In parallel, we performed various studies to investigate whether COX mutation presents a selective advantage/disadvantage upon growth, survival or function of pancreatic cells. Little is known about the location of pancreatic SCs and whether the progeny of these cells migrate in particular paths e.g. from exocrine ducts to islets. Studies in other tissues (e.g. gut) have successfully used this method to trace cell lineage relations by studying mtDNA mutations in individual laser-captured cells. The mitochondrial genome encodes 13 essential subunits of the respiratory chain and has remarkable genetics based on uniparental inheritance. Within human populations, the mitochondrial genome has a high rate of sequence divergence with multiple polymorphic variants. It has also been suggested that mtDNA mutations play a role in ageing and cancer, but the evidence for a causative role in these conditions is less clear. It is now known that mtDNA mutations occur in human colonic crypt SCs (Taylor *et al.* 2003, McDonald *et al.* 2006 [b], Greaves *et al.* 2006, Schon *et al.* 2003) and these mutations have been used to look at both the expansion of mutated clones in the colon and to provide a platform for lineage analysis in humans for really the first time.

1.1 Histology of the human pancreas

The pancreas is an elongated, tapered organ located at the back of the abdomen, behind the stomach. The right side of the organ, called the head, is the widest part of the organ and lies in the curve of the duodenum, the first section of the small intestine. The tapered left side, called the body, extends slightly upward and ends near the spleen, here called the tail. The pancreas has both endocrine and exocrine functions, but embryonically all the cells differentiate from the ductal epithelium. The endocrine cells migrate from the duct system and aggregate around capillaries to form isolated clusters of cells. These clusters of cells are called the islets of Langerhans (referred from herein as islets) and are found scattered throughout the exocrine glandular tissue that consists of closely packed secretory acini (see figure 1). There are approximately 1 million islets in a human pancreas, each composed of roughly 3000 cells of which 75 % are insulin producing β-cells (reviewed in Fellous *et al* 2006). The rest consists of α-cells (~5%), δ-cells (~20%) and PP-cells (1%) cells.



a b

Figure 1*. Haematoxylin-eosin stained normal pancreas tissue. (a) Overview, exocrine and endocrine. (b) Closer view of the exocrine part.*

1.2 Function of the human pancreas

The exocrine system is made up of acinar cells lined with secretory glandular cells (pyramidal-shaped) that feed into a network of ducts; intercalated ducts, intralobular ducts and interlobular ducts that finally empty into the ampulla of Vater, situated in the duodenum. The production of functional enzymes is induced by gastrointestinal hormones such as cholecystokinin, acetylcholine and secretin, which are released when fatty acids and amino acids enter the duodenum. The function of the acinar cells is to produce lipase, chymotrypsin and carboxypolypeptidase for digesting proteins, pancreatic amylase for digesting carbohydrates and the main enzymes for digesting fat is pancreatic lipase, cholesterolesterase and phospholipase. The proteolytic digestive enzymes are produced in an inactive form (trypsinogen, chymotrypsinogen and procarboxypolypeptidase) and become activated upon contact with enzymes in the intestinal tract, known as enterokinases. The intestinal mucosa secretes enterokinases as it comes in contact with chyme. Enterokinases lead to activation of trypsin, which in turn activates the other protein degrading enzymes. These function to prevent self-digestion of the pancreas, in addition to trypsin inhibitor, which is produced by the glandular cells. Acute pancreatitis can occur if these security controls fail and trypsinogen becomes activated at the sight of production (Guyton & Hall 2006 [a].).

The endocrine component constitutes approximately 1% of the mass of the pancreas, and regulates glucose storage and usage *via* secretion of insulin produced in the β-cells, glucagon produced in the α-cells, somatostatin made in the δ-cells (inhibits the endocrine pancreas) and pancreatic polypeptide produced in the PP cells (which inhibits the exocrine pancreas). Reduction in β-cell number will inevitably give rise to diabetes, one of the most common diseases in the western world. Diabetes occurs in two varieties, although both are caused by a relative lack of insulin to meet peripheral demands. Type I diabetes is due to an immune-mediated destruction of pancreatic insulin producing β-cells, whereas in type II the endocrine pancreas produces insufficient amounts of insulin to compensate for peripheral insulin resistance. The rate of normal pancreas proliferation declines dramatically with age. The pancreatic cells, although essentially quiescent in the normal adult, have a great capacity for proliferation in

response to injury; therefore it is reasonable to assume that the expression of SC markers will be greater in diseased pancreas. It is the renewal of β-cells that is of primary interest for the treatment of diabetes, a disease that currently afflicts around 200 million people worldwide (reviewed in Fellous *et al.* 2006).

1.3 Stem cells

The key properties of SCs, namely indefinite self-renewal and multilineage potential were first recognized in the bone marrow (reviewed in Burkert *et al.* 2006). In most tissues, SCs are rare (Reya *et al.* 2001). The ability of a single cell to give rise to a large family of descendants (clonogenicity), containing all the lineages normally found in that cells tissue of origin is often considered a reasonably robust proof of "stemness". To achieve self-renewal, on average, each SC division must give rise to one replacement SC and one transit amplifying cell (TAC) by asymmetric cell division. TACs are committed to differentiation and with each successive division they lose their proliferative potential (reviewed in Burkert *et al.* 2006). Eventually, TACs give rise to terminally differentiated cells (TDCs) (see figure 2) which are mitotically inactive. TDCs are the cells that form the bulk of the tissue e.g. in case of the small intestine, these comprise the enterocytes that line the villi.

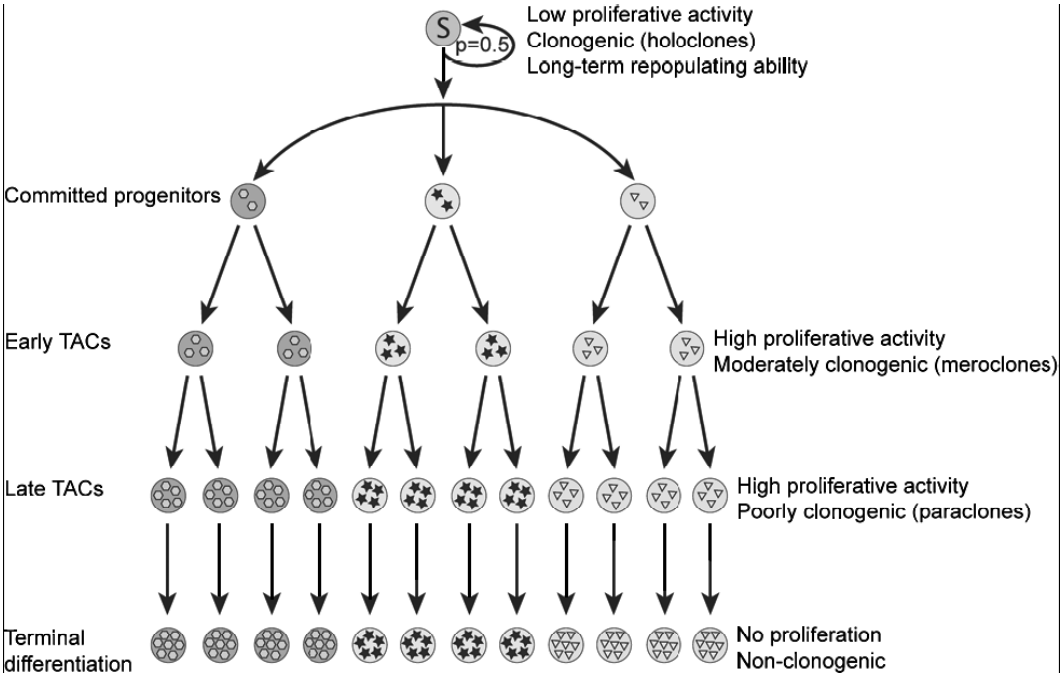


Figure 2. *Diagram showing the path of differentiation of a stem cell (Burkert et al. 2006). In most renewing systems, a cell hierarchy can be recognized in which a few self-renewing SCs give rise to a limited number of transit amplifying cells (TACs). TACs have limited proliferation potential and eventually give rise to reproductively sterile terminally differentiated cells.*

SCs are located in deterministic environments called niches (*Fr*. recess), composed of mesenchymal cells and extracellular matrix factors (Alison *et al.* 2006), wherein regulatory pathways such as Wnt/β-catenin, PTEN, Notch and

Hedgehog have a tight control on their renewal and maturation processes. Due to the hierarchical migratory nature of differentiating TACs, the SC niche is most likely to be situated at the origin of cellular flux, as interactions between the SCs and the niche cells are vital to the self-renewal process. For example, in a tissue like the small intestine, the niche is found close to the crypt base which is at the origin of the flux (reviewed in Burkert *et al.* 2006).

1.4 Cancer stem cells

A tumour's capacity for growth and propagation is suggested to be dependent on a small subset of cells within the tumour, the so-called cancer stem cells (CSCs) (Li *et al.* 2007). Cells that can re-initiate tumour growth in an immunocompromised animal are operationally defined as CSCs (Olempska *et al.* 2007). Reya *et al.* (2001), state that cancers of the haematopoietic system provide the best evidence that normal SCs are the targets of transforming mutations, and that cancer cell proliferation is driven by CSCs. They propose that newly arising cancer cells utilise the machinery that is normally expressed by SCs for self-renewal (Reya *et al.* 2001). There is also evidence that many of the pathways that classically are dysregulated in cancer, such as Wnt, Shh and Notch, may regulate normal SC behaviour during development (Reya *et al.* 2001). Therefore understanding SC self-renewal and its regulation is very important as this can give an understanding of the regulation of cancer cell proliferation, which can be seen as a disease/dysfunction of unregulated self-renewal (Reya *et al.* 2001). The existence of CSCs was first documented in haematological malignancies (Li *et al.* 2007) and was subsequently discovered in several solid tumours, including breast (Al-Hajj *et al.* 2003), prostate (Collins *et al.* 2005), brain (Singh *et al.* 2003), liver (Suetsugu *et al.* 2006), lung (Dome *et al.* 2006), melanoma (Grichnik *et al.* 2006) and colon (Guzman *et al.* 2002).

It has been shown that CSCs in some tumour types are more resistant to standard chemotherapeutic agents (Guzman *et al.* 2002). This might explain why chemotherapy can result in tumour shrinkage, but also why most tumours recur, implying that treatments that specifically target the CSC population may be more effective in treatments to eradicate solid tumours (Li *et al.* 2007).

Many markers such as CD44, CD133 and members of the Wnt signalling pathway including β-catenin and Tcf-4, have been proposed as markers of SC in several tissues (reviewed in Alison *et al.* 2006), although to date no definitive evidence of this has been documented in the adult human pancreas. CD133 is expressed by cells in solid tumours of the brain, prostate, colon and liver and is now increasingly accepted as a CSC marker, according to Olempska *et al.* (2007). CD44 is a cell surface glycoprotein involved in cell-cell and cell-matrix interactions. Increased expression of CD44 and its variants has been implicated in the transformation and progression of many malignancies (Burkert *et al.* 2006, Naor *et al.* 2002).

1.5 Stem cells in the pancreas

Though the existence of a specific self-renewing SC within the pancreas is still far from clear, a surprising variety of cells within the pancreas can differentiate towards a β-cell phenotype, with evidence that the ductular cells, periductular mesenchymal cells and β-cells themselves can all give rise to new β-cells. There is also evidence of an extra-pancreatic location of the pancreatic SC with evidence that cells in both the bone marrow and liver can differentiate and contribute to pancreatic β-cell mass (reviewed in Fellous *et al.* 2006).

In terms of pancreatic morphogenesis, the most probable location of pancreatic progenitor cells is the ductal epithelium, as it retains the ability to give rise to all the differentiated cell types of the pancreas. Data from the laboratory of Bonner-Weir *et al.* (2000) supports this suggestion, as they have shown that mature ductal cells can regress or dedifferentiate to become the main source for pancreatic growth and regeneration. As ductal cells account for about 80% of all pancreatic cancers, it seems likely that the CSCs will originate from the duct. Numerous studies have highlighted that ductal cells from mouse (Peck *et al.* 2002), rat (Kim *et al.* 2004) and human pancreas (Bonner-Weir *et al.* 2000) can generate all the pancreatic endocrine cell types under controlled *in vitro* conditions, but the identity of any multipotential SC is still lacking. Furthermore, the islets originate from the ductal epithelium and numerous studies in murine and human models have reported the differentiation of insulin producing cells *in vitro* and *in vivo* from cultured pancreatic ductal cells. Dor and colleagues (2004) suggest that new β-cells arise from self-duplication of pre-existing β-cells and that there are no SCs in the mouse pancreatic islets, although they didn't rule out the presence of a pancreatic SC population as they stated "the stem cells themselves may express insulin" (Dor *et al.* 2004). This is opposed by Oshima *et al.* (2007), who observed the differentiation of intra-islet progenitor cells into insulin producing cells *in vitro*, and therefore suggest the islets as a possible niche for SC. A considerable number of studies propose that islet cells regenerate from progenitor cells residing in the islets (Yalniz & Pour 2005). Hunziker and Stein (2000) reported a subset of nestin-expressing hormone negative cells within the pancreatic islet of a 4-week old mouse and proposed that these cells represent multipotential stem/precursor cells for the differentiated pancreatic endocrine cells. Subsequently, Zulewski *et al.* (2001), noted similar cells with an extended proliferative capacity, not only in the islets of the adult rat and human pancreas, but also in large, small and centrolobular ducts of the rat pancreas (Zulewski *et al.* 2001). The ability of insulin secreting β-cells to divide is recognized, but there is no universally accepted evidence for their derivation from adult pancreatic SCs (Bonner-Weir *et al.* 2000).

In addition to an intrapancreatic location of pancreatic progenitors, there has been a few suggestions of extra-pancreatic sources. Bone marrow SCs can seemingly abandon or alter their intrinsic programming for defined haematopoietic/mesenchymal lineage differentiation, and engraft and contribute to functional adult lineages within non-haematopoietic tissues, including the pancreas (reviewed in Fellous *et al.* 2006). Haematopoietic SCs in the bone marrow has been considered the potential source of pancreatic SCs, as they have

been found to reduce hyperglycaemia in diabetic mice after differentiation into insulin producing cells *in vitro* followed by cell transplantation (Voltarelli *et al.* 2007). Ianus *et al.* (2003), also claim that bone marrow cells are capable of transdifferentiating to produce fully functioning pancreatic β-cells. Other potential sources that have been proposed are mesenchymal SCs in the bone marrow (Sordi *et al.* 2005), spleen (Kodama *et al.* 2003) and liver (Grompe 2003, Shen *et al.* 2003, Wolf *et al.* 1990).

Zhang *et al.* (2005) showed that human foetal cells could differentiate to produce insulin, although this was a low production of insulin and the cells lacked specific transcription factors. Other recent studies claim that there are no progenitors in the pancreas (Brennand *et al.* 2007, Teta *et al.* 2007), as discussed later. All in all this gives us a variety of possible locations of SCs in the pancreas, but as said before, no definitive location has yet been found.

The prospect of curing diseases by replacing old, mutated SCs with new, healthy ones is very appealing (Bonner-Weir *et al.* 2000). Pancreatic cancer is usually diagnosed in an advanced state, which in addition with the lack of any effective therapies gives it the worst prognosis of any malignancy. Only 3% of patients have a 5 year expected survival (Li *et al.* 2007) and therefore cancer of the pancreas is the fourth leading cause of death in industrialized countries (Niederhuber *et al.* 1995, Olempska *et al.* 2007), thus highlighting the need for an early diagnosis (Niederhuber *et al.* 1995).

1.6 Stem cell markers

SC in the pancreas remain unidentified due to a lack of reliable markers of undifferentiated cells. But many different factors have been proposed as markers of pancreatic SCs including transcription factors Ngn3, Pax4, Nkx2.2, Nkx6.1, Pax 6 and Islet1, which are required for β-cell development. And indeed, according to Bonner-Weir and Sharma (2002) these factors are expressed in ducts and appear to be responsible for the rapid islet neogenesis that occurs after a 90% partial pancreatectomy in the rat (Bonner-Weir and Sharma 2002). Nestin has also been proposed as a pancreatic SC marker although this is controversial since nestin is also expressed by fibroblast like cells in the pancreatic stroma (Bonner-Weir and Sharma 2002). Suzuki *et al.* (2004) proposed c-Met as a marker of SC in the pancreas. However β-cells derived *in vitro* from these c-Met positive lineages were not fully functional and following transplantation of the c-Met-positive cells only very low levels of donor-derived pancreatic endocrine cells were observed. Other suggested pancreatic SC markers are the ABC transporters, such as ABCG2 and MDR-1, which are also expressed by nestin-positive islet-derived progenitor (NIP) cells (reviewed in Alison *et al.* 2006).

1.6.1 Label retaining cells (LRCs)

The slow-cycling nature of SCs allows them to be identified in animal models in tissues that undergo constant proliferation such as the gut and skin, following labelling with a marker of the cell cycle such as bromodeoxyuridine (BrdU) or triated thymidine (3HTdR) (reviewed in Burkert *et al.* 2006). If one can label a cell with a specific marker, then all the cells subsequently borne from this cell will

carry the marker and can thus be identified as offspring of the original labelled cell. Cells in the S-phase of the cell cycle use thymidine exclusively for the synthesis of DNA. Molecules of thymidine can be radioactively labelled to make 3HTdR or alternatively an analogue of thymidine in the form of BrdU can be used (Braun & Watt 2004). Thus all cells that have incorporated the labelled compound are recognised by either autoradiography in the case of 3HTdR, or by antibody detection of BrdU (Alison *et al.* 1997). With every cell division the label is diluted into daughter cells and so slowly cycling SCs can be LRCs. According to the Cairns immortal strand hypothesis SCs have template DNA strands that are always retained by the SC during asymmetric division (Cairns 1975). Another technique involves the use of labelling with a retrovirus containing the 'E coli β-galactosidase gene'. In this method, a retrovirus is used to incorporate the 'E coli β-galactosidase gene' into synthesising DNA and again all daughter cells will carry the label. As SCs in these tissues do not divide as rapidly as their transit amplifying progeny, the SCs retain their label, hence becoming 'label retaining cells' (Braun & Watt 2004, Alison *et al.* 2006). These techniques are only suitable for animal studies of cell lineage analyses as it would be highly unethical to use these techniques in human studies. As our study involves human tissue, but we still wanted to label and trace cells of common origin, we therefore applied a technique that utilises the random and natural accumulation of mutations in the mitochondrial genome.

1.7 Mitochondria and mtDNA

Mitochondria are membrane enclosed organelles, the energy producing components in all cells. The mitochondrion is a double-membraned organelle and is easily recognized due to the invaginations of the inner membrane. Mitochondria are present in all areas of each cell's cytoplasm though are more concentrated in those portions of the cell that are responsible for the major share of its energy metabolism. The total number of mitochondria per cell varies from less than a hundred up to several thousand, depending on the amount of energy required by the cell. Being the *powerhouse* of cells means conversion *via* oxidative phosphorylation of the reduced form of nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FADH) and nicotinamide adenine dinucleotide phosphate (NADP) into adenosinetriphosphate (ATP). ATP is the energy form used by the body for various physiological functions that are necessary to maintain and propagate life such as active transport of molecules across cell membranes, cell division and growth and various synthetic reactions that create hormones, cell membranes and many other essential molecules of the body. 90% of the carbohydrates utilized by the body are converted in to ATP via their digestion to glucose, fructose and galactose, with glucose representing approximately 80% of these. Glucose can diffuse into cells, but its uptake into cells is increased up to ten or more times more when insulin is secreted. In the absence of insulin the supply of glucose that is taken up by cells is negligible, with the exception of the cells in the brain and liver (Guyton & Hall 2006 [b].)
Impairment of mitochondrial function is intrinsically related to diabetes. Studies have observed a reduced rate of ATP synthesis in subjects with a family history of diabetes prior to the onset of impaired glucose tolerance (Petersen *et al.* 2004), indicating the significance of mitochondrial dysfunction in diabetes progression.

The presence of mtDNA results in the mitochondria being a self-replicative organelle, with mitochondrial division occurring independently of cell division following nuclear DNA replication. MtDNA is a maternally inherited circular double-stranded mtDNA composed of 16.5kb base pairs in humans. MtDNA comprises less than 1% of the total cellular nucleic acid (Cottrell & Turnbull 2000), although despite this mtDNA is essential for the normal survival of the mitochondria and hence the cell. MtDNA encodes 13 polypeptides, 22 tRNAs and 2 rRNAs, all components of the respiratory chain/oxidative phosphorylation system (Anderson *et al.* 1981) (see figure 3). Dysfunctional mtDNA results in an aberrant respiratory chain. At a single cell level, this defective oxidative phosphorylation becomes apparent when the level of mutated mtDNA exceeds 70% of the total mitochondrial population (Cottrell & Turnbull 2000). In older studies, the proposed percentage for the mutated phenotype to emerge is even higher, 85% according to Aspnes *et al.* (1997). MtDNA mutations frequently occur as mtDNA differs from normal DNA in its protective mechanisms. For example, mitochondria lack protective histones and have few and inefficient DNA repair mechanisms. This, in addition with the high rate of mitochondrial turnover and the exposure of mtDNA to free oxygen radicals from the electron transport chain makes the mtDNA more prone to somatic mutations which increase in incidence and accumulate with age (Brierley *et al.* 1997). MtDNA is estimated to be 16-fold more susceptible than nuclear DNA to oxidative modifications (Richter *et al.* 1988). However, mtDNA mutations are generally phenotypically passive, which could be crucial to the conclusions drawn from this study.

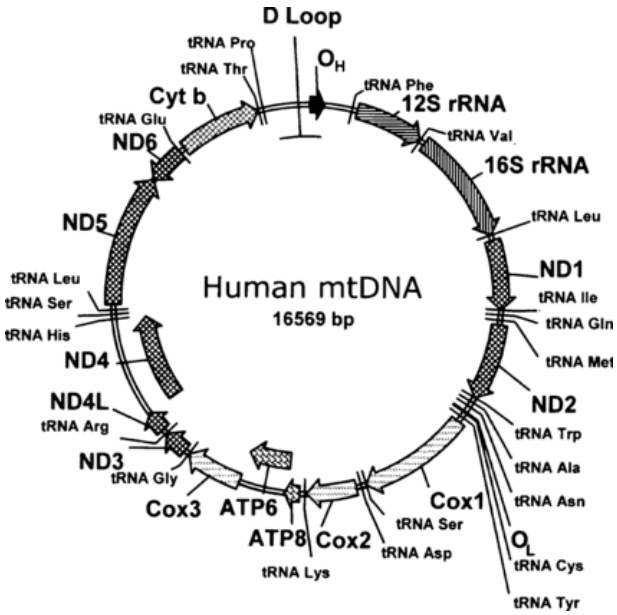


Figure 3. *Map of the mitochondrial genome.*

The electron transport chain, also called the respiratory chain, is an integral part of the inner mitochondrial membrane and consists of ATP synthetase, two mobile electron carriers and four electron-transporting protein complexes designated I-IV (Ritter 1996) (see figure 4). Cells deficient in COX, usually due to a truncating mutation, but with normal succinate dehydrogenase (SDH) activity are excellent biomarkers of mtDNA defects. COX is a large transmembrane protein which constitutes the fourth and last complex and also the second mobile electron carrier

of the respiratory chain. It receives an electron from each of four cytochrome c molecules (cytochromes are heme proteins), and transfers them to one oxygen molecule, converting molecular oxygen into two molecules of water. This redox reaction accounts for a significant portion of the water that is eliminated from the human body each day. In the process, it translocates four protons, helping to establish a chemiosmotic potential that the ATP synthase (seen as the fifth complex) then uses to synthesize ATP. Among the many classified mitochondrial diseases, those involving dysfunctional COX assembly are thought to be the most severe (Pecina *et al.* 2004). Complex IV is designed to prevent partially reduced O_2 from escaping, although some oxygen-containing free radicals do break away. Antioxidants and enzymes play a key role in protecting the body from these reactive agents (Ritter 1996). The three major catalytic subunits of COX (COX I, II and III) are encoded by mtDNA and so COX defiency can be used as a marker for mtDNA mutations. SDH on the other hand is entirely encoded by nuclear DNA and so is independent of mtDNA mutations. SDH is a enzyme functioning at the crossroads of the citric acid cycle (TCA) also called the Krebs cycle and the electron transport chain, and is the only TCA cycle enzyme that is membrane-bound. The enzyme complex is a heterotetramer divided into three domains: SDHA, the catalytic domain; SDHB, the electron transfer subunit; SDHC/SDHD, the dimeric membrane anchor that contains β-type heme (Ritter 1996).

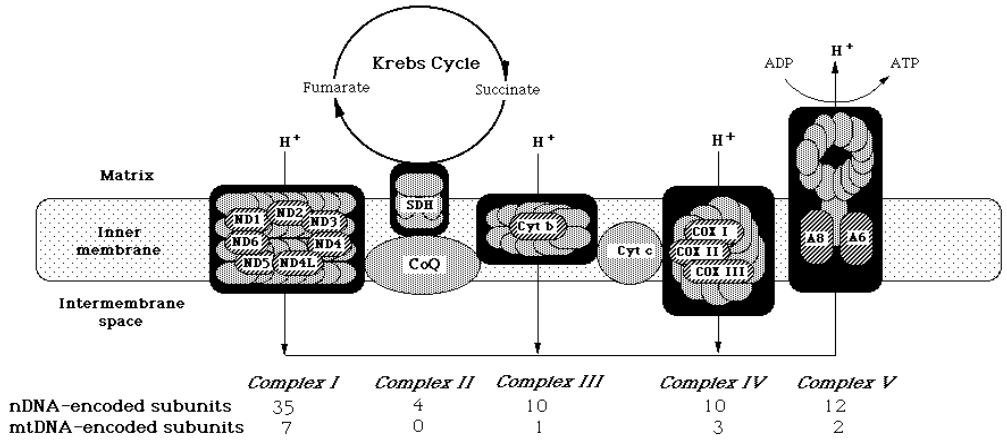


Figure 4*. The respiratory chain, its connection to the TCA and how the subunits are encoded.*

Different studies have shown that a characteristic feature of ageing and mtDNA disease is the presence of COX deficient cells in tissues, such as skeletal and cardiac muscle (Müller-Hoecker 1989, Müller-Hoecker *et al.* 1989, Müller-Hoecker *et al.* 1992, Brierley *et al.* 1997, Aspnes *et al.* 1997, Cottrell & Turnbull 2000).

If COX activity is absent, SDH activity should remain as both complexes are encoded by different DNA i.e. mitochondrial and nuclear DNA, respectively. It is possible to utilise this fact to detect cells that have a mutation in COX. In a dual COX/SDH assay, sections are incubated first in the COX-detecting medium and any cells that fail to become saturated with the brown reaction product, i.e. COX deficient cells, are turned blue by subsequent incubation in the SDH-detecting

medium. This method facilitates the visualisation of COX negative cells (for more information see 'materials and methods').

1.8 MtDNA mutations in the human colon

MtDNA mutations have been extensively studied in the human colon. Taylor *et al*. (2003), studied mtDNA mutations in COX deficient cells. The three major catalytic subunits of COX are encoded by mtDNA as mentioned above, so therefore COX deficiency can be used as a marker of mtDNA mutations (Taylor *et al.* 2003). The results of this study showed that human colonic crypts have a mixed phenotype. Ribbons of cells were observed to extend the entire length of the crypt from the site of the SCs in the crypt base, up to the intestinal lumen (see figure 5) (Taylor *et al.* 2003). Ribbons of COX-ve epithelial cells were frequently accompanied by ribbons of cells with a wild type phenotype i.e. no mutation in COX. Therefore, in this instance in each crypt there are at least two SCs, one with a COX deficiency due to mtDNA mutation, and the other expressing COX normally. A further study investigating mtDNA mutations in human colonic crypts was carried out by Greaves *et al*. (2006). In this study the sequences of the mitochondrial genome of COX-ve and COX-positive (COX+ve) cells were deduced, following laser microdissection of individual cells within patches of either phenotype. This was to identify whether COX-ve cells contained an identical mtDNA mutation, and were thus clonal in origin. Sequencing of these cells showed that the COX-ve cells within a single patch did indeed display identical mutations and are thus daughter progeny, derived from a clonally expanding SC (Greaves *et al.* 2006). Furthermore, it was demonstrated that COX-ve cells within neighbouring crypts expressed identical mtDNA mutations. The observation that identical mtDNA mutations are present in both arms of a bifurcating crypt confirmed that *crypt fission* is the process by which colonic crypts divide and spread (McDonald *et al.* 2006 [b], Greaves *et al.* 2006). The techniques used in this study can be used to investigate cellular behaviour in other tissues such as the human pancreas.

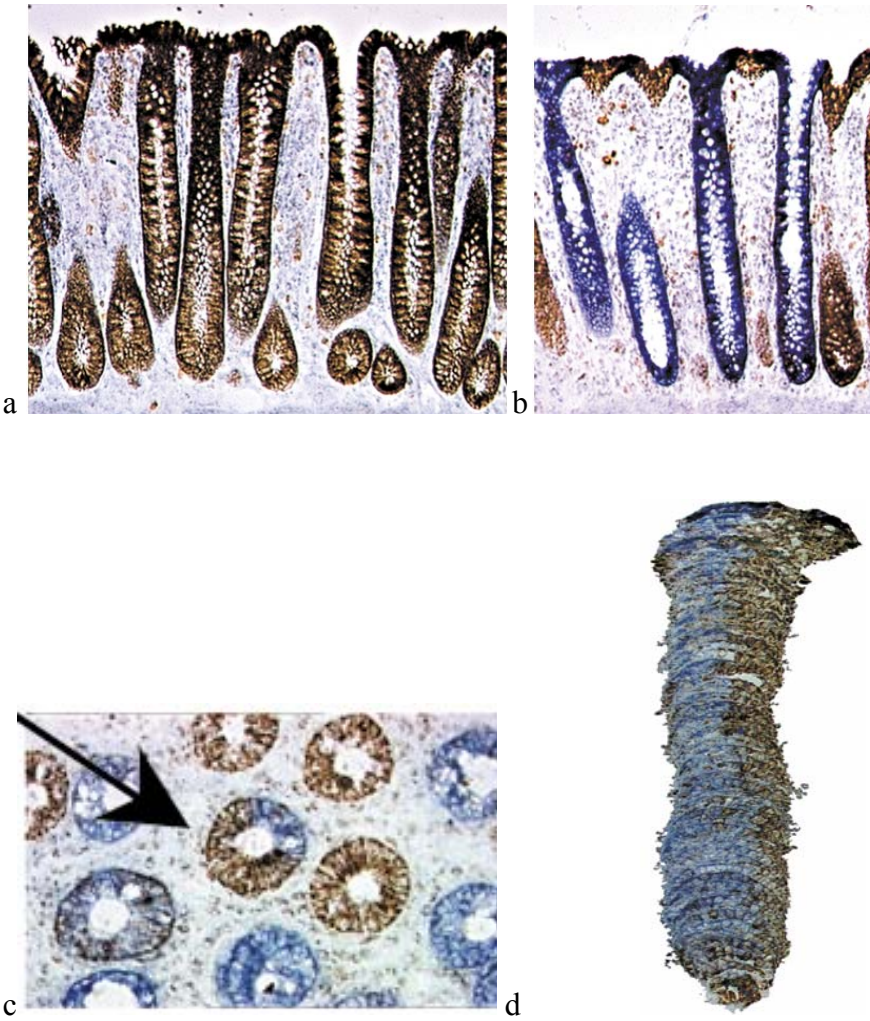


***Figure 5**. From Taylor et al 2006. (a) Normal COX activity (brown) in colonic crypts following a dual COX and SDH histochemistry. (b) COX deficient colonic crypts (blue) among crypts with normal COX activity (brown). (c) Partial COX deficiency in colonic crypt (arrow) following a dual COX and SDH histochemistry. (d) 3D representation of 59 serial sections, showing ribbons of cells that are derived from either a COX+ve SC (brown) or COX-ve SC (blue)*

2. HYPOTHESIS

In order to explore the possibility of a stem lineage within the human pancreas we will, in this study, take advantage of mutations that occur within the mitochondrial genome. If long-lived stem cells exist they will acquire a sufficient degree of mutated mtDNA to be recognised morphologically. If such cells produce a large family of progeny the resultant clone should be identifiable by the proposed technologies.

3. MATERIALS AND METHODS

3.1 Ethical approval to use human tissue

Ethical permission was granted from the Redbridge and Waltham Forest Local REC on 9th May 2006 to use human tissue from the Royal London Hospital REC. Permission was extended on 15th January 2007 to use tissue from the Hammersmith Hospital also (Hammersmith & Queen Charlotte's & Chelsea REC).

3.2 Immunohistochemistry

Immunohistochemistry is the only technique which can identify an antigen in its tissue or cellular location i.e. *in situ*. The definition of immunohistochemistry is the use of a labelled, and thus identifiable, antibody as a specific reagent to localise with high affinity and avidity to a specific tissue constituent (antigen) *in situ* (Polak & Van Norden 1997).

Immunohistochemistry using antibodies that specifically detected COX subunits was applied in this study to visualise patches of COX-ve cells within serial sections of normal human pancreas. Immunohistochemistry was also used to detect the expression of various antigens by COX-ve cells, to ensure that these cells were otherwise phenotypically normal. For all immunohistochemical protocols, appropriate positive and negative controls were used. Prior to routine use of any newly acquired antiserum, a serial dilution was tested on positive control sections of fixed tissue in order to establish optimal dilution for each antibody. Normal sera and secondary antibodies were used at the highest concentration recommended by the manufacturer. To eliminate background staining, the primary antibody was further diluted, or if unsuccessful, a serial dilution series of the secondary antibody was performed.

3.2.1 Tissue embedding and sectioning

Formalin-fixed, paraffin-embedded tissue blocks were obtained from the archives of the Royal London Hospital and Hammersmith Hospital. Embedded tissue blocks were sectioned at 4μm using a Leica sledge microtome, and then placed on a 57°C water bath to remove creases. Tissue sections were placed on glass microscope slides (Thermo Shandon COLORFROST®) and dried overnight at 37°C and then kept in sealed slide boxes. Tissue sections were cut by the Histopathology service, ICMS.

3.2.2 Dewaxing and blocking of endogenous peroxidases- paraffin sections

Tissue sections were deparaffinised/dewaxed by a 6 minute immersion in sulphur free xylene (BDH) followed by a 2-minute immersion in absolute ethanol and 1-minute in 70% ethanol. This was followed by a 15 minute immersion in 30% hydrogen peroxide (BDH) in methanol to block for endogenous peroxidases. Tissue sections were re-hydrated by 2 minute incubations in decreasing ethanol concentrations (100%, 100%, 95%, 70%) then rinsed in cold tap water and washed in PBS.

3.2.3 Immunohistochemical staining protocol

It is vital to ensure that tissue sections do not become dry during immunohistochemistry. Antigen retrieval was performed by microwave treatment in preheated sodium citrate buffer (10 mmol/L; pH 6, Fisher Chemicals) for 20 minutes at 700W, this is to destroy the cross-links (hydroxy-methylene bridges) that form during formalin fixture of tissues (34). Sections were then rinsed in tap water, and washed in PBS. Slides were then put into sequenzers (Shandon Sequenza, Thermo Electron Corporation) and rinsed with PBS (Sigma) containing 0.01% Tween-20. A mouse monoclonal antibody specific to human COX complex IV subunit I (Invitrogen Molecular Probes, USA) was used in combination with a staining kit, Super Sensitive Polymer-HRP Detection System (BioGenex, USA). 'Power block' from the kit was diluted 1 in 10 in H_2O and applied to slides for 10 minutes and then the COX antibody solution was added, diluted in PBS at a 1:500 concentration. Thereafter, the rest of the kit was applied in accordance with the manufacturer's recommendations (Super Enhancer solution for 20 minutes, followed by PBS rinse, HRP for 30 minutes, rinse with PBS, then finally an application of DAB chromogen solution to develop staining, followed by a final rinse in tap water).

Table 1*. Range of antibodies that were used in combination with the kit. The last four antibodies were stained courtesy to Prof Turnbull and Dr Ziabrevain, University of Newcastle.*

Antibody	Specificity	Species	Dilution	Source
COX IV subunit I	Human, rat, bovine, mouse	Mouse monoclonal IgG	1:500	Invitrogen
Ki-67	Human	Mouse monoclonal IgG	1:50	Novacastra
Porin	Human	Mouse monoclonal IgG	1:400	Invitrogen
COX I	Human	Mouse monoclonal IgG	1:100	Invitrogen
COX II-30 kDa	Human,	Mouse monoclonal IgG	1:200	Invitrogen
COX II-70 kDa	Human	Mouse monoclonal IgG	1:100	Invitrogen
COX IV	Human	Mouse monoclonal IgG	1:200	Invitrogen

3.2.4 Counterstaining protocol

Sections were counterstained with Coles haematoxylin (Pioneer Research Chemicals) for 1 minute after a bath in Copper (II) sulphate for 2 minutes (VWR International Ltd.). Then slides were rinsed thoroughly in cold tap water before immersion in acid alcohol (1% v/v concentrated HCl in 70% ethanol) for 10 seconds to get rid of excess haematoxylin. This was followed by immersion in Scott's tap water (30mM $LiCO_3$, 160 mM $MgSO_4$) for 20 seconds to fix the staining before rinsing in cold water for a final time. Sections were then rapidly rehydrated using graded ethanol (70% 15 sec, 93% 30 sec, 100% 30 sec, 100% 30 sec) before being immersed in xylene (BHD), finally sections were mounted using Permafluor mounting medium (Thermo Electron Corporation).

3.2.5 Serial Sectioning and Immunohistochemistry

Initial staining was performed on 30 blocks of normal human pancreas, and those containing patches of COX-ve cells were selected for further analyses (n=5). From each block, 50 x 4µm serial sections were cut. Immunohistochemistry for COX was performed on each section to allow visualisation of COX-ve patches of cells. This technique permitted us to envisage patch size, shape, phenotype of cells within the patch, possible direction of flux and interaction with morphological structures within the pancreas i.e. do patches of cells appear to emerge from or interact with the pancreatic ducts?

3.3 Immunofluorescence staining

3.3.1 Immunofluorescent double label staining – paraffin sections

As initial staining for COX revealed that the majority of COX-ve cells formed patches within the exocrine pancreas, further staining was performed where COX immunoreactivity was combined with amylase antigen expression, both of which were detected using fluorescence immunohistochemistry. For this technique, deparaffinisation of tissue sections and microwave antigen retrieval were performed as above. Non-specific antibody binding was minimized using normal swine serum (DAKO) in a 1:25 dilution for 15 minutes. The antibodies were applied in the following sequence, first primary; first secondary; second primary; second secondary (refer to tables 1 and 2). All antibodies were diluted in PBS pH 7.4 and incubated for 1 hour at room temperature, with 3 x 5 minute washings in PBS between each layer of antibody. A second block for non-specific antibody binding was done after the incubation with the first secondary antibody. After the final wash, the slides were mounted under glass coverslips using Vectashield Hardset with DAPI mounting medium (Vector Laboratories).

3.3.2 Immunofluorescent double labelling staining – frozen sections

The frozen sections were removed from the freezer and allowed to dry completely before staining. Antigen retrieval was performed by immersing slides in cooled ethanol 100% for 20 minutes. Slides were then washed in cold PBS for 5 minutes before washing in PBS at room temperature and blocking for non-specific binding using protein block (Dako Cytomatikon, Denmark). The antibodies were applied in the following sequence, first primary; first secondary; second primary; second secondary (refer to table 1 and table 2). All antibodies were diluted in PBS pH 7.4 and incubated for 1 hour at room temperature, with 3 x 5 minutes washing in PBS

between each layer of antibody. A second block for non-specific antibody binding was done after the incubation with the first secondary. After the final wash, the slides were mounted under glass coverslips using Vectashield Hardset with DAPI mounting medium (Vector Laboratories).

Table 1. *Primary antibodies used in the immunofluorescence protocols*

Antibody	Specificity	Species	Dilution	Source
Anti-Cox	Human	Mouse monoclonal IgG	1:500	Invitrogen
Amylase	Human	Rabbit monoclonal IgG	1:50	Sigma

Table 2. *Secondary antibodies used in the immunofluorescence protocols*

	Antibody	Fluorescence excitation/emission	Source
First secondary	488 conjugated goat anti-mouse IgG	FITC	Alexa fluor
Second secondary	555 conjugated goat anti-rabbit IgG	Cy 3	Alexa fluor

As the fluorescent stains are light sensitive the slides were kept in the dark during the process after adding the first secondary antibody to prevent bleaching of fluorophores.

3.4 Principles of enzyme histochemistry

It is not possible to isolate cells with mutant DNA from immunostained paraffin pancreas sections, thus another technique is required to detect COX activity in frozen pancreas. Succinate dehydrogenase (SDH) is entirely encoded by nuclear DNA, therefore independent of mtDNA mutations. In the dual COX/SDH double stain, when COX activity detected as a brown colour is absent nuclear SDH activity remains, staining blue (McDonald *et al.* 2006 [a]). This is another method of visualising COX-ve cells in frozen tissue allowing subsequent laser capture microdissection of individual cells. This method uses diaminobenzidine tetrahydrochloride (DAB) as an electron donor in the respiratory chain at the level of cytochrome *c,* which is then subsequently reduced by COX. Oxidation of DAB generates an insoluble indamine polymer. This reaction can only take place if COX is present to oxidise cytochrome *c* allowing it to accept electrons from (DAB). The SDH medium uses NitroBlue tetrazolium (NBT) as an electron acceptor in the electron transport chain at the level of SDH. When it is reduced, NBT creates an insoluble blue formazan. Phenazine methosulphate (PMS) is required to act as an electron carrier between SDH and NBT, thus acting as a catalyst and accelerating the rate of reaction (Mc Donald et al. 2006 a).

3.4.1 COX/SDH histochemistry staining protocol

Serial sections of 20µm thick frozen human pancreas were cut and mounted on polyethylenenaphthalate slides (Leica Microsystems UK). Sections were first

incubated in COX incubation medium (100μM cytochrome *c*, 4mM DAB, 20μg/ml catalase in 0.2M phosphate buffer pH 7.4) for 50 min. Sections then underwent a 3 x 5 minute wash in PBS followed by incubation in SDH medium (130mM sodium succinate, 200μM PMS, 1mM sodium azide, 1.5mM NBT in 0.2M phosphate buffer pH 7.4) for a further 45 minutes. This was followed by a further 3 x 5 min wash in PBS followed by dehydration in graded ethanols (70%, 95%, 100%) for 2 minutes each, followed by a final 100% ethanol for 10 minutes. Sections were cleared in 2 changes of Histoclear (National diagnostics, USA) then air-dried for 1 hour. All washes were in PBS pH 7.4 and incubations at 37^{o}C.

3.5 Visualization of slides

Tissue sections stained using DAB were visualized using a Nikon Eclipse E600 multi-headed microscope. Fluorescently stained slides were viewed using an Olympus BX61 fluorescence microscope/ Leica DM5000 fluorescence microscope (Leica Microsystems UK). Fluorescent images were captured using Elpac QImaging MicroPublisher 5.0. All images were captured at 72 pixels/inch using the image analysis software 'Qwin'. Imaging processing was performed using Adobe Photoshop software.

4. RESULTS

4.1 Results of COX immunohistochemistry

Immunohistochemistry for COX complex IV subunit I was initially performed on several tissue blocks (n=30) to identify COX deficient cells in the normal human pancreas. Based upon this 5 blocks were then selected which revealed the presence of COX-ve patches distributed throughout normal pancreas (see figure 6 a-c). Interestingly these patches were often in close contact with the epithelial ducts (see figure 6 a and c). Staining revealed that in most patches there are a small number of COX+ve cells dispersed throughout the mutant patches (see figure 6 e). It is possible that these cells are a distinct lineage from those COX-ve cells in the patch e.g. stellate cells, and thus are of a different clonal origin. Also we could see some cells with very high COX expression (see figure 6 d), these generally occurred throughout the exocrine pancreas, outwith the COX-ve patches and occurred in small clusters.

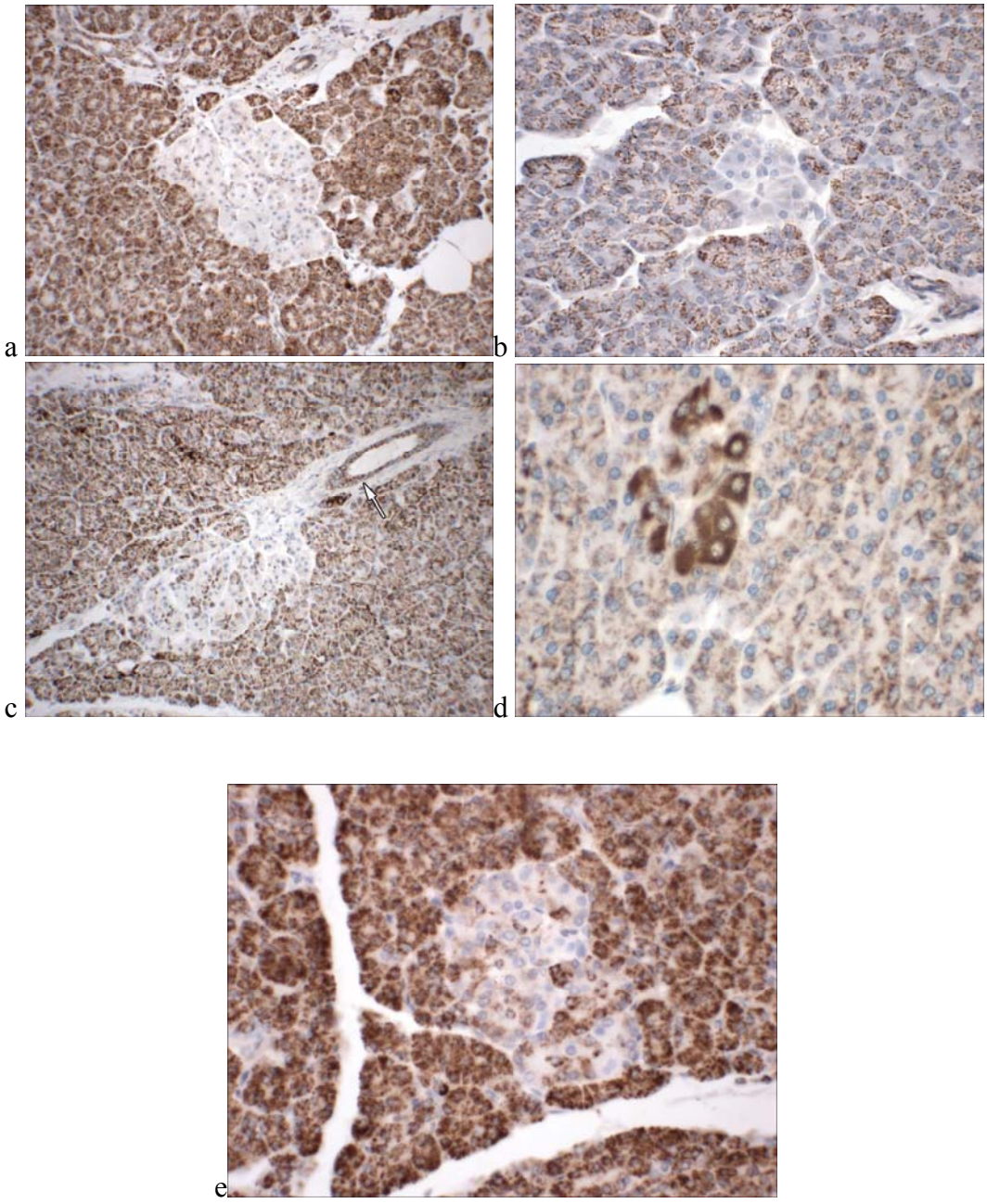


Figure 6. *Photomicrographs of immunohistochemistry for COX Complex IV subunit I in normal pancreas. (a-c) show patches of mutated cells in the exocrine pancreas and often near ducts (arrow, c). (d) Some cells have very high COX expression. (e) Shows the infiltration of COX+ve cells in a negative patch.*

4.2 Results of COX immunohistochemistry on serial sections of human pancreas.

Immunohistochemistry for COX complex IV subunit I was performed on 50 serial sections of human pancreata (n=5: aged 21, 21, 33, 34, 72) that had previously been selected for the presence of patches to observe size, shape and migration of the COX-ve colonies through the tissue. Staining revealed that the size and shape of the patches varied considerably (see figure 7 and 8). Movement of the patch could be seen and was followed throughout the series. Most patches was present for about 20 serial sections and did change shape, perhaps reflecting the sectioning through a more or less spheroid patch. Each patch was composed of numerous acini and individual acini that were only partially affected were never observed. Often patches appeared to be infiltrated by whole acini that were homozygous for COX expression.

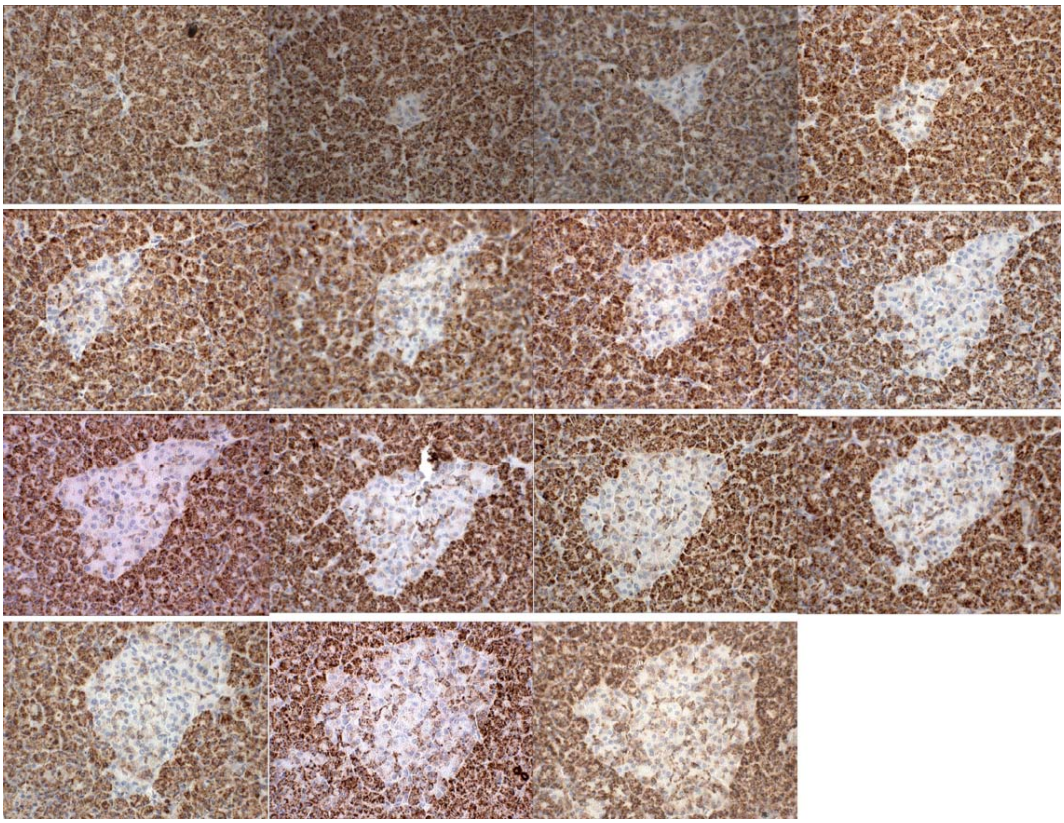


Figure 7. *Photomicrographs of immunohistochemistry for COX complex IV subunit I on 50 serial sections of human pancreas. Here a patch is followed for 18 serial sections.*

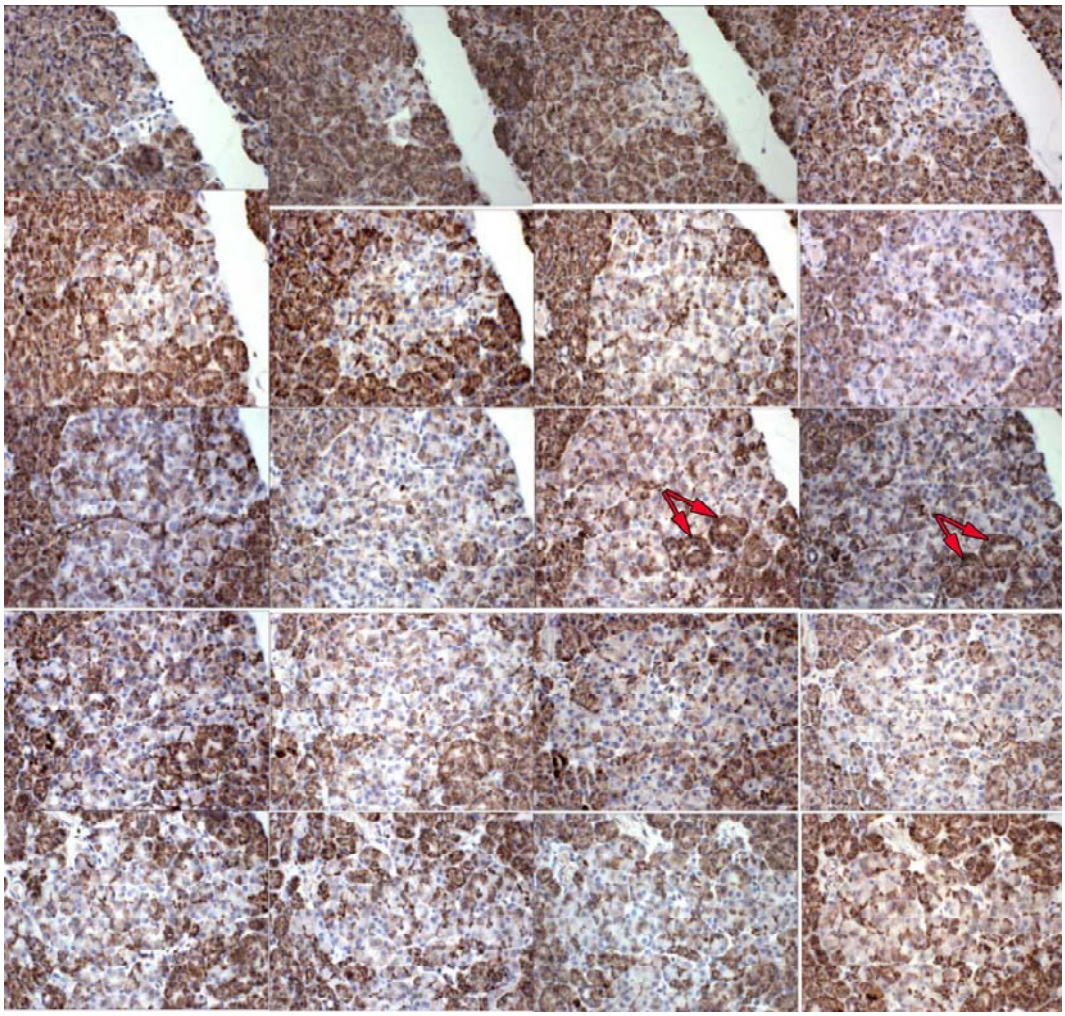


Figure 8. *Photomicrographs of immunohistochemistry for COX complex IV subunit I on 50 serial sections of human pancreas. Here a patch is followed for 20 serial sections. Arrows show presence of whole acini that are COX+ve.*

4.3 Results of immunohistochemistry for Ki-67

Immunohistochemistry for Ki-67, a cellular marker of cell proliferation, was performed on human pancreas sections. A COX-ve patch was identified, then on the following sequential section, the same tissue was stained for Ki-67. The COX-ve patch stained negative for Ki-67, suggesting that these COX-ve patches are non hyperplastic.

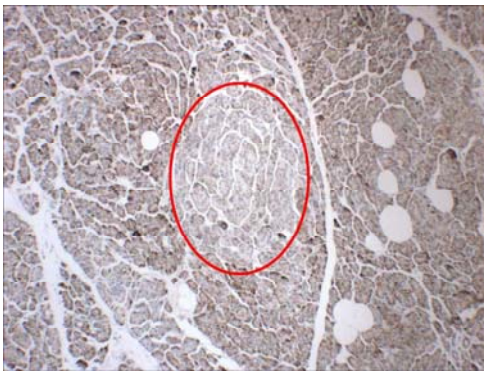
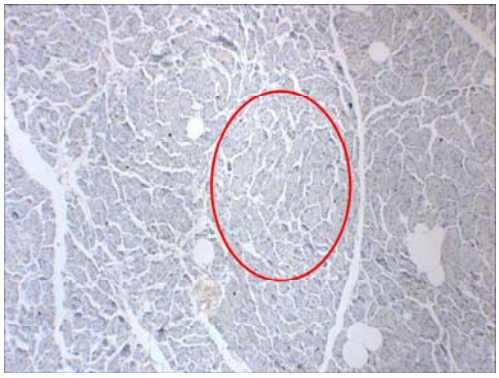
a  b 

Figure 9. *Immunohistochemistry for Ki-67 (a) Stained for COX and 9b) stained for Ki-67. No sign of over-expression of Ki-67 was seen in the COX-ve patch (circled).*

4.4 Results of immunofluorescent histochemistry

An immunofluorescent dual stain was performed on human pancreas sections for COX/amylase to test if patches of COX mutated cells are otherwise normal i.e. produce amylase. Normal pancreas was stained as a control and can be seen in figure 10. We observed patches that produced less amylase, which therefore implied that the synthetic function was reduced in the COX-ve cells making up a patch (see figure 11 and 12). However, interestingly we also found patches that were a mixture, containing cells that were COX-ve but produced amylase to the same degree as the surrounding COX+ve cells and one part where there was reduced for amylase expression (see figure 13). When viewed using the aqua-channel of the microscope, the cell morphology of the patches is not different to that of the surrounding cells (see figure 14) also confirming that the patches are not an artefact of the staining protocol as they cannot be viewed at other wavelengths. We also observed strongly immunoreactive cells by this method (see figure 15), interestingly these do not express amylase. Another interesting finding was that the ducts were COX+ve, COX-ve or a mixture of both (see figure 16).

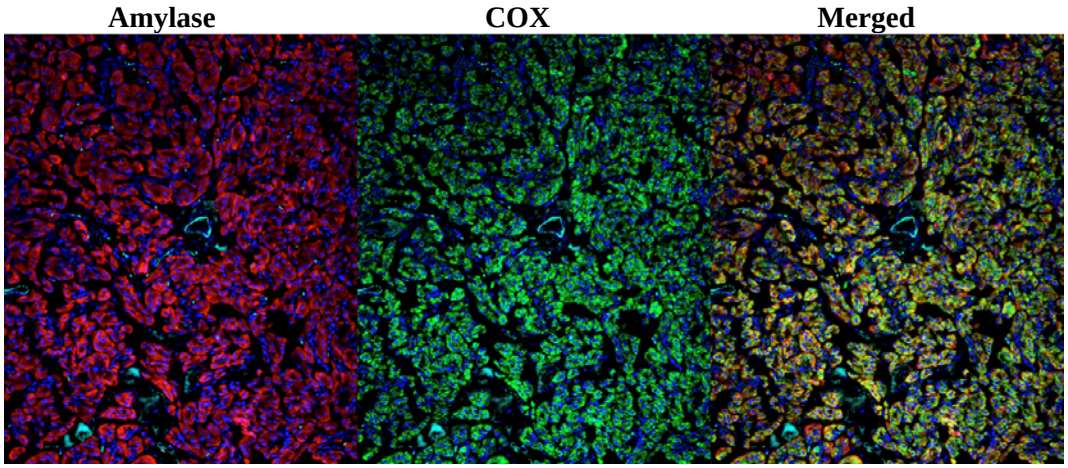


Figure 10. *Dual immunohistochemistry staining of normal pancreas. Block where no patches could be seen.*

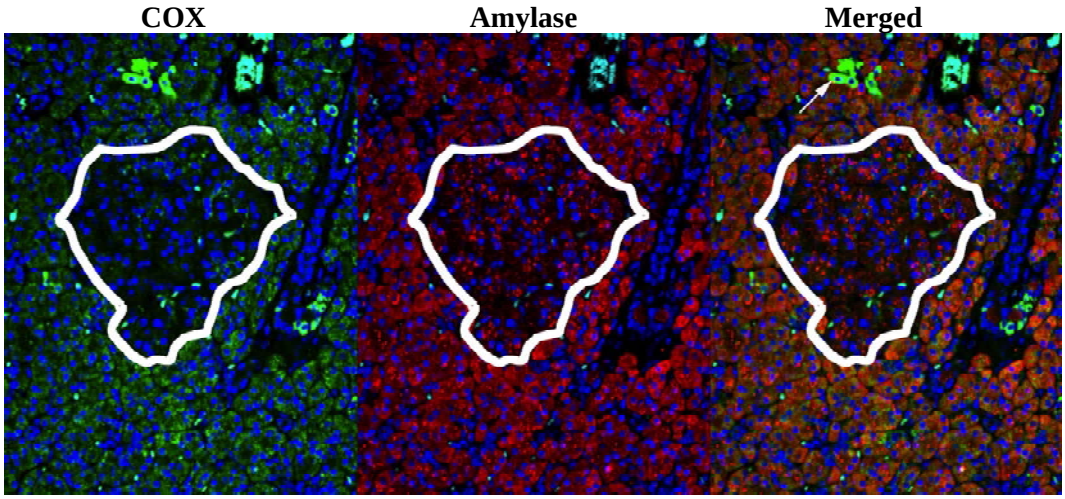


Figure 11. *Dual immunohistochemistry staining of normal pancreas. Green shows COX expression and the loss of it in the patch, red shows amylase expression which is reduced in the area of the COX-ve patch. Also one cluster of strong COX+ve cells can be seen (arrow). Due to DAPI in the mounting media the nuclei stain blue.*

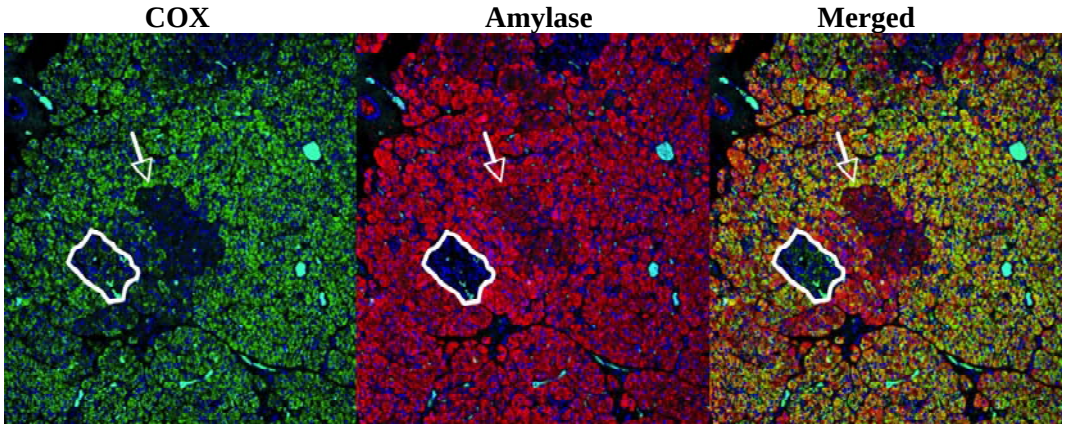


Figure 12. *Dual immunohistochemistry staining of normal pancreas. Patch with reduced amylase expression (arrow). Of interest, this patch is in close proximity to an islet (circled), which produces COX, but not amylase, as expected.*

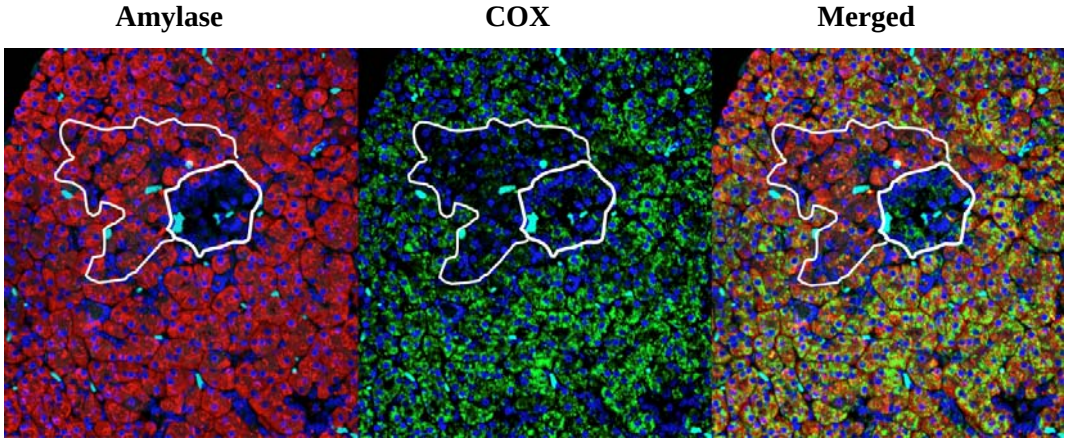


Figure 13. *Dual immunohistochemistry staining of normal pancreas. COX-ve patch with mixed amylase expression.*

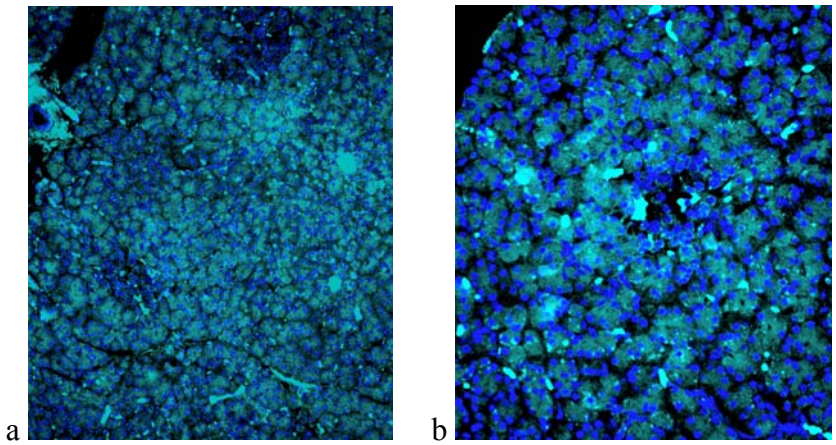


Figure 14. *Cell morphology viewed using the aqua channel of the fluorescent microscope. (a) With the patch with reduced amylase expression in figure 11 (b) with the mixed patch shown in figure 12.*

Amylase *COX* *Merged*

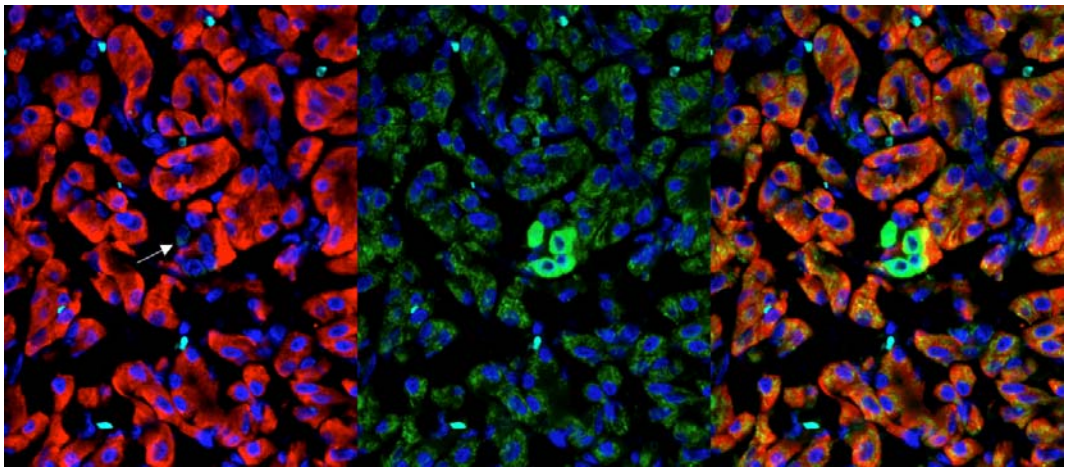


Figure 15. *Cells expressing COX very strongly in normal pancreas, although interestingly these do not express amylase (arrow).*

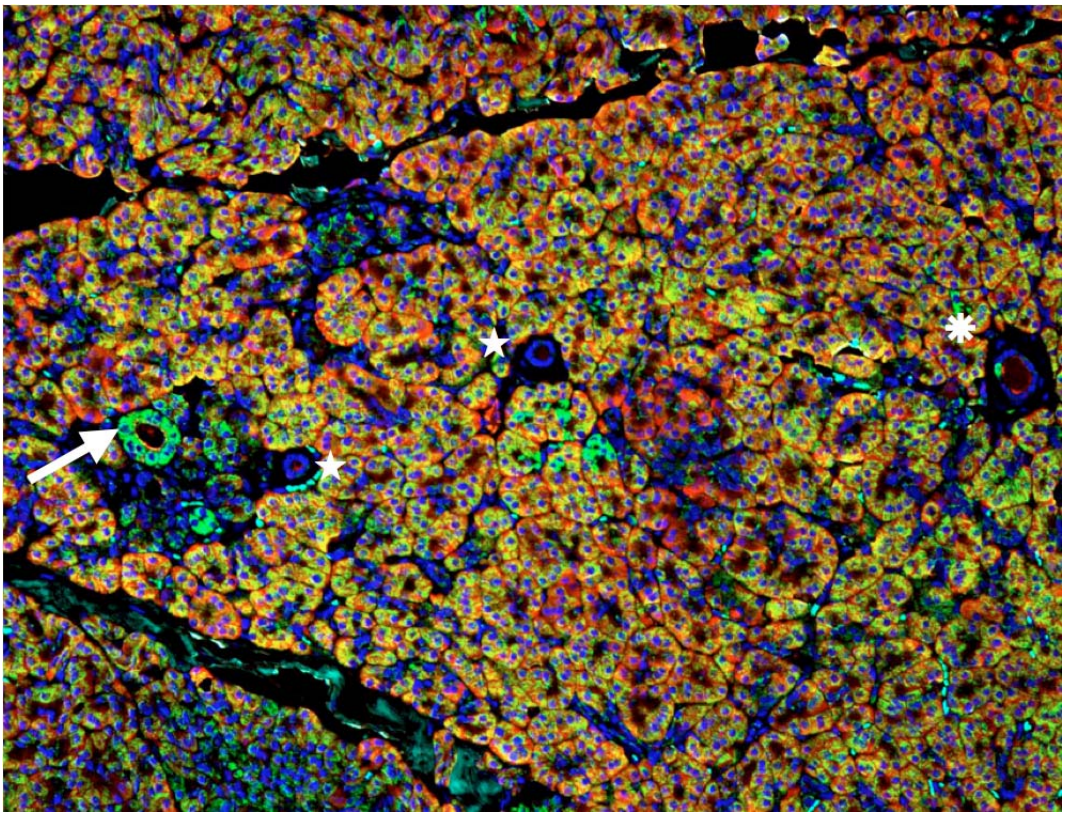


Figure 16. *Dual immunohistochemistry staining. Ducts are COX+ve (arrow) COX-ve (stars) or mixed (✱).*

4.4 Results for immunohistochemistry for Porin

The porin staining showed us that the mitochondrial density of the acinar cells in the patches was noticeably lower than in normal pancreatic acinar cells (see figure 17).

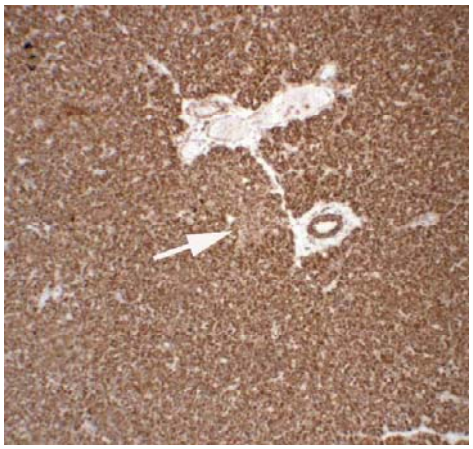


Figure 17. *Normal density of mitochondria in comparison to the patch (arrow) where less immunoreactivity with the anti-Porin antibody suggests fewer mitochondria.*

4.5 Results for immunohistochemistry for COX subunit 1, COX subunit IV and Complex II 30 kDa and Complex II 70 kDa

As expected/predicted the staining for COX complex I and complex IV showed us the same patches that we had previously detected when using another antibody for the COX complex IV (see figure 18 a-b). But also a patch could be seen when staining for Complex II of the respiratory chain, the complex that is totally encoded by nuclear DNA (see figure 18 c-d)

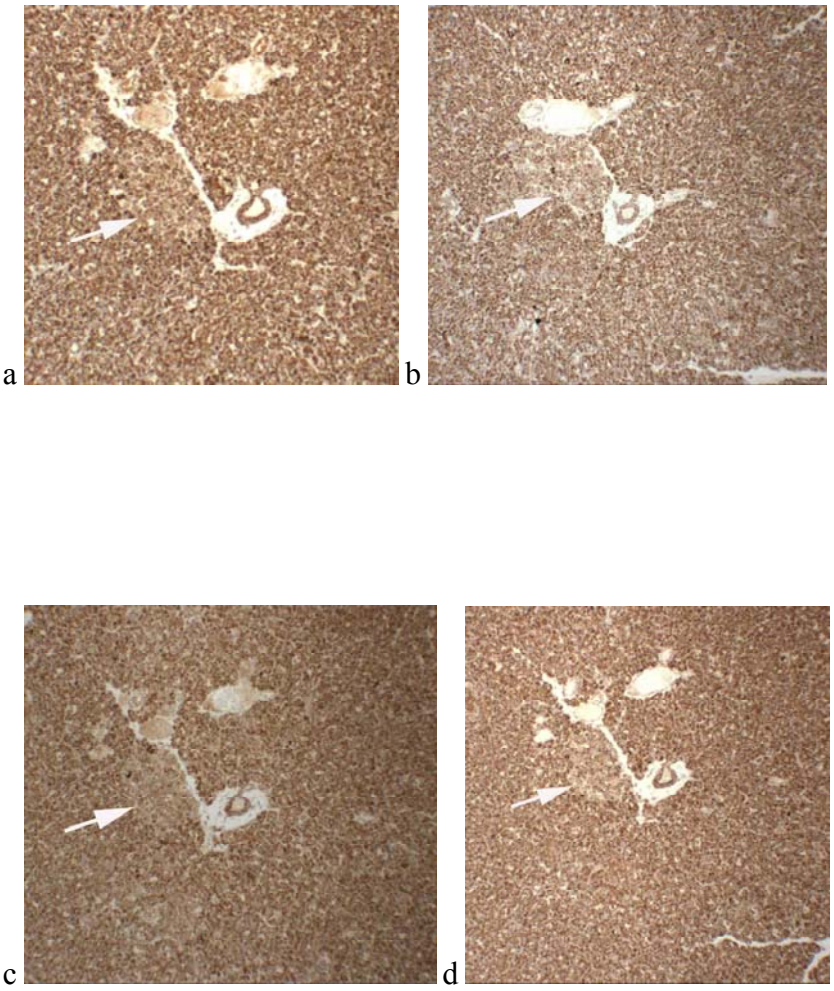


Figure 18. *Immunostaining for different parts of the respiratory chain in serial sections of the same block that is stained in Figure 18. Arrows show that the patches are clearly present for the; (a) COX subunit I (b) COX subunit IV (c) complex II 30 kDa (d) complex II 70 kDa.*

4.6 Results of enzyme histochemistry

An excellent way to show mtDNA defects is to double stain for COX and SDH. Cells that are deficient in COX will still stain positively for SDH. To ensure histochemical staining was specific, control experiments were performed on frozen pancreas sections, using specific mitochondrial enzyme inhibitors. To inhibit COX, sodium azide was used and sodium malonate was used to inhibit SDH. This was made as control experiment to confirm that the reaction is specific for COX activity and that the negative staining truly does reflect a decrease in enzyme activity (see figure 19).

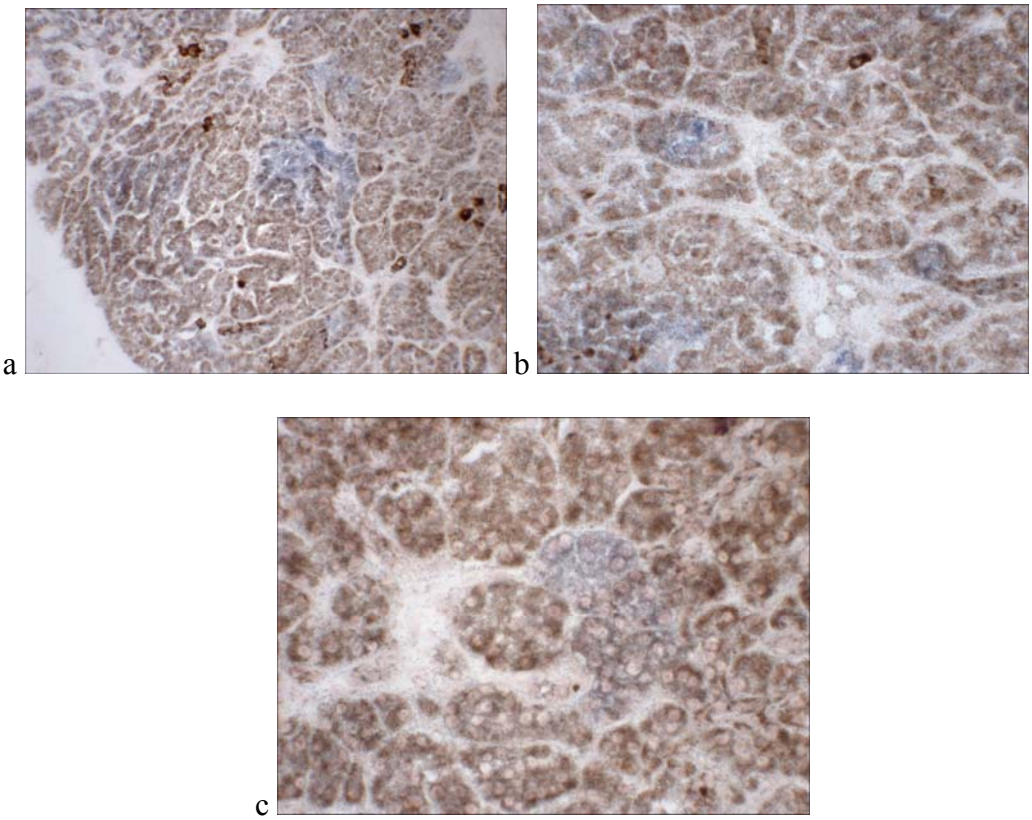


Figure 19. *Photomicrograph of dual COX/SDH histochemistry on human pancreas.*
(a-c) Cells stained blue are COX deficient and have therefore become stained with SDH

5. DISCUSSION

SC are multipotent cells with the capacity for long-term self-renewal and multilineage differentiation and therefore they represent a great source for replacement of dysfunctional cells in many diseases; such as β-cells in the pancreas for diabetes. However, due to the lack of specific surface markers required to isolate these tissue-specific SC cells, their recognition has been limited to a few organ systems (Suetsugu *et al.* 2006). According to two new studies that were published recently, there are no SCs in the pancreas. Both of them use methods to mark cells and then follow how the marker spreads/dilutes in the renewed tissue. Teta *et al.* (2007) used double labeling *via* DNA substrates to try and show cell lineages descending from SCs. Brennand *et al.* (2007) looked at early postnatal growth using two different approaches to labelling: a histone protein that was incorporated into many β-cells and another one that only labelled a few cells. Their conclusion was that neither could show signs that progenitor cells do exist. Both studies came to the conclusion that all β-cells are equally competent in proliferating. In this study we used human adult pancreata in order to investigate whether we could follow cell lineage progression *via* mtDNA mutations that occur naturally in our cells as we age. We used COX deficiency as our marker of mtDNA mutations as this is readily detected histologically, and has been used with great success previously in the human colon (Taylor *et al.* 2003, Greaves *et al.* 2006). We showed patches of COX deficient cells in the pancreas using the same method that Greaves *et al.* (2006) used to show the existence of SCs in the colon. Interestingly, though we didn't see the same need for a certain level of age of a patient for these mutations to become detectable. It is known that a certain percentage of the mitochondria in one cell has to be mutated in order for it to be detected phenotypically. Taylor *et al.* (2003), demonstrated that human colonic crypts can be a mixture of COX+ve and COX-ve cells. Greaves et al. 2006 claimed that COX mutations only reached a detectable level of heteroplasmy in the colon in patient greater than 40 years of age. The tissues that we chose blindly, according to the presence of patches were actually from patients between 21 and 72 years of age. From the initial results of the immunohistochemical analyses it appeared that clonal division from a SC was very likely to be the cause of the appearance of COX-ve patches, as they showed the same phenotype, not just for mutations in the COX complex but also for mutations in the second complex of the respiratory chain. The immunohistochemistry showed COX+ve cells within the negative patches. The reason for these could be that they either express the wildtype COX complex or that they are heterogeneous, containing both wild type and COX-ve mitochondria although at a level that is too low to be detectable by this method. Unfortunately in the present study we were not able to complete the sequencing of the mtDNA to prove the clonal origins of these cells in the patches but this will be done in further work.

It was vital to test that COX-ve cells were functionally normal. Therefore we investigated whether COX-ve pancreatic cells were functionally similar to their wild type counterparts. Ki-67 is a prognostic marker for some human cancers as it can be detected in nuclei undergoing interphase i.e. is present during all active phases of the cell cycle (G1, S, G2) but absent during cell arrest (G0). We showed Ki-67 was not present in an increased number of COX-ve cells compared to COX+ve cells, and therefore we can say that they were not proliferating abnormally.

Our staining to show the normal synthetic function of the COX-ve cells didn't give us the results we had expected. However they are still very interesting and have given, rise to several further studies, outlined below. We showed that COX-ve cells in the exocrine pancreas produce variable amounts of amylase and thus may have reduced enzyme production and function. Slides are currently being stained to see if trypsinogen production is affected in these cells, another measure of pancreatic exocrine cell function. We next stained for mitochondrial loading, i.e. using an antibody for mitochondrial porin (VDAC). Porins are proteins which cross a cellular membrane and act as a pore through which molecules can diffuse. They are prevalent in the outer membrane of the mitochondria and Gram-negative bacteria. In the same series we stained with antibodies for complex II, the so-called SDH complex in the respiratory chain. The results from this showed us that not only the COX complex, but also the SDH complex was affected. This was not expected as all subunits in that complex are encoded for by nuclear DNA and therefore shouldn't be affected by mtDNA mutations. If this shown pattern is due to less mitochondrial loading or due to mutations in the DNA (either mtDNA or nuclear DNA) coding for the complexes, will be shown by the sequencing.

5.1 Conclusion

Using mtDNA mutations to track cells with related progeny it was possible to identify clonal units within the human pancreas. We observed clusters of cells with the same phenotype, i.e. altered COX expression. This is strong evidence that these patches of cells are a result of proliferation from a common progenitor. Our results showed that the COX-ve cells in these patches had abnormal function in their reduced production of enzymes, although as these cells are the same phenotype, this in fact supports our theory that COX-ve patches are clonal proliferation. This theory would be further supported by sequencing the mtDNA from multiple cells within a COX-ve patch, and showing that each cell has an identical mutation. When we apply this to the *odds ratio* theory, it would be virtually impossible for these cells to have the same mutation without being related. Due to time constraints, I was unable to complete this sequencing work, although it is currently underway in the lab.

Further work within this field could lead to a useful means of studying pancreatic diseases e.g. changes in cell proliferation, migration of cancer, and identification of the site of origin of a clone of mutated cells.

5.2 Further studies

Sequencing will be done to see if clonality is the cause of these cells having the same COX expression. The case of two cells having the same mutation at the same position by chance or by a random event is very small and as said in *Greaves et al.* (2006), this fact can be demonstrated *via* calculating odds ratio for this event to occur. This is done by taking the amount of base pairs multiplied times 3 possible transition mutations. As DNA is double-stranded it has to be calculated for two strands and that gives us the following set up: (16,600 x 3) x

(16,600 x 3) making 2.48 x 10^9:1 being the odds ratio. This calculation does not take into account mutations that are due to insertion or deletion as it is not possible using a set factor for these events to occur.

3D modelling, as described by Taylor *et al.* (2003) in the human colon, will be continued in the lab so that the shape and movement as well as the interaction of the patch with surrounding structures can be visualized in the pancreas. Studies will continue to look at the strong COX+ve cells, e.g. possible endocrine progenitor cell function. As seen in figure 16 these cells don't produce amylase, although resemble acinar cells morphologically. So it would be very interesting to see what their synthetic production is, e.g. if they express insulin.

Pancreatic carcinoma has a large mortality rate, thus if a putative SC population can be identified in the pancreas, it will allow novel and specific therapeutic targeting as well as provide an alternative source of functional and viable β-cells for diabetic patients, thus helping millions of people worldwide. The techniques used in this study were proposed to work in any tissues that contain mitochondria, to show a novel way of tracing cells of related origin. This study didn't give us the expected results, but if the continued work with sequencing shows that the cells are clonal then maybe this staining might be a way of showing cells in a pre-pathological state and might be used as marker for pre cancerous lesions.

5.3 Limitations

As work was performed on human tissues ethical approval was required to use diagnostic blocks. Therefore we were limited in the amount of sections we could get from each block. Some of the tissues that contained patches we were unable to get more sections from to continue our work. Ongoing sequencing is being done on frozen tissues, whilst the immunostaining was done in paraffin sections. So we will never be able to prove that the findings we have in the sequencing will be the same mutation in the paraffins, unless we manage to get both frozen and paraffin sections from the same patients.

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