Molecular reprogramming of hepatocytes into beta-like cells

Thowfeequ, Shifaan

Award date:
2009

Awarding institution:
University of Bath

Molecular Reprogramming of Hepatocytes into Beta-like Cells.

Shifaan Thowfeequ

Thesis for the degree of Doctor of Philosophy.

Department of Biology and Biochemistry
University of Bath, UK.

March 2009

Supervised by: Dr. David Tosh and Prof. Jonathan M.W. Slack.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

** Figures illustrating results are given at the end of the results section of the chapter.*

ACKNOWLEDGEMENTS

To misuse a famous quotation, ‘one could only see further by standing on the shoulders of giants’; this work would not have been possible without the intellectual, technical and moral support of many others. I would like to thank my supervisors David and Jonathan for guidance throughout the course of the PhD and helping me secure the ORS studentship to fund my education. I gratefully acknowledge the assistance and professional guidance lent by all the past and present members of the Tosh and Slack labs, most notably special thanks to Michael, whose research provided the foundation on which this work is based; Daniel for all sorts of technical help especially with the lineage tracing; Iryna, Chris and the staff at the Annex for technical assistance; Zoë, Rebecca and Amani for many valuable discussions and Kathy for being a great colleague. I would also like to thank my other colleagues in the department (Pete, Sam, Nick, Jo, Al, Jane, Mike, Anastasia, Emily, Kathy, Phil, Rob, Neal, Mark and Tom to name some) for making it such an enjoyable place to work. Finally special thanks go to my family for the continuous encouragement and unconditional support.

Declaration of Material from Previously Submitted Work.

In Chapter 5, Sections 5.2 to 5.4, the effect of betacellulin treatment on pancreatic development is based on preliminary observation reported in a Master of Research dissertation (entitled: Betacellulin Regulates Endocrine and Exocrine Differentiation During Pancreatic Development by Shifaan Thowfeequ) for the University of Bath, MRes in Biociences programme. Research was later continued during the course of the PhD and the results were published as:-

THOWFEEQU, S., RALPHS, K. L., YU, W. Y., SLACK, J. M. W. & TOSH, D. (2007) Betacellulin inhibits amylase and glucagon production and promotes beta cell differentiation in mouse embryonic pancreas *Diabetologia* 50:1688-97.

Figures 5.1 to 5.11 in Chapter 5 are taken from the above publication.

ABSTRACT

The conversion of one differentiated cell-type into another, challenges our long-held view of differentiated cells being in a fixed terminal stage of differentiation, incapable of reversal or change. Such conversions are most favourable between developmentally related cell-types such as hepatocytes from the liver and cells of the pancreas. The main drawback of culturing hepatocytes *in vitro* has been their limited life span and their rapid de-differentiation in culture. However, the de-differentiation of hepatocytes, and the progressive diminution in the transcription levels of liver-specific genes, is in fact associated with a concomitant induction of a pancreatic gene expression profile. In this 'primed' state, it is possible to convert these de-differentiated hepatocytes more efficiently into beta-like cells by the transduction of the pancreatic master switch gene, Pdx1, in rat, mouse and human hepatocytes. An extensive screen of several other key-regulatory pancreatic transcription factors has yielded that along with Pdx1, the transcription factors Neurogenin3, NeuroD, Pax4 and MafA are crucial for achieving efficient reprogramming. The 'beta-phenotype' of the cells generated can be further enhanced by using the extrinsic factors betacellulin, Activin A and nicotinamide. Many of these factors, most notably betacellulin can promote endocrine differentiation in favour of beta cells, as was demonstrated using an explant model of pancreatic development. The beta-like cells generated by the molecular reprogramming of hepatocytes in addition to expressing a wide array of beta cell-specific genes, also exhibit many beta cell functions. The beta-like cells can process pro-insulin into the mature insulin protein and secrete insulin in a regulated manner in response to glucose stimulation. These cells therefore could serve as an attractive alternative for generating autologous beta cells for transplantation as a means of a cell-based therapy for type 1 diabetes mellitus.

LIST OF ABBREVIATIONS

AFP	α-foetoprotein
β-gal	β-galactosidase
BMP	bone morphogenic protein
C/EBP	CCAAT/Enhancer binding protein
CAR	coxsackievirus B and adenovirus receptor
Cdx	caudal type homeo box genes
ChrA	chromogranin A
CK	cytokeratins
CMV	cytomegalovirus (promoter)
CPE	cytopathic effect
CpE	carboxypeptidase E
CPS	carbamoyl phosphate synthetase
Dex	dexamethasone
dpc	days post coitus
dpp	days post partum
E	embryonic stage/day
EGF(R)	epidermal growth factor (receptor)
ES	embryonic stem (cells)
Exe4	exendin4
FGF	fibroblast growth factor
Fox	forkhead box containing genes
GCK	glucokinase
GFP	green fluorescent protein
GLP1	glucagon-like peptide 1
GLUT	glucose transporter
GS	glutamate synthetase
GSIS	glucose-stimulated insulin secretion
HA	haemagglutinin
HGF	hepatocyte growth factor
HNF	hepatic nuclear factor
IAPP	islet amyloid polypeptide
IH(C)C	immunohisto(cyto)chemistry
iPS	induced pluripotent stem (cells)
Isl	islet LIM/homeodomain 1 genes
kDa	kilo Daltons
KGF	keratinocyte growth factor
Kir	K^+ channel subunit
LAP	liver activation protein
LIP	liver inhibitory protein
LIF	leukaemia inhibitory factor
M.O.I	multiplicity of infection
Maf	musculoaponeurotic fibrosarcoma oncogene homolog A genes
MODY	maturity onset diabetes of the young
NeuroD	neuronal determination factor
Ngn3	Neurogenin3
Nic	nicotinamide
Nkx	natural killer cell transcription factor genes
NOD	non-obese diabetic (mice)
Pax	paired box genes
Pbx1	pre-B cell leukaemia transcription factor genes
PC	prohormone convertases
Pdx1	pancreatic and duodenal transcription factor 1 (a.k.a Ipf1)
PH3	phospho-histone 3
RT-PCR	reverse transcriptase polymerase chain reaction
SCID	severe combined immunodeficient (mice)
Shh	sonic hedgehog
Sox	SRY(sex-determining region Y)-box containing genes
STZ	streptozotocin
SUR	sulphonylurea receptor subunit
TFN	transferrin
TGF	transforming growth factors
TTR	transthyretin
v/v	volume by volume
w/v	weight by volume
(e)YFP	(enhanced) yellow fluorescent protein
Zebu	zebularine
-/-	double knockout

Chapter ONE

Introduction

1A MOLECULAR REPROGRAMMING OF CELLS

The emergence of multicellularity was a fundamental step in the evolution of complex organisms. This step was facilitated by the ability to derive a multitude of different cell types from a single cell such as the fertilised egg. Initiated upon fertilisation, mammalian development proceeds through a hierarchical route from pluripotent embryonic stem cells of the blastocyst, via germlayer- and tissue-specific- progenitors to an array of 'terminally' differentiated cell-types. It was thought that this developmental pathway was programmed, irreversible and unchangeable. However, recent discoveries have shown that deviations from this hierarchical programme of development, is quite the norm in some organisms and during some pathological processes. What was observed in nature and in disease can now be experimentally recapitulated for therapeutic benefits. Embryonic stem cells can be encouraged to differentiate along specific lineages. Similarly, fate-committed progenitor cells and precursor cells can be altered to give rise to cell-types atypical of their normal developmental pathway. Furthermore, the long held view that terminally differentiated cells are just that; in their final stage of differentiation, and that this state is fixed and cells are not capable of reversal or change, has been challenged. The remarkable cellular plasticity of differentiated cells has now been demonstrated by the ability to reprogramme them into other cell-types (including induced pluripotent stem cells) by molecular and genetic manipulation.

1A.1 The differentiated state of cells.

All cells that make up an organism have a common developmental origin- the pluripotent cells of the inner cell mass within the blastocyst (Fig 1.1). However throughout development, the differentiation pathways delineate, committing progenitor cells to distinct fates until fully differentiated and functional cell-types are established. A differentiated cell is characterised by a distinct phenotype that demarcates it from other cells, either by functional,

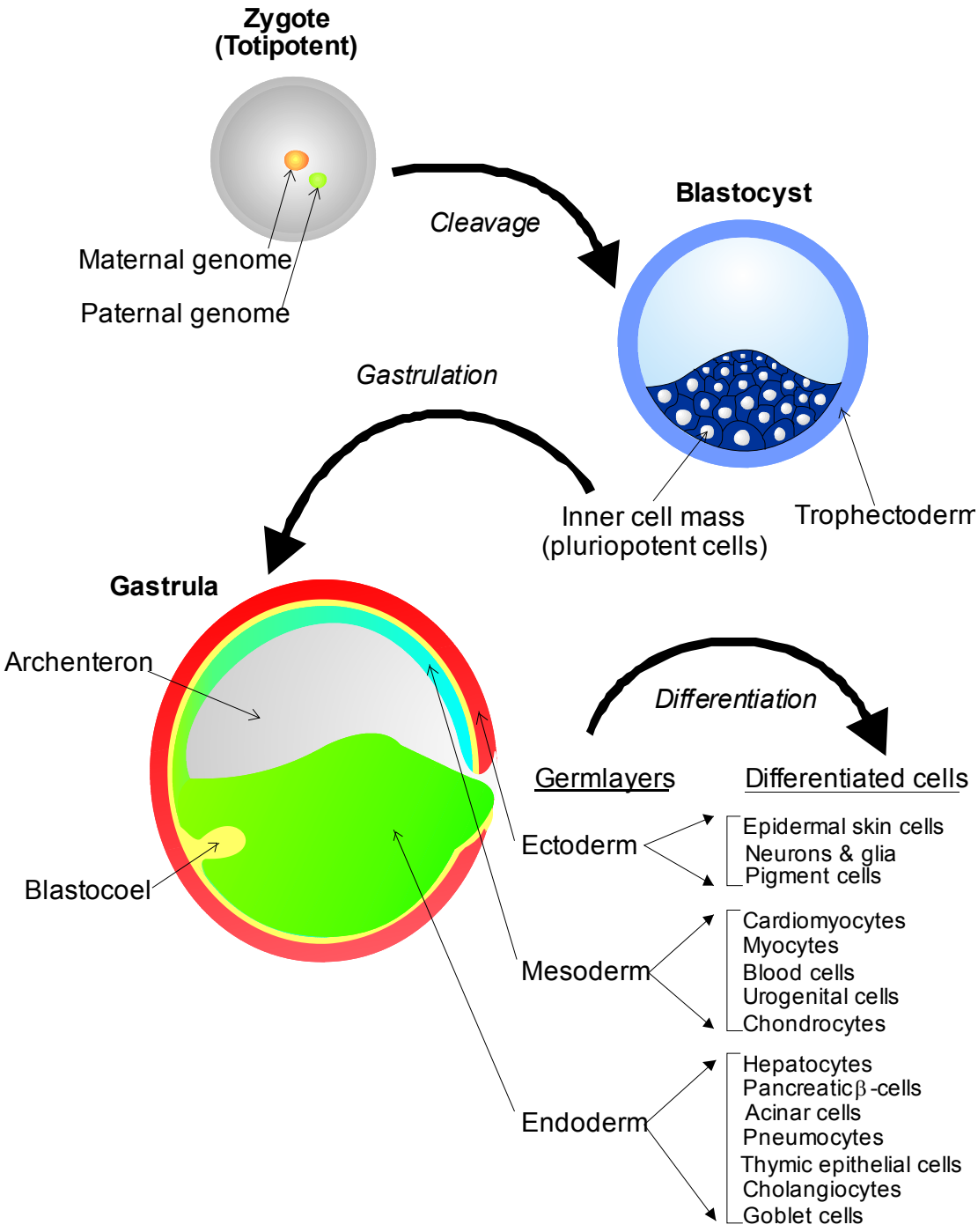


Figure 1.1. The developmental hierarchy of differentiation. The newly fertilised zygote is totipotent and capable of producing all the cell types within the embryo as well as all the extra-embryonic tissues. In the blastocyst the inner cell mass consists of cells that are pluripotent and differentiate into the three germlayers after gastrulation. As development proceeds, fully differentiated cell types of ectodermal, mesodermal and endodermal are derived from the specific multipotent stem/precursor cell compartments of each germlayer (adapted from Gilbert, 2006 and Slack, 2006).

morphological or biochemical differences. Despite the homogeneity of the genetic makeup of virtually all cells, morphological and functional heterogeneity is achieved due to differential gene expression. This differential gene expression is brought about by differential responses to gradients of morphogens mediated by transcription factors that are activated or repressed by the signal. The mechanisms underlying differential gene expression are not due to alterations in the DNA sequence itself, but due to epigenetic modifications providing discriminative accessibility of the genetic material (reviewed in Jaenisch and Bird, 2003).

In the nuclei of all cells, DNA is compacted and packaged in the form of chromatin in association with proteins (Fig. 1.2). The basic repeat element of chromatin is the nucleosome composed of DNA wrapped around an octamer of histone proteins (H2A, H2B, H3 and H4). The nucleosomes wrap around histone H1 and are further compacted into the 30nm fibre that make up the active chromatin or is organised into chromosomes. Epigenetic modifications to the DNA or the associated histone proteins ensure that genes can be turned on in one lineage but off in another. Firstly DNA methylation triggers heritable gene silencing, either by interfering with transcription factor binding or by the recruitment of co-repressor complexes by methyl-CpG binding domain proteins (Levenson and Sweatt, 2005, Kiefer, 2007). Secondly, post-translational modification by acetylation, phosphorylation, ubiquitination and methylation of the N-terminal tails of histones can alter the structural and functional state of chromatin. It is believed that specific combinations of histone modifications are responsible for coordinating differential gene expression (Strahl and Allis, 2000, Li, 2002). Collectively, epigenetic marks ensure that for a given differentiated cell-type, in addition to expressing ubiquitous genes, a whole array of cell-type specific genes are expressed while those not characteristic of the cell-type are repressed. For example in a fully differentiated hepatocyte, *albumin*, *transferrin* and *haptoglobin* genes are expressed along with genes coding for liver-specific transcription factors that are responsible for maintaining the hepatic phenotype. Therefore, cell-type specific transcription factor expression ultimately modulates the composition of the whole transcriptome of a differentiated cell.

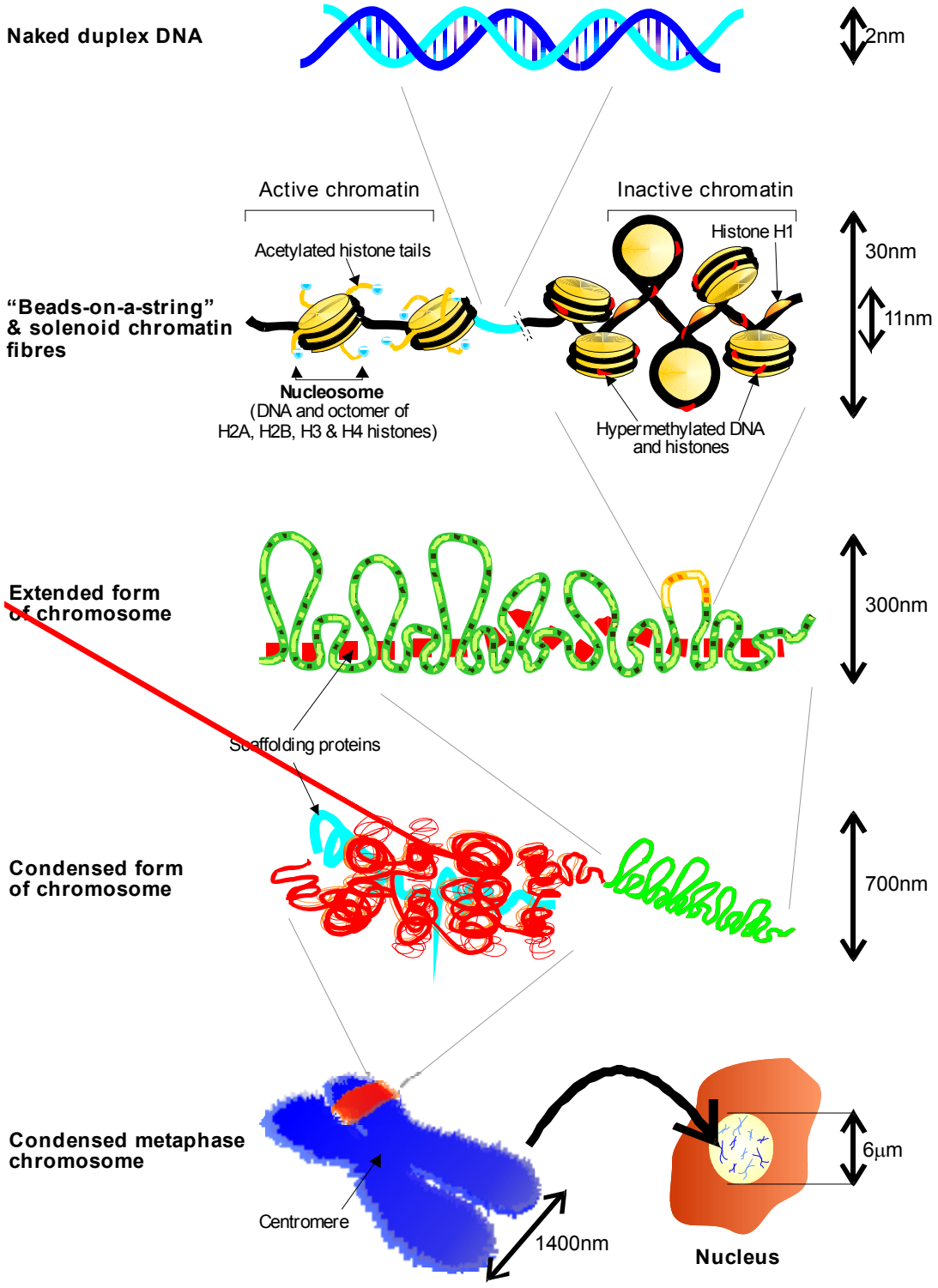


Figure 1.2. Compaction of DNA and the structure of chromatin. In eukaryotic cells, DNA is compacted with heterodimers of core histone proteins to form a nucleoprotein complex called a nucleosome. The transcriptional activity of genes are in turn determined by the methylation and acetylation state of DNA and histone tails respectively. Inactive chromatin is further compacted in association with scaffolding protein to form chromosomes (adapted from Lodish et al., 2003).

1A.2 The phenomenon of de-differentiation.

De-differentiation refers to two quite distinct processes. The first is the process by which a terminally differentiated cell loses its cell-specific properties and the second, refers to the process by which the cell reverses the normal developmental processes and once again becomes a precursor or stem-like cell. The study of de-differentiation has mostly been driven by the curiosity to understand the mechanisms underlying the regenerative capabilities of various plants and vertebrates. De-differentiation is marked by the re-entry into the cell cycle and at the same time deviating from a differentiated state to a state that confers multi- or pluripotentiality (Williams et al., 2003). During limb and tail regeneration in urodele amphibians, multinucleate myofibres lose their striated nature and dissociate into mononucleated cells to contribute to the blastema which forms beneath the wound epithelium. Fluorescently-labelled and retrovirally-tagged myotubes implanted into the balstema change into mononucleated cells, proliferate and by four to six week after implantation give rise to muscle and cartilage tissue of the regenerating structure (Lo et al., 1993). This suggests that the myofibres are capable of de-differentiating and acquiring multipotentiality before re-differentiating into its original or an entirely novel lineage. *In vivo* de-differentiation has also been observed during the regeneration of the jaw (Ghosh et al., 1994), spinal cord (O'Hara et al., 1992) and cardiac muscle (Oberpriller et al., 1995) in amphibians.

The de-differentiation of cells in culture has also been noted and is often considered a hindrance to their long term *in vitro* maintenance. Both hepatocytes and cells of the kidney epithelium are known to de-differentiate in long term culture (Forino et al., 2006, Guillouzo, 1986). Similarly human pancreatic beta cells (Ouziel-Yahalom et al., 2006), but not their murine counterparts (Atouf et al., 2007), can expand *in vitro* following a process that resembles an epithelial-mesenchymal transition event. Recently, attempts have been made to induce the de-differentiation of cells in hope of deriving pluripotent cells capable of re-differentiating into other lineages (Odelberg, 2002, not reproducible). Only limited data is available on the molecular mechanisms that govern de-differentiation in mammalian cells. However,

work in amphibians and plants have revealed the importance of chromatin decondensation and other epigenetic changes in the process of de-differentiation (Zhao et al., 2001). Understanding the molecular mechanisms underlying the phenomenon is imperative in order to utilise de-differentiation as step in the molecular reprogramming of differentiated cells into other cell-types.

1A.3 Cell-type switching in development and disease.

Cells pass through a hierarchy of developmental decisions during the course of embryonic development from stem-like cells to fully differentiated cell-types. However, those that do not conform to this hierarchical developmental pathway are less common, but nonetheless are seen in nature (Fig. 1.3). Such changes include switching between cells of different tissue types (broadly referred to as metaplasia and includes the interconversion of fully differentiated cell-types sometimes referred to as transdifferentiation), derivation of cells with abnormal phenotypes (dysplasia) and also the reversal of the differentiated state (de-differentiation, as discussed above). Collectively all changes that do not conform to the normal developmental pathway will be referred to as cell-type conversions, cell-type switching, metaplastic changes or cellular reprogramming (if it is induced). Although it may seem unconventional to think so, metaplasias are not necessarily always associated with errors made during development or tissue repair. Though rare, the phenomenon is observed across the metazoan phylogeny. For example, during mammalian development, epithelial cell-type switches from columnar to stratified squamous and from pseudostratified to squamous during the development of the oesophagus and the female reproductive system respectively, have been long identified (Cunha, 1976, Yu et al., 2005). In non-embryonic states, cell-type conversions are thought to be used as a mechanism for tissue repair which excludes the need to resort to undifferentiated reserves of cells such as stem cells. It is also used during vast tissue respecification processes associated with metamorphosis and the normal progression through stages of the lifecycle (Thowfeequ et al., 2007b).

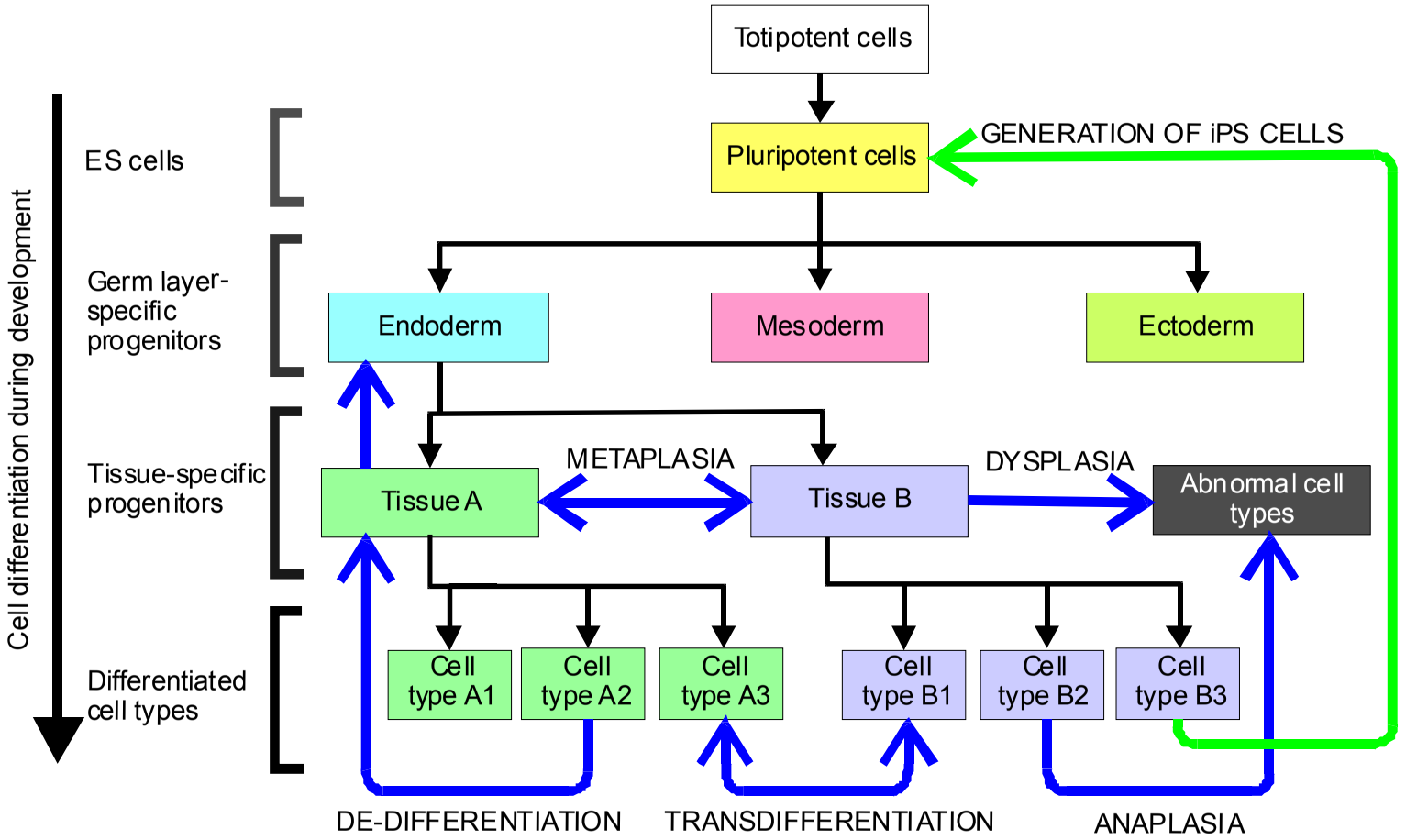


***Figure 1.3*. Deviations from the normal developmental hierarchy.** Several variations of tissue-type switching can be seen in nature (blue arrows) and others can be experimentally induced (green arrow). While some changes including de-differentiation and transdifferentiation can be seen during the normal development of organisms, others such as metaplasia, dysplasia and anaplasia can predispose to neoplasia. Note that only interconversions in the endodermal lineage are shown, although the same is expected for the mesodermal and ectodermal lineages (amended from Slack, 2007).

Differentiated striated muscle cells of hydrozoan jellyfish in culture, can be multipotent with the ability to convert into various cell-types including smooth muscle cells and nerve cells positive for the neuropeptide FMRFamide (Alder and Schmid, 1987, Schmid et al., 1988). Tissue respecification is more commonplace during metamorphosis of arthropods and during regeneration of body parts by members of the echinoderm phylum. However, some vertebrates are also capable of restoring by regrowth, parts of their body that have been lost to injury or by autotomy. In the process of Wolffian lens regeneration in urodele amphibians, cells of the dorsal iris proliferate, undergo de-pigmentation and re-differentiate into keratinocyte-like crystallin containing cells of the lens (Tsonis et al., 2004, Yamauchi et al., 1984). It is also possible to redirect the neuronal retina and cells of the outer cornea into a lens epithelium fate in this manner (Opas and Dziak, 1998). The homeodomain transcription factor *Six3* may be the master switch gene in the iris-lens conversion process. Transfection of *Six3* accompanied by inhibition of the BMP (bone morphogenic protein) signalling pathway can induce the transdifferentiation of the ventral iris (which does not normally take part in lens regeneration) into lens tissue (Grogg et al., 2005). Cellular reprogramming has also been suggested to play a role in amphibian limb and tail regeneration, where epidermal, neuronal and muscle tissues are replaced following amputation (Brockes and Kumar, 2002). However, most of the evidence suggests that the cell-type conversions in this case, are facilitated via a de-differentiated intermediate rather than being a direct cell-type conversion that can be labelled as a transdifferentiation event.

Postnatal cell-type switching is not a basic, primordial attribute of only a few vertebrates. Although less well understood, it has also been observed in mammals. Tissues that constantly renew throughout life such as hair follicles, the gut and blood have an adequate reservoir of dedicated adult stem cells. However, many organs are not believed to maintain a resident undifferentiated stem cell population and either have to rely on the proliferation or transdifferentiation of existing differentiated cells to replenish the cell mass after injury. The lung for example, is an organ exposed to the external environment and therefore to pathogens and toxicants. Hence the

lung epithelium needs to respond rapidly and effectively to cell damage repeatedly caused by these agents. During repair and maintenance of the lung epithelium, in addition to mobilising stem cells and promoting the proliferation of differentiated cells, various cell-type conversion including direct transdifferentiation is also thought to occur (Rawlins and Hogan, 2006). Following damage to the bronchiolar epithelium, ciliated cells respond by spreading and undergoing a sequential squamous to cuboidal to columnar transition and eventually change into distinct epithelial cell-types to repair the damaged airway epithelium. During the process, expression of several key regulatory transcription factors characteristic of the developing lung, including β-catenin, Foxa2, Foxj1, and Sox family members are reactivated (Costa et al., 2001, Rawlins and Hogan, 2006). During pancreatic regeneration in the Vervet monkey, foci of hepatocytes are found in the pancreas (Wolfe-Coote et al., 1996). It is likely that the ectopic hepatocytes could have arisen from differentiated cells of the exocrine pancreas as has previously been observed in other experimental systems such as in copper-depleted rats (Rao et al., 1986). Similarly, the reverse transformation or foci of acini and pancreatic ductules in the human liver have been found; but is associated with severe posthepatitic cirrhosis (Wolf et al., 1990).

There are numerous examples of metaplasia in human histopathology and some are more clinically significant than others in that they predispose to the development of neoplasia. Metaplasias are implicated in the development of various cancers including lung, prostate, vaginal and breast cancer (Auerbach et al., 1961, Bjerkvig et al., 2005, Nelson et al., 2002, Quinlan et al., 2007). A clinical example of cell-type switching is the premalignant condition of Barrett's oesophagus or Barrett's metaplasia. Barrett's metaplasia is a known precursor to oesophageal adenocarcinoma (Hameeteman et al 1989). A patient is said to have developed Barrett's when the normal squamous lining of the oesophagus is converted to an intestinal type columnar epithelium normally at the junction of the oesophagus and stomach (Barrett, 1957). Research suggests that the metaplasia is induced by chronic acid reflux from the stomach into the oesophagus (Spechler, 2002). The master switch gene responsible for inducing Barrett's metaplasia,

is thought to be the *Cdx2* gene, a member of the parahox cluster (Ferrier et al., 2005). Cdx2 is involved in intestinal epithelial differentiation and distinguishes the upper and lower epithelium of the alimentary canal; furthermore *Cdx2* expression has been found to be upregulated in adenocarcinomas of the intestine (De Lott et al., 2005). Experiments have shown that ectopic expression of *Cdx2* can induce intestinal metaplasia in the stomach (Li et al., 2005b). The exact mechanism by which metaplasia is induced in Barrett's is still unclear.

Other clinical examples where cell-type switching has been implicated include liver fibrosis leading to cirrhosis, interstitial fibrosis of renal disease, and aerosolised allergen-induced mucous metaplasia. Knowledge obtained from studying metaplasias and other cell-type conversions as a result of a natural phenomenon or as part of a disease is important for a number of reasons. Such studies will help answer questions regarding normal development, regeneration and the etiology of certain diseases. Furthermore it will enable us to devise potential therapeutic modalities for various degenerative diseases, where in addition to alleviating the symptoms may also make it possible to permanently replace the damaged tissue.

1A.4 Inducing cell-type switching for cell-based therapies.

Inducing the conversion of one differentiated cell type to another cell type for therapeutic applications holds a distinct advantage over using stem cell technology; as the utilisation of stem cells in cellular therapy is often hindered by the ethical controversies and oncogenic concerns centred around the use of embryonic stem cells and the practical difficulties of identifying and isolating adult stem cells (Samson and Chan, 2006). Early experiments showed that the transfection of 5-azacytidine treated mouse fibroblasts with cDNAs from myocyte cDNA libraries can induce their conversion into myoblasts (Davis et al., 1987). cDNAs that induced muscle differentiation in fibroblasts have homology to the myc-like regions of MyoD and Myf-5. These are members of the MRF (Myogenic Regulatory Factor) family of transcription factors which were later found to be essential for

myogenesis (Kitzmann et al., 1998). It is thought that cell-type conversions may occur naturally and can be induced following changes in expression of such 'master switch genes'. These are genes which encode transcription factors that induce a cell to differentiate along a particular developmental pathway.

A much celebrated example of an induced cell-type conversion for which lineage analysis is available is the stepwise conversion of B-cells into macrophages. The CCAAT/Enhancer binding protein family transcription factors C/EBPα and C/EBPβ, can act synergistically with the transcription factor PU.1 in converting isolated B-lymphocytes into macrophages (Xie et al., 2004, Slack, 2007). This conversion is accompanied by a loss of the B-cell specific marker CD19 and the induction of the macrophage marker MAC1. The presence of rearranged immunoglobulin genes in the reprogrammed macrophages, indicate their origin from cells that have already undergone normal B-cell differentiation. Furthermore, in transgenic mice where *Cre* is driven off the *CD19* promoter (which permanently labels B-cells with the eYFP reporter), the origin of the macrophages from B-cells can be followed in real time (Xie et al., 2004).

Likewise, the developmental pathways of liver and pancreas are similar; potentially similar enough that cell-type conversion may be induced between the two tissue types for therapeutic purposes using ectopic expression of one or a few master switch genes. In the case of treatment of liver diseases, the clear problem involving transplantation is the current lack of available donor tissue, despite the fact that liver can be obtained from living donors (Ridgway et al., 2005). Sources of hepatocyte-like cells for transplantation include salivary gland cells. SGP-1 cells derived from duct-ligated submandibular salivary glands can be induced to form hepatic (AFP (α-foetoprotein) and/or albumin positive) clusters when cultured on type-1 collagen (Okumura et al., 2003). Similarly, current work on the conversion of pancreatic cells to hepatocytes raises the possibility that liver disease could be treated using reprogrammed cells from the patients' own pancreas. The conversion of the pancreatic cell line AR42J-B13 (B13) to a hepatic phenotype can be induced following addition of the synthetic glucocorticoid, dexamethasone (Dex)

(Shen et al., 2000). Treatment with Dex causes the downregulation of the pancreatic marker amylase and induction of liver markers including, transferrin, α1-antitrypsin, transthyretin, glucose-6-phosphatase, α-foetoprotein, cytokeratin 8, P-glycoprotein and phenol sulphotransferase. Later work demonstrated that not only could the new hepatocyte-like cells express early liver markers but also expressed genes involved with Phase I and II detoxification pathways, ammonia detoxification and glucose metabolism, indicating that the cell-type conversion was accompanied with the induction of genes associated with a mature phenotype after treatment with Dex and Oncostatin M (Burke et al., 2006, Tosh et al., 2002, Wang et al., 2005). It has been proposed that the molecular mechanism controlling the conversion of the B13 cells to hepatocytes involves the activation of the glucocorticoid receptor by Dex, which activates transcription of a master switch gene a member of the C/EBP family of transcription factors (Shen et al., 2000). An upregulation of C/EBPβ along with α-foetoprotein is seen during the Dex-induced reprogramming of primary rat pancreatic exocrine cells into hepatocytes (Lardon et al., 2004).

Based on the same concept that the liver and the pancreas are developmentally related and that during development the two tissues are distinguished by the expression of one master switch gene, recent work has focused on the conversion of hepatocytes into pancreatic beta cells for diabetes therapy. Pdx1 is thought to be the master switch gene for the conversion of liver cells to pancreatic cells and is discussed in more detail in Section 1C.2. All examples of cell-type switching, be they natural or induced, demonstrate that the differentiated cells are not ‘fixed’ but rather retain the ability to undergo genetic reprogramming.

1B THE PANCREATIC BETA CELL

The mature pancreas is both morphologically and physiologically divided into two distinct compartments- the exocrine pancreas and the endocrine pancreas. The exocrine portion is organised into acini that produce and secrete digestive enzymes such as amylase, and the ductal network that secretes bicarbonate and transports the enzymes to the intestine. Alternatively, the endocrine pancreas is devoted to the production and secretion of a variety of hormones directly into the blood stream. Within the endocrine compartment that comprises about 1% of the pancreas, the hormone-producing cells are organised into individual, highly vascularised and highly innervated micro-organs called the islets of Langerhans (see Fig. 1.4). The insulin-producing beta cells are the most abundant cell-type in the islet (65-80%), followed by glucagon-producing alpha cells (15-20%), somatostatin producing delta cells (3-10%), pancreatic polypeptide-producing PP cells (3-5%) and the ghrelin-producing epsilon cells (<1%) (Edlund, 2002). The shear abundance of beta cells reflects the importance of their function in responding synchronously to stimulatory concentrations of blood glucose by releasing insulin to maintain glucose homeostasis. Therefore it is important to know the developmental origin of the beta cell, how their numbers are maintained and more importantly how they function.

1B.1 Developmental origins of the beta cell.

Before exploring the mechanisms of endocrine lineage segregation and the origination of beta cells, it is essential to understand the fundamentals of early pancreatic development. Pancreatic development is a highly coordinated process involving morphogenesis and differentiation, orchestrated by extracellular signals and transcription factor interactions. Like the liver and the intestine, the pancreas is an organ derived from the definite endoderm (Slack, 1995). Following gastrulation, signals from the

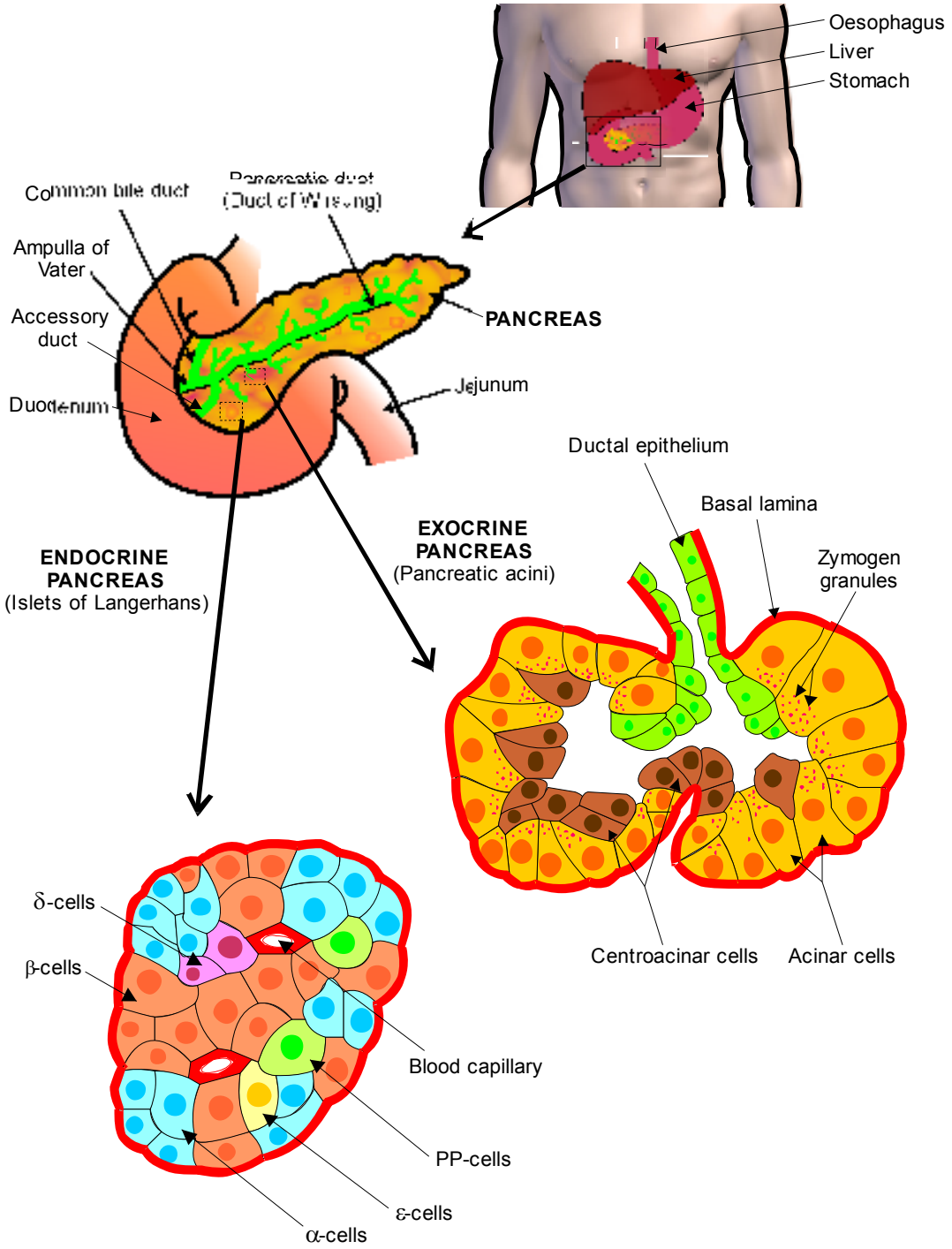


Figure 1.4. The structure and organisation of the pancreas. The pancreas is located behind the stomach in the abdominal cavity. It secretes digestive juices into the duodenum and endocrine hormones directly into the blood stream via capillaries. The exocrine tissue makes up most of the pancreatic mass and is made up acinar, centroacinar and ductal cells. The endocrine portion of the pancreas comprises of the Islets of Langerhans in which the different endocrine cell types are grouped together (based on figures by the University of Wisconsin Hospital and Clinical Authority; from the website: http://www.uwhealth.org).

mesoderm and ectoderm are responsible for the anterior-posterior patterning of the endoderm (Wells and Melton 2000). However, induction of pancreatic development and the commitment to a pancreatic fate is set up by an Activin βB- and FGF2-mediated repression of *Sonic hedgehog* (*Shh*) expression in the domain that becomes the prepancreatic endoderm (Hebrok et al., 1998, Hebrok et al., 2000; Fig. 1.5). It is believed that the signals arise from the adjacent mesodermal structures such as the notochord, endothelia of the dorsal aorta, cardiogenic mesoderm and the septum transversum (Kim et al., 1997, Lammert et al., 2001). The competence of the foregut endoderm to respond to the instructive mesodermal signal is primarily mediated by the preceding expression of the transcription factors *HNF1β*, *FoxA1*, *FoxA2*, *HNF4* and *HNF6* (*Onecut1*), as well as *GATA4, 5* and *6* (reviewed in Wilson et al., 2003, Cereghini, 1996, Zaret, 1999). Although, the signals are common to both the dorsal and ventral pancreatic anlagen, the induction strategies are different. While the dorsal foregut endoderm defaults to intestine in the absence of mesodermal signals, the default pathway for the ventral foregut endoderm is to become pancreatic. It is only BMP4 from the septum transversum working in concert with FGFs from the cardiac mesoderm that can drive hepatogenesis- the alternative fate of the bipotential precursors of the ventral foregut endoderm (Kim and MacDonald, 2002, Rossi et al., 2001, Zhou et al., 2007). Following *Shh* repression, a consequent activation of *Pdx1* expression by HNF1β and FoxA2 in the pancreatic precursor population is seen (Lantz and Kaestner, 2005).

The early prepancreatic endoderm expresses two parahox transcription factors- *Pdx1* (McKinnon and Docherty, 2001, Offield et al., 1996) and *Hlxb9* (Harrison et al., 1999). *Pdx1* expression is seen in two pancreatic buds as well as the adjacent duodenum and antral stomach (Ohlsson et al., 1993). The interaction of Pdx1 with Pbx1 is thought to be important for the expansion of the pancreatic buds as the impairment of pancreatic development in the absence of Pbx1, mimics that seen in *Pdx1* null embryos (Kim and MacDonald, 2002). The early epithelial progenitor epithelium which is positive for the transcription factors HNF1β, Onecut1, FoxA2 and Sox9 (which controls progenitor cell identity by regulating the former three),

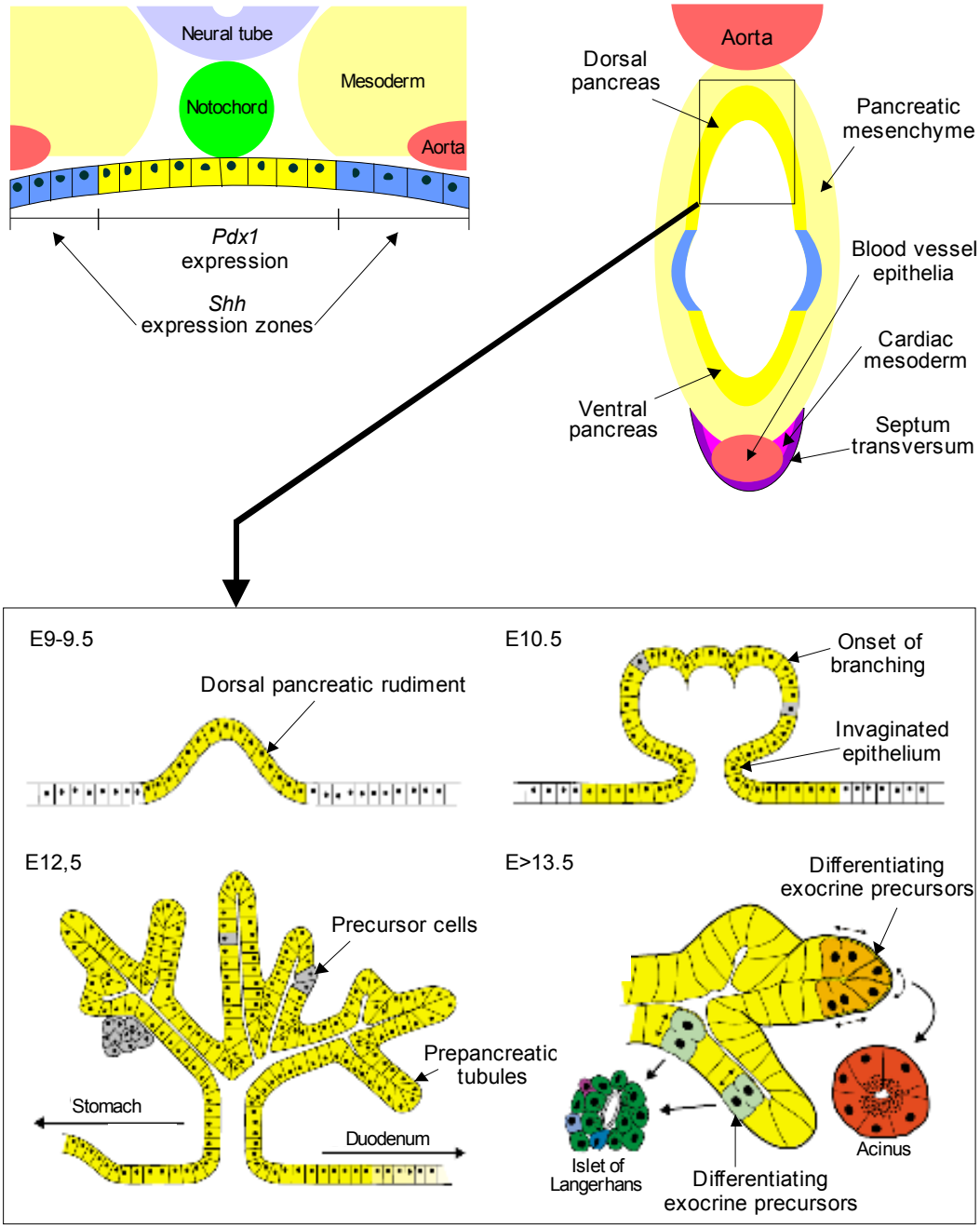


Figure 1.5. Schematic diagram of pancreatic development. At E8 (top left), the region of the midline endoderm of the dorsal pancreatic anlagen directly in contact with the notochord express Pdx1 whereas the adjacent Shh positive domains remain Pdx1 negative. These Pdx1 negative regions later come into contact with the cardiac mesoderm and adopt a hepatogenic fate. Around E9.5, the blood vessels and the septum transversum provide signals that direct the pancreatic gene expression in the adjacent endoderm. In the boxed figures, yellow represents the Pdx1 expressing domains and grey represents early endocrine precursor cells. These cells migrate out of the epithelium and cluster to form the islets of Langerhans around E12.5. The exocrine acini are formed from cells at the tip of the prepancreatic tubules (from Kim and MacDonald, 2002).

undergoes expansion and branches into duct-like structures (Lynn et al., 2007). This is followed by the secondary transition, which is characterised by synchronised waves of differentiation. Several other transcription factors come into play in order to determine the specification of the endocrine and exocrine lineages. In an analogous scheme to neuronal development, in a subset of cells, notch inhibition releases the Hes1 imposed repression of *Neurogenin3* expression (Murtaugh et al., 2003). The expression of *Neurogenin3* is scattered and transient, and regulated by an auto-repressive feedback loop (Fig. 1.6); however it is necessary and sufficient to initiate endocrine differentiation of the pancreas (Schwitzgebel et al., 2000). Downstream of Neurogenin3, the pro-endocrine basic helix-loop-helix (bHLH) transcription factor NeuroD is directly activated (Gradwohl et al., 2000). In contrast, cells of the prepancreatic endoderm, where Neurogenin3 expression is extinguished by persistent notch signalling, are destined to become the exocrine pancreas driven by the pro-exocrine transcription factor p48/Ptf1a (Kawaguchi et al., 2002, Rose et al., 2001).

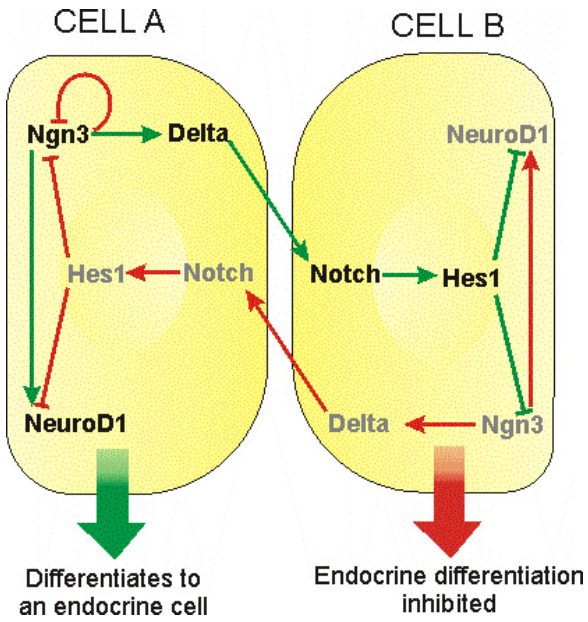


Figure 1.6. **Lateral inhibition and the role of the Notch signalling pathway during beta cell development.** In endocrine precursors, on the removal of Hes1 imposed Neurogenin3 (Ngn3) expression NeuroD is activated. Ngn3 also activates the Notch ligand Delta, which in an adjacent epithelial cell can activate Hes1 via Notch. Thus Hes1 represses endocrine differentiation in this adjacent cell. Ngn3 expression itself is controlled by an autoregulatory feedback loop (from Skipper and Lewis, 2000).

Once committed to an endocrine fate, the next step involves the segregation of the distinct endocrine lineages including beta, alpha, delta and PP. It is believed that the default pathway for the endocrine progenitors is to become alpha cells, as ectopic expression of Neurogenin3 almost exclusively results in this outcome (Schwitzgebel et al., 2000). However it does not mean that all lineages start off as glucagon-expressing cells and become multihormonal cells before being respecified into other lineages (see Herrera, 2000). Therefore additional signals are necessary to deviate the fate of the progenitor cells to becoming other endocrine cell-types such as beta cells before they go onto become alpha cells by default. This is modulated by the selective sequential expression of a number of transcription factors. These include the early factors such as Pax4, Nkx2.2 and Nkx6.1 expressed alongside Neurogenin3 in endocrine progenitors and the late factors including Pax6, Isl1, Pdx1 and MafA expressed in more mature beta cells (Fig. 1.7). Much of what we know about the roles of these transcription factors in pancreatic development has been elucidated by the study of their expression profiles and the phenotype of transgenic animals. For example, Nkx2.2 deficient mice lack fully differentiated beta cells and have reduced number of alpha and PP cells (Sussel et al., 1998). Nkx2.2 null mice also fail to express Nkx6.1. Furthermore, mice homozygous for Nkx6.1 mutations completely lack beta cells and double homozygous mutants for both the NK-homeodomain transcription factors are phenotypically similar to Nkx2.2 null mice (Sander et al., 2000). Collectively, these studies propose both the NK-homeodomain transcription factors as being essential for complete beta cell differentiation with Nkx2.2 acting upstream of Nkx6.1. Table 1.1 summarises the data from other similar studies. Although most of the information relates to the function as known in rodents, the same transcription factors are implicated in the development of the human foetal endocrine pancreas (Lyttle et al., 2008).

The final stage of endocrine pancreatic development involves isletogenesis or the organisation of the islets. It is generally accepted that the migration of endocrine precursors out of the endodermal epithelium, takes place prior to completion of their differentiation. The migration events are mediated via the

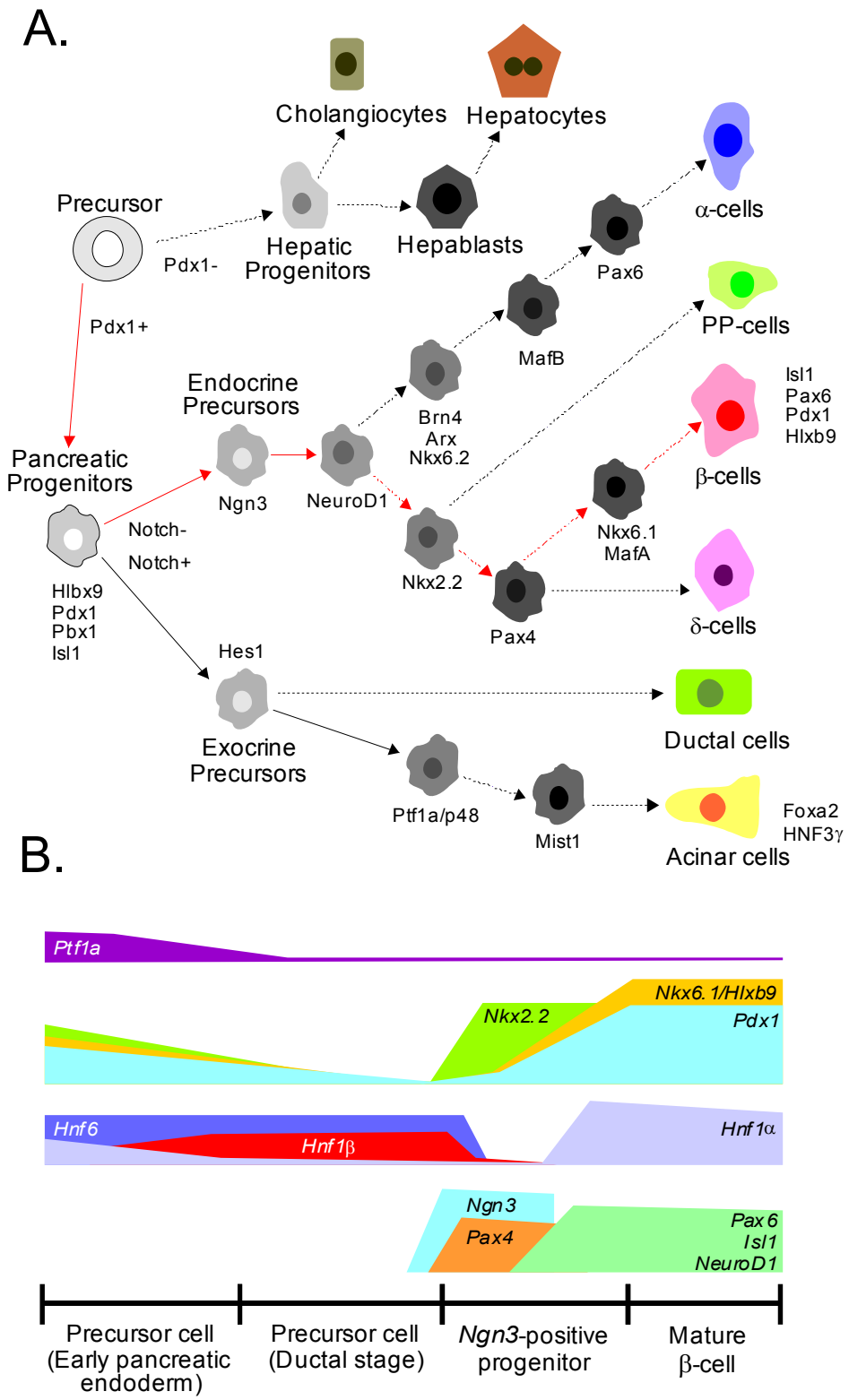


Figure 1.7. Gene expression during beta cell differentiation. A- the gene expression cascade involved in deriving differentiated pancreatic cell types and liver cell types from a common precursor population during development (amended from Samson and Chan, 2006 and Wilson et al., 2003). B. The relative expression patterns of different pancreatic transcription factors during the differentiation of beta cells from precursor cells of the early pancreatic endoderm (from Servitja and Ferrer, 2004).

Table 1.1. Transcription factors and the pancreatic phenotype genetic mouse models suggesting providing evidence for their importance in regulating pancreatic development and function.

Factor	Genetic model	Phenotype	References
Hlxb9	*Hlxb9*$^{-/-}$	Dorsal pancreas agenesis; reduced islet size and beta cell numbers in the ventral pancreas.	(Harrison et al., 1999)
IA1/Insm1	*Insm1*$^{LacZ/LacZ}$	Endocrine precursors formed but reduced numbers of mature insulin-positive beta cells.	(Gierl et al., 2006)
Isl1	*Isl1*$^{-/-}$	No dorsal endocrine cells or mesenchyme.	(Ahlgren et al., 1997)
MafA	*MafA*$^{-/-}$	Post natal glucose intolerance; impaired secretagogue response; beta cell numbers not changes.	(Zhang et al., 2005)
NeuroD1	*NeuroD1*$^{-/-}$	Disorganised islets; secretion-defective acinar cells, hyperglycaemic at birth; perinatal mortality.	(Naya et al., 1997)
Ngn3	*Ngn3*$^{-/-}$	Absence of intestinal and pancreatic endocrine lineages; abnormal acinus.	(Gradwohl et al., 2000)
Nkx2.2	*Nkx2.2*$^{-/-}$	Absence of differentiated beta cell; reduced alpha and PP cell numbers; increased numbers of ghrelin cells; hyperglycaemic at birth; perinatal mortality.	(Sussel et al., 1998)
Nkx6.1	*Nkx6.1*$^{-/-}$	Reduced islet size; reduced number of beta cells after secondary transition.	(Sander et al., 2000)
Pax4	*Pax4*$^{-/-}$	Reduced beta and delta cell numbers; increase in alpha and ghrelin cells numbers.	(Sosa-Pineda et al., 1997)
Pax6	*Pax6*$^{-/-}$	Disorganised islets; absence of alpha cells.	(Sander et al., 1997)
Pdx1	*Pdx1*$^{-/-}$	Pancreatic agenesis; small pancreatic rudiment with few alpha cells.	(Jonsson et al., 1994)
	Pdx1$^{+/-}$	Glucose intolerance and impaired GSIS; reduction in beta cell mass and survival with age.	(Johnson et al., 2003)
Ptf1a/p48	*Ptf1a*$^{-/-}$	Hypoplastic pancreas; absence of exocrine cells in dorsal pancreas; normal endocrine cells and cell numbers.	(Krapp et al., 1998)
Sox9	*Pdx1-Cre/ Sox9*$^{loxp/loxp}$	Hypoplastic pancreas; reduced amylase-positive acinar cells; reduced proliferation of Pdx1-positive progenitors.	(Seymour et al., 2007)

integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) regulated by mesenchymal derived TGFβ while E- and N- cadherins control the aggregation of the endocrine cells into islets (Dahl et al., 1996, Docherty, 2001, Miralles et al., 1998). At this point pancreatic organogenesis is complete. However it is important to note that most of the late transcription factors are also crucial for the postnatal maintenance and the functioning of the mature beta cells.

1B.2 Beta cell maintenance and survival.

Persistent expression of several late transcription factors regulate the expression of key beta cell genes and are important for the maintenance of the mature beta cell phenotype. This is clinically attested in maturity onset diabetes of the young (MODY) syndromes where heterozygous mutations in some of the genes encoding these transcription factors leads to progressive impairment in beta cell function and eventually diabetes (Fajans et al., 2001). Furthermore, recent advances in Cre-Lox technology have enabled us to look at the role of transcription factors in the mature beta cell independent of their function during development. For example, though Pdx1 is downregulated following the induction of pancreogenesis, Pdx1 expression reappears confined to more differentiated beta cells where it directly binds to and regulates the expression of several genes including *insulin*, *GLUT2, IAPP* and *Nkx6.1* (Cerf, 2006, Docherty et al., 2005). Similarly, the transcription factor NeuroD is thought to regulate *glucokinase* expression (Moates et al., 2003) while MafA plays a key role in beta cell function (Zhang et al., 2005). Foxa2 on the other hand, is an upstream regulator of both *Pdx1* and the ATP-sensitive K^+ channel, *Kir6.2* (Sund et al., 2001). Emerging evidence also suggests the importance of the FoxO proteins in maintaining the beta cell phenotype. FoxO-dependent mechanisms are thought to enhance beta cell survival and help them accomplish their endocrine function by: 1- increasing their resistance to oxidative stress. 2- conditioning beta cells to cope with changes in fuel abundance (reviewed in Glauser and Schlegel, 2007). Since, beta cell-specific deletion of most of these genes only results in a diminution but not a complete loss of beta cell function, a

considerable degree of redundancy is apparent within the transcriptional network governing the mature beta cell.

1B.3 Beta cell replication and neogenesis.

An organism needs to be able to control its beta cell mass in accordance with its insulin needs. A stable and sufficient pancreatic beta cell mass is necessary for efficient functioning of the endocrine pancreas. Beta cell hypertrophy can increase beta cell mass without the necessity of making new cells. However, new beta cells can be formed either by the mitotic division of pre-existing beta cell (replication) or by the differentiation of a pool of undifferentiated precursor/stem cell population (neogenesis). Much controversy still lingers regarding which of these two mechanisms remain accountable for the origin of the majority of beta cells in adult life.

The rate of replication of beta cells in the adult pancreas is generally low (2-3% per day) but can undergo dynamic shifts to compensate for changes in body mass or during pregnancy to match increased insulin demand (Ackermann and Gannon, 2007, Bouwens and Rooman, 2005, Finegood et al., 1995). Using a lineage tracing model to label the initial beta cell population with human placental alkaline phosphatase, it has been shown that beta cell replication dominantly contributes to beta cell homeostasis in the adult (Dor et al., 2004) as well as during beta cell regeneration following beta cell loss (Nir et al., 2007). Several other studies have independently confirmed this (Teta et al., 2007). The importance of beta cell proliferation in the maintenance of the adult beta cell mass has also been shown in *cyclin D2*$^{-/-}$ mice. In these mice, the islets show normal prenatal development but in the adults the beta cell mass is almost undetectable (Georgia and Bhushan, 2004, Kushner et al., 2005).

The minimal role of progenitor cells especially in beta cell regeneration does not remain undisputed. It is accepted that in the foetus 10-20% of the beta cells arise by beta cell duplication but the remaining 80% or more is attributed to neogenesis from undifferentiated precursors (Bouwens and Rooman, 2005). The presence of putative precursor/stem cells in the adult

pancreas has been suggested (Weir and Bonner-Weir, 2004), however their localisation and possible contribution to beta cell homeostasis remains undetermined. Recent studies have shown, that in the partial duct ligated pancreatic injury model, regeneration occurs via neogenesis of beta cell from a Neurogenin3 positive progenitor (O'Neill et al., 2008, Xu et al., 2008). In the case of type 2 diabetics, beta cell neogenesis seems to be the predominant mechanism for beta cell mass regeneration while the beta cell replication rates remain relatively unchanged. Nonetheless, the neogenesis of beta cells from precursor cells in the undisturbed healthy adult pancreas remains debatable (Butler et al., 2003).

1B.4 Insulin- from gene to secretory granules.

In the adult, insulin is a protein that is produced almost exclusively in the beta cells of the islets of Langerhans, notwithstanding the low levels of extra-pancreatic insulin that has been detected in the brain (Devaskar et al., 1994), thymus (Pugliese et al., 1997), salivary (Vallejo et al., 1984) and lachrymal glands (Cunha et al., 2005). The well characterised function of the insulin released from the beta cells is to induce most of the body's cells (including myocytes and adipocytes) to take up glucose from the blood, storing the excess as glycogen in the liver and muscle, and minimise the use of fat as an energy source. However the role of the extra-pancreatic insulin is unclear. It has been suggested that it may play a role in the hormonal communication networks necessary to maintain overall energy balance or help establish immune tolerance (Hay and Docherty, 2006, Porte et al., 2005).

Most mammals have one copy of the insulin gene (*ins*). In the case of humans, the *INS* gene is located on chromosome 11 at position p15.5 (Harper et al., 1981). However few species including the rat and the mouse have two non allelic-variants of the insulin gene, namely *ins1* and *ins2*, of which *ins2* corresponds to the single copy in other species. It is thought that i*ns1* was retroposed from the partially processed *ins2* mRNA (Hay and Docherty, 2006, Shiao et al., 2008). Both the *ins* genes share strong homology to each other and for this reason are believed to be subjected to

the same regulatory pathways (Roderigo-Milne et al., 2002). The *ins* gene is small, consisting of three exons and two introns with the sequence immediately upstream of the transcription initiation site being defined as the *ins* promoter (German and Wang, 1994). Control of insulin gene expression is largely exerted at the transcriptional level through well defined elements located within this promoter region that bind to beta cell restricted as well as ubiquitous transcription factors (Fig. 1.8). The compactness of the *ins* promoter and the close proximity of the regulatory elements requires complex additive and synergistic interactions between the binding factors in order to achieve efficient transcription regulation. Furthermore, overlapping of regulatory elements introduces another level of control through binding competition between transcription factors (Docherty et al., 2005). The highly conserved A, E and C boxes act as *cis*-acting elements with no significant transcriptional activity of their own, but can dramatically boost *ins* promoter activity once bound by the transcription factors Pdx1, the E47/NeuroD heterodimer and MafA respectively (Docherty et al., 2005). The formation of functional transcriptional activator complexes are completed by the non-DNA binding co-activator-mediated stabilisation of the DNA-bound transcriptional factors (Qiu et al., 2002). Several upstream transcription factors such as Nkx2.2 are not essentially part of the transcription complex. However they regulate *ins* gene expression by influencing the expression of factors that are part of the complex (Ohneda et al., 2000a).

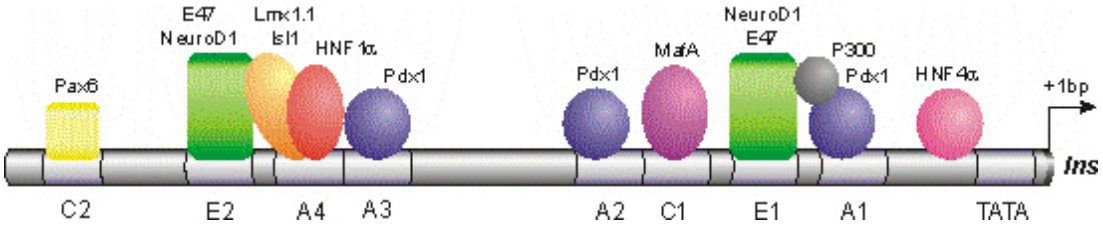


Figure 1.8. **The rat *ins1* promoter.** The well characterised DNA elements (between C2 and A1) of the rat *insulin* promoter are shown in grey. The major transcription factor complexes known to bind to these elements and activate the promoter are also shown (amended from Chakrabarti and Mirmira, 2003).

A further level of regulation of the *ins* gene is provided by the long-term nutritional and hormonal control of transcription. Glucose for instance, influences *ins* gene expression by affecting Pdx1 in multiple ways. Glucose can promote the nuclear inclusion of Pdx1, acutely stimulates Pdx1 binding to elements A1-A3 within the *ins* promoter and increases the activation potential of the Pdx1 activation domain (MacFarlane et al., 1994, Petersen et al., 1998). The cyclic binding of MafA and NeuroD to the *ins* promoter are however unhindered by changes in glucose concentration (Barrow et al., 2006). Fatty acids on the other hand inhibit *ins* transcription. Fatty acid metabolism results in *de novo* ceramide synthesis which can directly inhibit Pdx1 nuclear translocation and MafA gene expression (Hagman et al., 2005, Poitout et al., 2006). Once transcribed, the stability of the *ins* mRNA can be affected by several other factors such as somatostatin, glucose and L-leucine (Docherty and Clark, 1994, Philippe, 1993).

Successful translation of the *ins* mRNA results in the large precursor preproinsulin molecule which is bound to the ribosome. Following the association of the ribosome with the endoplasmic reticulum (ER) and subsequent ER entry via the sec61 translocon, the signal peptide is cleaved releasing the proinsulin molecule into the ER. Glucose can increase the rate of transfer of the free ribosomes bearing the nascent preproinsulin from the cytoplasm to the ER membrane (Wolin and Walter, 1988). The proinsulin molecule consists of the distinct carboxy-terminal A- and amino-terminal B-chains linked together by the connecting peptide (C-peptide). Proinsulin undergoes post-translational preoteolysis in ER and during the formation of the insulin secretory granule at the trans-Golgi network, to yield the mature functional hormone, insulin (Fig. 1.9). Proinsulin processing is a multi-step process. Firstly, the action of type 1 (prohormone convertase (PC) 1/3) and type 2 (PC2) endopeptidases cleave at the B-chain/C-peptide junction and the C-peptide/A-chain junction respectively. This is followed by the removal of the exposed basic amino acids at the C-terminus by the exoproteolytic action of carboxypeptidase E (Halban, 1994, Hutton, 1994). The remaining A- and B-chains are bound together by disulphide bonds and condensation

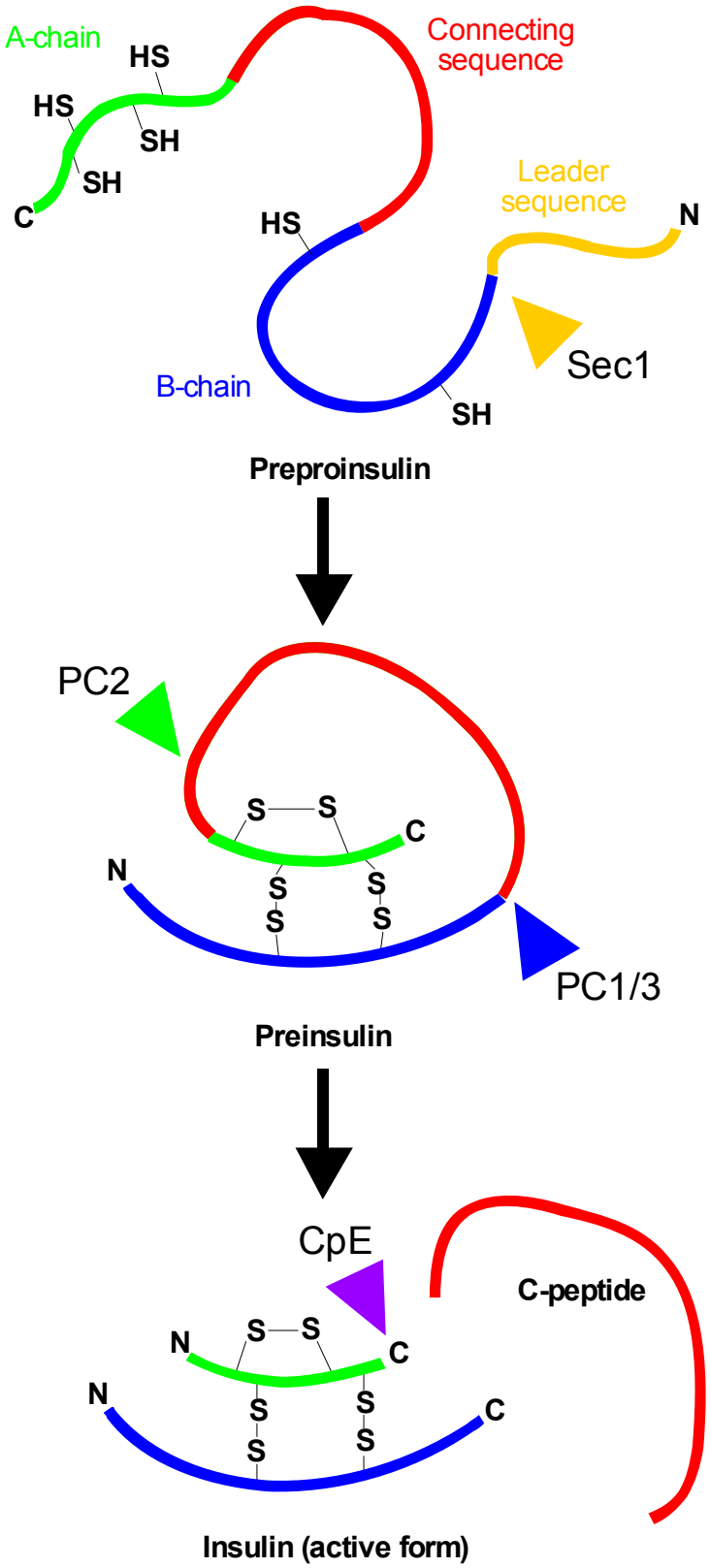


Figure 1.9. The enzymatic processing of insulin. Mature insulin protein is derived from preproinsulin molecule via serial steps of cleavage involving several endopeptidases (prohormone convertases, PC) and exopeptidases (carboxypeptidase E) . The connecting c-peptide is removed and the A and B chains are linked by disulphide bonds (based on figures in Halban, 1994).

of the insulin molecule takes place. The secretory granules (containing mature insulin) accumulate in the cytoplasm and are stored until a stimulus for secretion is received. Multiple levels of regulation from the transcription of the *ins* gene to post-translational modifications of the proinsulin molecule, ensure that a sufficient amount of functional and mature insulin is always available to meet demand. The efficient mobilisation of this insulin by the beta cells is the next step essential for maintaining glucose homeostasis.

1B.5 Beta cell function.

The two features that underlie the adequate functioning of a fully-differentiated beta cell are the ability to sense changes in external glucose concentration and the ability to release the required amount of stored insulin accordingly. In comparison to glucose signalling in yeast and plants, glucose signalling in mammalian pancreatic beta cells requires extensive glucose catabolism (reviewed in Rolland et al., 2001). Glucose is transported into the beta cell through GLUT2, a facilitated glucose transporter. Glycolysis then takes place inside the cell, where phosphorylation, initiated by the enzyme glucokinase (with an islet specific homolog), acts as the rate limiting step for the reaction. However, neither GLUT2 nor glucokinase act as direct glucose sensors. Instead it is the increase in the ATP:ADP ratio as a result of glycolysis that inhibits the hetero-octomeric ATP-dependent transmembrane K^+-channel (composed of the Kir6.2 subunits and the sulphonylurea receptor (SUR1) subunits) which causes membrane depolarisation (Dunne, 2000). Consequently, voltage gated Ca^{2+} channels open and Ca^{2+} flows into the beta cell. The intracellular Ca^{2+} concentration is further increased by IP3 (inositol 1,4,5-triphsophate)-mediated Ca^{2+} induced Ca^{2+} release from ER stores. A significant increase in the cytosolic Ca^{2+} concentrations then causes the insulin secretory vesicles to move towards the plasma membrane, dock, fuse and release their content into the circulating blood by exocytosis (Pertusa et al., 1999; Fig. 1.10). Both glucose metabolism and Ca^{2+} levels oscillate resulting in pulsatile but synchronised insulin secretion by all the beta cells with a periodicity of 3-6 minutes (Gilon et al., 2002). It is

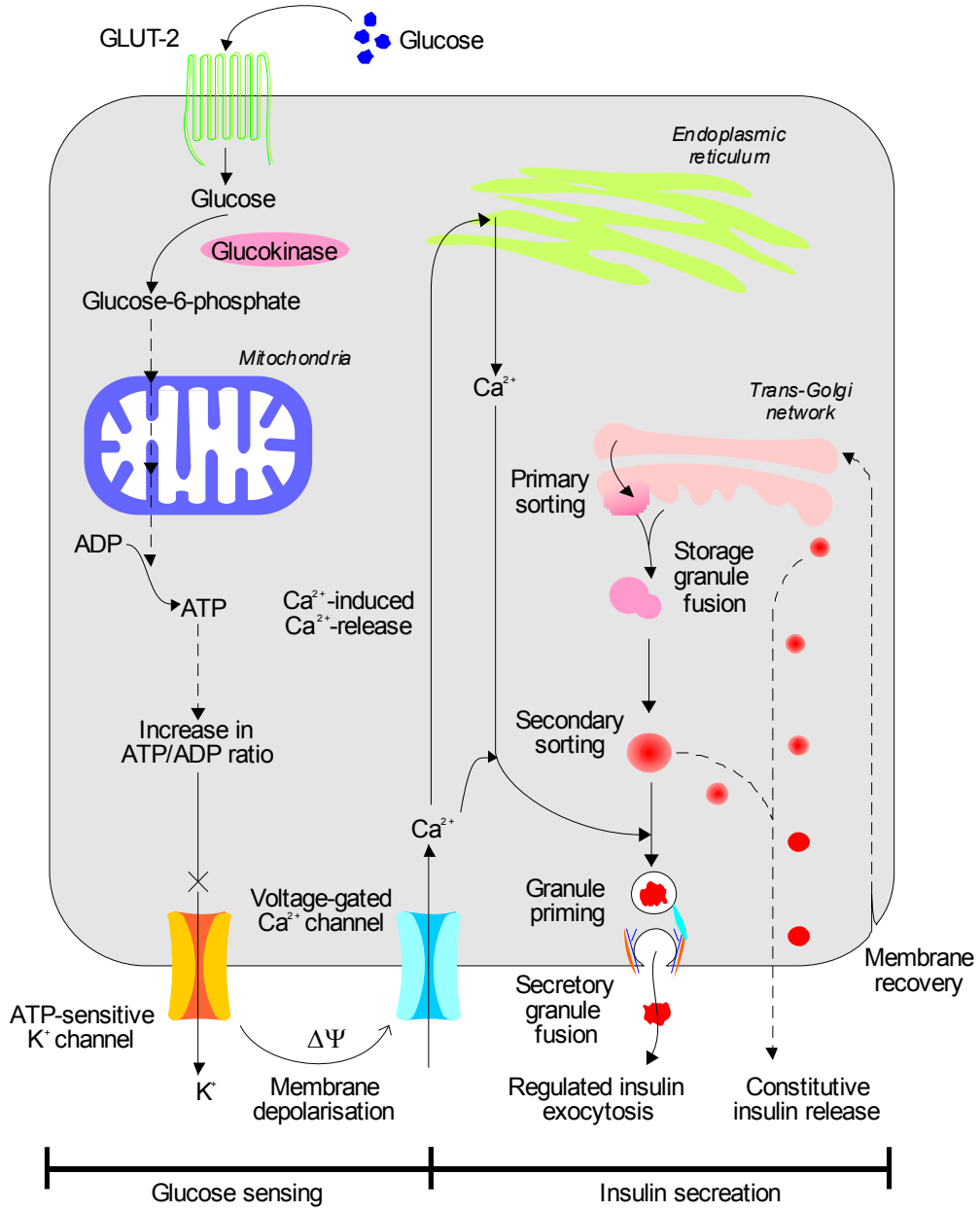


Figure 1.10. The glucose sensing and insulin secretion mechanisms in the pancreatic beta cell. Glucose that is internalised into the cell via the Glut2 transporter is phosphorylated mainly by glucokinase during glycolysis. This results in the production of ATP from ADP and a resultant increase in the ATP/ADP ratio which in turn inhibits ATP-sensitive K+ channels causing membrane depolarisation (ΔΨ). This is thought to activate voltage-gated Ca2+ channels leading to IP3-mediated Ca2+-induced Ca2+-release from Ca2+ stores in the endoplasmic reticulum. The increase in intracellular Ca2+ levels causes the fusion of the insulin secretory granules with the plasma membrane in a regulated manner. Secretory granules are formed by the condensation of the secretory product in the trans-Golgi network. The initial sorting event is followed by granule fusion, vesicular budding and maturation. Insulin protein that is not sorted in this manner appears to be secreted constitutively. Membrane proteins associated with both regulated and constitutive exocytosis are thought to be recycled back to the trans-Golgi network (based on figures in Rolland et al., 2001 and Hutton, 1994).

through this regulated insulin secretion pathway that most of the insulin of a fully-differentiated beta cell is secreted. However, some insulin is secreted by the beta cells through the constitutive insulin secretion pathway without stimulation by glucose. It is now widely accepted that low levels of constitutive insulin secretion is necessary for the beta cell to maintain sensitivity to glucose and also for the glucose-stimulated regulated insulin release (Srivastava and Goren, 2003).

In addition to glucose and insulin itself, several other factors modulate glucose-stimulated insulin secretion. They include circulating fatty acids (Stein et al., 1996), acetyl CoA and protein kinase C levels (Yaney et al., 2000). Furthermore, transcription factors such as MafA can play an important role in regulating insulin secretion. Although the regulation might not be direct, it is possibly mediated by the ability of the transcription factors to influence the expression of many genes such as *Glut2* and *glucokinase* that are involved in glucose-stimulated insulin secretion. For example, MafA has been implicated as a key regulator of insulin release since MafA-deficient mice show impaired glucose-stimulated insulin secretion and are glucose intolerant (Zhang et al., 2005). Similarly, a modest reduction in Pdx1 levels can result in the dysregulation of normal glucose sensing and insulin release in beta cells by affecting glycolysis, mitochondrial stability as well as intracellular Ca^{2+} mobilisation (Brissova et al., 2002). Such changes can manifest in the form of diabetes mellitus.

1C DIABETES MELLITUS

Diabetes mellitus is a multifactorial disease characterised by disordered glucose metabolism resulting in high blood glucose levels or hyperglycaemia. It can be broadly classified as those caused by an insufficient production of insulin (type 1 diabetes mellitus and maturity-onset diabetes of the young/ MODYs) and those due to the diminished response of the body to insulin (type 2 diabetes mellitus and gestational diabetes). While the MODYs are

caused by autosomal dominant inheritance of mutations in individual genes (*Hnf4α*, *glucokinase*, *Hnf1α*, *Pdx1*, *Hnf1β* and *NeuroD*), polygenic recessive mutations underlie the susceptibility to type 1 and type 2 diabetes. Since it is type 1 diabetes that is primarily associated with beta cell failure and loss, it will be dealt with in more detail. In its various forms, diabetes affects over 200 million people worldwide, of which 10% of cases correspond to type 1 diabetes (Wild et al., 2004).

1C.1 Pathophysiology of Type 1 diabetes

Type 1 diabetes is regarded as an autoimmune disease whereby the beta cells of the body are destroyed by autoimmune attack. The trigger for the autoimmune attack is not yet identified, but a possible induction by viral infections has been postulated (Oldstone et al., 1991). Various other environmental triggers including dietary factors such as (antibodies raised against) cow's milk proteins, vitamin D_3 deficiency and pharmacological agents have also been suggested (reviewed in Knip and Akerblom, 1999). However it is the complicated interplay between environmental triggers and genetic disease predisposition that leads to the progression to clinical type 1 diabetes.

Following the autoimmune destruction of the beta cells, the deficiency of the hormone insulin, results in inefficient glucose homeostasis and hyperglycaemia. When blood glucose concentrations overshoot the renal threshold, incomplete reabsorption of glucose, results in glycosuria and polyuria, leading to polydipsia. Polyuria, polydipsia and polyphagia manifest as the classical triad symptoms of diabetes. Prolonged hyperglycaemia can lead to long term complications of diabetic retinopathy, nephropathy, neuropathy and cardiomyopathy. It is believed that non-enzymatic glycosylation of proteins under herperglycaemic conditions underlies many of the chronic complications of diabetes. Increases in the amount of endothelial surface glycoproteins lead to basement membrane disruption and damage to the vasculature primarily in the retina, renal glomeruli and macrovessels (King et al., 1994, Brownlee, 2001, Nathan, 1996). Glycosylation of

erythrocyte proteins (haemoglobins), plasma protein (albumin, low-density lipoproteins and immunoglobulins), nerve proteins (myelin-associated proteins), lens proteins (crystallins), and some extracellular matrix components (collagens) can further exacerbate the complications (Kennedy and Baynes, 1984). There is growing evidence to suggest that in addition to hyperglycaemia, some of the complications might also be caused due to altered fatty acid, protein and nitric acid metabolism, altered NAD^+/NADH ratios and insulin deficiency itself (Brownlee, 2001, Nathan, 1996).

Despite the individual mechanisms involved, many of the complications of diabetes are chronic and if left untreated patients can succumb to death by heart failure and/or ischemic stokes. Therefore developing a therapy and a potential cure for diabetes as a means of alleviating effects of hyperglycaemia is of paramount importance.

1C.2 Therapies for type 1 diabetes.

Insulin dependency of type 1 diabetic patients requires exogenous insulin supplementation to control hyperglycaemia. This is the most widely used means of controlling blood glucose levels in type 1 diabetics as well as late-stage type 2 diabetics with reduced beta cell mass (Limbert et al., 2008). However, exogenous administration of insulin does not ensure satisfactory or continuous control of blood glucose. Hyperglycaemia and hypoglycaemia events are common due to incorrect dosage. Therefore, ideal therapies and potential cures for type 1 diabetes are aimed towards the rejuvenation or the replacement of the depleted beta cell mass.

Restoring the regenerative capacity of the pancreas, and in particular beta cell rejuvenation, is an interesting avenue of research for a potential diabetes therapy. As already mentioned above (in Section 1B.3) the mechanism by with the beta cell mass is regulated postnatally is still debatable. Although beta cell replication may be the predominant mechanism for beta cell maintenance in the normal adult pancreas, the situation could prove to be different in the type 1 diabetic pancreas (as is the case for the type 2 diabetic pancreas). In several studies, various extrinsic factors have been utilised to

promote beta cell proliferation and neogenesis *in vitro*. Human islets have been expanded *in vitro* using a combination of serum-free culture media, growth factors and insulinotropic agents such as nicotinamide (Beattie et al., 2002, Gao et al., 2003). However, a combination of senescence and de-differentiation in culture leads to the loss of beta cell function in long term culture and after mass expansion. Glucagon-like peptide 1 (GLP1) can inhibit apoptosis and increase the proliferation of beta cells both *in vitro* and *in vivo* thus proving to be a potential candidate to be used for diabetes therapy (Brubaker and Drucker, 2004). Similarly, administration of a combination therapy of EGF and gastrin has been shown to enhance the expansion of the beta cell mass *in vivo* in non-obese diabetic (NOD) mice (Suarez-Pinzon et al., 2005). However, the protection of the rejuvenated beta cell mass from ongoing autoimmune destruction is a challenge and requires rigorous immunomodulatory regimes.

An alternative to regenerating the beta cell mass is to find functional substitutes for the beta cells lost to autoimmune attack. One option is to transplant islets isolated from cadaveric pancreata. Islet transplantation using the Edmonton protocol has enabled many type 1 diabetic patients to achieve long-term insulin independence and restore normoglycaemia. The protocol involves the isolation of islets of the donor using enzymatic digestion, prior to infusion into the patient's portal vein (Shapiro et al., 2000). Islet transplantation is however not a cure for type 1 diabetes and by 5 years after transplantation only 10% of patients attain glycaemic control with the average duration of insulin independence being 15 months (Ryan et al., 2005). Compared to whole pancreas transplantation, islet transplantation is technically simpler, has lower morbidity and allows the harvested islets to be stored long-term in cryopreservation if immediate utilisation is not required. In spite of this, allogenic transplantations necessitate strict immunosuppressive therapies, frequently accompanied by an array of adverse side effects making it unattractive, especially for paediatric applications. Moreover, the source of islets is limited by the number of donors, which is exacerbated by the fact that the Edmonton protocol requires cells (11000 islets per kg of body weight of patient) from up to three donors for successful transplantation

(Limbert et al., 2008). The scarcity of pancreatic tissue from human donors has spurred the search for alternative sources of beta cells for transplantation. Xenogenic tissue such as porcine islets are more readily available and has been considered a good source of cells for transplantation since the generation of transgenic pigs lacking xenogenic surface antigens (Phelps et al., 2003). However the transmission of xenozoonotic viruses still remains a possibility.

The use of *in vitro*-derived cells for transplantation engenders the relatively novel discipline of cell-based therapies for degenerative diseases. The 'non-beta' sources of insulin-producing cells can be broadly divided into four categories: the use of, 1- insulin-producing cell lines, 2- insulin-producing cells derived from embryonic stem cells, 3- insulin-producing cells differentiated from adult stem cells and progenitor cells, 4- use of cells derived from differentiated cells either by direct molecular reprogramming or by using induce-pluripotent stem cell technology. Most of these studies are still in their infancy in regards to clinical application and have only been tested in rodent models.

Strict criteria have been laid down to outline what unequivocally characterises a complete *in vitro* differentiation event of a 'non-beta' cell into an autologous beta cell safe for clinical applications (Lechner and Habener, 2003). Firstly, the original source of cells from which the beta cells are to be derived should be fully characterised. Upon deriving the autologous beta cells *in vitro*, the following criteria needs to be met: 1- the expression of beta-, islet- and endocrine-specific markers (other than insulin) should be demonstrated, 2- the presence of insulin secretory granules should be confirmed by ultramicroscopic studies, 3- *in vitro,* beta cell function in terms of glucose responsiveness, the responsiveness to other secretagogues and incretins and electrophysiological properties should be reminiscent of their natural counterparts, 4- *in vivo,* a reproducible and durable attenuation of the diabetic phenotype should be demonstrated following the transplantation of the autologous beta cells along with the reappearance of the diabetic phenotype upon their removal. Additionally, the tumourigenicity of the end

product as well as the immune response towards the transplanted cells should be thoroughly examined.

With these criteria in mind, one source for deriving insulin-producing cell lines that is commonly used, is the pancreatic beta cells themselves. As stated previously the long-term culture of isolated beta cells is thwarted by their de-differentiation in culture and loss of beta cell properties. However, reversibly immortalised beta cell lines (such as NAKT-15) can now be created from isolated human beta cells and maintained *in vitro* by retrovirus-mediated insertion and subsequent excision of the immortalising genes simian virus 40 large T-antigen (SV40T) and human telomerase reverse transcriptase (hTERT) from the genome (Narushima et al., 2005). Similarly the human PANC-1 cell line and the rat ARIP cell line can be induced to differentiate into insulin-secreting endocrine cells by exposing them to GLP1. However this does require the expression of Pdx1 which is not normally expressed in the PANC-1 or ARIP cells (Hui et al., 2001). The surrogate beta-like cells generated in this manner can rescue alloxan- and/or streptozotocin-induced diabetic mice after transplantation. Irrespective of how closely a modified cell line resembles primary beta cells, all of their phenotypic features are not defined. Therefore one should approach with caution when using such cells for cell based therapy.

The derivation of insulin-producing cells from embryonic stem (ES) cells has also been achieved with variable success (reviewed in Docherty et al., 2007). The first studies utilised the nature of ES cells to spontaneously differentiate into a large variety of cell types. Using a cell trapping strategy, the spontaneously generated insulin expressing cells were selected by driving the neomycin resistance gene off the *ins* promoter (Soria et al., 2000). A more directed approach to differentiating ES cells has also been employed such as using a modified protocol for ES cell to neuron differentiation, by deriving an enriched population of nestin-postive intermediates from embryoid bodies. By manipulation and supplementation of the culture medium (Lumelsky et al., 2001), ectopic expression of Pax4 (Blyszczuk et al., 2004) and PI3K inhibition (Hori et al., 2002), insulin-positive cells have been derived from ES cells. However, the levels of expression of insulin in

these beta-like cells were inadequate and the efficiency of the protocols in terms of the number of cells generated was in each case extremely low. Therefore recently, a more directed approach to ES cells to beta cell differentiation has been pursued, recapitulating the normal developmental biology of the pancreas. Using such an approach, ES cell differentiation is directed through various checkpoints corresponding to undifferentiated ES cells, definitive endoderm, posterior foregut endoderm, pancreatic endoderm, islet precursors and finally differentiated beta cells, each expressing a unique array of markers specific to that particular stage (Fig. 1.11). Subsequent studies have used directed differentiation of human ES cells, first to generate definite endoderm then endocrine hormone expressing islet cells including beta cells, which when transplanted were capable of restoring normoglycaemia in diabetic mice (D'Amour et al., 2005, D'Amour et al., 2006, Kroon et al., 2008). Nonetheless, the turmurogenic potential of ES cells in giving rise to teratomas and teratocarcinomas in humans makes them a less desirable option for clinical application.

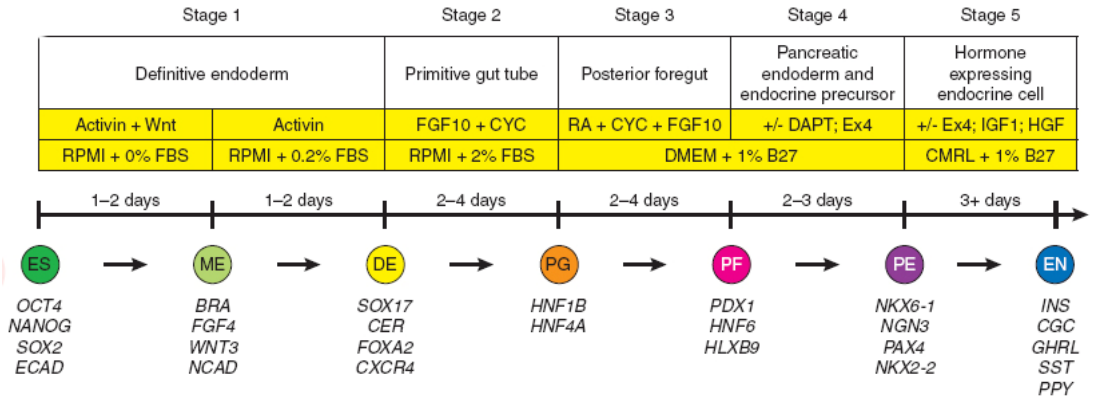


Figure 1.11. **A protocol for deriving endocrine cells from human ES cells.** The figure schematically outlines how human ES cells can be differentiated *in vitro* into hormone-expressing endocrine cells. The culture media composition and growth factor supplementation at each stage is given in the yellow boxes. The protocol involves five stages of differentiation and aims to achieving a specific identifiable endodermal intermediate at the end of each stage. The lower panel indicates the markers characteristic of each stage of differentiation (D'Amour et al., 2006).

Adult stem cells and progenitor cells are present in the body throughout life and are more ethically sound than ES cells, constituting an attractive source

of cells for cell therapy. However, adult stem cells are rare, have low proliferative rates and reside in stem cell niches that are hard to identify. Pancreatic stem or progenitor cells have been isolated from adult ducts and islets by a combination of flow cytometry and clonal analysis identified by the expression of Neurogenin3, c-met and/or nestin (Seaberg et al., 2004, Suzuki et al., 2004). Alternatively insulin-expressing cells have been derived from more well defined extra-pancreatic stem/progenitor cells such as mesenchymal stem cells (MSC). Despite the low levels of insulin production, MSC-derived beta-like cells are still capable of rescuing diabetes in streptozotocin-treated mice (Tang et al., 2004). Transplanted MSC themselves have been shown to enhance the number of endogenous beta cells in non-obese diabetic severe combine immunodeficient (NOD/SCID) mice (Lee et al., 2006).

The use of differentiated cells for the generation of insulin producing beta-like cells often fails to fulfil the rigorous criteria that define a *bone fide* transdifferentiation event. Yet it is still of immense interest to cell-based therapeutic modalities. Functional beta-like cells have also been generated from cultured adult exocrine pancreatic cells treated with leukaemia inhibitory factor (LIF) and epidermal growth factor (EGF) (Baeyens et al., 2005). Similarly by over-expressing *Neurogenin3, Pdx1* and *MafA,* adult murine pancreatic exocrine cells have been converted into beta-like cells that can ameliorate hyperglycaemia (Zhou et al., 2008). Furthermore, based on the concept that liver and pancreas arise from the same region of the developing embryo, recent work has focused on the conversion of hepatocytes to beta cells. Most approaches have been based on over-expression of *Pdx1*, the master gene controlling pancreatic development. A modified form of the *Pdx1* homolog *Xlhbox8* carrying the *VP16* transcriptional activation domain from *Herpes simplex*, has been used to convert liver to pancreas in transgenic *Xenopus* tadpoles. The *Xlhbox8-VP16* construct was driven off the *transthyretin* (*TTR*) promoter in order to direct expression in the liver and contained a *GFP* tag under the pancreatic *elastase* promoter (Beck and Slack, 1999, Horb et al., 2003). Transient expression of *Xlhbox8-VP16* may induce formation of ectopic whole pancreas, in an otherwise normal animal,

giving rise to both endocrine and exocrine cell-types (Horb et al., 2003). It is thought that the appearance of ectopic pancreata in this case is the result of a metaplastic conversion of at least partially differentiated liver cells rather than simply changing the developmental fate of undifferentiated endodermal cells. This investigation also discovered that the same construct could also induce similar effects in the human hepatoma HepG2 cell line (Li et al., 2005a). Pdx1 can also induce the expression of pancreatic genes in many other cell-types including foetal liver progenitor cells and in a liver stem line-the WB cells (Zalzman et al., 2003, Tang et al., 2006). *In vivo* transformation of hepatocytes into insulin-producing cells has been successfully achieved by the intravenous injection of adenoviruses encoding *Pdx1* (with and without *VP16*) and/or various other key regulatory pancreatic transcription factors including *NeuroD, Neurogenin3* and *MafA* (Ber et al., 2003, Imai et al., 2005, Kaneto et al., 2005a, Kaneto et al., 2005b, Koizumi et al., 2004, Kojima et al., 2003, Shternhall-Ron et al., 2007). Beta-like cells produced from liver cells were able to rescue streptozotocin induced diabetes in mice. Likewise, adult rat and human hepatocytes have been transformed *in vitro* and subsequently transplanted into diabetic mice, correcting hyperglycaemia for various lengths of time (Sapir et al., 2005, Meivar-Levy et al., 2007). Compared to the *in vivo* approaches, *in vitro* transformation of cells has the added advantage of overcoming the narrow tropism of the viruses especially for organs such as the liver. It also reduces vector toxicity by minimising systemic infections.

The derivation of induced pluripotent stem (iPS) cells from both adult mouse (Takahashi and Yamanaka, 2006, Okita et al., 2008) and human (Takahashi et al., 2007) somatic cells could potentially serve as an easily accessible and immunotolerant source of cells to generate beta cells *in vitro* for transplantation. Nevertheless, understanding the immunological causes of diabetes is an area of research that should be addressed alongside the search for new sources of beta cells for cell-replacement therapy. New avenues of research that are being actively pursued in regards to a immuno/cell-therapy for type 1 diabetes include monocyte derived dendritic

cells and autologous cord-blood infusion, and autologous nonmyeloablative haematopoietic stem cell transplantation.

1D AIMS AND OBJECTIVES.

The work outlined in this thesis was aimed to develop a protocol for the generation of fully differentiated beta-like cells by the *in vitro* molecular reprogramming of primary hepatocytes.

The objective was to identify the specific culture conditions, the transcription factors and extrinsic factor combinations necessary for such a hepato-pancreatic conversion. The cells derived by hepato-pancreatic reprogramming should be able to closely resemble *bone fide* beta cells in terms of gene expression and protein synthesis as well as function. Therefore based on their performance in functional tests, additional rectifications and amendments to the protocol were undertaken to improve the quality of the beta cell phenotype achieved.

Chapter TWO

Materials and Methods

2A MATERIALS

All chemicals were dissolved in distilled water unless stated otherwise in the manufacturer's instructions. See Appendix A for a full list of all commercial suppliers.

2A.1 General laboratory chemicals

NaCl, KCl, ethylenediaminetetraacetic acid (EDTA), ethanol, methanol, isopropanol, acetone, chloroform, paraformaldehyde (PFA) and Tris(hydroxyl methyl) aminomethane (Tris Base) were purchased from Fisher Scientifics. $CaCl_2$, CsCl, K_2HPO_4, sodium azide, D-glucose, D-sucrose, formaldehyde, phenol red, Tween-20, Triton x-100, tricine, glycine, sodium dodecylsulphate (SDS) and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), were all obtained from Sigma. Electrophoresis grade agarose (Invitrogen) and phosphate buffered saline (PBS; Autogen-Bioclear) were also used.

2A.2 Laboratory equipment

Spectrafuge 16M (Jencon-PLS) was used as the routine bench-top centrifuge. Allegra® 25R swinging bucket centrifuge, L-80 Ultracentrifuge (Beckman) and ALC PK110 centrifuge (Thermo) were used for specific purposes (see 2B: methods). SSM3 mini-gyro rockers, Denley Spiramix 5 rollers and a PMS-1000 Bio-Gen platform shaker (Jencon-PLS) were used during long incubation steps.

For microscopy, $MZ7_5$ dissection microscopes, a DM*IRB* inverted microscope, a DMRB microsope (all Leica) and LSM 510 and LSM 510-META confocal microscopes (Zeiss) were used (see 2B.13 for details). A DU® 530 UV/Vis Spectrophotometer (Beckman) was used to measure DNA and RNA concentrations. A Spectra Rainbow Thermo microplate spectrophotometer (Tecan) was used to measure protein concentration.

2A.3 Cell culture reagents and media

Table 2.1. Tissue culture cell lines and their passaging densities.

Cell lines	Passage	Source
Rat insulinoma (RIN-5F)	1/5	European Collection of Cell Cultures, Porton Down, UK
Murine insulinoma (MIN6)	1/3	
Human embryonic kidney epithelium (HEK293)	1/12	
Rat pancreatic exocrine (AR42J-B13)	1/10	Itaru Kojima, Junetendo University School of Medicine, Tokyo, Japan
Bipotential mouse embryonic liver (BMEL)	1/15	George Yeoh, University of Western Australia, Pert, Australia

Table 2.2. Composition of media used for cell culture.

Media	Composition
Hepatocyte Attachment Medium	William's Medium E (Sigma) with 10% (v/v) FBS, 10U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine
Hepatocyte Differentiation Medium (KS)	Keratinocyte Serum Free Medium (KSFM; Gibco) supplemented with 2mM L-glutamine, 10U/ml penicillin, 100µg/ml streptomycin, 100µg/ml amphotericin B, 50µg/ml gentamycin, 50µg/ml bovine pituitary gland extract (bPGE) and 5ng/ml human recombinant EGF (rhEGF).
Hepatocyte Differentiation Medium (KdS)	Same as KS medium, supplemented with 10nM dexamethasone.
Hepatocyte De-differentiation Medium (DS)	Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% (v/v) FBS, 2mM L-glutamine, 10U/ml penicillin, 100µg/ml streptomycin, 100µg/ml amphotericin B, 50µg/ml gentamycin, 50µg/ml bPGE and 5ng/ml rhEGF.
Dissection Medium	Minimal Essential Medium Eagle's with Hank's salts (MEM-E; Sigma) and 10% (v/v) FBS.
Embryonic Pancreas Culture Medium	Basal Medium Eagle's with Earle's salts (BME; Sigma), 20% (v/v) FBS, 2mM L-glutamine, 10U/ml penicillin and 100µg/ml streptomycin.
RIN-5F Culture Medium	RPMI1640 Medium (Sigma), with 10% (v/v) FBS, 2mM L-glutamine, 10U/ml penicillin and 100µg/ml streptomycin.
MIN6 Culture Medium	High glucose (25mM) DMEM with 10% (v/v) FBS, 2mM L-glutamine, 10U/ml penicillin and 100µg/ml streptomycin.
HEK293 & B13 Culture Medium	DMEM with 10% (v/v) FBS, 2mM L-glutamine, 10U/ml penicillin and 100µg/ml streptomycin.
BMEL Growth Medium	William Medium E with 10% (v/v) FBS, 2mM L-glutamine, 10U/ml penicillin, 100µg/ml streptomycin, 100ng/ml amphotericin B, 30ng/ml IGF2, 20ng/ml human recombinant EGF, 10µg/ml insulin (humulin; Sigma)

Heat-inactivated foetal bovine serum (FBS; Gibco^{TM}/Invitrogen) was used routinely for all cell culture media, except with HEK293 cells where γ-irradiated FBS was used instead. Stock solutions of 200U/ml penicillin, 2mg/ml streptomycin, 40mM L-glutamine (Sigma), 2mg/ml amphotericin B, gentamycin, trypsin-EDTA (Gibco^{TM}/Invitrogen) were used. 1mM dexamethasone was made up in 100% ethanol and stored at -20°C. 0.4% (w/v) trypan blue (Sigma) in 0.81% (w/v) NaCl, 0.06% (w/v) K_2HPO_4 was diluted 1:1 to assess cell viability.

2A.4 Adenoviral vectors

Table 2.3. List of adenoviral vectors used for gene transduction.

Adenoviral construct	Titre (iu/ml)	Source
Ad-CMV-Null	2.0×10^8	Harry Heimberg, Vrije Universiteit, Brussels, Belgium.
Ad-CMV-Pdx1-eGFP	1.0×10^{10}	
Ad-CMV-Pdx1VP16-eGFP	1.2×10^{11}	
Ad-CMV-HA-Ngn3-eGFP	7.7×10^{11}	
Ad-CMV-Pax4	3.8×10^{10}	
Ad-CMV-Pax6	3.7×10^{10}	
Ad-CMV-HA-Nkx6.1-eGFP	4.6×10^{10}	
Ad-CMV-Nkx2.2	7.0×10^{10}	
Ad-CMV-IA1-eGFP	1.3×10^{12}	
Ad-CMV-NeuroD	5.2×10^{11}	Vector Biolabs.
Ad-CMV-Sox9-IRES-eGFP	5.2×10^{10}	Made from a plasmid construct from Neil Hanley, University of Southampton, Southampton, UK.
Ad-CMV-p48-IRES-GFP	9.9×10^9	James Dutton, Stem Cell Institute, Minneapolis, MN, USA.
Ad-CMV-p63	7.6×10^{11}	Wei-Yuan Yu, University of Bath, UK.
Ad-RSV-GFP	1.0×10^{10}	Emma Regardsoe, University of Oxford, Oxford, UK.
Ad-CMV-LacZ	7.0×10^9	Andrew Byrnes, University of Oxford, Oxford, UK.
Ad-CMV-LIP	1.8×10^{11}	Hiroshi Sakaue, Kobe University, Kobe, Japan.
Ad-CMV-MafA-eGFP	2.8×10^8	Takaaki Matsuoka, Osaka University, Osaka, Japan.
Ad-TTR-Xlhbox8	1.5×10^{12}	Mark Hornsey, University of Bath, Bath, UK.
Ad-TTR-Xlhbox8VP16	1.3×10^{12}	

CMV- Cytomegalovirus promoter; RSV- Rous-sarcorma virus promoter, TTR- transthyretin promoter; eGFP- enhanced green fluorescent protein; HA- haemagglutinin tag.

2A.5 Antisera for immunocytochemistry

Table 2.4. List of primary antibodies used for immunocytochemistry.

Antibody	Species	Supplier	Dilution
α-foetoprotein (AFP)	rabbit	DAKO	1:100
Albumin	rabbit	DAKO	1:100
Amylase	rabbit	Sigma	1:200
Carbamoyl phosphate synthetase (CPS)	rabbit	*Gift*[a]	1:1000
Chromogranin A	rabbit	DAKO	1:100
C-peptide	rabbit	Acris	1:100
Cytokeratin 19	mouse	*Gift*[b]	1:1
Cytokeratin 20	mouse	Abcam	1:100
E-cadherin	rat	Epitomics	1:100
Ghrelin	rabbit	Phoenix	1:50
Glucagon	mouse	Sigma	1:100
Glut2	rabbit	Biogenesis	1:100
Glutamate synthetase	mouse	Transduction Labs	1:200
Haemagglutinin	mouse	Santa Cruz	1:100
Haptoglobin	rabbit	Sigma	1:100
HNF4α	rabbit	Santa Cruz	1:100
Insulin	guinea pig	DAKO	1:300[f], 1:100[g]
Insulin	mouse	Sigma	1:300[f], 1:100[g]
MafA	rabbit	Abc am	1:100
NeuroD	goat	Santa Cruz	1:100
Neurogenin3	rabbit	*Gift*[c]	1:100
OV6	mouse	*Gift*[d]	1:200
Pancreatic Polypeptide	rabbit	Zymed	1:50
Pdx1	rabbit	*Gift*[e]	1:100
PECAM-1	rat	BD Pharmingen	1:100
PGP9.5	rabbit	AbD Sero Tec	1:50
Phospho-histone 3	rabbit	Upstate	1:200
Smooth muscle actin (SMA)-Cy3 conjugate	mouse	Sigma	1:300
Somatostatin	rabbit	DAKO	1:100
Sox9	rabbit	Chemicon	1:100
Thy1	rabbit	Santa Cruz	1:50
Transferrin	rabbit	DAKO	1:500
Transthyretin	rabbit	DAKO	1:100
UDP-glucuronosyl transferase (UGT)	sheep	Cypex	1:200
Vimentin	mouse	Sigma	1:100

a- Wouter Lamers, University of Amsterdam, Amsterdam, The Netherlands; b- Birgit Lane, Institute of Medical Biology, Singapore; c- Michael German, UCSF, CA, USA; d- Stewart Sell, Ordway Research Institute & Wadsworth Centre, Albany, NY, USA; e- Jonathan Slack, University of Bath, UK; f- for pancreas; g- for all other tissue.

Table 2.5. List of secondary antibodies used for immunocytochemistry. All secondary antibodies were used at a dilution factor of 1 in 200.

Antibody	Species	Conjugate	Supplier
Anti-rabbit IgG	goat	TRITC	Vector Laboratories
		FITC	
	donkey	HRP	
Anti-mouse IgG	horse	Texas Red	
		FITC	
		AMCA	
		HRP	
Anti-sheep IgG	rabbit	Texas Red	
Anti-rat IgG	rabbit	FITC	
Anti-goat IgG	rabbit	FITC	
Anti-guinea pig IgG	goat	FITC	
	rabbit	TRITC	Sigma
	goat	HRP	Abcam

TRITC- tetramethylrhodamine isothiocyanate; FITC- fluorescein isothiocyanate; HRP- horse radish peroxidase; AMCA- aminomethylcoumarin acetate.

The conjugated compounds *Amanita phalloides* phalloidin-TRITC (Sigma) and *Dolichos biflorus* agglutinin (DBA)-FITC (Vector Biolabs) were used at dilutions of 1/1000 and 1/400 respectively. 4,6-diamidino-2-phenylindole (DAPI, Sigma) was used at 1:1000 to visualise the nuclei.

2A.6 Antisera for western blot analyses.

Table 2.6. List of primary & *HRP-conjugated secondary antibodies used.

Antibody	Supplier	Species	Dilution
Insulin	Santa Cruz	rabbit	1:100
Glucagon	Sigma	mouse	1:2000
Amylase	Sigma	rabbit	1:2000
Somatostatin			1:500
Ghrelin	Phoenix Pharmaceuticals		1:5000
HNF4α	Santa Cruz		1:1000
Pdx1	*Gift*[a]		1:100
Ptf1a	Abcam		1:300
Sox9	Chemicon		1:1000
pStat3^{Tyr705}	Cell Signalling		1:1000
CK19	*Gift*[b]	mouse	1:1000
α-tubulin	Sigma		1:4000
*Anti-rabbit IgG	Vector Laboratories	donkey	1:2000
*Anti-mouse		Sheep	1:2000

IgG			

2A.7 Primers for polymerase chain reaction.

The PCR primers were designed using the online Primer3^{TM} software (Rozen and Skaletsky, 2000) and purchased from MWG Primers service, UK.

Table 2.7. List of PCR primers and their annealing temperatures T_m.

	Gene	Forward/ Reverse (5' to 3')	T*m*/ °C	Product/ bp
Rat	α1-antitrypsin (AAT)	TGTCCCCTATGACCATCACC/ TCACTGTCTGGCCTGTTGAG	56	376
	Albumin	GCCCTACCCACAAAGCCTCAG/ AGCCCCTTCATATCACAGAGCA	56	543
	Alpha-foetoprotein (AFP)	ACCAAGTGTGGATTCTCAGG/ ATTGATGCTCTCTTTGTCTG	54	276
	Amylase	GCCTACTGACAGAGCCCTTG/ TGGTCCAATCCAGTCATTCA	58	200
	Beta-actin	TCCGTAAAGACCTCTATGCC/ AAAGCCATGCCAAATGTCTC	56	477
	Cdx2	TCCCTAGGAAGCGAAGTGAA/ CTGCGGTTCTGAAACCAAAT	57	187
	C/EBPα	GTTCCACATCGCACACTGCG/ TGACCAAGGAGCTCTCAGGC	55	426
	Chromogranin A	ACTAAGGTGATGAAGTGTGT/ TCTCTACAGTGTCCTTGGAG	58	353
	Chymotrypsin (Crtb)	GATCGCGAGGTCTTTAGAA/ CATCGACGTTGGGTGACAC	55	298
	CK19	ACCATGCAGAACCTGAACGAT/ CACCTCCAGCTCGCCTTAG	59	312
	c-kit	AGCAAGAGTTAACGATTCCGGAG/ CCAGAAAGGTGTAGGTGCCTCCT	60	340
	Carbamoyl phosphate synthetase 1 (CPS1)	CGTCCAAGATTCCTTGGTGT/ ATGGAAGAGAGGCTGGGATT	57	158
	Elastase 1	GGAACCATCCTGGCTAACAA/ ACACACCATGGTCGTCTTCA	59	170
	FoxA3	GGCTTGTACACGGAAAGGAA/ TCATTGAAGGACAGCGAGTG	57	226
	Ghrelin	CCCAAGCTTAGGCCATGGTGTC/ CCGGAATTCCAGTGGTTACTTGTT	62	212
	Glucagon-like peptide 1 receptor (GLP1-R)	TCTCTTCTGCAACCGAACCT/ CTGGTGCSGTGCSSGTGTCT	58	351
	Glucagon	GACCGTTTACATCGTGGCTG/ GGTTCCTCTTGGTGTTCATCA	58	247
	Glucokinase (islet-specific/ isl-GCK)	AGGCCACCAAGAAGGAAAAG/ TTGTCTTCACGCTCCACTGC	65/60	265
	Glucokinase (liver-specific/ liv-GCK)	GAGCCCAGTTGTTGACTCTG/ TTGTCTTCACGCTCCACTGC	65/60	364
	Insulin 1	ACCTTTGTGGTCCTCACCTG/ CCAGTTGGTAGAGGGAGCAG	56	230
	Insulin 2	AGTGCCAAGGTCTGAAGGTC/ CAGCACCTTTGTGGTTCTCA	58	163
	Isl1	CGGGAGGATGGGCTTTTCTG/ AGCTGCTTTTGGTTGAGCACAG	56	211

Table 2.7. *(continued).*

Rat	Islet Amyloid Polypeptide (IAPP)	GGCTGTAGTTCCTGAAGCTT/ AAGGTTGTTGCTGGAGCGAA	58	260
	Kir6.2	ACCACGCTGGTGGACCTCAAG/ GCACCACCTGCATATGAATGG	58	481
	MafA	TCAACGACTTCGACCTGATG/ GGGCAGAGTGATGATGGTG	58	209
	Nestin	TGCAGCCACTGAGGTATCTG/ CAGTTCCCACTCCTGTGGTT	60	449
	NeuroD	CTTGGCCAAGAACTACATCTGG/ GGAGTAGGGATGCACCGGGAA	59	228
	Neurogenin3	CTTCACAAGAAGTCTGAGAACA/ CTGCGCATAGCGGACCACAGCT	58	211
	Nkx2.2	CAGCAGCGACAACCCCTAC/ AAGAGCACTCGGCGCTTCC	58	203
	Nkx6.1	ATGGGAAGAGAAAACACACCAG/ TAATCGTCGTCGTCCTCCTCG	58	279
	Pancreatic Polypeptide (PP)	AACAGAGGGCTCAATACGAA/ GAGACAGAAGGGAGGCTACA	56	212
	Pax4	CTGCCTGTGCTGGAAGAAT/ TAGGTTGATGGCGCTTGTC	58	231
	Pax6	GAGACAGATTACTCTCCGAGG/ ACCACACCTCTATCCTTGCTTAGG	59	464
	Pdx1	TCGCTGGGAACGCTGGAAC/ CTTTGGTGGATTTCATCCACG	58	223
	PGP9.5	GCCCAGCATGAAAACTTCAG/ AGGGGACAACTTCTCCGTTT	57	209
	Prohormone convertase 1/3 (PC1/3)	TCAGGGAATGGGGGTCGTCAAG/ CCAAATCGGCTGTTCACCATCAAG	63	400
	Prohormone convertase 2 (PC2)	CTGAGGCTGGTGTGGCTAC/ AGCTGGCGTGTTTGCATTA	57	366
	SDF1a	ATTCTTTGAGAGCCATGTCGC/ CCTTGAGCTGAGTGACTCTCG	60	638
	Somatostatin (SS)	ACCCCAGACTCCGTCAGTTT/ CTCCAGCCTCATCTCGTCCT	60	173
	Sox9	TGAAGAAGGAGAGCGAGGA/ CAGAGCTTGCCCAGAGTCTT	57	245
	Sulphonylurea Receptor 1 (SUR1)	AAGATCATGCACTTGTCTACT/ AGACAGCAGGAACAGCGGTGT	58	591
	Surfactant associated binding protein-B (SURF-B)	CTGTGCCAAGAGTGTGAGGA/ TGGCTTTGGGTTTAATCTGG	59	201
	Thy1	GCAGATGTCCCGAGGACAGA/ GGCAGTCCAGTCGAAGGTTCT	56	187
	Vimentin	CACCAACGAGAAGGTGGAAT/ GTGCCAGAGAAGCATTGTCA	58	349
Mouse	Albumin	GCAGAGGCTGACAAGGAAAG/ TTCTGCAAAGTCAGCATTGG	56	182
	Beta-actin	TACGCATGTCAACGTCACAC/ AAGAGCTATGAGCTGCCTGA	55	159
	Betacellulin	CCAAGCAGTACAAGCATTACTG/ GAACACCACCATGACCACTATC	60	184
	CK19	AAGACCATCGAGGACTTGCG/ CTATGTCGGCACGCACGTCG	62	545
	E-cadherin	GCTGGACCGAGAGAGTTA/ TCGTTCTCCACTCTCACAT	57	380
	ErbB1	GTGCCCCCGAAACTCGTGGTGAC/ CTAGAGGGGAGTGCGCGTGAAAG	55	272

Table 2.7. *(continued).*

Mouse	ErbB4	CGGACGGCGGCGGAGAAGG/ GTGGCGGGGGAGGTGGTAGAA	60	303
	Insulin 1	TAGTGACCACCTATAATCAGAG/ ACGCCAGGTCTGAAGGTCC	56	217
	Insulin 2	AGGTCTGAAGGTCACCTGCT/ CCCTGCTGGCCCTGCTCTT	56	212
	Kir6.2	CCAAGCCCAAGTTTAGCATC/ TGGGGCCSGAAATAGCATAG	58	235
	SUR1	TGAAGCAGACCAACGAGATG/ GCAATGGGGATAGCTGTGTT	56	222
	Vimentin	ATGCTTCTCTGGCACGTCTT/ AGCCACGCTTTCATACTGCT	57	268
Human	Albumin	CTCAAGTGTGCCAGTCTCCA/ TGGGATTTTTCCAACAGAGG	58	272
	Beta-actin	AGAAAATCTGGCACCACACC/ GGGGTGTTGAAGGTCTCAAA	58	142
	Insulin	AGCCTTTGTGAACCAACACC/ CGGCCAAGTTCTTCAACAAT	58	245
	Kir6.2	GAGATCATCGTCATCCTGGAA/ GTAGGCTGTGGTCCTCATCAA	58	221
	SUR1	TGACCTCCATCTCCAACTCC/ TGGATCTGGATCTTCCCTTG	58	195
	Vimentin	CCCTCACCTGTGAAGTGGAT/ TCCAGCAGCTTCCTGTAGGT	58	261

2B.8 Extracellular factors and other compounds.

Table 2.8 Concentrations of compounds used for various treatments.

Compound	[Stock]	[Working]	Supplier
Alloxan[a]	1M	5mM	Sigma
Betacellulin	10mg/ml	10ng/ml	
Growth hormone	25ng/ml	10nM	
Metformin[b]	1M	10mM	
Nicotinamide	2M	20mM	
Prolactin	7mg/ml	500ng/ml	
$NaC_4H_5O_2$ (butyrate)	10mM	0.5mM	
Streptozotocin[c]	250mM	2.5mM	
Trichostatin A	10mM	20µM	
Zebularine	10mM	100µM	
γ-secretase inhibitor IV	25mM	25µM	Calbiochem
PI3K inhibitor (LY294002)	10mM	10µM	
Activin A	1mM	10nM	R&D Systems
BMP4	10µg/ml	10ng/ml	
EGF	100µg/ml	50ng/ml	
FGF7/KGF	10µg/ml	10ng/ml	
FGF10	25µg/ml	100ng/ml	
TGF-α	100µg/ml	10ng/ml	
TGF-β1	10µg/ml	10ng/ml	
VEGF	10µg/ml	50ng/ml	

a- 1,3-Diazinane-2,4,5,6-tetrone; b-*N*,*N*-dimethylimidodicarbonimidic diamide; c- 1-methyl-1-nitroso-3-[2,4,5-trihydroxy-6- (hydroxymethyl) oxan-3-yl]-urea.

2B METHODS

2B.1 Hepatocyte Isolation

All laboratory animals used were kept under standard laboratory conditions, at 25°C, on a 12:12 h light-dark cycle and given *ad libitum* access to food and water. The animal studies were performed under the regulations of the UK Home Office Licence and the use of human material was undertaken with the approval of the University of Bath Ethics Committee.

2B.1.1 Rat hepatocytes

The method for rat hepatocyte isolation is based on a modification of the two-step collagenase perfusion technique previously described by Tosh et al. (1988). Rats of the albino Wistar strain, weighing 280g-320g (University of Bath) were anesthetised with 4.5% isoflorane (Baxter Healthcare Ltd., UK) in oxygen, flowing at a constant rate of 1500ml/minute. When the rat was fully anaesthetised (indicated by a lack of the pedal reflex), the animal was maintained so, with 3% isolfluorane delivered via a face mask. The animal was placed supine on a flat tray and the abdominal fur was disinfected with 70% ethanol. Laparotomy was performed by making a 'U' shaped incision from the lower abdomen to the lateral aspects of the rib cage. The skin was folded over the chest, fully exposing the viscera. The intestine was then displaced, to reveal the portal vein. The portal vein was elevated by placing a flat matchstick underneath it and excess adipose tissue was removed with the blunt side of a curved forceps. A non-absorbable suture was loosely tied around the portal vein (4-0; Ethicon^{TM} Mersilk). An 18GA cannula (BDVenflon, Franklin Lakes, NJ) was inserted into the portal vein (see Fig. 2.1A) 3-5mm distal to the suture and the inner metal stylet of the cannula was then removed, while slightly advancing the cannula into the vein. The suture was then firmly tightened around the cannula and the abdominal blood vessels were cut along with the spinal cord, whereupon the death of the animal occurs by exsanguination. The cannula was connected to the pump and perfusion was initiated with perfusion buffer 1 [0.05 (w/v) KCl in

10mM HEPES buffer, 5mM D-glucose, 200μM EDTA, 0.001% (v/v) phenol red, in 500ml of sterile phosphate buffered saline (PBS), pH 7.4] pre-warmed to 39^{o}C. The inferior vena cava was then cut and the abdominal blood vessels clamped with a Spencer-Well's artery forceps, thereby establishing a continuous flow (25-30ml/minute) of the buffer from the cannula in the portal vein, through all the lobes of the liver and out through the vena cava into the thoracic cavity. After perfusing the liver with 250ml of perfusion buffer 1, perfusion was continued with 200ml of pre-warmed perfusion buffer 2 [0.05 (w/v) KCl in 10mM HEPES buffer, 20mM HEPES buffer, 5mM D-glucose, 1mM $CaCl_2$, 0.001% (v/v) phenol red, in 500ml of sterile PBS, pH 7.4] in which 50mg of type 2 collagenase (Worthington, Lakewood, NJ) was dissolved.

The above procedure isolates a total population of viable hepatocytes which includes cells from the periportal, intermediate and perivenous zones of the hepatic acinus. In order to isolate enriched populations of periportal and perivenous hepatocytes, 6-8ml of a 4mg/ml solution of digitonin (Sigma, made up in 150mM NaCl, 6.7mM KCl., 50mM HEPES, pH7.5 by heating to 55°C) was injected through the thoracic portion of the vena cava (the hepatic vein drains into the vena cava) or portal vein respectively. For periportal isolations, when the selective zone 2 and 3 destruction pattern of the perivenous hepatocytes by digitonin was apparent, the perfusate was switched back to perfusion buffer 1 and the perfusion was carried on as usual in the orthograde (portal to caval) direction (Fig. 2.1C). For perivenous isolations, when selective zone 1 destruction pattern of the periportal hepatocytes was visible, the digitonin was washed away by perfusion with perfusion buffer 1 in the retrograde direction (caval to portal) for 10 minutes before resuming the perfusion in the orthograde direction as before (Fig. 2.1D).

Following perfusion, the whole liver was removed and dissociated in a class II microbial safety cabinet using two pairs of curved forceps. The cells were filtered through a 70μm mesh (Gore-Tex, Newark, DE) and dead and dying cells were removed by serial differential centrifugation at 50*g* for 2 minutes

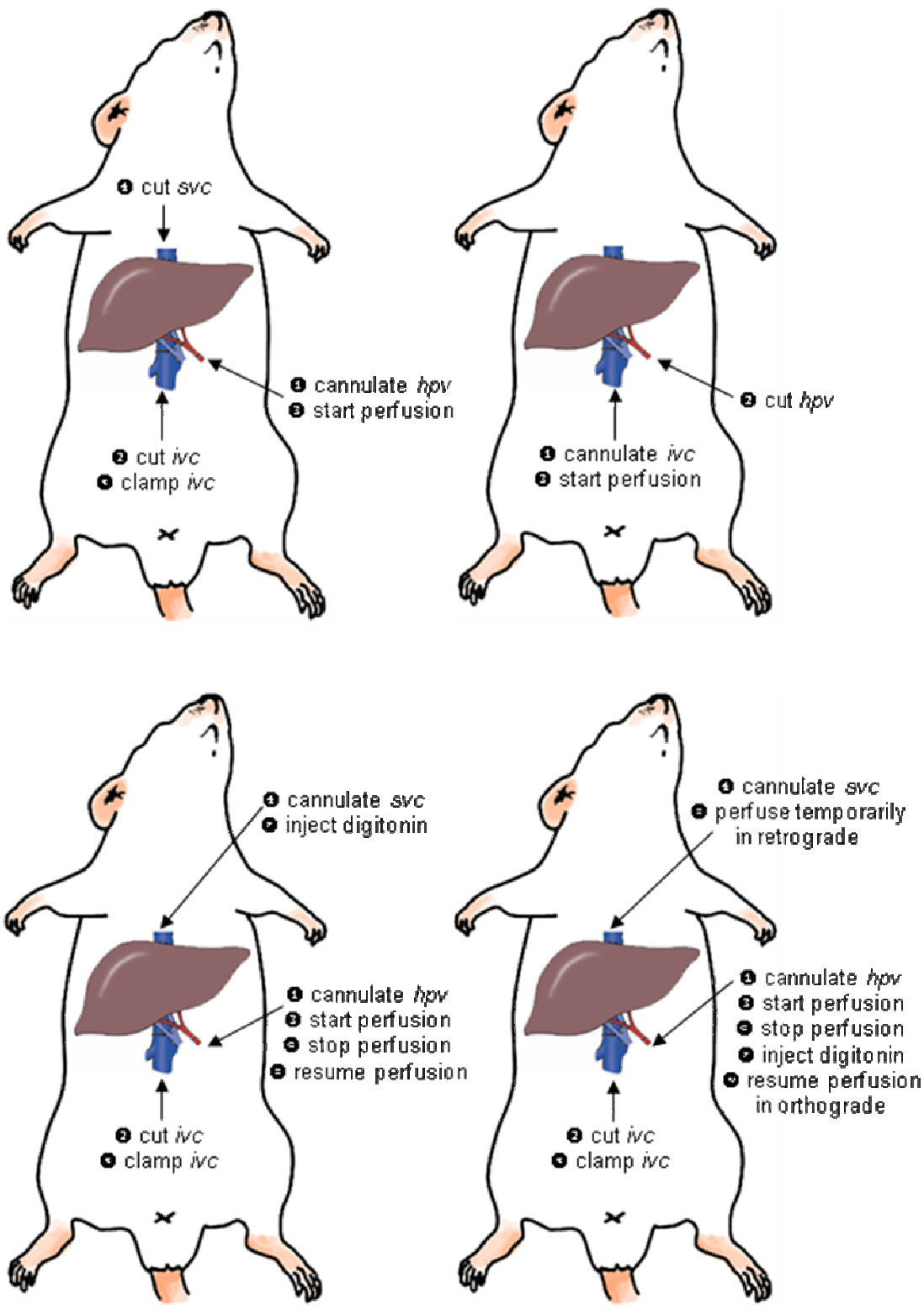


Figure 2.1 Steps in the procedure for isolation of A- total hepatocytes from rats, B- total hepatocytes from mice, C- periportal hepatocytes from rats and D- perivenous hepatocytes from rats. [*svc*- superior vena cava; *ivc*- inferior vena cava; *hpv*- hepatic portal vein].

each time in perfusion buffer 2 or attachment media [William's Medium E (Sigma), 10% (v/v) foetal bovine serum (FBS), 100units/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine]. The cell viability was determined at 85% viable using 0.4% (w/v) trypan blue (Sigma). The hepatocytes were inoculated in attachment media at a final density of $4.5x10^5$ cells/ml into 35mm, 60mm and 100mm vented plastic culture dishes (Nunclon). The cells were left to attach for 5-6hours at 37°C in 5% (v/v) CO_2 and then transferred to the different culture media.

2B.1.2 Mouse hepatocytes

Hepatocyte isolation from CD1, Rosa26 LacZ and C57BL/6 *Alb-Cre x R26R* LacZ mice (University of Bath, UK) were carried out as described above for the rat but with the following modifications. Extra care was taken while anaesthetising C57BL/6 *Alb-Cre x R26R* LacZ mice as they were notably more sensitive to isoflorane compared to the other strains. After displacing the intestine, excess abdominal fat around the inferior vena cava was removed with the blunt side of a curved forceps. A flat matchstick was then inserted underneath the vena cava just posterior to the renal veins. A non-absorbable suture was loosely tied around the vena cava and a 24GA cannula (Terumo) connected to the perfusion tubing was inserted into the vena cava and advanced just anterior to the renal veins (Fig. 2.1B). The inner metal stylet was removed making sure there were no air bubbles in the cannula and the suture was tightened around it. The portal vein was then cut and the perfusion tubing was reconnected to the cannula maintaining a flow rate of 5-12ml/minute. The liver was initially perfused with 100ml of perfusion buffer 1 followed by 8-10 minutes of perfusion with perfusion buffer 2 (containing 50mg of type 2 collagenase per 150ml of buffer).

After dissociation of the liver, the cells were filtered through a 100µm mesh and viable hepatocytes were isolated by serial differential centrifugation as described above. Generally more centrifugation steps are needed compared to rat hepatocytes and the viability of the cells can be judged under the microscope after each step. The hepatocytes were inoculated in attachment media and left to attach for 5-6 hours.

2B.1.3 Human hepatocytes

Human hepatocytes were obtained from the UK Human Tissue Bank (De Montfort University, Leicester, UK). The hepatocytes were isolated by *in vitro* perfusion of liver resections from male and female donors between the ages of 66 and 70. Human hepatocytes were incubated overnight in attachment media before being transferred to their respective culture media.

2B.2 Embryonic pancreas dissections

Embryos were obtained from CD1 strain albino mice (University of Bath, UK) 11.5 days postcoitum (dpc). The day of the vaginal plug was considered as 0.5dpc. The pregnant female mice were killed by cervical dislocation and the uteri were surgically removed by the horn of the uterus. The uteri were transferred to 100mm Petri dishes containing ice-cold phosphate buffered saline (PBSA) where individual uteri were separated using surgical scissors. The embryos were removed from their deciduas using fine forceps. The embryos were then transferred to 60mm Petri dishes containing dissection medium (see Table 2.2). The gut was dissected out of the body with fine forceps. Using a tungsten needle, the liver buds and the dorsal pancreatic bud were removed from its location in the posterior region of the stomach adjacent to the duodenum, (see Fig. 2.2). The pancreata and liver buds were collected for tissue culture in 35mm Petri dishes containing the dissection medium.

The cultures were set up as described in Percival and Slack (1999) with the following modifications. In a tissue culture hood, subbed glass coverslips (Fisher Diagnostics), which have been sterilised by baking for 2 hours at 180^{o}C, were placed in 35mm Petri dishes. To culture pancreatic buds for immunohistochemistry, a 40µl drop of 50µg/ml bovine plasma fibronectin (Invitrogen) was placed at the centre of each coverslip and left to dry. 2ml of the embryonic pancreas culture medium (See Table 2.2) was added to each Petri dish. A plastic cloning ring of 3mm internal diameter was then placed over the fibronectin-coated area of each coverslip and the pancreatic buds

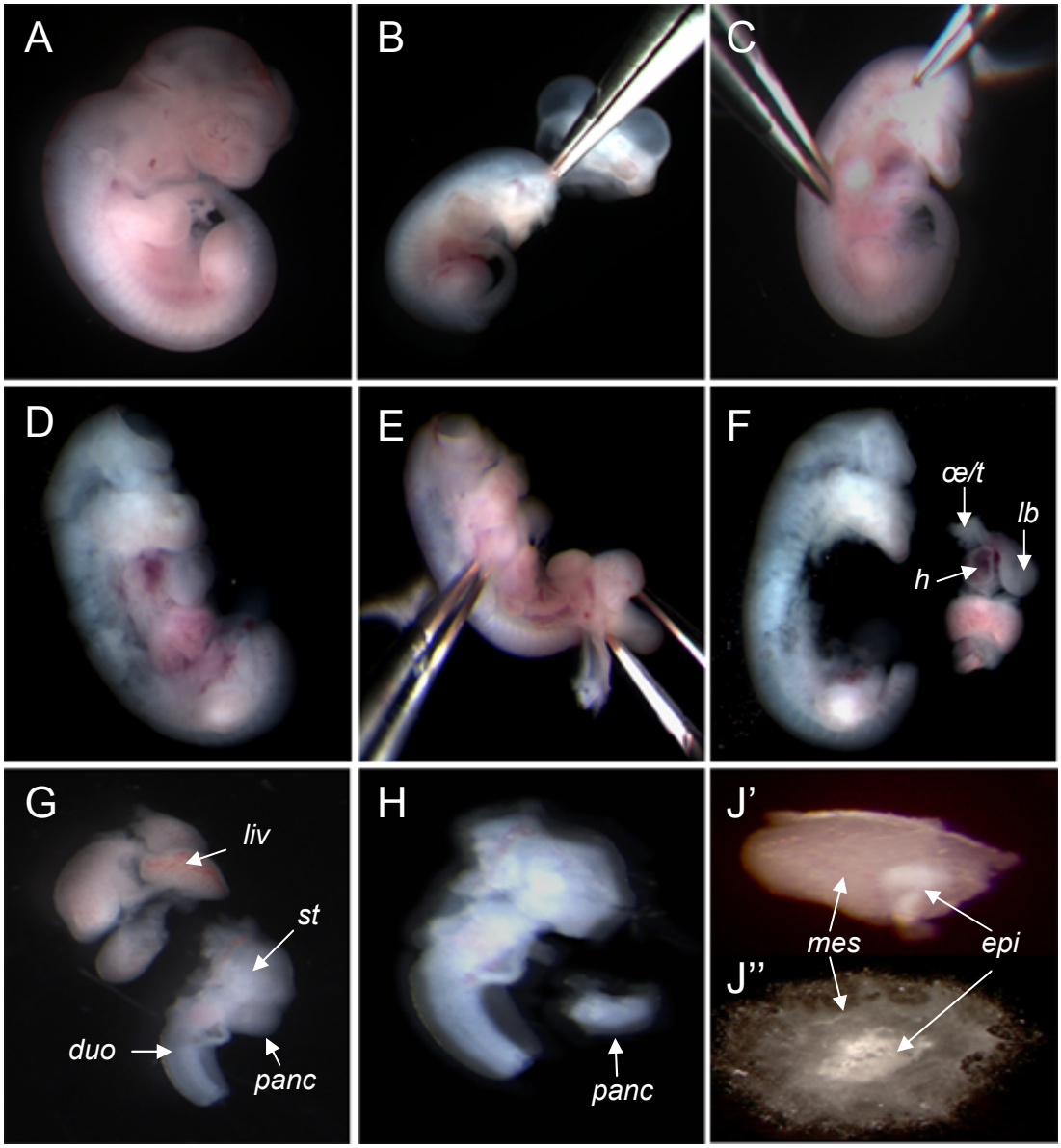


Figure 2.2 Steps in the dissection of the dorsal pancreatic bud from E11.5 mouse embryos. A- E11.5 mouse embryo; B- decapitation of the embryo; C to F- opening up of the embryo and removal of the internal viscera; G- separation of the liver from the stomach; H- separation of the dorsal pancreatic bud from the base of the stomach; J- the dorsal pancreatic bud before (J') and after attachment (J'') onto the fibronectin coated coverslip. [*œ*- oesophagus*; t*- trachea; *lb*- lung buds*; h*- heart*; liv*- liver*; st*- stomach*; duo*- duodenum*; panc*- dorsal pancreatic bud*; mes*- pancreatic mesenchyme*; epi*- pancreatic epithelium].

were dropped into the centre of each cloning ring. To ensure the spreading of the tissue during culture, the pancreatic buds were oriented using fine tungsten needles, so that they were in the centre of the cloning rings with their cut face lying down. To culture pancreatic buds for protein extraction and protein assays, up to six buds were cultured on the same coverslip wholly coated with fibronectin.

2B.3 Cell Culture

2B.3.1 Primary cultures of hepatocytes

The hepatocytes were cultured in different culture media as indicated in Table 2.2, at 37°C in 5% (v/v) CO_2 for up to 8 days. The cultures were set up in 35mm dishes containing glass coverslips, 60mm and 100mm dishes for immunocytochemistry, RNA extraction and protein extraction respectively. The media was changed every two days. Whenever swapping between different media, the cells were briefly rinsed with pre-warmed PBS before the new media was added. The different extracellular factor treatments were initiated 24 hours after attachment, unless stated otherwise (see Table 2.8).

2B.3.2 Culture of cell lines: maintenance, storage and revival

A number of different cell lines were used as positive controls for various genes that were being investigated. Table 2.2 summarises the different culture media for the murine insulinoma cell line MIN, rat insulinoma cell line RIN-5F, the rat pancreatic-exocrine AR42J-B13 cell line, human embryonic kidney epithelial cell line HEK293 and the bipotential mouse embryonic liver cell line BMEL. All the cell lines were passaged by the same method when 70-80% confluent. The cells were washed with PBS and then trypsinised with 5ml of trypsin-EDTA for 3-5 minutes at 37°C. The trypsin was neutralised with an equal volume of supplemented medium and the cells were re-suspended and centrifuged at 1000 rpm in a bench top PK110 centrifuge (ALC Intl., Milan, Italy) for 4 minutes. The supernatant was removed and the

cell pellet re-suspended in 1ml of fresh medium. The cell suspension was reseeded in 15ml of fresh medium in 75cm^2 flasks after making the necessary passaging dilutions for the specific cell line (see Table 2.1).

For cell storage, after centrifugation the cell pellets were stored in cryovials containing 1ml freezing medium [10% (v/v) dimethyl sulfoxide (DMSO; VWR International) in FBS] and then frozen in an isopropanol freezing chamber at -80°C overnight before being transferred to liquid nitrogen for long-term storage. The cells were revived by rapid thawing in a 37°C water bath, re-suspending the pellet in 1ml of fresh medium, followed by centrifugation at 1000 rpm for 4 minutes (except for RIN-5F and MIN6 cells which were put into culture straight after thawing). The new pellet was re-suspended in 1ml of fresh medium and then inoculated in 15ml of medium in 75cm^3 flasks. The medium was changed the following day and every other day thereafter.

2B.3.3 Maintenance of embryonic organ cultures

All organ cultures were maintained for 7 to 10 days at 37°C in 5% CO_2. On day 1, the cloning rings were removed and the culture medium was changed every 24 hours then on. For betacellulin treated samples, 10ng/ml of human recombinant betacellulin (Sigma) was added to the culture medium after the cloning rings were removed.

2B.4 Lineage tracing of hepatocytes in culture

Hepatocytes of C57BL/6 *Alb-Cre x R26R* LacZ mice were used to trace the lineage hepatocytes in culture (Postic and Magnuson, 2000, Soriano, 1999). For X-gal staining, the cultures were fixed at day 8 after isolation, in 0.5% glutaraldehyde in PBS for 5 minutes at room temperature (RT) and stained at 37°C in 5-bromo-4-chloro-3indolyl-β-D-galactopyranoside (X-gal), 2mM $MgCl_2$, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS (pH 7.3) for 6 hours. Cultured hepatocytes of Rosa26 LacZ mice, expressing bacterial β-

galactosidase ubiquitously(Zambrowicz et al., 1997), and CD1 wild type mice also fixed at day 8, were used as positive and negative controls, respectively.

2B.5 Adenovirus preparation and titration

HEK293 cells were grown in vented 75cm^2 flasks (as previously described in 2B.3.2) until 70% confluence. 10µl, 1µl, 0.1µl and 0.01µl of virus stocks were added to the flasks and incubated at 37°C. Approximately 50% of the cells detach within 2-4 days, as a result of the cytopathic effect (CPE) of the virus and is indicative of good viral infection. The cells were harvested simply by shaking the flasks, collecting the media and centrifuging at 1000 rpm for 4 minutes. The resulting pellet was re-suspended in 1ml of HEK293 culture medium (*see* Table 2.2) and the cells were lysed by subjecting the cell suspension to four rapid freeze/thaw cycles, alternating between a dry ice/ethanol mixture and a 37°C water bath. The pre-stocks of the viruses were stored at -80°C.

For further amplification of the virus, HEK293 cells were grown in vented 225cm^3 culture flasks and infected with 125µl, 12.5µl and 2µl of the viral pre-stocks to ascertain the optimal volume (i.e. giving 50% CPE in 2-4 days) of pre-stock to add to each flask. Eight 225cm^3 flasks were infected with this optimal volume of viral pre-stock. 2-4 days later, the cells from all eight flasks were harvested and centrifuged at 2000 rpm for 10 minutes. The pellet was re-suspended in 5ml 100mM Tris-HCl, pH 8.0, and subjected to 4 rapid freeze/thaw cycles and centrifuged at 1000 rpm for 5 minutes. The supernatant was mixed with 0.6 times the volume of sterilised supersaturated CsCl [(Sigma); in 100mM Tris-HCl, pH8.0] and divided into two centrifuge tubes (Beckman). The tubes were sealed after discarding all air from the tubes and centrifuged at 65000 rpm for 4 hours at 22°C on an L-80 Ultracentrifuge (Beckman). The step gradient of the supersaturated CsCl solution concentrates the virus as a prominent bluish-white band, which was collected via a 25GA needle into a 10ml syringe. The virus was mixed with supersaturated CsCl and re-centrifuged at 65000 rpm overnight at 22°C. The new virus band was extracted as before and made up to 2.5ml with 10mM

Tris-HCl, pH7.5, and passed through an equilibrated PD-10 Sephadex^{TM} G-25 M desalting column (Amersham Biosciences). The column removes any CsCl and allows the virus to flow through. The virus particles were purified by filtering through a 0.22µm filter.

For the titration of the virus, a 12 well plate was seeded with HEK293 cells and grown to 70% confluence. The cells were infected at 37°C with 100µl of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions of virus in separate wells with the last five dilutions in duplicate. After 48 hours of infection, the medium was discarded, the plate was aspirated and the cells were fixed at -20°C for 10 minutes with ice-cold 100% methanol. The titration was carried out according to instructions provided with the Adeno-X^{TM} Rapid Kit (Clontech Laboratories) which uses a mouse anti-hexon to immunocytochemically localise the viral hexon protein expressing infected cells. A 3,3'-diaminobenzidine (DAB) chromogen kit (Vector) was used for development of the immunostaining. The infected cells were stained brown and the titre was estimated as the number of infectious units (iu)/ml:

$Titre_{(iu/ml)} = \frac{\bar{N} \times 573}{0.1 \times d}$, where $\bar{N}$ is the mean number of infected cells per field at 20x objective magnification (10x eyepiece) for the viral dilution factor *d*.

2B.6 Adenoviral infections

Table 2.3 summarises all the viruses used. The adenoviruses were initially diluted in DS medium to $1x10^8$ iu/ml. 72 hours after isolation, the hepatocytes were infected in the serum-containing DS medium at a total multiplicity of infection (M.O.I.; number of virus particles per cell) of 20-50 for 1 hour at 37°C. During multiple infections the individual M.O.I. for each virus was kept constant, using the *Ad-Null* virus to moderate the total M.O.I. Cells were maintained after infection for 5 more days before RNA and protein isolation or fixation for immunostaining was carried out. GFP expression in the cells was visualised under a Leica DM*IRB* inverted microscope, for GFP tagged viruses 24 hours after infection to assess the efficiency of infection.

2B.7 Qualitative and quantitative RT-PCR

2B.7.1 RNA extraction

The same RNA extraction protocol was used for the isolated of RNA for both qualitative and quantitative RT-PCR. Total RNA was extracted from cells on 60mm culture dish per each condition, using TRI REAGENT™ (Sigma) according to manufacturer's instructions. The RNA pellets were dissolved in DEPC-treated [0.0001% (v/v) diethylpyrocarbonate; Sigma] RNase-free water. The concentration of the extracted RNA was measured in µg/µl on a Beckman Spectrophotometer. Only RNA samples with 260nm/280nm absorbance ratio between 1.8 and 2.0 were used for reverse transcription. 4µg of RNA was treated with 0.1U/µl of RQ1 RNase-free DNase I (Promega) at 37°C for 30 minutes to get rid of any genomic DNA contamination. The DNase was inactivated by incubating at 65°C for 10 minutes.

2B.7.2 Reverse transcription

Messenger RNA (mRNA) was isolated from 2µg of DNAse-treated total RNA by using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) which involved the incubation of RNA with 0.5µg of Olido$(dT)_{12\text{-}18}$ oligonucleotides and 10mM *d*NTP mix at 65°C for 5 minutes. Complementary DNA (cDNA) was synthesised at 42°C for 52 minutes, using 10U/µl SuperScript™ II reverse transcriptase enzyme, 2U/µl RNaseOUT™ and 10mM dithiothreitol (DTT). The mRNA-cDNA hybrids were degraded by incubating with 0.1U/µl RNase H at 37°C for 20 minutes. All reagents for reverse transcription were obtained from Invitrogen.

2B.7.3 Polymerase chain reactions

The PCR reactions were carried out with 50ng of cDNA, 500ng of sense (forward) and antisense (reverse) primers (see Table 2.7 for a full list of primers) and 20µl of PCR ReddyMix™ Master Mix (AbGene, UK), in a DNA thermal cycler using the following conditions: denaturation at 94°C for 60 seconds, amplification at the annealing temperature T_m (see Table 2.7) for

60 seconds and elongation at 72°C for 60 seconds for 25-35 cycles. The rat GCK primer sequences were obtained from Heimberg et al. (1996), where PCR reactions were performed for 10 cycles of 94°C, 60 seconds; 65°C, 90seconds; 72°C, 90 seconds followed by 20 cycles of 94°C, 30 seconds; 60°C, 60seconds; 72°C, 90 seconds. The PCR products were analysed by electrophoresis on a 1.0-1.5% agarose gel alongside 100bp and 1kb DNA ladders (Invitrogen).

For quantitative real-time PCR, 50ng of cDNA, 500ng of sense and antisense probes were mixed with 10µl of 1x SYBR®Green Taq ReadyMix™ (Sigma) in LightCycler® capillaries (Roche, Germany). Real-time PCR was performed on the Roche LightCycler® 1.5 and the thermal cycling parameters were set as 45 cycles of denaturation at 95°C for 30 seconds, amplification at T_m for 5 seconds and elongation at 72°C for 20 seconds, followed by cooling at 40°C for 2 minutes. For real-time PCR all samples were amplified in triplicate.

2B.7.4 Data analysis for quantitative real-time PCR

The relative amounts of mRNA were calculated using the $\Delta\Delta C_T$ method. The C_T value of a gene is the cycle number during the PCR at which a certain threshold T of amplification is reached. The C_T values were generated using the Roche LightCycler® software after the completion of the reaction. The mean C_T values ($\bar{C}_T$) were calculated for the amplification of both, the target gene of interest (*target*) and a house keeping reference gene such as beta-actin (*reference*) for either a positive control or a non-treated control (*control*) and all the experimental samples (*experimental*).

Then, $\Delta C_T = \bar{C}_{T(\mathrm{target})} - \bar{C}_{T(reference)}$, and

$$\Delta\Delta C_T = \Delta C_{T(control)} - \Delta C_{T(\mathrm{experimental})}.$$

The level of mRNA of the gene of interest relative to that of the reference gene and normalised to the control was expressed as $N^{\Delta\Delta C_T}$, where N is the efficiency of the reaction taken as the exponent of amplification.

For calculation purposes it was assumed that $N = 2$, based on the theoretical dynamics of a PCR; unless the efficiency of the reaction was significantly lower, in which case the primers were redesigned. All comparisons were made with the $N^{\Delta\Delta C_T}$ for the control which was equal to 1, (as $\Delta\Delta C_{T(control)} = 0$), thus giving the result as the fold increase or decrease compared to the control.

2B.8 Immunocytochemistry.

Cells were fixed in 4% (w/v) paraformaldehyde (VWR International), pH7.4, in PBS for 20-30 minutes at room temperature (RT) or in ice cold 1:1 acetone:methanol for 5 minutes. Pancreas and liver organ cultures were fixed in MEMFA (3.8% (v/v) formaldehyde, 0.15M MOPS, 2mM EGTA, 1mM $MgSO_4$, pH7.4) for 20 minutes at RT. Antigen retrieval was performed in 1x citrate buffer or 1x EDTA buffer (Lab Vision) at 37^{o}C for 1 hour where necessary. Samples were permeablised in PBS with 0.1% (for cells) or 1% (for organ cultures) (v/v) Triton x-100 (Sigma) for 20 minutes and then blocked in 2% (w/v) blocking buffer (Roche, UK) for 1-2 hours. The samples were then incubated overnight at 4^{o}C with the indicated dilutions of the primary antibodies (Table 2.4). The samples were washed in PBS and then incubated at RT for 2 hours in the dark with the appropriate fluorochrome-conjugated secondary antibody (Table 2.5), avoiding simultaneous incubation with possible cross-reacting antibodies. When using fluorochrome-conjugated primary antibodies, lectins or phalloidin the samples were incubated at RT for 2 hours in the dark with the appropriate dilution of the conjugated compound. After several washes with PBS, the samples were mounted on superfrost slides (Mensel-Gläser, Braunschweig, Germany) using Gel-Mount^{TM} mounting medium (Biomeda, Foster City, CA). Where horseradish peroxidase (HRP)- conjugated secondary antibodies were used, the endogenous peroxidase activity was quenched by two 15 minute incubations at RT in 0.3% (v/v) H_2O_2 in methanol just after permeablisation. The samples were developed using a DAB immunolabelling kit (Vector) according to manufacturer's instructions. For cell counting

experiments, prior to mounting the coverslips were incubates with 500ng/ml 4,6-Diamidino-2-phenylindole (DAPI; Sigma, UK) for 20 minutes. Positive controls for all the primary antibodies used and the no primary controls for all the secondary antibodies are included in the Appendix B.

The same procedure as described above was followed for the immunofluorescent staining of freshly isolated hepatocytes in suspension. The staining was carried out in 1.5ml Eppendorf tubes which were pre-incubated with 2% blocking buffer at RT for 30 minutes to prevent antibody binding to the plastic. All the incubation steps were done on a bench-top nutator. After each step the cells were re-pelleted by centrifugation at 1000rpm for 4 minutes and then re-suspended in the subsequent solution.

2B.9 Tissue processing and histology

Adult and embryonic tissue used as positive controls for different antibodies were initially fixed in 4% (w/v) paraformaldehyde for 2 hours at 4°C followed by 3 serial PBS washes for 15 minutes each. The tissue was processed in a Leica TP1020 tissue processor, overnight through a series of 70-100% (v/v) ethanol and then histoclear (Fisher Scientific). The samples were impregnated in paraffin wax at a Leica EG1160 embedding station and then sectioned using a Leica RM2155 microtome into 5µm thick ribbons collected onto Menzel-Gläzer Polysine slides (Thermo Scientifics) which were dried overnight at 38°C. Before immunohistochemistry, the sections were de-waxed in histoclear and then rehydrated though an ethanol series.

For cryosectioning, the fixed samples were sequentially incubated with 5%, 15% and 30% (w/v) sucrose in PBS at 4°C on rollers, until the tissue attained neutral density with each solution. The samples were mounted with Optimal Cutting Temperature (OCT) compound (Leica) on a trough of dry ice and isopropanol. The frozen blocks were cut into 10-15µm thick sections using a Leica CM1850 cryostat run at -20°C to -27°C and collected onto pre-warmed SuperFrost Plus® glass slides (Thermo Scientific). The slides were washed in PBS at RT prior to immunostaining, to dissolve the OCT mounting media.

2B.10 Protein extraction and Western blot analysis

2B.10.1 For all proteins larger than 10kDa

Tissue samples and cells were washed in ice-cold PBS, transferred to lysis buffer [150mM NaCl, 1mM EDTA, 2mM dithiothreitol (DTT; Sigma), 20mM HEPES, 1% (v/v) Triton x-100, pH7.6] containing 1:100 protease inhibitor cocktail for mammalian tissues (Sigma) and the supernatant was collected from cellular debris by centrifuging at 4^{o}C on 13,000rpm for 10 minutes. Protein concentrations in the lysates were assayed using the Bio-Rad protein assay based on the Bradford dye-binding procedure. If necessary the sample solutions were further concentrated by ultrafiltration using a Centricon YM-100 Centrifugal Filter Device (Millipore). The samples were stored at -80^{o}C.

The protein lysates were mixed with equal volumes of 2X sample buffer [125mM Tris-HCl (pH6.8), 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 0.2mM DTT and 0.02% (w/v) bromophenol blue (all Sigma)] and then heated on a 100^{o}C heat block for 5 minutes. A volume of sample corresponding to 10µg of protein alongside a Precision Plus Protein Rainbow Standard^{TM} ladder (BioRad) was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) on 4-20% gradient, 10% or 15% Tris-HCl Criterion^{TM} Precast Gels (BioRad) in 1 x running buffer [25mM Tris, 192mM glycine and 0.1% (w/v) SDS] at 200V. The protein from the gels were then electrophoretically transferred to a nitrocellulose membrane (Pall) by blotting for 2 hours at 200mA in 1 x transfer buffer [25mM Tris-HCl, 192mM glycine and 20% (v/v) methanol]. The membranes were stained in Ponceau S (Sigma) to visualise the bands and then blocked in 5% (w/v) skimmed milk (Marvel) in PBS with 0.1% (v/v) Tween-20 (PBST; Sigma). Incubation with the primary antibodies (Table 2.6) was done overnight at 4^{o}C with gentle constant agitation on a bench top rocker at 70 rpm. After stringent washes in PBST, the membranes were incubated with the secondary antibodies (Table 2.6)

for 2 hours at RT. The immunoreactive proteins were visualised by the ECL or ECL plus Western blotting analysis systems (Amersham) followed by autoradiography on Hyperfilm^{TM} (Amersham), or colorimetrically by using a DAB immunolabelling kit according to manufacturer's instructions (Vector). All antibodies were prepared in 3% (w/v) skimmed milk (Marvel) in PBST except when a biotinylated secondary had to be used, in which case they were prepared in 5% (w/v) bovine serum albumin (BSA: Sigma) in PBST.

The membranes were stripped with stripping buffer [62.5mM Tris-HCl, 100mM β-mercaptoethanol and 2% (w/v) SDS, pH6.7] for 30 minutes at RT in a fume hood and re-probed if necessary. The membranes were washed with distilled water, dried and stored, wrapped in cling film at 4°C for future use.

2B.10.2 For insulin and other proteins smaller than 10kDa

Protein was extracted from de-differentiated hepatocytes and RIN-5F cells by lysing and homogenising the cells in 0.18M HCL in 35% (v/v) ethanol containing 1:100 protease inhibitor cocktail for mammalian tissues (Sigma). A total of 400µl of lysis reagent was used per 100mm dish. The cell lysates were rotated overnight at 4^{o}C, centrifuged at 13,000 rpm and the supernatants were stored for future use at -80^{o}C. The HCl in the cell lysate was neutralised using equimolar NaOH before the protein content was measured using the Bio-Rad protein assay as described above. A total of 50µg of protein (10µg for RIN-5F), mixed with an equal volume of 2 x sample loading buffer [200mM Tris-HCl (pH 6.8), 40% (v/v) glycerol, 1% (w/v) SDS, 0.01% (w/v) Coomassie Brilliant Blue R250] was denatured by heating to 100^{o}C for 5 minutes. The proteins were then separated on a pre-cast Criterion^{TM} 16.5% Tris-Tricene gel (Bio-Rad, Watford, UK) in running buffer [0.1M Tris-Hcl, 0,1M Tricene and 0.1% (w.v) SDS)] along side an ultra-low range molecular weight marker (1.06-26.6kDa; Sigma). The blot was run at 50V for 10 minutes followed by 120V for 90 minutes, both at RT. The protein

bands were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) with a current of 50V for 30 minutes at 4^{o}C in 1x transfer buffer [25mM Tris-HCl, 192mM glycine and 20% (v/v) methanol]. The membranes were blocked and probed as mentioned above (2B.10.1). Rabbit polyclonal anti-insulin antibody was used at a concentration of 1:100 followed by anti-rabbit HRP (1:1000) and developed using the ECL Western blotting analysis system followed by autoradiography.

The ultra-low range molecular marker and the total protein on the membranes were visualised by using a Coomassie stain. After blotting, the gels were fixed in a Tris-Tricene gel fixative [0.2% (v/v) glutaldehyde, 30% (v/v) ethanol and 0.2M sodium acetate] for 1 hour at RT with gentle rocking and then stained with Coomassie staining solution [0.025% (w/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol. 7% (v/v) acetic acid] overnight at RT with gentle agitation on a rocker. Background stain was removed by serial 10 minute washes in de-staining solutions I [40% (v/v) methanol and 7% (v/v) acetic acid] and II [5% (v/v) methanol and 7% (v/v) acetic acid]. The stained gels were stored at RT in de-staining solution II with 1% (v/v) glycerol.

2B.11 Glucose Stimulated Insulin Secretion (GSIS)

Cells cultured in 10mm dishes were washed with PBS and then incubated with 10ml of phenol red-free normal glucose (5.5mM) or high glucose (25mM) DMEM with 100units/ml penicillin and 100µg/ml streptomycin, for 1 hour at 37°C. The media was collected and then concentrated using a Centricon YM-3 Centrifugal Device (nominal molecular weight cut-off limit of 3kDa; Millipore) by centrifuging at 4000 *g* for 40 minutes on a swinging bucket centrifuge. Approximately 200µl of protein concentrate was recovered and mixed with 1:100 proteinase inhibitor cocktail. The insulin concentration of the concentrated media and cell lysates (see 2B.10.2) was measured using an ultra-sensitive rat insulin ELISA kit (Mercodia) according to manufacturer's instructions. All the standards and samples were analysed in triplicate.

Any insulin lost during the protein extraction process was determined by spiking the initial lysis buffer with a known concentration of insulin while extracting the total protein from differentiated hepatocytes (that otherwise do not contain insulin), and measuring the percentage recovered after the extraction process. The total insulin content of cells was calculated as the amount of insulin per total amount of protein, accounting for any insulin lost during the extraction process. The amount of insulin released into the media was expressed as the percentage of total insulin content of cells released during the one hour period of glucose stimulation.

2B.12 Quantification of C-peptide levels

The C-peptide content of cell lysates were measured using a high precision Rat C-peptide ELISA kit (Mercodia) according to manufacturer's instructions. A total sample volume of 10µl was used per well and all the samples were measured in triplicate and the experiment was repeated three times. The C-peptide content of cells were calculated as pmoles of c-peptide per gram of total protein. Any samples with a measurement below the threshold detection limit for the kit (i.e. ≤100pmol/L) was discounted.

2B.13 Image processing

Images of cells in culture, were collected on a DM*IRB* inverted microscope using a SPOT^{TM} camera operated with the SPOT^{TM} Advanced software. Fixed and stained fluorescent specimens were observed under the Leica DMRB microscope. Zeiss LSM 510 and LSM 510-META microscopes were used for confocal microscopy (the latter was used for high magnification images where the DAPI staining had to be collected along with other fluorochromes). For measurements of cell size and area, the measuring tools on the Zeiss LSM Image Browser software were used. The final figures were assembled using Adobe Photoshop 7.0 software.

2B.14 Statistics

All numerical results are expressed as the mean ± SEM. The statistical significance between different values were determined with a student's t-test performed using the GraphPad Prism™ 3.02 software.

Chapter THREE

Hepatocyte De-differentiation & the Induction of Pancreatic Genes

BACKGROUND

Hepatocytes are highly differentiated epithelial cells that constitute up to 80% of the liver mass (Mitaka, 1998). They have several major functions that help maintain body homeostasis. Hence, primary hepatocytes (both rodent and human) maintained in culture have been used as an *in vitro* model system for the study of xenobiotic metabolism, liver physiology, as well as understanding the biology of certain viral pathogens and parasites (Elaut et al., 2006). Cultured hepatocytes are also proving to be an attractive candidate to make cell-based extracorporal liver support systems (Court et al., 2003). However, the use of primary hepatocytes for any form of *in vitro* experimentation or therapeutic application has long been hindered by their limited life-span and de-differentiation in culture (Guillouzo, 1986). The distinguishing feature of the phenomenon of de-differentiation is the withdrawal from a given differentiated state and in the case of plants, invertebrates and amphibians, the attainment of a state that confers pluripotency (Odelberg et al., 2000, Zhao et al., 2001, Grafi, 2004). In hepatocytes however, de-differentiation is characterised by a progressive loss of liver-specific gene expression, function and distinct hepatocyte morphology. Such changes have been attributed to the diminished expression of liver-enriched transcription factors upon isolation. This in turn is due to the loss of signals emanating from the extracellular matrix, blood and via intercellular contacts with nonparenchymal cell types that otherwise maintain the hepatic phenotype *in vivo* (reviewed in Elaut et al., 2006).

With the recent description of a culture medium that can maintain the hepatic phenotype in long-term culture (Li et al., 2007), it is possible for the first time to directly compare differentiated and de-differentiated hepatocytes *in vitro*. The experiments described in this chapter will aim to assess the changes that take place during the process of hepatocyte de-differentiation. Furthermore the resulting potentiality of the de-differentiated hepatocytes, in particular of becoming pancreatic, will also be addressed.

3.1 Isolation and characterisation of primary rat hepatocytes.

Primary rat hepatocytes were isolated as described in Chapter 2 (2B.1.1) and the viability was estimated at greater than 85% using trypan blue exclusion test, to be considered suitable for culture. Freshly isolated hepatocytes, if viable have uninterrupted shiny membranes and the nuclei are often visible (Fig. 3.1A). The occurrence of cell surface blebbing was common, partly due to oxidative stress during the isolation process and the high Ca^{2+} content of perfusion buffer 2. However, since cell surface blebbing is Ca^{2+}-dependent, and if the blebbing was not too extensive, the majority of the cells recovered during the first few hours of culture in the attachment medium. Once the cells attach to the glass or plastic substrates of the culture dish, the cells flatten out and the hepatic morphology is evident (Fig. 3.1B). Hepatocytes are hexagonal in shape with the majority being binucleate and bile cannaliculi are often seen between adjacent hepatocytes in culture.

To ascertain the purity of the initial hepatocyte population, freshly isolated hepatocytes were fixed and the expression of various cell-type specific markers was examined by immunocytochemistry (Fig. 3.2). The results indicate that the initial starting population is a relatively pure population of parenchymal hepatocytes all expressing the hepatocyte-enriched iron transporter protein transferrin. The hepatocyte population comprised cells from both the periportal and perivenous zones of the liver, indicated by the differential expression of the urea cycle enzymes, carbamoyl phosphate synthetase (CPS) and glutamine synthetase (GS) respectively. The proportions of CPS and GS positive cells (Fig. 3.3) found in the isolated hepatocytes were reminiscent of that found *in vivo*, in the liver. The cells stained negatively for the endothelial marker PECAM-1, the connective tissue marker smooth muscle actin, the non-parenchymal liver cell marker Thy1 and the (intra-hepatic) duct-specific lectin (DBA) binding α-N-acetylgalactosamines, confirming that there was no contamination by any of these cell types in the isolate (Fig. 3.2D-G).

The only contaminating cell-type found was a sub-population of cells positive for the intermediate filament vimentin, which comprised of 0.039 ± 0.009% of

the total cells (Figs 3.2H & 3.3). These cells were most often found within clumps of hepatocytes and their numbers were reduced by ensuring a single cell suspension of hepatocytes was obtained during the centrifugation steps.

After attachment and by 24 hours into culture, all the hepatocytes have typical hepatocyte morphology as previously described, and express an array of hepatocyte-specific proteins (Fig. 3.4). They include the plasma proteins albumin and transthyretin, the oxyhaemoglobin binding glycoprotein haptoglobin, the iron transporter protein transferrin and the liver-specific enzymes UDP-glucuronosyltransferase (UGT), CPS and GS. The expression of such proteins is a hallmark feature of a differentiated hepatocyte.

3.2 De-differentiation of primary rat hepatocytes.

The isolated hepatocytes were cultured long term (up to 11 days) in either the KS medium to maintain the differentiated state or the DS medium to initiate de-differentiation. Hepatocytes cultured in the KS medium, maintained their hepatic morphology, whereas those cultured in the DS medium underwent de-differentiation and showed rapid loss of their hepatic epithelial-like morphology (Fig. 3.5A-B). During the 7 day culture period in the DS medium, the cells became more elongated and attained a mesenchymal morphology, which was associated with a dramatic rearrangement of their actin cytoskeletal network (Fig 3.5C). The change in morphology was apparent from day 2 onwards.

In addition to the change in morphology, by day 7, de-differentiation was also accompanied by the loss of hepatic gene expression including albumin, transferrin, UGT and the liver enriched transcription factor hepatic nuclear factor 4α (HNT4α) which were otherwise maintained under KS culture conditions (Fig. 3.6). Following de-differentiation, genes that were not typically expressed in hepatocytes were also induced. Such genes included vimentin, smooth muscle actin and cytokeratin 20 (CK20). A population of cells with DBA binding sites also appeared after de-differentiation.

Following the cells through the course of de-differentiation over 8 days revealed that the loss of the hepatic gene expression and the induction of non hepatic genes were not sudden but gradual (Fig 3.7). By day 3, while the expression of the Glut2 transporter was being lost from hepatocytes, there was already overt expression of vimentin and CK20 which concurrently increased during the next 5 days of culture.

3.3 Lineage analysis during de-differentiation.

Whenever a change of phenotype is observed in cultured cells, as during de-differentiation, it is necessary to demonstrate that the effect is not due to selection of a small population of contaminating cells. This is because a rapidly growing minority of cells could become a significant proportion of the total cells after an 8 day culture period. There is indirect evidence to suggest that the mesenchymal-like cells were derived from the epithelial hepatocytes during the de-differentiation process (Fig. 3.8). For example, low residual levels of transferrin, UGT and HNF4 protein expression was seen in de-differentiated cells at day 7, long after the change in morphology had taken place. Furthermore, some de-differentiated cells that were expressing vimentin or smooth muscle actin were also binucleate- which is a feature common to hepatocytes but atypical of fibroblasts or other mesenchymal cell types. However, to draw more conclusive evidence to demonstrate that the de-differentiated cells arose from hepatocytes, a lineage tracing experiment was devised.

The low efficiency with which primary hepatocytes can be transfected with any conditional labelling construct made it necessary that, transgenic animals were employed for lineage analysis. The impracticability of generating transgenic rats and the fact that mouse hepatocytes also show the same de-differentiation phenomenon as rat hepatocytes (discussed later in Section 3.8), meant that *Alb-Cre R26R* LacZ mice could be used for the lineage tracing experiments. In the *Alb-Cre R26R* LacZ mice, Cre recombinase is expressed only in cells that have activated the *albumin* promoter but not in other cell-types. The Cre excises the stop sequence from

the R26R gene, leading to the permanent expression of the *E. coli* β-galactosidase gene under the control of the *cytomegalovirus* (*CMV*) promoter. The non-hepatocyte cells that might be present in small numbers in the original culture will not be labelled in this way as an active *albumin* promoter is specific to the hepatocyte population. Hepatocytes were isolated from these mice as described in Chapter 2 (2B.1.2), and cultured in the DS medium. The cells were fixed and stained with X-gal to visualise β-galactosidase expression. By day 8 of culture, the vast majority of cells with mesenchymal morphology were X-gal positive and therefore derived from hepatocytes that once expressed albumin, rather than having arisen from a sub-population of a contaminating cell-type (Fig. 3.9). Hepatocytes from Rosa26 LacZ mice that express bacterial β-galactosidase ubiquitously and those from CD1 wild type mice were used as positive and negative controls respectively, for the X-gal staining. A few of the cells with mesenchymal-like morphology were X-gal negative, but this is also true on the positive control cultures set up from Rosa26 LacZ mice in which all cells express β-galactosidase constitutively from the same promoter. The lack of X-gal staining in all cells could be due to a number of factors. The recombination at the *R26R* locus may not be 100% efficient, or the β-galactosidase levels in some cells may fall below the detection threshold of the X-gal staining or there may be a downregulation of the *R26R* locus during de-differentiation.

3.4 Hepatocyte de-differentiation resembles an epithelial-mesenchymal transition event.

Epithelial-mesenchymal transition (EMT) is a phenomenon that is associated with organogenesis during development and the initiation of metastatic cancer. During EMT, epithelial cells lose their epithelial phenotype, become more mesenchymal like and show increased cell mobility. EMT has also been observed *in vitro* in cells that undergo rapid expansion by cell proliferation.

The de-differentiation of hepatocytes cultured in the DS medium is characteristic of an EMT. *In vivo*, hepatocytes are normally mitotically

quiescent and stay under G0 arrest. However they are unique among differentiated parenchymal cells in that, they retain the ability to proliferate when triggered to do so. This underlies the remarkable regenerative capacity of the liver in response to acute injuries that diminish hepatic mass. During de-differentiation, in addition to losing their epithelial morphology and becoming mesenchymal-like, the hepatocytes also started to proliferate by re-entering the cell cycle at the G1 mitotic phase. This can be seen as an increase in the number of cells expressing phosphorylated histone 3, which plays a role in chromatin remodelling and chromosome condensation associated with cell cycle re-entry (Fig. 3.10). However, during the de-differentiation process an equal number of cells were seen undergoing replication, both before and after becoming vimentin positive (Fig. 3.11).

The other feature characteristic of an EMT, which was also observed in de-differentiated hepatocytes was increased cell mobility. Cells were cultured in KS and DS medium. 24 hours after attachment, a 1cm^2 area of cells from the centre of the coverslip was scraped off using a cell scraper. The cells were washed to get rid of any floating and partly attached cells, the media was changed and the cultures were continued for 10 more days. In the KS medium, where the hepatocytes maintained their differentiated state, only minimal cell migration and subsequent invasion of the clear area was seen even at 10 days after cell scraping. In the DS medium, where hepatocytes de-differentiated, the cells were more mobile. Cell migration was observed at 2 days after cell scraping and by day 10, almost 50% of the clear area was occupied by migrating cells (Fig 3.12). However, limited cell mobility can also be induced in 2 day KS cultures by treating the cells with 10ng/ml TGF-β1 that has been shown to induce EMT in other primary culture systems (Forino et al., 2006).

3.5 De-differentiation and the induction of pancreatic gene expression.

In addition to losing the hepatic gene expression and gaining vimentin expression, hepatocytes cultured under DS conditions also started to

express a pancreatic gene expression profile (Fig. 3.13). The pancreatic genes that were induced included several characteristic of endocrine beta cells, such as the transcription factor *Pdx1*, the genes for the hormones insulin (*ins1* and *ins2*) and islet amyloid polypeptide (*IAPP*), the neuroendocrine secretory protein *chromogranin A* and the genes encoding the two subunits of the pancreatic beta cell K^+-ATP channel- sulphonylurea receptor 1 complex (*SUR1* and *Kir6.2*). Genes characteristic of a more mature beta cell phenotype, such as *pancreatic glucokinase* (*islet GCK*), were also induced while the gene encoding the liver-specific isoform of the enzyme (*hepatic glucokinase; liver GCK*) was downregulated in a complementary fashion. The pancreatic exocrine maker *amylase* was also expressed when cells were cultured in the de-differentiation medium.

In order to determine if it was only a pancreatic gene expression profile that was being induced following de-differentiation and not that of any other endoderm-derived organ, the expression of lung and intestine specific genes were investigated. However, neither the gene encoding the lung-specific pulmonary surfactant associated protein B (*Sftp-B*), nor the marker for intestinal-type differentiation- the caudal type homeobox transcription factor 2 (*cdx2*)- were induced as a consequence of de-differentiation (Fig 3.14).

The cells cultured in the DS medium, by day 8, comprise a heterogeneous population (Fig. 3.15). There are three types of vimentin positive cells that are noticeable in this population: 1- mesenchymal like cells derived from de-differentiated hepatocytes; 2- large multinucleate cells (with 3 or more nuclei) formed either by the fusion of de-differentiated cells or incomplete cytokinesis following karyokinesis during hepatocyte replication; 3- a few contaminating cells remaining from the initial isolation that are much smaller in size than the other cells. To distinguish whether the pancreatic genes (such as *insulin*) were being induced in the de-differentiated hepatocytes and not in the contaminating population, the cells were cultured in the KS medium for 3 days to suppress fibroblast growth prior to initiating de-differentiation in the DS medium. This culture routine removed of most of the small vimentin positive cells arising from the initial contaminantion (Fig. 3.16A-B). The resulting population still showed the induction of *ins1* gene at

levels comparable to hepatocytes that were cultured in the DS medium continually for 5 or 8 days (Fig. 3.16C), suggesting that the pancreatic genes were being induced, at least in part, in the de-differentiated hepatocytes rather than predominantly in the contaminating population.

3.6 The reversibility of the de-differentiated phenotype.

To determine if the de-differentiated phenotype was reversible, hepatocytes were cultured under de-differentiating DS conditions for 3 days and then transferred to the KS medium and maintained under these conditions for a further 5 days (Fig. 3.17). Initially after the transfer, the de-differentiation (as indicated by vimentin expression) and the levels of the induced pancreatic genes were downregulated. However, this suppression of gene expression was only temporary and by 3 days after transfer to KS conditions, de-differentiation had resumed although at a much slower rate compared to those cells cultured throughout in the DS medium. More dramatic was the upregulation of liver gene expression (as seen by levels of *albumin* and *cps*) in de-differentiated hepatocytes following the transfer to the KS medium. The expression of the liver genes appeared to be more sustained during the 5 day culture period that followed in the KS medium. Therefore, the de-differentiated phenotype is not entirely reversible. Although liver-specific genes can be re-induced, a true hepatic phenotype cannot be achieved just by swapping the media and without the use of other exogenous factors.

3.7 Are progenitors induced during hepatocyte de-differentiation?

During embryonic development, pancreatic and liver lineages arise from different progenitors derived from a common endodermal domain. After the completion of organogenesis, it is widely accepted that some cells with progenitor-like properties reside within the organs well into adult life in order to contribute to tissue mass during post-embryonic organ growth and innate regenerative responses of the body. Markers for such tissue-specific

progenitors have been identified and several more have been proposed. In order to find out if any of these hepatic or pancreatic progenitor markers were induced during the de-differentiation process, after loss of the hepatic phenotype and prior to the induction of the pancreatic phenotype, RT-PCR was carried out (Fig. 3.18). The expression of the embryonic liver markers *alpha-foetoprotein* (*AFP*) and *stromal cell derived factor 1a* (*SDF1a*), and the oval cell marker *c-kit* were all seen during de-differentiation. Additionally, the genes encoding the protein gel product 9.5 (*PGP9.5*) and the winged helix transcription factor *FoxA3* (or *Hnf3γ*) were also expressed. These genes are otherwise expressed in the early endodermal epithelium and neuroendocrine progenitors. *Nestin*- a postulated marker for pancreatic progenitors, *CK19*- a marker of the pancreatic ductal epithelium in which the beta cell precursors are thought to reside and *Sox9*- a transcription factor expressed in cells from which the pancreatic endocrine cells are derived, were all induced following de-differentiation.

The expression of both the progenitor markers and the differentiated pancreatic cell-type markers at common time points does not necessarily imply co-expression. The total population might comprise cells at several different stages of differentiation. The progenitor markers that can be seen at an immunodetectable level are the pancreatic and hepatic oval cell maker OV6 and the early pancreatic progenitor marker, Sox9 (Fig. 3.19). Both these proteins were only detected in de-differentiated hepatocytes in the DS medium and not in freshly isolated hepatocytes or hepatocytes maintained in the KS medium. This suggests that a heterogeneous hepatic/pancreatic progenitor-like population is produced during de-differentiation.

3.8 Induction of pancreatic genes in de-differentiated mouse and human hepatocytes.

It is important to establish whether the de-differentiation phenomenon and the induction of pancreatic genes observed with rat hepatocytes extends to other rodent hepatocytes (e.g. from the mouse) and more importantly to human hepatocytes. Mouse and human hepatocytes can be cultured under

KS and DS conditions as previously described for the rat hepatocytes. Mouse hepatocytes are slightly larger than rat hepatocytes (Fig. 3.20 A and B) and in the DS medium, the mouse hepatocytes also lose their hepatic morphology and gene expression. The downregulation of hepatic gene expression is accompanied by the induction of *vimentin* expression and also of several pancreatic genes including *ins1, Kir6.2 and SUR1* (Fig. 3.21).

Freshly isolated human hepatocytes were found to contain more lipid than the rodent hepatocytes partly due to the age of the human cell donors. Although human hepatocytes started to express vimentin following culture in the DS medium, the change in morphology of the human hepatocytes was not as pronounced as that for rat hepatocytes undergoing de-differentiation (Fig. 3.32). However, a similar loss of liver gene expression was seen both at mRNA and protein levels (Figs 3.23 and 3.24). By day 7, the expression of albumin, transferrin, UGT and HNF4 were all downregulated. It was also observed that, unlike for the rodent hepatocytes the KS medium was not sufficient enough to maintain high levels of hepatic proteins in human hepatocytes cultured for 7days (Fig. 3.23). However, the induction of pancreatic gene expression following de-differentiation was also observed in human hepatocytes, but the levels of induction was slightly lower compared to de-differentiated rodent hepatocytes (Fig. 3.24).

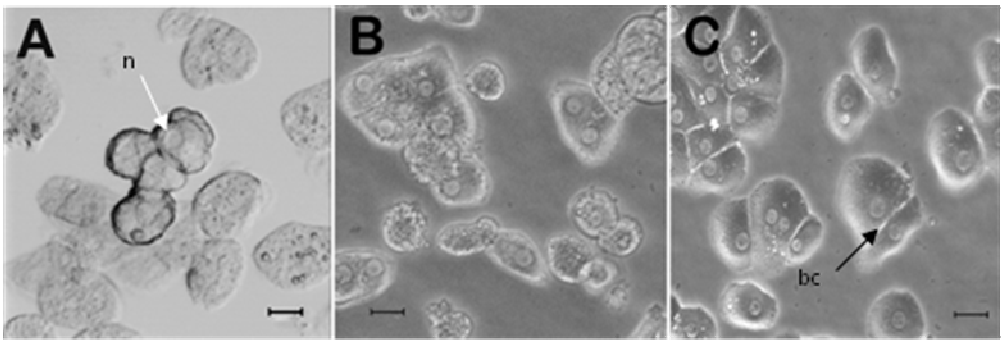


***Figure 3.1*. The isolation and culture of primary rat hepatocytes.** Primary rat hepatocytes were isolated using the two-phase collagenase based liver perfusion protocol. Primary rat hepatocyte as seen immediately after isolation (A), hepatocytes after 5 hours in the attachment medium (B) and after 24 hours of culture (C) in the KS medium that maintain hepatocyte morphology. Note the presence of binucleate cells and bile cannaliculi in between cells in the cultured hepatocytes. *n*- nucleus; *bc*- bile cannaliculus. The scale bar represents 10µm.

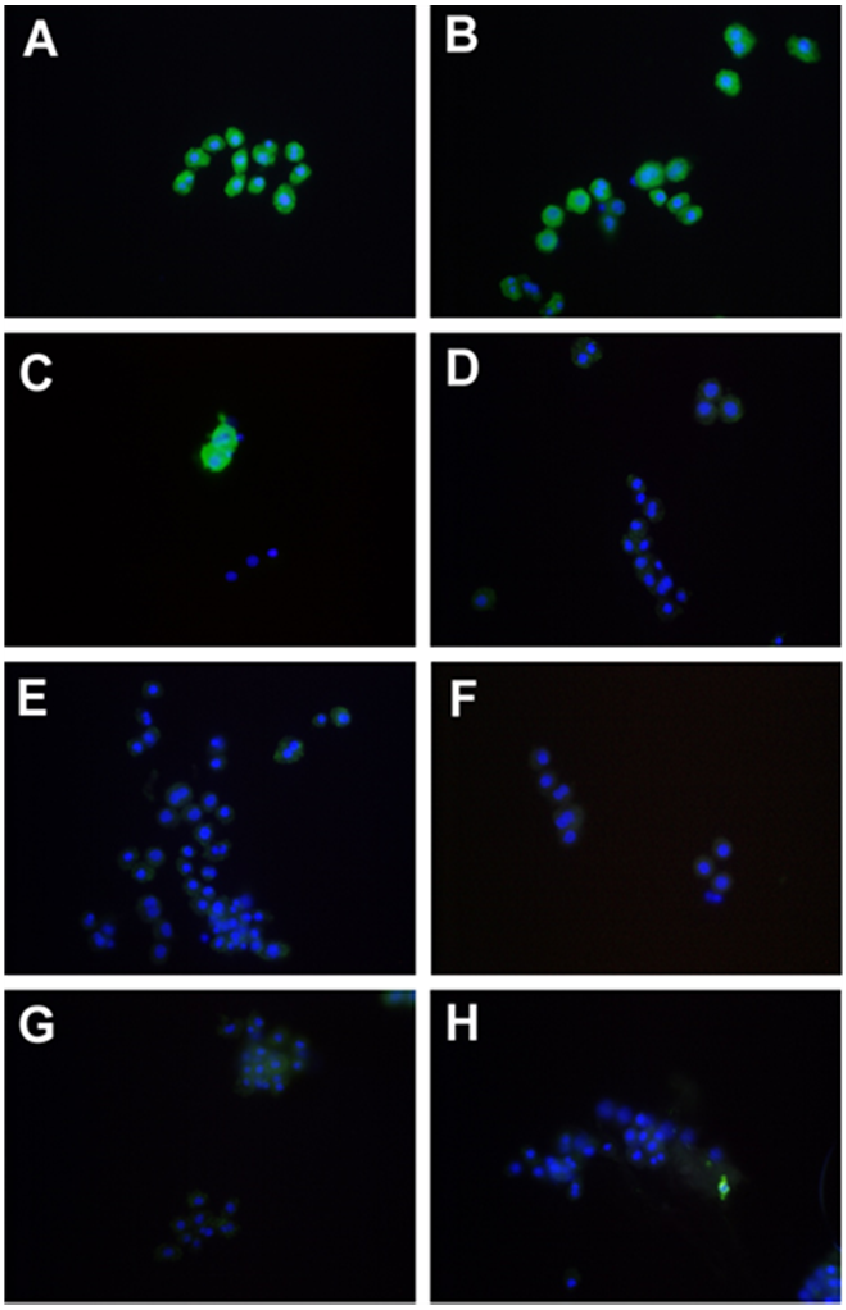


Figure 3.2. **The isolated cells constitute a relatively homogenous population of parencymal hepatocytes.** The expression of the hepatocyte markers transferrin (A), CPS (B) and GS (C) was seen in freshly isolated hepatocytes. The cells were negative for the markers of connective tissue, SMA (D), non-parenchymal liver cells, Thy1 (E), DBA-binding (intra-hepatic) duct-specific α-N-acetylgalactosamines (F), and the endothelial cell marker, PECAM-1 (G) suggesting the absence of these cell types in the isolate. However few cells expressing vimentin (H) were seen within clusters of freshly isolated hepatocytes. All markers when present are in green and the nulcei are stained with DAPI (blue). The scale bars represent 20μm. (See Appendix B for positive controls).

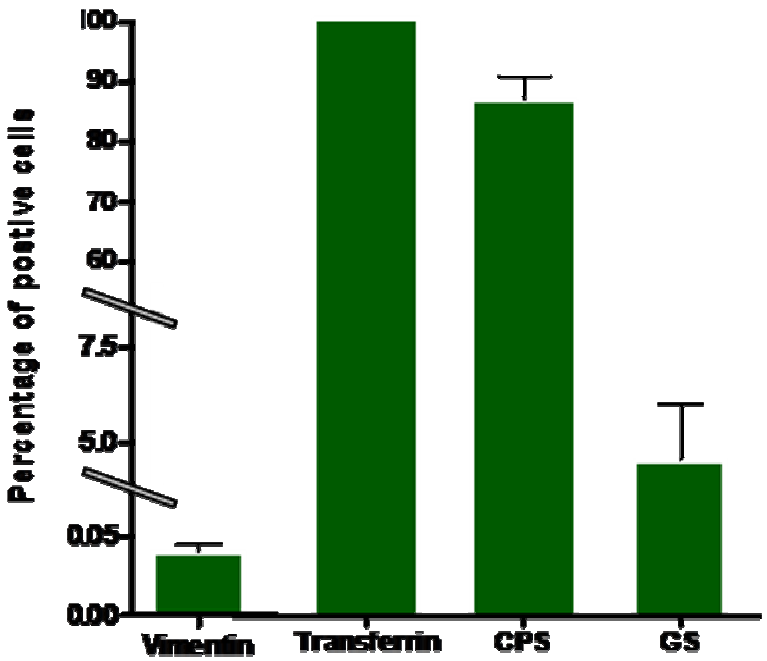


Figure 3.3. **The isolated hepatocyte population has relatively few contaminating cells.** The number of cells expressing the liver markers transferrin, CPS and GS, as well as the intermediate filament protein vimentin were counted after immunostaining the freshly isolated hepatocyte population in suspension. The proportions of CPS-expressing periportal hepatocytes and the GS-expressing perivenous hepatocytes are representative of numbers found *in vivo*. Note that the data is represented against a broken y-axis in order to accommodate all the bars on the same graph. n=3.

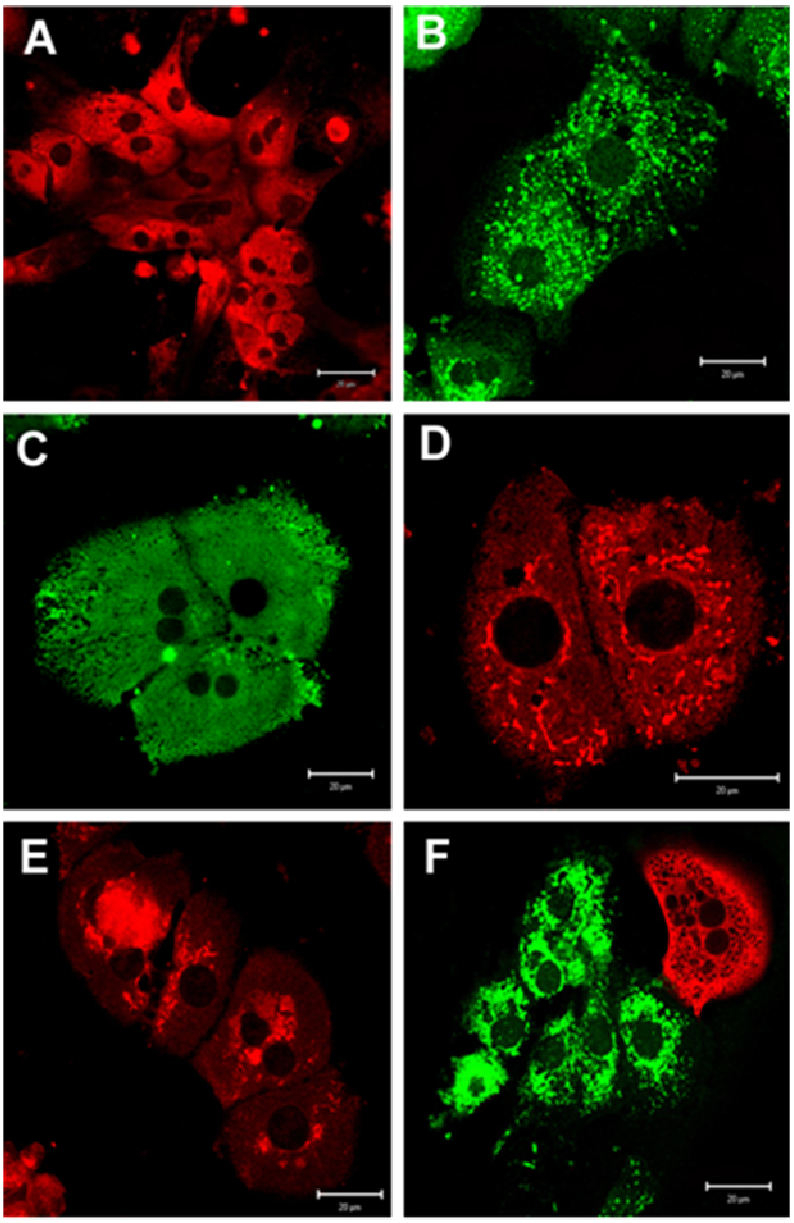


Figure 3.4. **Expression of hepatic proteins in cultured rat hepatocytes.** The expression of proteins specific to differentiated hepatocytes can be seen in cells that have been cultured in the KS medium for 24 hours. These include A- albumin (red); B- transthyretin (green); C- UGT (green); D- transferrin (red); E- haptoglobin (red); F- CPS (green) and GS (red). The scale bars represent 20µm.

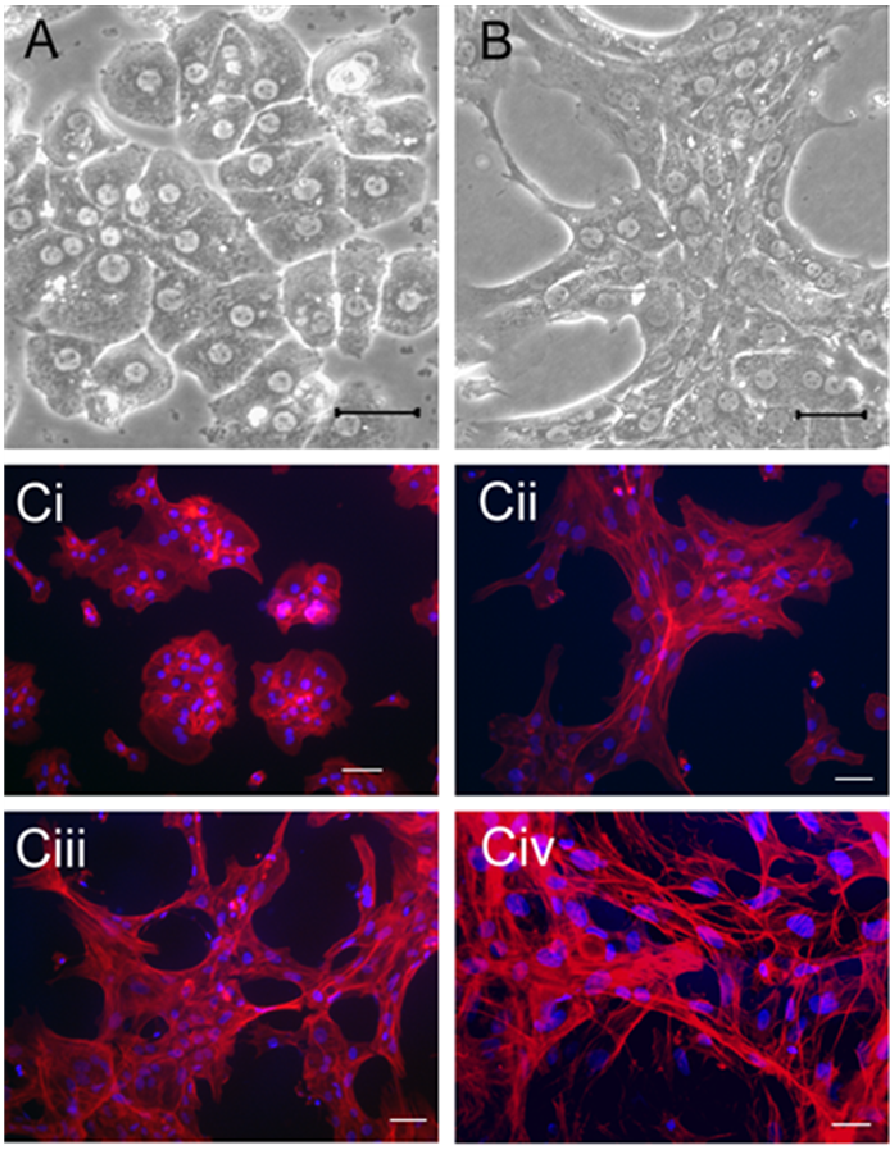


Figure 3.5. **Hepatocytes change morphology during de-differentiation.** The differences in morphology of hepatocytes cultured in the KS medium (A) and those cultured in the DS medium (B) for up to 7 days. The rearrangement of the cytoskeletal components can be seen during the de-differentiation time course in the DS medium (C) through day1 (i), day 3 (ii), day 5 (iii) and day 8 (iv). Phalloidin (red) shows the actin cytoskeletal network of the cells and the nuclei are stained with DAPI (blue). The scale bars represent 20µm.

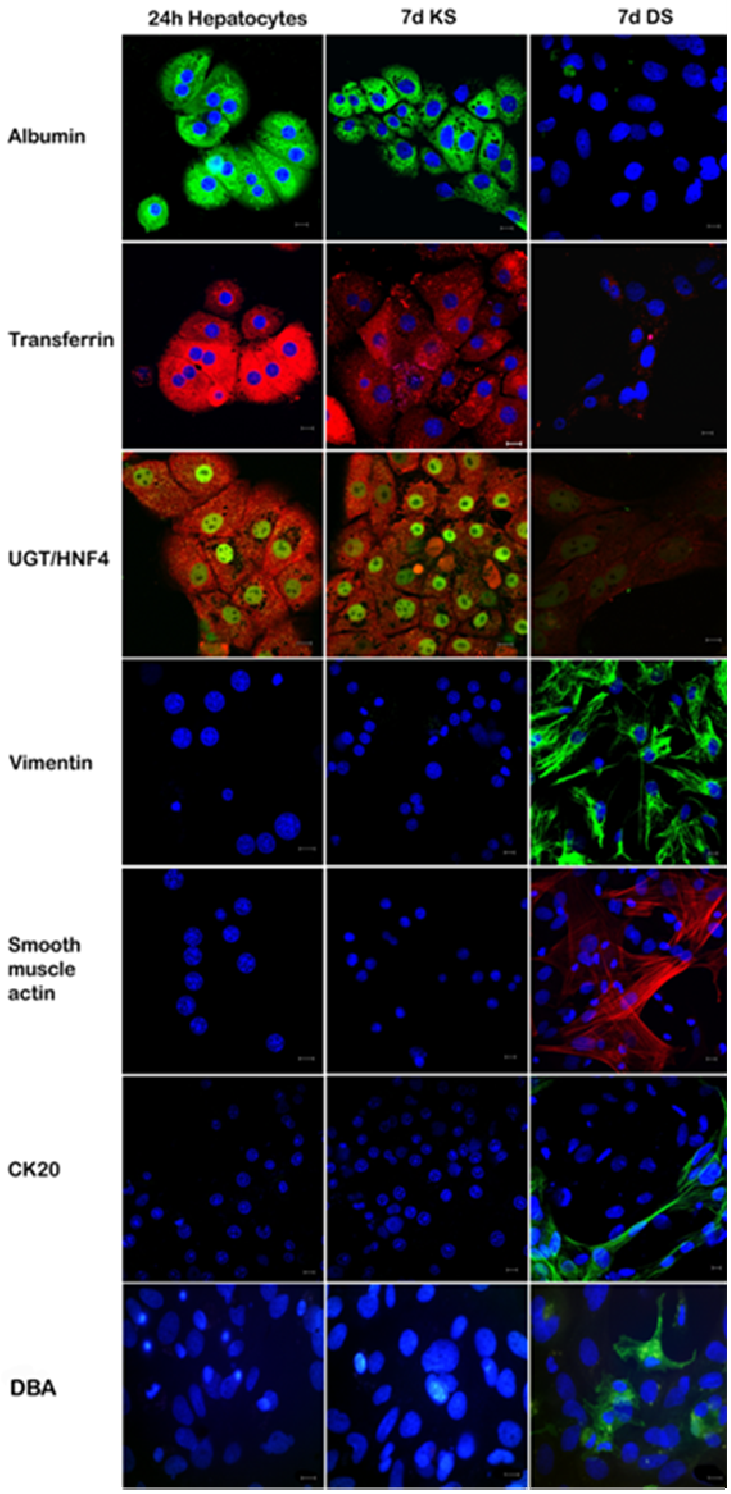


Figure 3.6. **The change in protein expression during de-differentiation.** The downregulation of various hepatic markers (rows 1-3) during 7 days of de-differentiation in DS medium compared to hepatocytes cultured for 24 hours and 7 days in KS medium. The induction of various other markers (rows 4-7) is also seen following de-differentiation: Albumin, HNF4, vimentin, CK20, DBA (green) and transferrin, UGT and SMA (red). The scale bars represent 20µm.

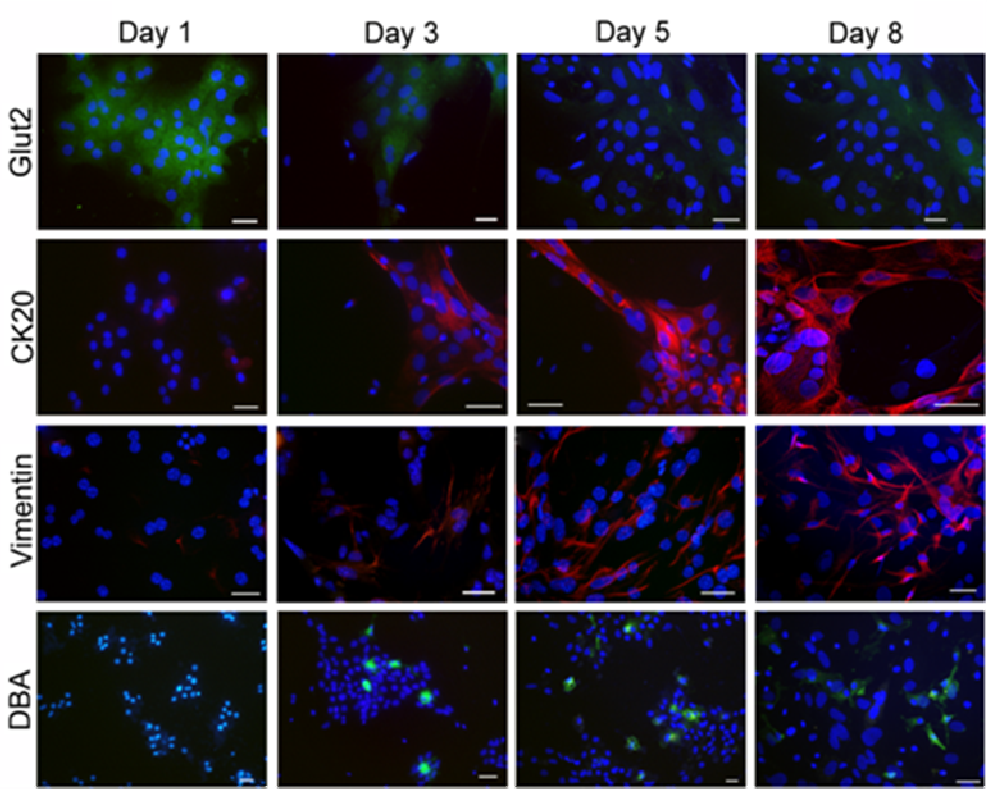


Figure 3.7. **The expression of specific proteins change gradually during the course of de-differentiation.** The induction and upregulation of CK20 (red) and vimentin (red) expression as well as the increase in the number of DBA (green) positive cells during the time course of de-differentiation in the DS medium. This accompanies a downregulation of liver gene expression such as that of the glucose transporter Glut2 (green). The nuclei are stained with DAPI (blue). The scale bars represent 20µm.

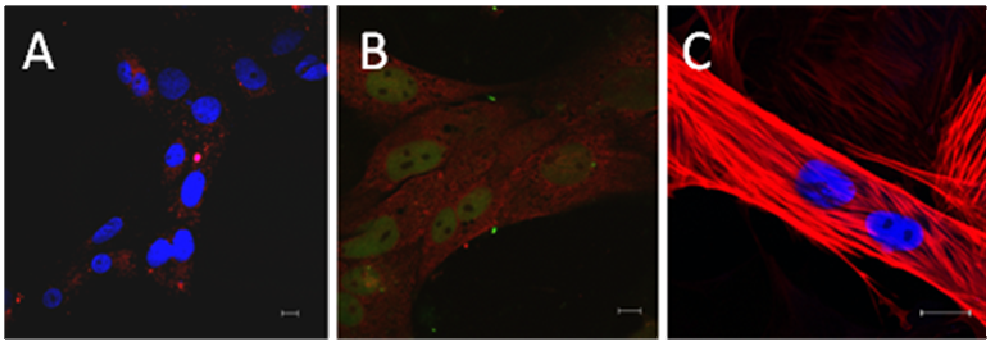


Figure 3.8. **De-differentiated cells show many signs of their origin from differentiated hepatocytes.** The residual expression of some liver proteins in de-differentiated hepatocytes-transferrin (A; red), UGT (B; red) and HNF4 (B; green) can be seen. The expression of smooth muscle actin (C; red) is also seen in binucleate cells which are likely to be hepatocyte-derived. The nuclei are stained with DAPI (A, C; blue). The scale bars represent 10μm.

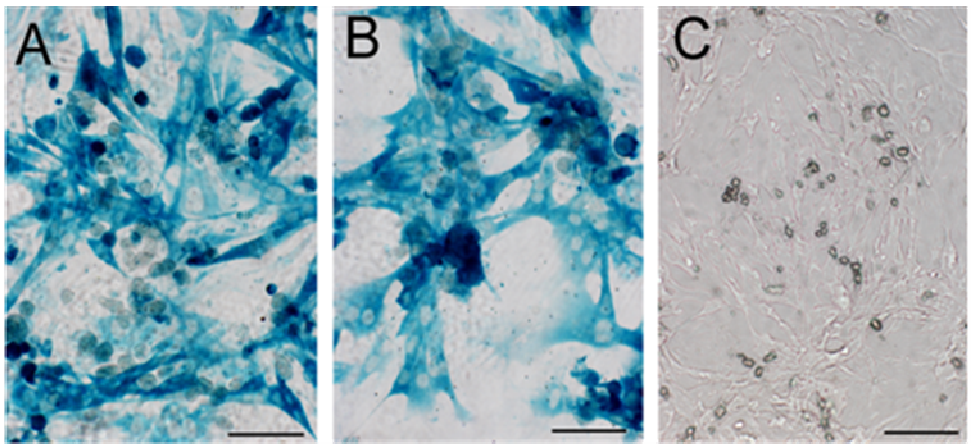


Figure 3.9. **Lineage tracing shows that de-differentiated cells are derived from differentiated hepatocytes.** Hepatocytes were isolated from Alb-Cre x R26R LacZ mice (A) expressing bacterial β-galactosidase in hepatocytes, Rosa26 LacZ mice ubiquitously expressing β-galactosidase (B) and CD1 wild type mice (C). Cells were cultured in DS medium for 8 days, then fixed and stained with X-gal. The Rosa26 LacZ and CD1 cells were used as positive and negative controls respectively. The fibroblast like cells in the 8 day hepatocytes culture from Alb-Cre x R26R LacZ mice (A) being X-gal positive suggest their origin from albumin-expressing hepatocytes. The scale bars represent 20μm.

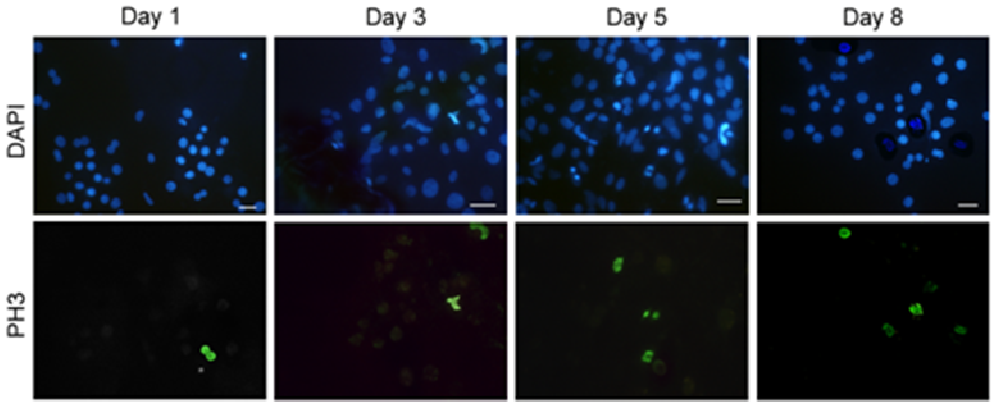


Figure 3.10. **De-differentiated hepatocytes proliferate in DS culture.** The increase in the number of proliferating cells through the course of de-differentiation over 8 days shown by PH3 expression (green). The scale bars represent 20µm.

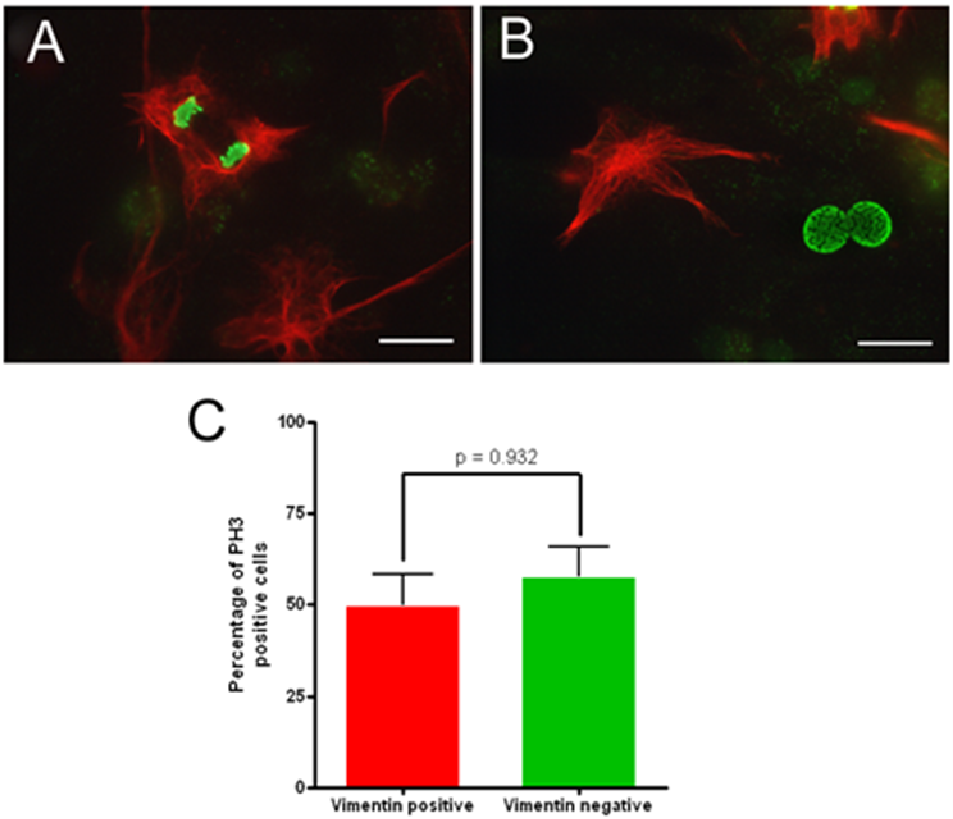


Figure 3.11. **De-differentiated hepatocytes can proliferate before and after becomming vimentin positive.** The expression of PH3 (green) in cells that are both vimentin (red) positive (A) and vimentin negative (B) can be seen. C- The relative proportions of cells with vimentin-PH3 co-localisation compared to those without, shows that de-differentiated hepatocytes are able to divide both before and after the acquisition of vimentin expression. The scale bars represent 20µm (A, B). n=3 (C).

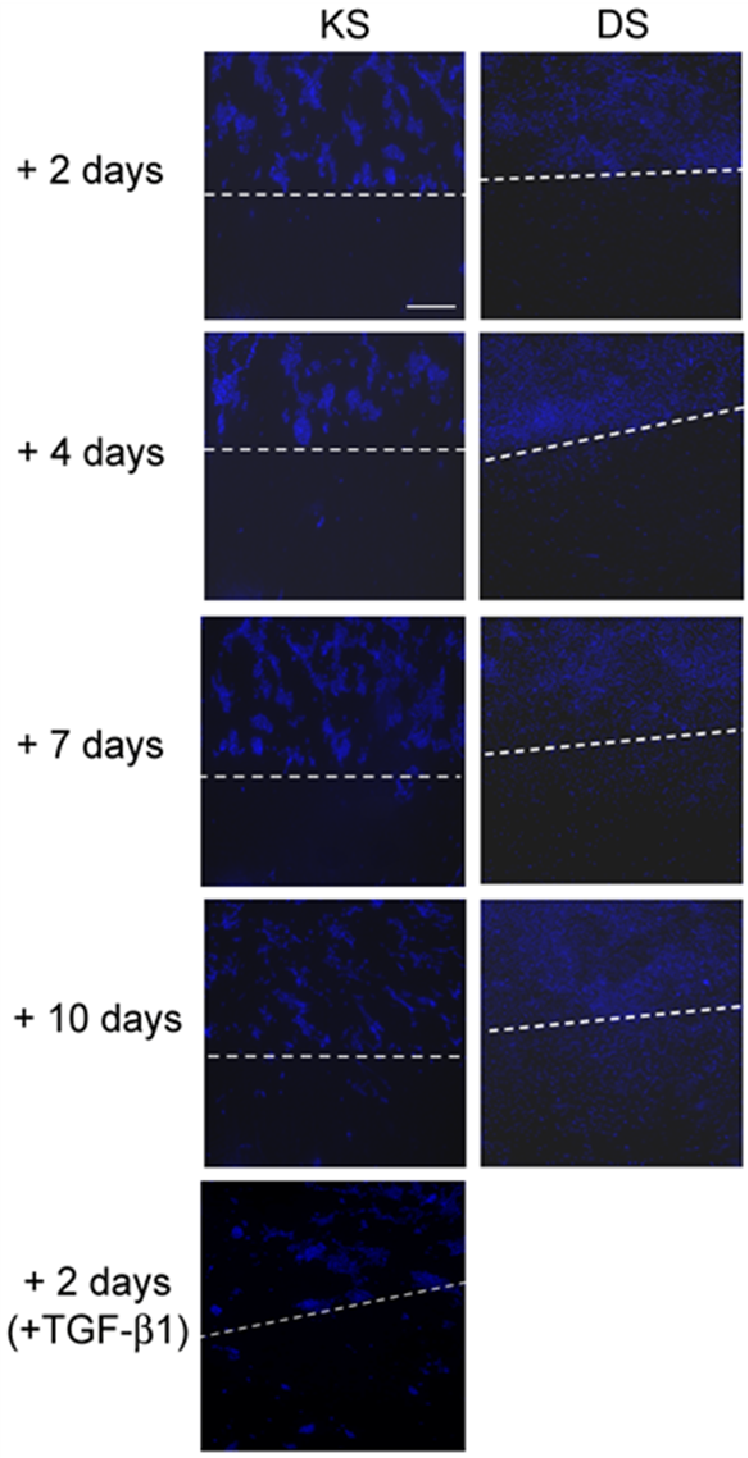


Figure 3.12. **De-differentiated hepatocytes are more motile compared to their differentiated counterparts.** Evidence for increased cell motility in hepatocytes cultured in the DS medium as opposed to those cultured in the KS medium at different time points after cell scraping. The white dotted line indicates the line below which the cells were scraped off. The nuclei of cells are stained with DAPI (blue). TGF-β1 can induce cell migration in KS cultures. 2 days after treatment. The scale bars represent 100μm.

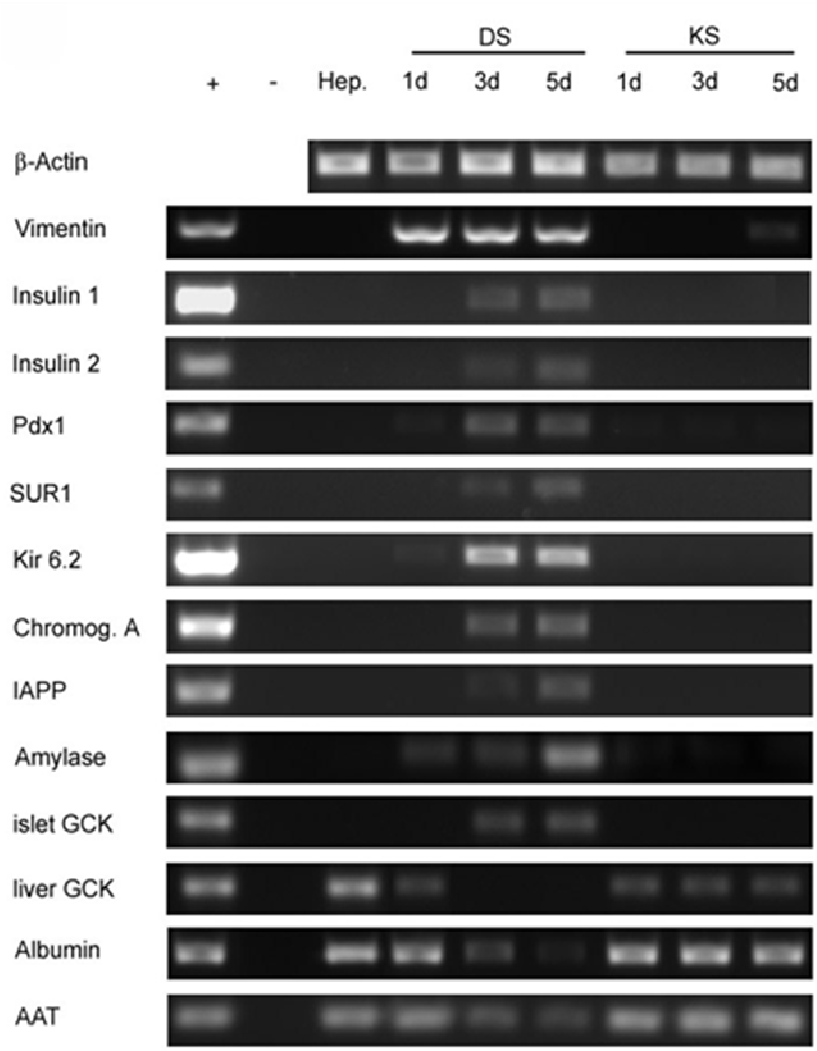


Figure 3.13. **Hepatocyte de-differentiation is accompanied by the induction of pancreatic genes.** Hepatocytes were cultured in DS or KS medium for 1, 3 and 5 days. The expression of a panel of pancreatic and hepatic genes was examined by RT-PCR. cDNA from RIN cells, rat skin, rat liver and rat exocrine AR42J-B13 cells were used as positive control for endocrine genes, *vimentin*, hepatic genes and *amylase* respectively.

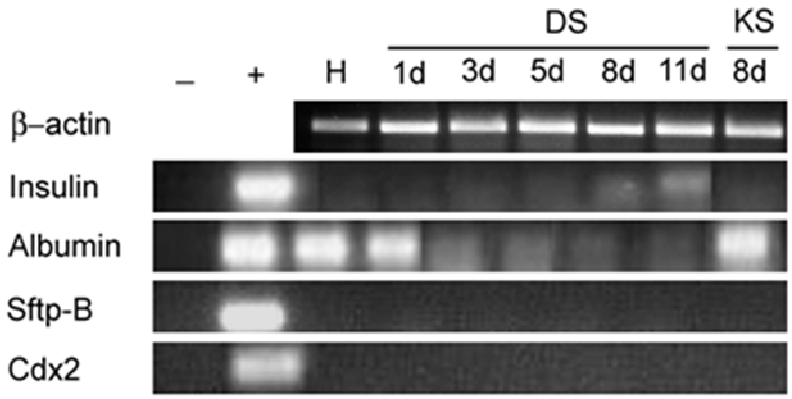


Figure 3.14. **Only a pancreatic gene network is induced upon de-differentaition.** The evidence for only a pancreatic gene profile being induced following de-differentiation and not that of any other endodermal lineage such as that of the lung (*Sftp*-B expression) or the intestine (*Cdx2* expression) was examined by RT-PCR. The hepatocytes were cultured under DS conditions for up to 11 days and compared with those cultured under KS conditions for 8 days. cDNA from RIN cells, rat liver, rat lung and rat intestine were used as positive control.

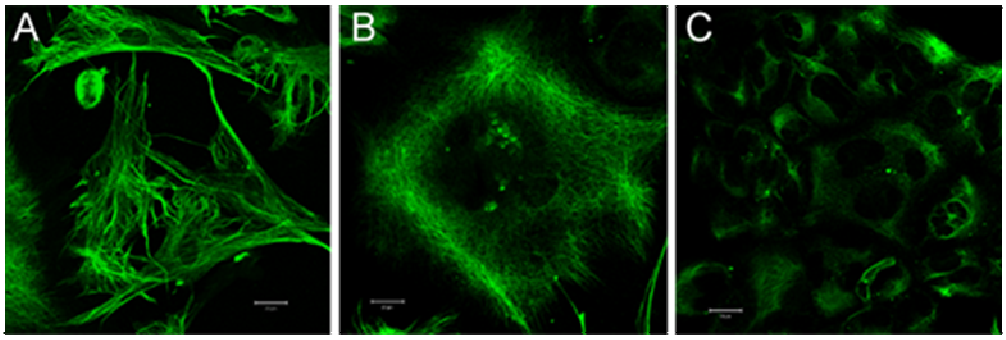


Figure 3.15. **The de-differentiated population is heterogenous.** The vimentin (green) expressing de-differentiated population comprise of individual de-differentiated hepatocytes (A), large cells formed by the fusion of de-differentiated cells or their incomplete cytokinesis (B) and small vimentin positive cells derived from the initial contaminating population (C). The scale bar represents 10µm.

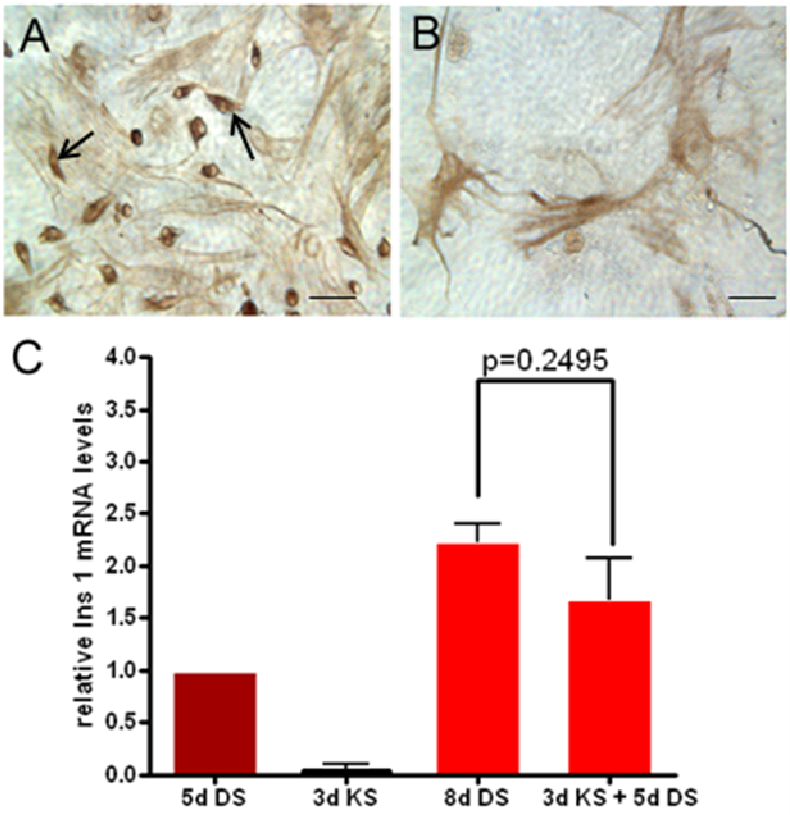


Figure 3.16. **The pancreatic genes are induced in the fibroblast-like vimentin positive cells and not primarily in the other cell types.** The small vimentin (DAB/ brown) expressing cells seen during 8 days of DS culture (A) can be eliminated by a culture regime involving 3 days of culture in KS followed by 5 days in DS (B). Cells cultured in this way still show insulin expression (C) when analysed by real time RT-PCR. The scale bar represents 10µm (A, B). n=3 (C).

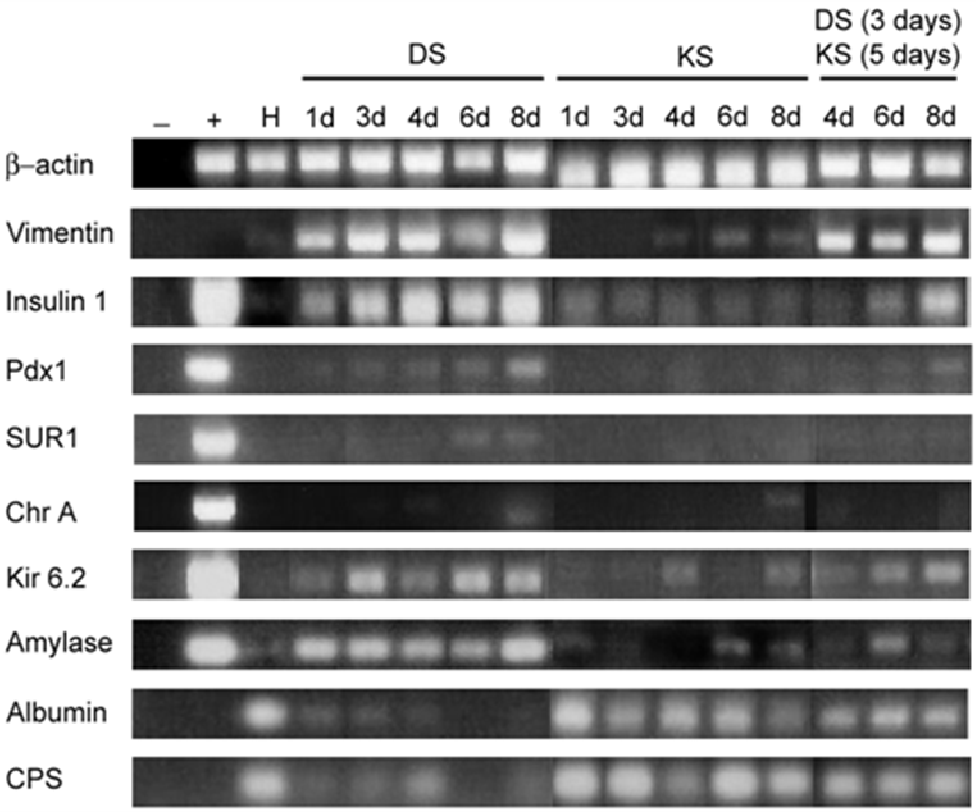


Figure 3.17. **The de-differentiated phenotype of hepatocytes is reversible.** Reversibility of the de-differentiated phenotype can be shown by swapping cells cultured in the DS medium back into KS medium after 3 days. There was a slight diminution of pancreatic gene expression and a complete regain of hepatic gene expression as seen by RT-PCR. cDNA from RIN cells, rat skin, rat liver and rat exocrine AR42J-B13 cells were used as positive control for endocrine genes, *vimentin*, hepatic genes and *amylase* respectively.

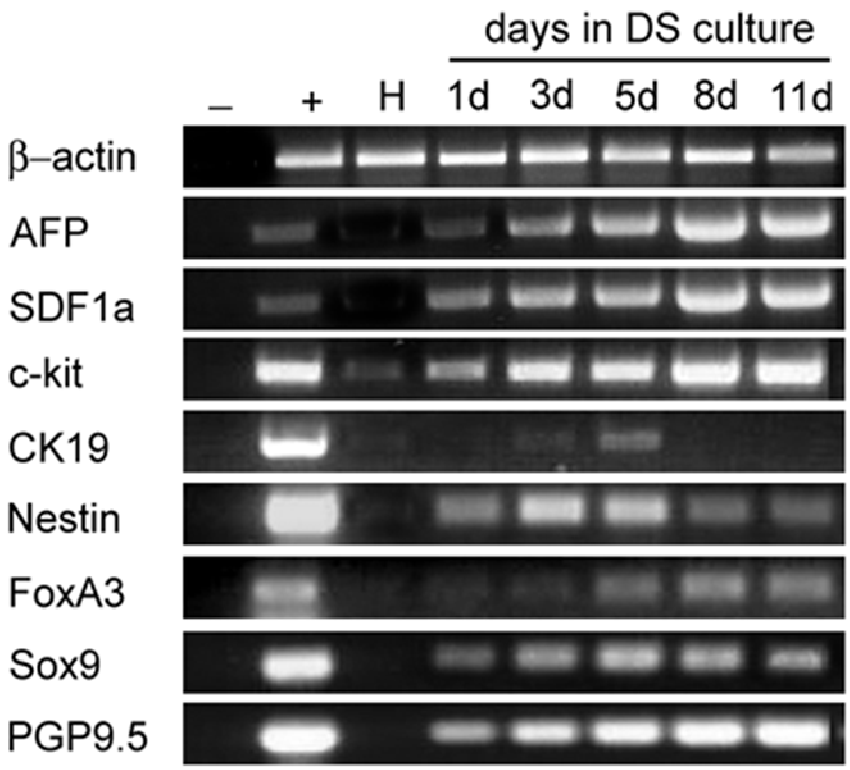


Figure 3.18. **Progenitor-specific markers are induced in de-differentiating hepatocytes.** The expression of hepatic and pancreatic progenitor markers in de-differentiated hepatocytes was analysed by RT-PCR. cDNA from the following organs were used as positive controls: E15.5 embryonic rat liver (*AFP, c-kit*), rat femoral bone marrow (*SDF1α*), rat pancreatic duct (*CK19*), E15.5 rat pancreas (*nestin,FoxA3*), rat testes (*Sox9*) and E15.5 embryonic rat brain (*PGP9.5*).

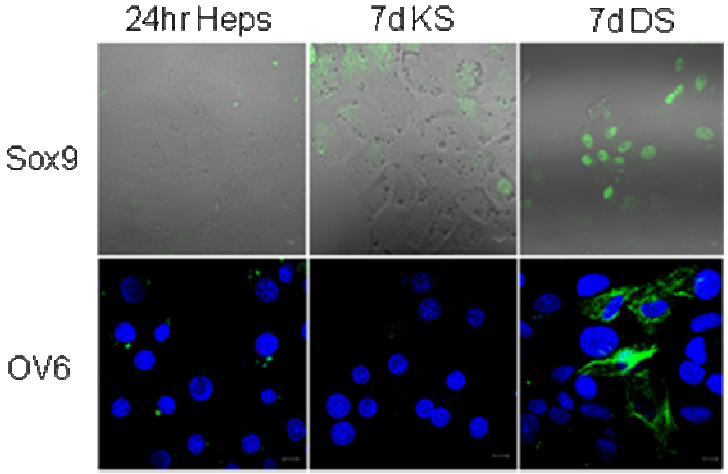


Figure 3.19. **Sox9 and OV6 is induced during de-differentiation.** Expression of the pancreatic progenitor marker Sox9 (green) and hepatic oval cell marker (OV6; green) was seen during the de-differentiation in DS medium for up to 7 days. The nuclei are stained with DAPI (blue). Scale bar shows 10μm.

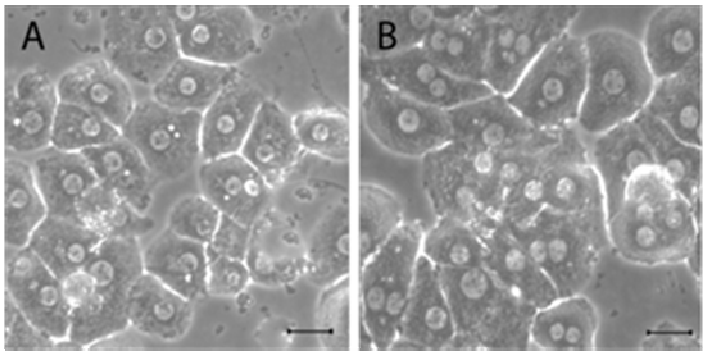


Figure 3.20. **Mouse hepatocytes are morphologically different from rat hepatocytes.** The difference in morphology of freshly attached rat hepatocytes (A) and mouse hepatocytes (B) as seen 6 hours after isolation and initial culture in the attachment medium. The mouse hepatocytes are larger than the rat hepatocytes.. The scale bar represents 10µm.

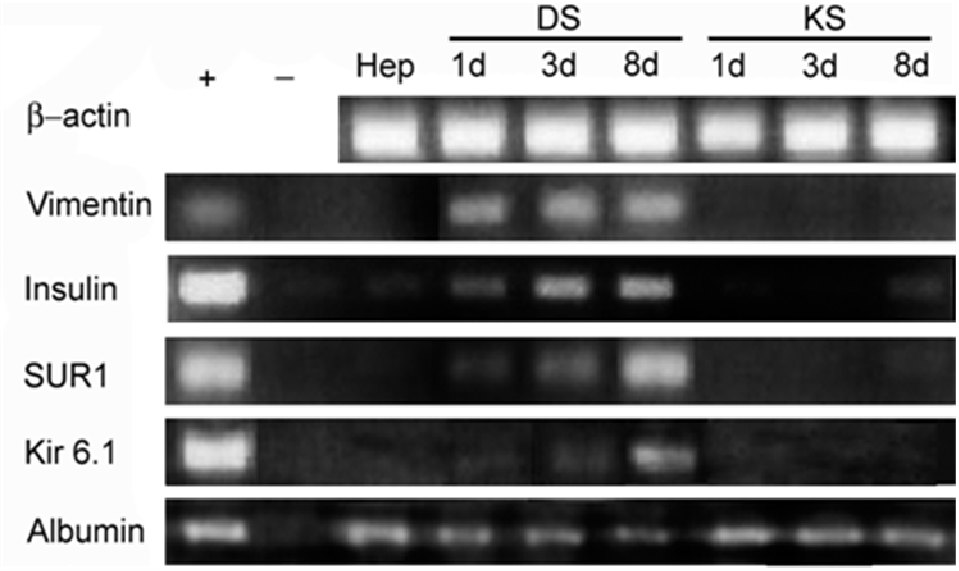


Figure 3.21. **Hepatocyte de-differentiation in the mouse is also accompanied by the induction of pancreatic genes.** Isolated mouse hepatocytes were cultured under DS and KS conditions for up to 8 days. The change in expression of the de-differentiation marker *vimentin*, the hepatic marker *albumin* and the pancreatic markers *insulin 2*, *SUR1* and *Kir6.1* were analysed by RT-PCR. cDNA from MIN cells, mouse skin and mouse liver were used as positive control for endocrine genes, *vimentin* and *albumin* respectively.

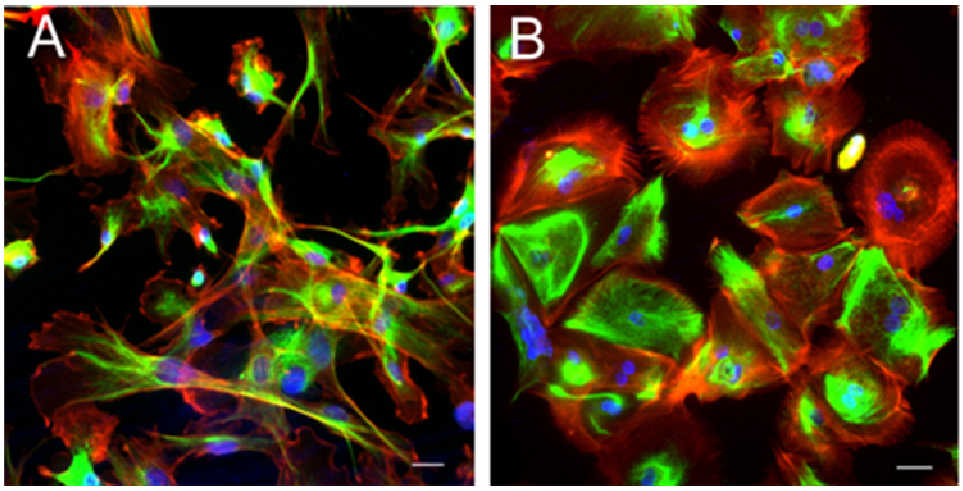


Figure 3.22. **Human hepatocyte de-differentiation is not accompanied by dramatic changes in morphology.** Comparison of the change in morphology during rat (A) and human (B) hepatocyte de-differentiation at 8 days in DS medium. Although vimentin expression (green) is seen in both, the human hepatocytes do not become mesenchymal-like in morphology. The actin network is shown by phalloidin binding (red) and the nuclei are stained with DAPI (blue). The scale bars represents 10μm.

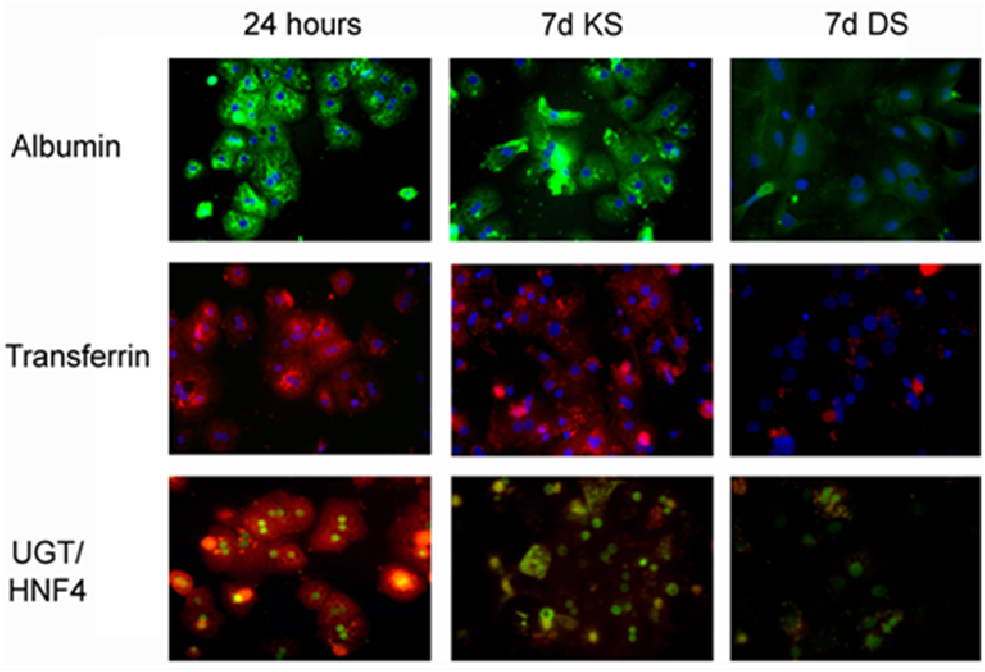


Figure 3.23. **The change of protein expression during the de-differentiation of human hepatocytes.** The down regulation of hepatic markers albumin (green), transferrin (red), UGT (red) and HNF4 (green) in de-differentiating human hepatocytes after 7 day DS culture, compared to those cultured in KS medium for 24 hours and 7 days. The nuclei are stained with DAPI (top two rows, blue). The scale bars represents 20μm.

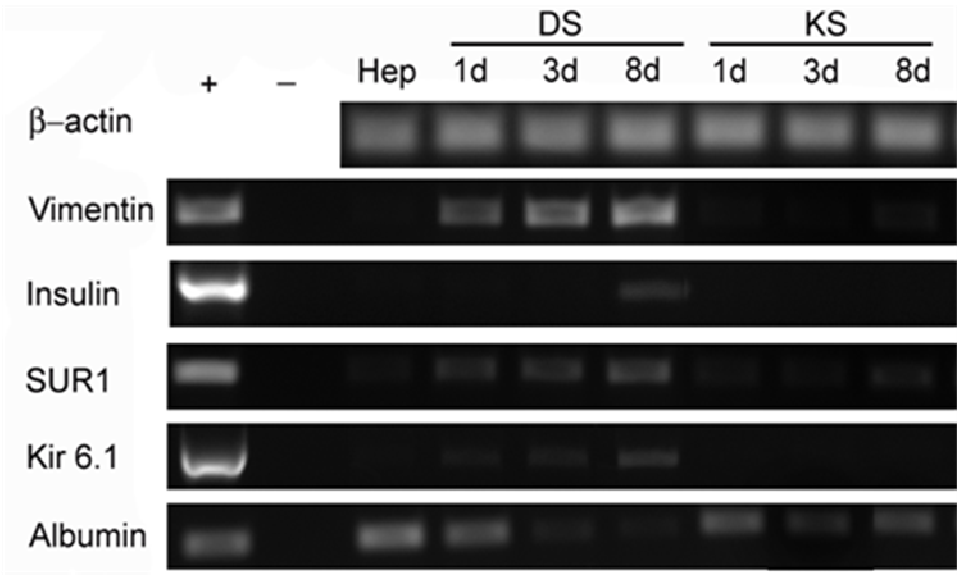


Figure 3.24. **The de-differentiation of human hepatocytes is also accompanied by an induction of pancreatic gene expression.** The expression of pancreatic and hepatic genes in human hepatocytes cultured under DS and KS conditions for up to 8 days was examined by RT-PCR. cDNA from human pancreatic endocrine tissue, de-differentiated human hepatocytes and freshly isolated human hepatocytes were used as positive control for endocrine genes, *vimentin* and hepatic genes respectively.

DISCUSSION

The morphological changes that are initiated upon hepatocyte isolation and during their subsequent culture have been previously described (Guillouzo, 1986). The ascertaining the homogeneity of the initial population is vital and it has confirmed that the phenotypic changes seen, both in morphology and gene expression are directly due to de-differentiation of hepatocytes. Furthermore, the changes associated are too rapid to be accounted for by selective overgrowth of a very small population of undifferentiated precursors. For example, the most abundant contaminant is the vimentin positive cells at 4 cells per 10000 hepatocytes. These would need to divide 13 times, in the total absence of division by the hepatocytes, before they became a majority of the culture. The PH3 staining indicated that this was not the case. We have also confirmed the phenomenon of hepatocyte de-differentiation by the lineage tracing with *Alb-Cre R26R* LacZ cells, which shows that most of the cells at 8 days are derived from the original hepatocytes. Therefore it is important to understand the mechanisms that provoke de-differentiation of hepatocytes and how it applies to our system.

Epithelial-mesenchymal transition events that resemble hepatocyte de-differentiation have also been reported for several other cell-types such as pancreatic beta cells and kidney tubular epithelial cells maintained in long-term primary culture (Gershengorn et al., 2004 (remitted), Ouziel-Yahalom et al., 2006, Forino et al., 2006). The potential molecular mechanisms underlying the de-differentiation processes involve disruption of the *in vivo* mechanisms that maintain the differentiated phenotype. For hepatocytes these can be divided into, 1- soluble endogenous substances, growth factors and hormones circulating in the blood, 2- autonomic hepatic innervations, 3- the extracellular matrix, and 4- intercellular communication mediated via paracrine factors and cell junctions (such as adherens junctions and connexin containing gap junctions). Most of the above mentioned mechanisms can influence the transcriptional regulation of hepatic genes by directly or indirectly interacting with nuclear receptors and recruiting liver-enriched transcription factors such as HNFs, C/EBPs and DBP (Cereghini,

1996, Lekstrom-Himes and Xanthopoulos, 1998, Hayhurst et al., 2001). Most liver-specific promoters have consensus sequences to which these transcription factors bind to, in order to regulate gene expression and ultimately hepatic function. Thus the liver is an organ which highlights the importance of the histoarchitecture, the soluble environment and the transcriptional machinery in maintaining a differentiated phenotype *in vivo*.

During the hepatocyte isolation process and subsequent dissociation of the liver, the collagenase-mediated digestion of the extracellular matrix and the disruption of normal tissue integrity triggers de-differentiation (Elaut et al., 2006). Hepatic ischemia/reperfusion (I/R) injury is also considered one of the major causes of hepatocyte de-differentiation, during the isolation process itself (Fondevila et al., 2003). Our protocol for liver perfusion *in situ* and prior oxygenation of the perfusion buffers minimises the duration of oxygen deprivation. Nonetheless the hepatocytes are still susceptible to I/R injury. This can account for the slightly low levels of hepatic mRNA in freshly isolated and 24 hour cultured hepatocytes compared to RNA isolated from a non-perfused liver. The presence of PH3 positive cells as early as 24 hour into cultures could also be explained by I/R stress, as the G0 to G1 phase transition can take place during the isolation itself (Su et al., 2002).

The other phenotypic alterations seen during the culture of hepatocytes under DS conditions can be summarised as the change in morphology, induction of vimentin expression and further downregulation and near abolition of hepatic gene expression. During culture as a monolayer, hepatocytes depolarise partly due to the loss of tight and gap junctions, and also the absence of gradients of signals that otherwise maintain cell polarity (Arterburn et al., 1995, Luttringer et al., 2002). Under these depolarised conditions, the need for the cells to re-establish connectivity and adapt to the new culture environment calls for major changes to the cytoskeletal architecture resulting in a physical change in cell shape (Baker et al., 2001). This was seen as the cells changed morphology by cytoskeletal rearrangement (visualised by phalloidin binding) and became more fibroblast or mesenchymal-like when cultured in the DS medium.

In addition to changes in morphology, acquisition of vimentin expression is considered a sign of de-differentiation. Unlike the cytokeratins which are epithelial intermediate filaments, vimentin is an intermediate filament primarily expressed in cells of mesenchymal origin (Traub, 1985). The reason for the induction of vimentin expression in de-differentiated hepatocytes remains obscure. However it is known that in hepatocytes the keratins are found attached to desmosomes and hemidesmosomes at points of cell-cell and cell-extracellular matrix adhesion sites respectively in order to maintain epithelial integrity. Following isolation, such adhesion junctions are lost. Thus the induction of vimentin expression (and this could also apply to the acquisition of smooth muscle actin expression) might relate to cells adapting to new conditions of being cultured in a more two-dimensional and flattened monolayer compared to the three dimensional environment *in vivo*. There is evidence to suggest that the vimentin filaments are laid along the existing cytokeratin filaments before completely replacing them (Pagan et al., 1996). The results also indicate that where hepatocytes clusters were present, the cells in the periphery changed morphology most drastically, had lamellar projections and showed highest levels of vimentin expression, while cells at the centre of clusters remained more epithelial like. This is in line with the evidence that the cells at the centre with the most cell-cell contacts, maintain their cytokeratins, compared to those at the periphery. Vimentin expression might also explain the increased cell motility seen in DS cultured, as vimentin is a more dynamic intermediate filament compared to the cytokeratins. It is yet uncertain, which factors in the KS medium enables the cells to override most of these effects in order to maintain a differentiated state and the expression of liver enriched transcription factors long-term. However, it was observed that cell death during the first 24 hours of cultivation is greater under serum-free KS conditions perhaps favouring the survival of epithelial clusters with greater cell-cell contacts as opposed to individual cells with no cell-cell contacts (which otherwise survive under serum-containing DS conditions). This can be seen in Fig. 3.12, where the nuclei appear more clustered in KS cultures. The reversibility of the de-differentiated phenomenon on swapping to KS medium from DS can also be

explained by such a selection of epithelial clusters against individual de-differentiated cells under KS conditions.

In addition to I/R stress, the absence of growth factors (including FGFs and EGF) that might otherwise maintain the hepatic phenotype, contribute to the further downregulation of hepatic gene expression. However, why a pancreatic gene network and not any other gene network is induced following the immediate departure from a hepatic state remains an enigma. Once scenario is that the de-differentiated cells recapitulate normal endodermal differentiation during embryonic development. As previously mentioned (in Chapter 1), during embryonic development the default pathway for the bipotential population of cells in the foregut endoderm is to attain a pancreatic fate (Deutsch et al., 2001). During *in vitro* culture however, FGF, BMP and Wnt signals that otherwise push the progenitor cells into a hepatic fate would not be present. Therefore in the absence of such signals, the cells, once de-differentiated, can follow their default differentiation pathway to become pancreatic. However, we have to bear in mind that neither are the signals essential for true pancreatic differentiation present in our undefined culture medium to the best of our knowledge. This is presumably why the transcriptional levels of the pancreatic genes in de-differentiated hepatocytes are low compared to any fully differentiated pancreatic cell-type. The other possibility is that pancreatic gene activation takes place as a consequence of chromatin remodelling during de-differentiation itself. Recent evidence suggests that in the liver, most pancreatic promoters are in a bivalent state of methylation with both activating and repressive histone modifications present concurrently (Ulupi Jhala, UCSD; unpublished). This would make the activation of such promoters in hepatocytes much easier compared to any other non-pancreatic cell-type. If this is true for the de-differentiated hepatocytes, activation of pancreatic genes might simply be a consequential outcome of the chromatin remodelling during de-differentiation and pancreatic promoters preferentially becoming more accessible to the transcriptional machinery. In a comparable situation, it has been shown that treatment of bone marrow cells with

trichostatin A, which opens chromatin by inhibition of histone deacetylases, can provoke some pancreatic gene expression (Tayaramma et al., 2006).

In addition to the activation of pancreatic genes, a number of genes that define pancreatic and liver progenitors are also expressed during de-differentiation. Therefore we cannot entirely rule out the possibility of the attainment of pancreatic gene expression by a sequential procession down a differentiation hierarchy. That is, from an undifferentiated, to a progenitor and finally a differentiated state. This can be attributed to the absence of particular signals such as FGFs and BMPs, and potentially (though not definitely) the presence of other signals. However the exact lineage down which the cells expressing the pancreatic genes are derived from de-differentiated hepatocytes, cannot be known for certain, unless immunodetectable levels of the progenitor-specific and pancreas-specific proteins are obtained. This would also enable us to elucidate the true heterogeneity of the de-differentiated and 're-differentiating' population and discern which markers are co-expressed at different stages of differentiation.

Finally, with a view to possible future uses of de-differentiated hepatocytes to produce beta-like cells for transplantation, it is important to establish whether the same effects occur in human cells. The results show that the phenomenon of de-differentiation can be confirmed for human hepatocytes. The only elementary difference was that an overt cytoskeletal rearrangement and morphological change was not seen as for the rodent hepatocytes. One possibility is that human vimentin protein is able to maintain the integrity of the cytoplasm and cytoskeletal interactions at a different level compared to that of rodents. Such differences are commonplace between human and rodent cells in primary culture. For example, the reverse is true for beta cells in culture whereby cultured human beta cells undergo an EMT while those from mice do not (Billestrup and Otonkoski, 2008). Despite the conservation of morphology, pancreatic genes were still being induced. However, the reprogramming from hepatic to pancreatic is far from complete. Moreover, unfavourable expression of pancreatic exocrine genes were also seen at low levels. Thus, the next steps involved enhancing the expression of the already induced pancreatic genes, particularly in favour of a beta cell phenotype.

Chapter FOUR

Transcription Factors & the Enhancement of the Pancreatic Phenotype

BACKGROUND

An intricate, pervasive, and combinatorial interplay of several sequence-specific DNA binding factors or transcription factors ensure that the right genes are expressed in the right cells, at the right time during embryonic development. The robust spatio-temporal patterns of gene expression generated by multiple transcription factor-DNA interactions, ultimately orchestrate cellular differentiation and tissue organisation essential for organogenesis (Chen and Rajewsky, 2007). The pancreas like the liver and the intestine, is derived from the definitive endoderm (Slack, 1995). Firstly the expression of the master regulator of pancreatic development, Pdx1, marks a precursor cell population for pancreatic development within the foregut endoderm. Then a hierarchical cascade of pancreatic developmental transcription factor expression, in the pancreatic progenitor cells, gives rise to the endocrine and exocrine cell populations and the distinct cell types within each of these populations (Jensen, 2004, Wilson et al., 2003). For example, transcription factors such as Neurogenin3 and NeuroD are essential for early endocrine lineage determination (Chakrabarti and Mirmira, 2003, Jensen, 2004, Watada, 2004). Furthermore, sequential expression of Nkx2.2, Pax4, Nkx6.1, Pdx1, Pax6 and MafA determine insulin producing beta cell fate, while others such as Brn4, Arx1, Nkx6.2 and MafB promote the glucagon producing alpha cell fate (Chakrabarti and Mirmira, 2003, Jensen, 2004, Samson and Chan, 2006).

Understanding the transcription factor hierarchy involved in pancreatic development and maturation of beta cells has proved useful in reprogramming other cell types such as hepatocytes into beta cells for diabetes therapy. In theory, this can be achieved by the over-expression of pancreatic transcription factors in hepatocytes. However, the genetic transformation of hepatocytes both *in vivo* and *in vitro* has been rather challenging. In recent years, recombinant adenoviruses have become an attractive gene delivery vehicle for primary cells such as hepatocytes (Fig. 4.1). Adenoviruses can enter cells, facilitated by the coxsakievirus B and adenovirus receptor (CAR) that normally functions as a cell-cell adhesion

molecule on the basolateral surface of epithelial cells (Honda et al., 2000, Howitt et al., 2003). The ability to grow recombinant viruses to high titres, the high capacity for transgene insertion, the non-integrating nature of the transgene to the host genome, and the efficient transduction of quiescent and dividing cells make adenoviruses a popular gene delivery vehicle for molecular reprogramming purposes (McConnell and Imperiale, 2004).

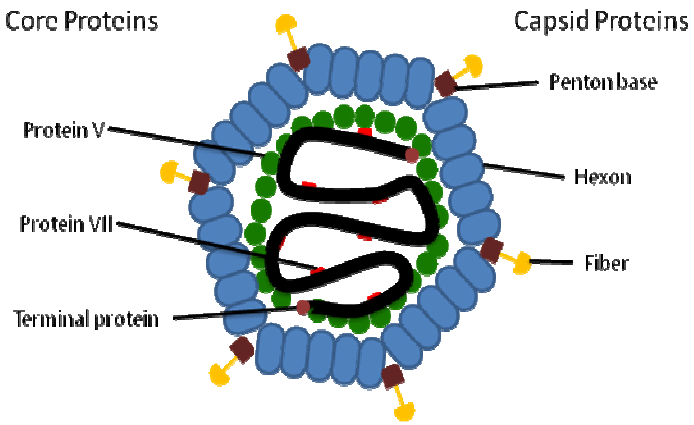


Figure 4.1. A schematic illustration of the structure of an adenoviral particle (adapted from Shenk, 1996).

Previously, several different groups have independently transduced hepatocytes both *in vivo* and *in vitro* using adenoviral vectors encoding pancreatic transcription factors such as Pdx1 (eg:- Ber et al., 2003, Sapir et al., 2005), Neurogenin3, NeuroD (Kaneto et al., 2005b), and MafA (Kaneto et al., 2005a), achieving varying degrees of insulin expression and diabetes rescue. Using *in vitro* adenovirus-mediated genetic manipulation of cultured cells and their subsequent transplantation minimises the adaptive immune responses and systemic toxicities that are otherwise associated with a systemic *in vivo* administration of adenoviruses (reviewed in Liu and Muruve, 2003). However an ideal combination of transcription factors for efficient and reproducible hepatic to beta cell conversion, thus far has not been elucidated. Furthermore, much doubt still remains regarding the feasibility of adenoviral infections in hepatocytes, the stability of the newly acquired phenotype following transcription factor over-expression, as well as the importance of the individual pancreatic transcription factors themselves. This chapter will attempt to answer such questions using the cultured rat hepatocyte model.

4.1 Optimisation of adenoviral infections.

Freshly isolated hepatocytes are readily infected with adenoviruses. However cell mortality due to the cytopathic effect of the adenovirus was high during the early stages of culture. Therefore to avoid this, the primary hepatocytes were allowed to recover in culture for 3 days after isolation prior to adenoviral infection. Since the ultimate aim was to transduce hepatocytes with multiple pancreatic transcription factors, the probability of infection with individual adenoviruses during a multiple infection was determined. In order to do this, three viruses, each with a different reporter, were used. They were: *Ad-CMV-GFP* coding for the green fluorescent protein (GFP) which can be detected by fluorescence, *Ad-CMV-LacZ* with the *E. coli β-galactosidase* gene detectable by X-gal staining and *Ad-CMV-HA-Ngn3* with the haemagglutinin tag (-HA-) that can be detected by immunocytochemistry using an anti-HA antibody. When visualised 5 days after a multiple infection, more than 90% of cells were infected with one, two or all three viruses and it was easy to detect those cells infected with more than one viral type (Fig. 4.2A-B).

The multiplicity of infection (M.O.I.) is the theoretical number of virus particles per cell; making the two assumptions that all the cells have an equal probability of being infected and that all the virus particles based on the measured titre are infectious. When the cells were infected with a single adenovirus (*Ad-CMV-GFP*) at an M.O.I. of 4 (i.e. a mean of 4 adenoviral particles per cell), 86% of the cells were infected (Fig. 4.2C). The observed ratio of infection by single and multiple viruses, during double and triple infections is summarised in the form of Venn diagrams in Fig. 4.2C. The infectibility of the three viruses were slightly different from the expected values (i.e. an equal proportion of the total infected cells to be infected by each virus). Such differences in infectibility can be accounted for by inaccuracies in determining the viral titres as well as the reduction in viral titres since titration and during long-term storage. Although the infectibility of *Ad-CMV-HA-Ngn3* was considerably lower compared to the other two viruses used at the same M.O.I., a total of 7% of cells were infected with all the three viruses during a multiple infection. This was a significant proportion

of the total cells and the probability can be further improved by increasing the M.O.I.

In order to find out the probability of infection by each virus during sequential infections, once again three different viruses were used. The hepatocytes were first infected with *Ad-CMV-GFP* on day 3 after isolation, followed by *Ad-CMV-LacZ* and *Ad-CMV-HA-Ngn3* on days 4 and 5 respectively (Fig. 4.3A). The cells were maintained for 3 more days before analysis. The number of cells infected with the first virus outnumbered those infected with the other two (74% compared with 47% and 21%). Similarly, cells co-infected with the first two viruses were more than those infected with any other combination. The percentage of cells infected with all three viruses after a sequential infection was also 2% less than with a simultaneous infection (Fig 4.3B). The differential infectibility of the adenoviruses mentioned above can partly account for these differences. However it is also possible that the hepatocytes develop some form of resistance to adenoviral infection after each subsequent infection, by the internalisation of the tight-junction coxsackievirus-adenoviral receptors (CAR) for instance.

As outlined in chapter 3, the isolated hepatocyte population consists of hepatocytes from both the periportal and perivenous zones of the liver. Periportal and perivenous hepatocytes express the enzymes CPS and GS respectively. Enriched populations of periportal and perivenous hepatocytes can be isolated by digitonin-mediated selective destruction of the liver prior to collagenase perfusion (see chapter 2 2B.1.1 and Fig. 2.1). Following the destruction of the perivenous cells with digitonin, a relatively pure population of CPS expressing hepatocytes can be obtained with no perivenous hepatocytes present (Fig. 4.4C-D). In the contrary, perivenous isolations have some periportal contamination (Fig. 4.4E-F), but the number of GS expressing cells are significantly increased compared to a normal hepatocytes isolation (Fig. 4.4A-B). To test if both periportal and perivenous hepatocytes can be infected with adenoviruses, cells from an enriched perivenous isolation was used as it contains comparable numbers of both CPS and GS expressing cells. The cells were infected with *Ad-CMV-LacZ* on the third day after isolation at an M.O.I. of 4 and then the cells were fixed and

stained with X-gal as well as antibodies for CPS and GS (Fig. 4.5). The results indicate that both the CPS and GS expressing populations are infectible with adenoviruses with equal efficiency.

As a final step in the optimisation of adenoviral infections, the adenoviruses to be used were tested to see if cells transduced with the viruses could produce the protein encoded for by the virus. It is sometimes possible that during serial viral amplification steps, mutations and di-cistronic rearrangement can occur in the viral construct, making the gene of interest non-functional while still keeping the reporter intact. Thus the reporter is a good indicator of infectibility but not necessarily of an effective virus. Using immuocytochemistry, Pdx1, Neurogenin3, NeuroD, Sox9 and MafA coding viruses (those viruses coding for proteins with antibodies available against them) were confirmed as being able to produce immunodetectable levels of these transcription factors in hepatocytes infected with them (Fig 4.6).

4.2 Enhancement of the pancreatic phenotype by Pdx1.

Previous work has suggested that the transduction of the master switch gene for pancreas development, Pdx1, can induce the expression of pancreatic genes such as *insulin* in cultured hepatocytes. Since *insulin* can be induced by de-differentiation alone, it was important to know how much of an effect Pdx1 transduction itself had on hepato-pancreatic reprogramming. Hepatocytes were cultured under DS conditions that trigger de-differentiation and KS conditions that maintain the hepatic phenotype. The hepatocytes were infected on day 3 with *Ad-CMV-Pdx1-eGFP* at a total M.O.I. of 20. RNA was isolated from the cells 5 days after and the levels of *insulin 1* were compared to those in non-infected hepatocytes by real time RT-PCR (Fig. 4.7). Freshly isolated hepatocytes and hepatocytes cultured in the KS medium up to 8 days do not express any *insulin.* In the contrary, those cultured in the DS medium show induction of *insulin* as a result of de-differentiation alone. However, the levels of the induced *insulin* expression were further enhanced by Pdx1 transduction by 9.8 fold in DS medium. Furthermore, insulin expression can also be induced by Pdx1 at low levels in

hepatocytes cultured in the KS medium. Therefore, though Pdx1 can induce pancreatic gene expression itself, de-differentiation synergises the effect of Pdx1. The levels in both KS and DS cells were nonetheless 10^6 fold lower than *insulin 1* levels in RIN cells (Fig. 4.7).

To further ascertain if this enhancement of pancreatic gene expression was due to Pdx1 and not an artefact of viral infection, exogenous protein expression or transcription factor over-expression, both KS and DS hepatocytes were infected with *Ad-CMV-null* (empty vector with no protein coding gene), *Ad-CMV-GFP* and *AD-CMV-p63* (a non-pancreatic transcription factor) respectively (Fig. 4.8). An induction of pancreatic gene expression (including that of *insulin 1, insulin 2, endogenous Pdx1, SUR1, Kir6.2, chromogranin A, IAPP, amylase* and *islet-specific glucokinase*) in KS hepatocytes was only seen with Pdx1 and not with any of the other viruses. Similarly, an enhancement of the already induced pancreatic gene expression in DS hepatocytes was seen only with Pdx1. It was also observed that Pdx1 transduction further facilitated the de-differentiation process in DS hepatocytes and induced de-differentiation in KS hepatocytes by the downregulation of hepatic gene expression (seen as reduced levels of *albumin* and liver-specific *glucokinase*). This downregulation of hepatic gene expression was not replicable with any of the other viruses. However, none of the viruses, including Pdx1, were capable of affecting the levels of *vimentin* expression.

Similar to rat hepatocytes, de-differentiated human hepatocytes can also be infected with the *Ad-CMV-Pdx1-eGFP* virus with good efficacy (Fig. 4.9A). Pdx1 transduction can induce pancreatic gene expression in human hepatocytes cultured under KS conditions and further enhance the induced pancreatic genes such as *insulin*, *Kir6.2* and *SUR1* in human hepatocytes cultured under DS conditions (Fig. 4.9B).

The *CMV* promoter is a strong promoter that can be expressed ubiquitously irrespective of cell type. As discussed in Chapter 3, the cells cultured under DS conditions comprise a mixed population with a majority of de-differentiated hepatocytes and also few cells derived from the initial vimentin expressing contaminants. To find out, if the effect of Pdx1 was specifically on

the hepatocytes and not on any of the non-hepatic contaminating cell types, the *Ad-TTR-Xlhbox8* virus was used, where the *Xenopus laevis* Pdx1 homolog, *Xlhbox8,* was driven off the hepatocyte specific *transthyretin* (*TTR*) promoter. With this virus a similar enhancement of *insulin 1* expression was seen as with the *Ad-CMV-Pdx1* virus (Fig. 4.10). However, the levels of enhancement was slightly reduced, possibly because the *TTR* promoter is weaker compared to the *CMV* promoter and furthermore the downregulation of hepatic genes (including *transthyretin*) would have already been initiated by day 3 which is when infections are carried out. Nonetheless the effects are enough to conclude that the observed effect of Pdx1 (and Xlhbox8) is on de-differentiated hepatocytes and not predominantly on any other cell type.

It has been suggested that the effect of a transduced gene can be enhanced by using a construct containing the *VP16* trans-activating domain from the *Herpes simplex* virus, downstream of the gene of interest. When de-differentiated hepatocytes were infected with *Ad-CMV-PdxVP16* and *Ad-TTR-Xlhbox8VP16*, the levels of *insulin 1* mRNA were increased compared to those infected with *Ad-CMV-Pdx1* and *Ad-TTR-Xlhbox8* respectively (Fig. 4.11). However, the increase was not statistically significant for Xlhbox8.

4.3 Determining an optimal transcription factor combination for hepato-pancreatic reprogramming.

During development, Pdx1 is the earliest transcription factor to mark the endodermal domain for pancreatic organogenesis. However, several other key regulatory transcription factors play vital roles in delineating the different pancreatic lineages including that for beta cells, alpha cells and exocrine cells. To find out which of these transcription factors are vital for the effective molecular reprogramming of de-differentiated hepatocytes into pancreatic beta-like cells, an extensive screen of different pancreatic transcription factors with pro-endocrine expression profiles was carried out. The screening strategy involved, transducing de-differentiated hepatocytes with different combinations of candidate transcription factors on day 3 after isolation. The 10 transcription factors included in the screen were Pdx1, Neurogenin3,

NeuroD, Nkx2.2, Nkx6.1, Pax4, Pax6, IA1, Sox9 and Ptf1a (p48). All the viral infections used for the transcription factor screening were performed with an individual M.O.I. of 5 for each transcription factor with the total M.O.I. always moderated at 50 using the *Ad-CMV-null* virus. The effect of each combination was scored based on the relative amount of *insulin 1* mRNA, analysed by real time RT-PCR. When used individually, Pdx1, Neurogenin3 and NeuroD were the only transcription factors to enhance the amount of *insulin 1* expression by 9.8-, 7.8- and 7.3-fold respectively. All possible combinations of 1, 2, 7, 9 and 10 viruses were tested (a full list of data is included in Appendix C). The results showed that no single combination gave a considerably high level of *insulin 1* expression in de-differentiated hepatocytes compared to the others The mean level of *insulin 1* expression for all the combinations tested was 8.85×10^{-6} times the amount in the adult rat pancreas. However, a few combinations repeatedly scored much higher than the other combinations (i.e. more than the predetermined arbitrary threshold of 2×10^{-5} times the amount in the adult rat pancreas). In all the combinations that scored higher than this predetermined threshold, above the average, Pdx1 was present (Fig. 4.12). Apart from Pdx1, the transcription factors Neurogenin3, NeuroD and Pax4 frequently appeared in the high scoring combinations at frequencies greater than 77.8%. The combination of these four transcription factors (Pdx1, Neurogenin3, NeuroD and Pax4) were selected as the ideal combination, as infections with more than four viruses reduces the probability of being infected with all the viruses and also increases the competition by the transcription factors to occupy pancreatic promoters, making it less efficient.

De-differentiated hepatocytes transduced with the combination of Pdx1, Neurogenin3, NeuroD and Pax4 showed 2- and 1.3-fold increase in the amount of *insulin 1* and *insulin 2* respectively compared to de-differentiated hepatocytes transduced with Pdx1 alone (Fig. 4.13). The same trend was observed at the protein level for insulin, with the highest expression being in de-differentiated hepatocytes transduced with all the four transcription factors, compared to those infected with Pdx1 alone (Fig. 4.14).

4.4 The feasibility of sequential transcription factor transductions.

The sequential expression of pancreatic transcription factors during the development of the mouse embryo is well documented. However, the information regarding embryonic pancreatic development in the rat is more limited. Pancreata were dissected from rat embryos at stages 11.5 to 22.5 days post dp (dpc) and 1 day post partum (dpp). The levels of expression of the transcription factors *Isl1, NeuroD, Ngn3, Nkx2.2, Nkx6.1, Pax4, Pax6* and *Pdx1* were analysed by RT-PCR (Fig. 4.15). The expression pattern was similar to that established for mouse pancreatic development with *Isl1, Pdx1* and *Neurogenin3* expression occuring much earlier on compared to other transcription factors such as *Pax4* or *Nkx6.1*.

The sequence of transcription factor expression during a hepato-pancreatic conversion was examined after de-differentiated hepatocytes were transduced with Pdx1 (Fig. 4.16). The pattern was similar to that during normal pancreatic development, with *Isl1, Pdx1, Neurogenin3* and *NeuroD* again being expressed much earlier compared to *Pax4, Pax6, Nkx2.2* and *Nkx6.1*. However, the expression pattern did not exactly resemble that of normal pancreatic development. The endogenous *Pdx1, Neurogenin3* and *NeuroD* were seen to be expressed in a biphasic manner, being downregulated between day 7 and 10 before being upregulated again.

In an attempt to mimic the developmental expression pattern of Pdx1, Neurogenin3, NeuroD and Pax4, de-differentiated hepatocytes were transduced sequentially with Pdx1 on day 3 after isolation, Neurogenin3 on day 4, and NeuroD and Pax4 on day 5 (each at an M.O.I. of 10). However, sequential infections reduced the viability of cells and the number of transformed cells that survived up to day 8 (Fig. 4.17A). Furthermore, there was a 2.7-fold reduction in the levels of *insulin 1* (Fig. 4.17B). This data suggests that it is necessary to take measures to reduce the cytopathic effects of the adenovirus on hepatocytes, if sequential infections are to be carried out successfully

4.5 Facilitating de-differentiation and chromatin accessibility prior to adenoviral transduction.

While de-differentiation facilities the molecular reprogramming process, there are several other factors that antagonise the effects of Pdx1 and the molecular reprogramming from a hepatic to a pancreatic phenotype. Firstly, liver-enriched transcription factors play an important role in maintaining the hepatic phenotype by ensuring hepatic gene expression is sustained. Such liver-enriched transcription factors include C/EBPα and β, HNF1α and 4α, DBP and RXRα (Costa et al., 2003, Hayhurst et al., 2001). Although the expression of most of these transcription factors are downregulated during the course of de-differentiation, the process of de-differentiation and initiation of pancreatic gene expression can be further enhanced by minimising the effects of such liver-enriched transcription factors. Secondly, the identity of each cell-type is maintained by its unique gene expression profile. Epigenetic modifications ensure that genes that define the cell type (such as hepatic genes in hepatocytes) are expressed and remain active, while those non-characteristic of the cell type (such as pancreatic genes in hepatocytes) remain inactive. Such epigenetic changes include but are not restricted to DNA methylation and post-translational histone modifications, which can pack the DNA at the promoter regions of inactive genes so as to make them inaccessible to transcription factors. Therefore, cellular reprogramming requires the remodelling of these epigenetic marks. Recently there has been mounting interest in the use of DNA demethylating agents and histone deacetylase inhibitors to activate genes by mediating global epigenetic changes in chromatin structure.

With the aim of facilitating and speeding up the de-differentiation process prior to pancreatic transcription factor transduction, hepatocytes cultured in the DS medium were infected with the *Ad-CMV-LIP* virus 24 hours into culture. *LIP* encodes the liver inhibitory protein- a dominant-negative isoform produced by alternative splicing of C/*EBPβ* mRNA. Even at sub-stoichiometric proportions, LIP can heterodimerise with LAP (liver activation protein isoform of C/EBPβ) thereby negating the transactivation capabilities of LAP (Descombes and Schibler, 1991, Ossipow et al., 1993). Hepatocytes

over-expressing LIP showed more rapid changes in morphology compared to uninfected cells, becoming mesenchymal like within 2 days after infection (Fig. 4.18A-B). In an attempt to bring about chromatin remodelling and increase the accessibility of pancreatic promoters to pancreatic transcription factors, *Ad-CMV-LIP* infected, de-differentiated hepatocytes were treated with 100μM zebularine starting at 24 hours into culture. Zebularine is an effective inhibitor of DNA methylation. Zebularine attenuated the rapid morphology change brought about by LIP and allowed the infected hepatocytes to sustain an epithelial morphology for much longer (fig. 4.18C). However neither LIP transduction nor zebularine treatments were capable of enhancing the effect of Pdx1 on its own or in combination with Neurogenin3, NeuroD and Pax4, analysed in terms of *insulin 1* expression (Fig. 4.18D). In fact, zebularine treatments notably reduced the amount of *insulin 1* expression.

4.6 Using MafA to further promote the beta cell phenotype.

One of the pancreatic transcription factors that was not included in the initial screen (see 4.3) was the basic-leucine zipper transcription factors MafA. It is expressed later on during pancreatic development around E13.5, only in those insulin positive cells that go onto mature into islet beta cells (Matsuoka et al., 2004). Therefore MafA is thought to be essential for the maturation of beta cells in the differentiation process. When MafA was included in the transcription factor combination along with Pdx1, Neurogenin3, NeuroD and Pax4, a 1.56 fold increase in *insulin 1* gene expression was seen compared to the combination that did not include MafA (Fig. 4.19). Insulin protein expression was also analysed by immunocytochemistry using a peroxidase conjugated secondary and DAB staining, as this protocol is known to be more sensitive than immunofluorescence. However, even with MafA, the levels of insulin protein expression were far too low to be immunocytochemically detectable (Fig. 4.20).

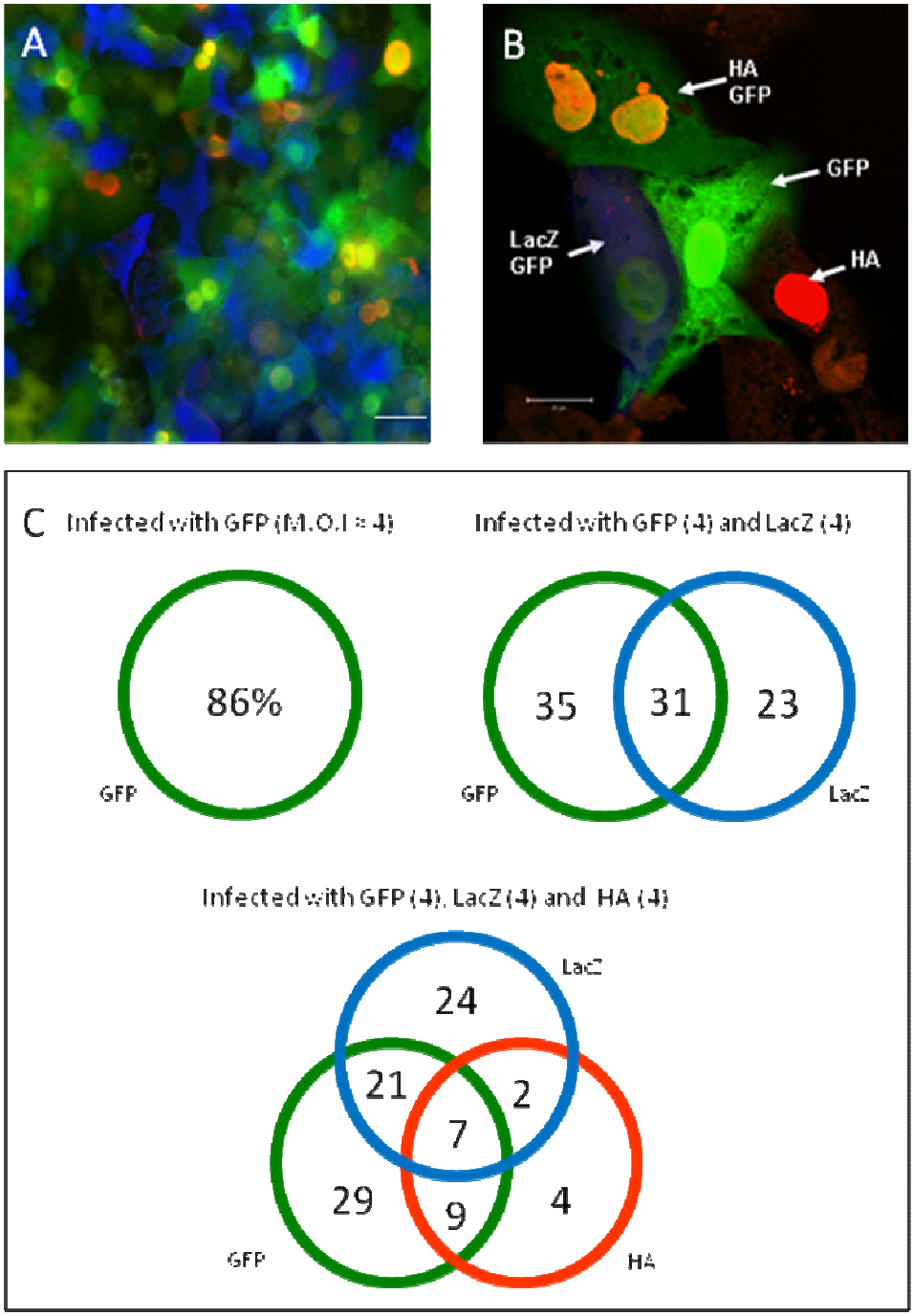


Figure 4.2. **A single hepatocyte can be transduced with multiple adenoviral constructs using a simultaneous multiple infection protocol.** A & B show low and high magnification images of cells infected with three different viruses with three different reporters: GFP (green), HA (red) and LacZ (blue by X-gal staining and under transmitted light; the colours have been inverted and false colour added by channel swapping in order to visualise blue with the fluorescent tags). The scale bar represents 20µm. C- the typical observed percentage of infection with 1, 2 and 3 viruses during single, double and triple infections determined by cell counting.

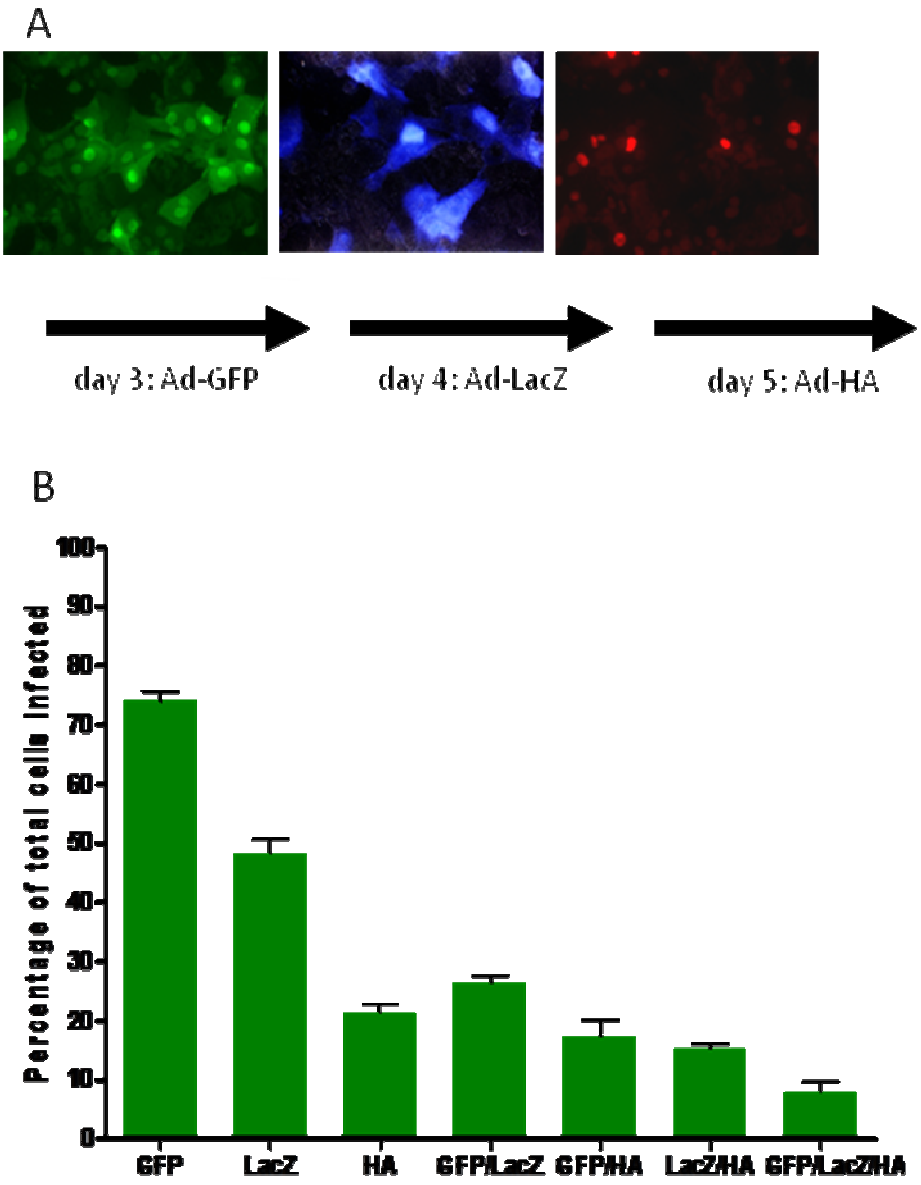


Figure 4.3. **The percentage of cells infected with multiple adenoviruses is reduced when using a sequential multiple infection protocol.** A shows the timeline for the sequential infection of hepatocytes by three different viruses. Infections were carried out with Ad-GFP (green) on day 3 after isolation, followed by Ad-LacZ (blue) and Ad-HA (red) on day 4 and 5 respectively.The scale bar represents 20µm. B represents the observed percentage of total cells infected with 1, 2 and 3 viruses after a series of sequential infection with three viruses each at an M.O.I. of 4.

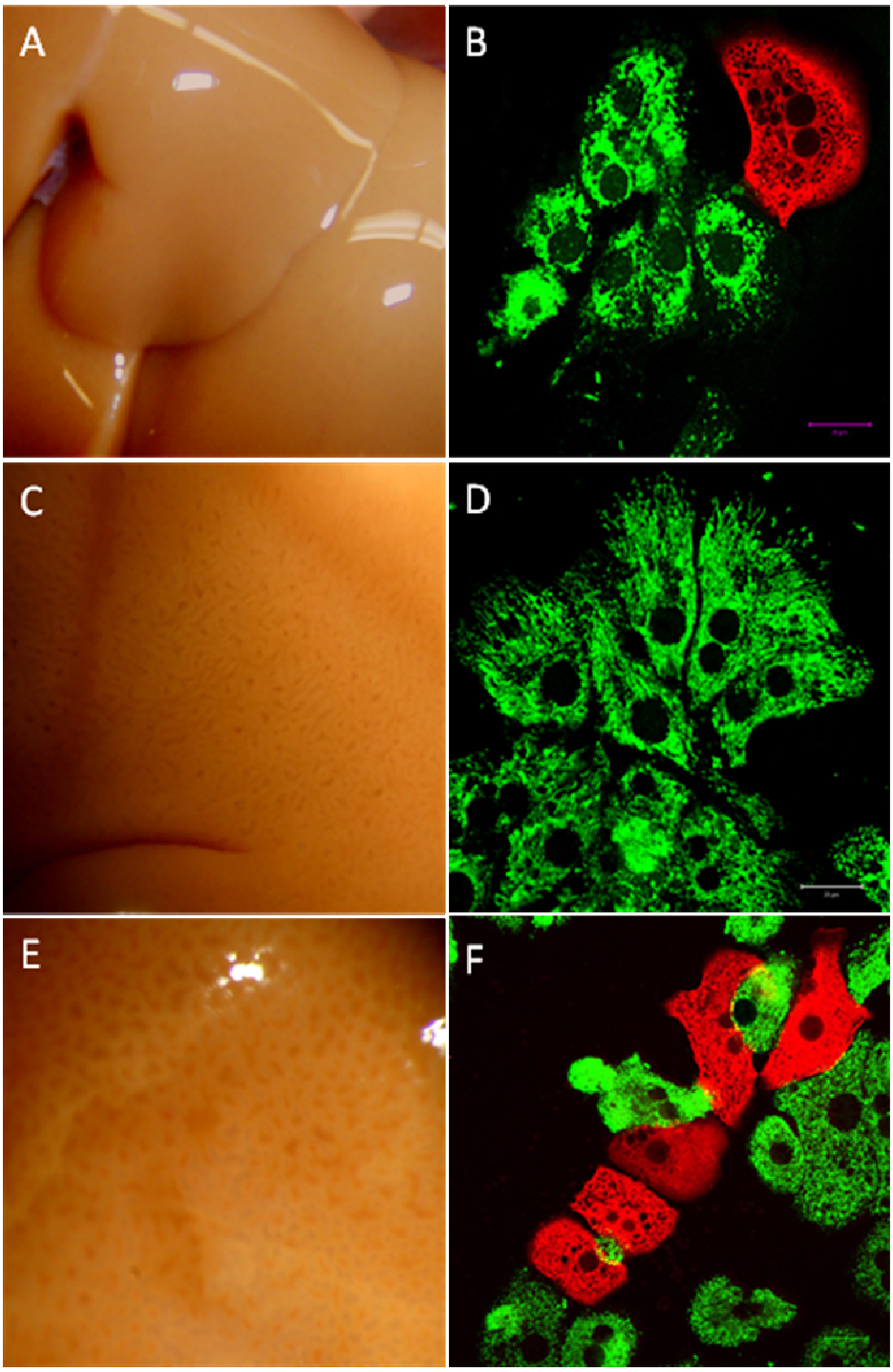


Figure 4.4. **Enriched populations of periportal and perivenous hepatocytes can be isolated using digitoin-mediated selective destruction prior to digestion.** Selective destruction patterns of perivenous (C) and periportal (E) hepatocytes can be clearly seen. CPS (green) and GS (red) are used as markers of periportal and perivenous hepatocytes respectively. No perivenous contamination was seen in periportal isolations (D), but periportal hepatocytes were seen in perivenous isolations (F). A and B show the digestion pattern of the liver and cells isolated by a normal hepatocyte isolation. The scale bar represents 10μm.

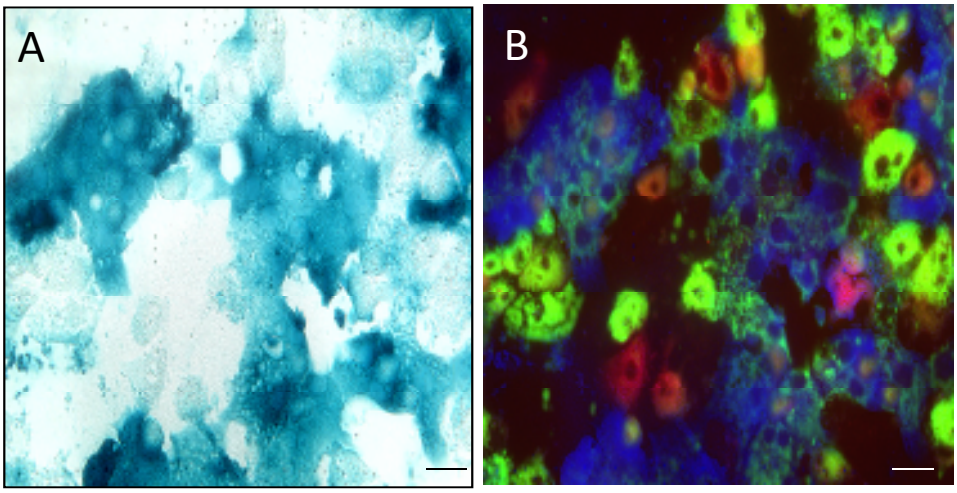


***Figure 4.5*. Both perivenous and periportal hepatocytes can be transduced with adenoviral constructs.** An enriched population of perivenous rat hepatocytes (with contaminating periportal hepatocytes) was isolated using digitonin-mediated selective destruction of the periportal zone. The cells were cultured in KS medium and then infected with the Ad-LacZ virus on day 3 after isolation (A). The infected cells are seen in blue by X-gal staining. The infected cells include both GS (red) positive perivenous hepatocytes and CPS (green) positive periportal hepatocytes (B) showing the infectibility of both populations. Note that LacZ appears blue by X-gal staining and under transmitted light (A); the colours have been inverted and false colour added by channel swapping in order to visualise blue with the fluorescent tags (B). The scale bar represents 20µm.

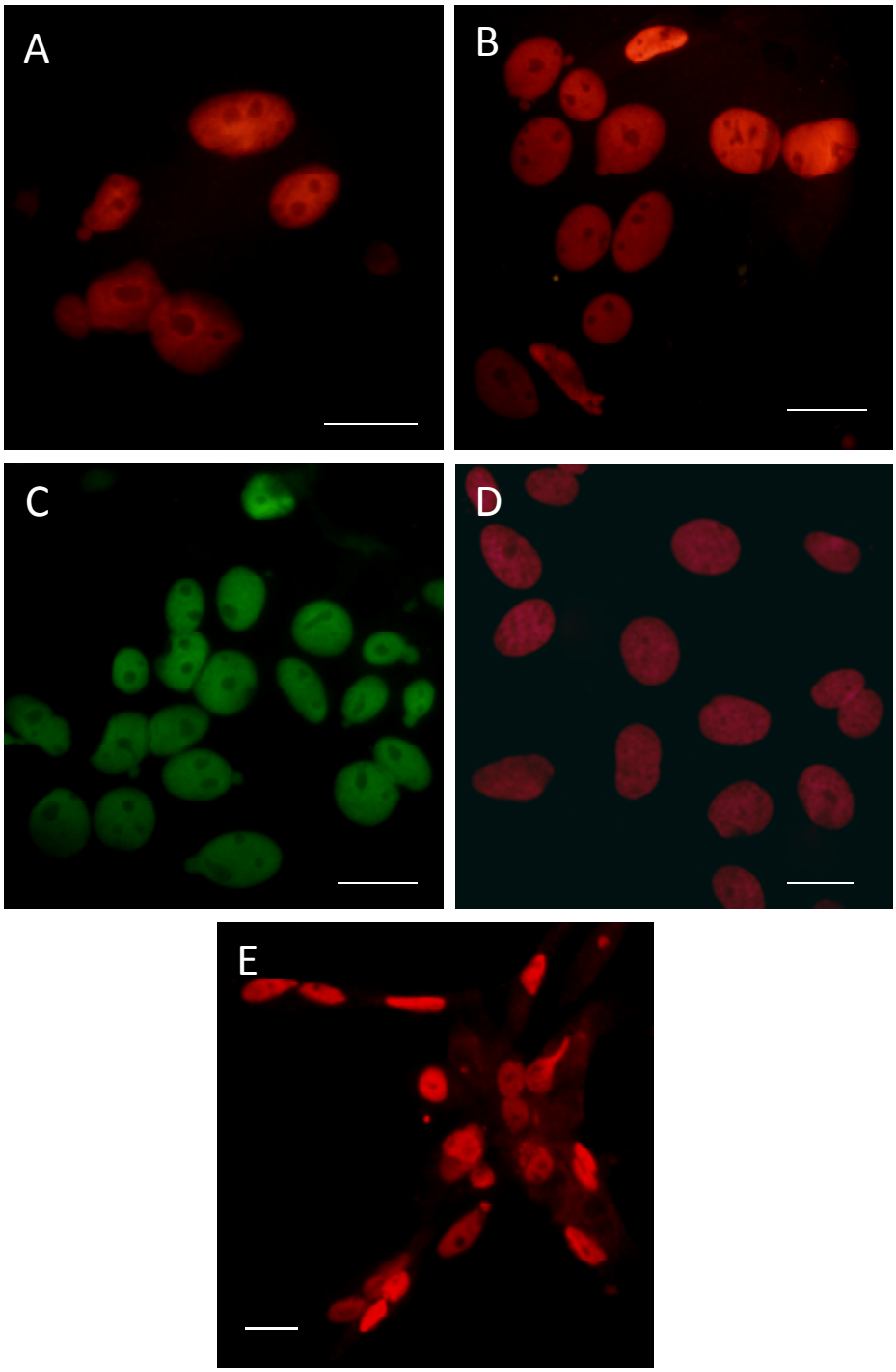


Figure 4.6. **Adenoviruses used express the proteins coded for by their constructs.** Testing the viruses by immunocytochemically analysing transcription factor expression in the nuclei of hepatocytes that have been infected with adenoviruses coding for Pdx1 (A, red), Neurogenin3 (B, red), NeuroD (C, green), Sox9 (D, red) and MafA (E, red). These were the only transcription factors against which working antibodies were available. The scale bar represents 10µm.

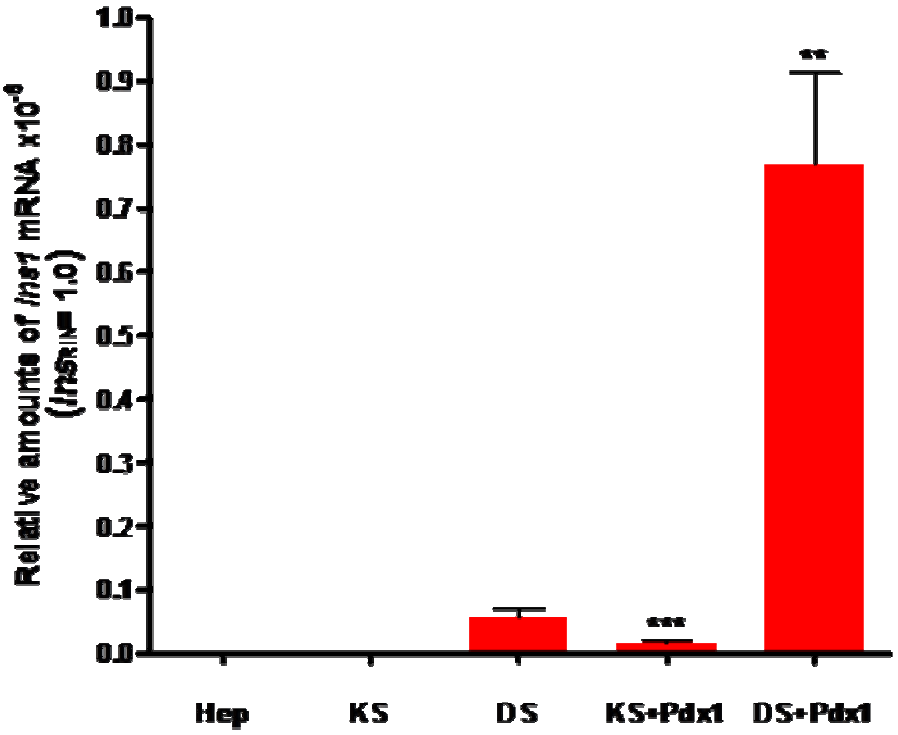


Figure 4.7. **Pdx1 enhances the levels of *insulin* expression in de-differentiated hepatocytes.** The relative amounts of *insulin 1* expression in hepatocytes cultured in the DS and KS medium with and without Pdx1 transduction was analysed using real time RT-PCR. Cells were cultured for a total of 8 days in either KS or DS medium, and the viral infections were carried out on day 3 after isolation. No *insulin 1* mRNA was detected in freshly isolated hepatocytes (Hep) and those cultured in the KS medium for 8 days. Viral infections were done at an M.O.I. of 10. $^{**}p=0.0079$, $^{***}p< 0.0001$, n=3.

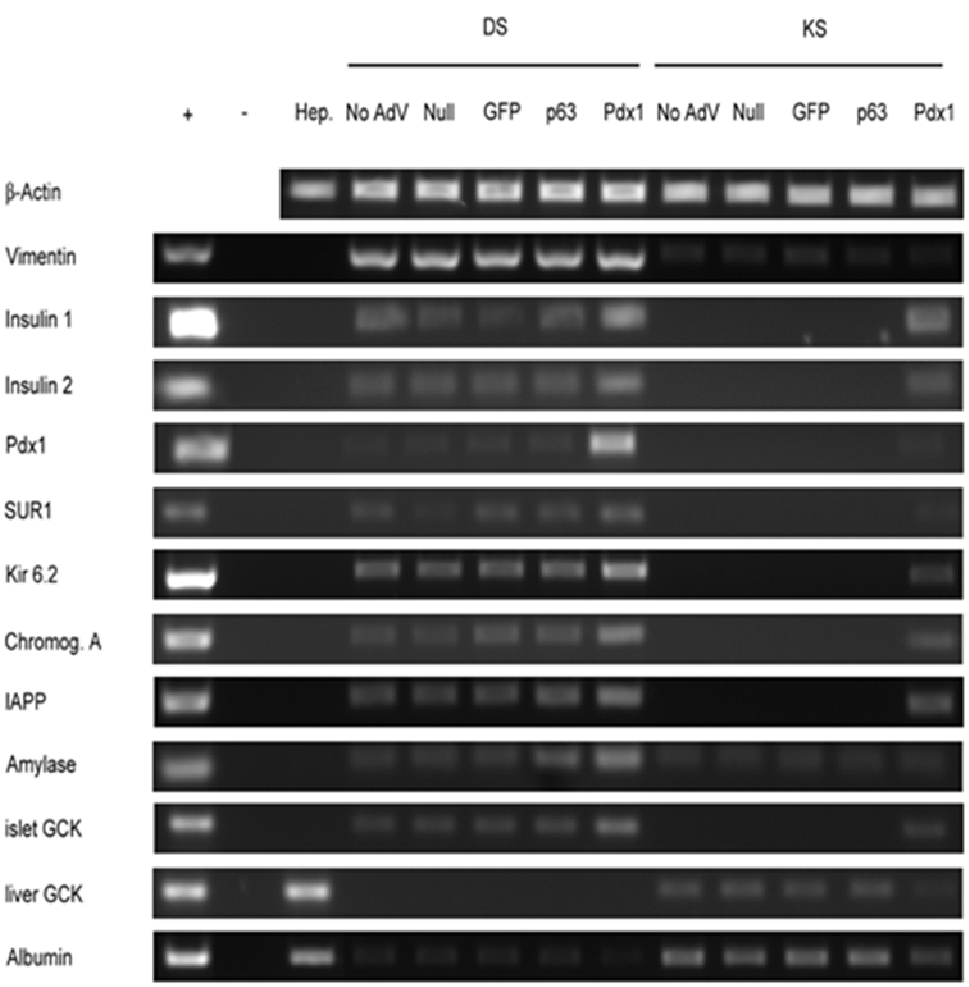


Figure 4.8. **The enhancement of pancreatic gene expression by Pdx1 is not an artefact of viral infection or exogenous protein/transcription factor expression.** Overexpression of *Pdx1* can induce pancreatic gene expression in hepatocytes, with a greater effect on de-differentiated hepatocytes (DS). This was not brought about by *Ad-Null*, *Ad-GFP* and *Ad-p63* infections. The cells were cultured for 2 days in their respective medium, infected with different adenoviruses for 1 hour on the third day and then maintained for 5 more days before RNA isolation and gene expression analysis by RT-PCR. cDNA from RIN cells, rat skin, rat liver and rat exocrine AR42J-B13 cells were used as positive control for endocrine genes, *vimentin*, hepatic genes and *amylase* respectively.

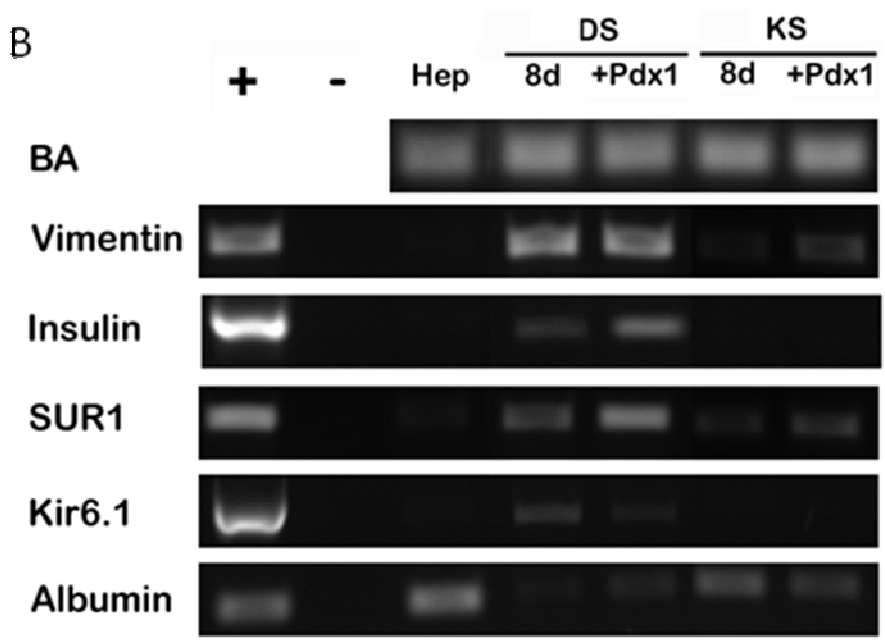


Figure 4.9. **De-differentiated human hepatocytes show enhanced pancreatic gene expression following Pdx1 transduction.** A- Primary human hepatocytes in culture can be infected with the Ad-CMV-Pdx1-eGFP virus with good efficiency at an M.O.I. of 10. The scale bar represents 10μm. B- Similar to rat hepatocytes, the pancreatic gene expression seen in the DS medium was enhanced by Pdx1 transduction. In the KS medium low levels of pancreatic gene expression was induced following Pdx1 transduction with a concurrent down-regulation of *albumin* expression. cDNA from de-differentiated hepatocytes, human pancreatic endocrine tissue and freshly isolated human hepatocytes were used as positive controls for *vimentin*, pancreatic genes and *albumin* respectively.

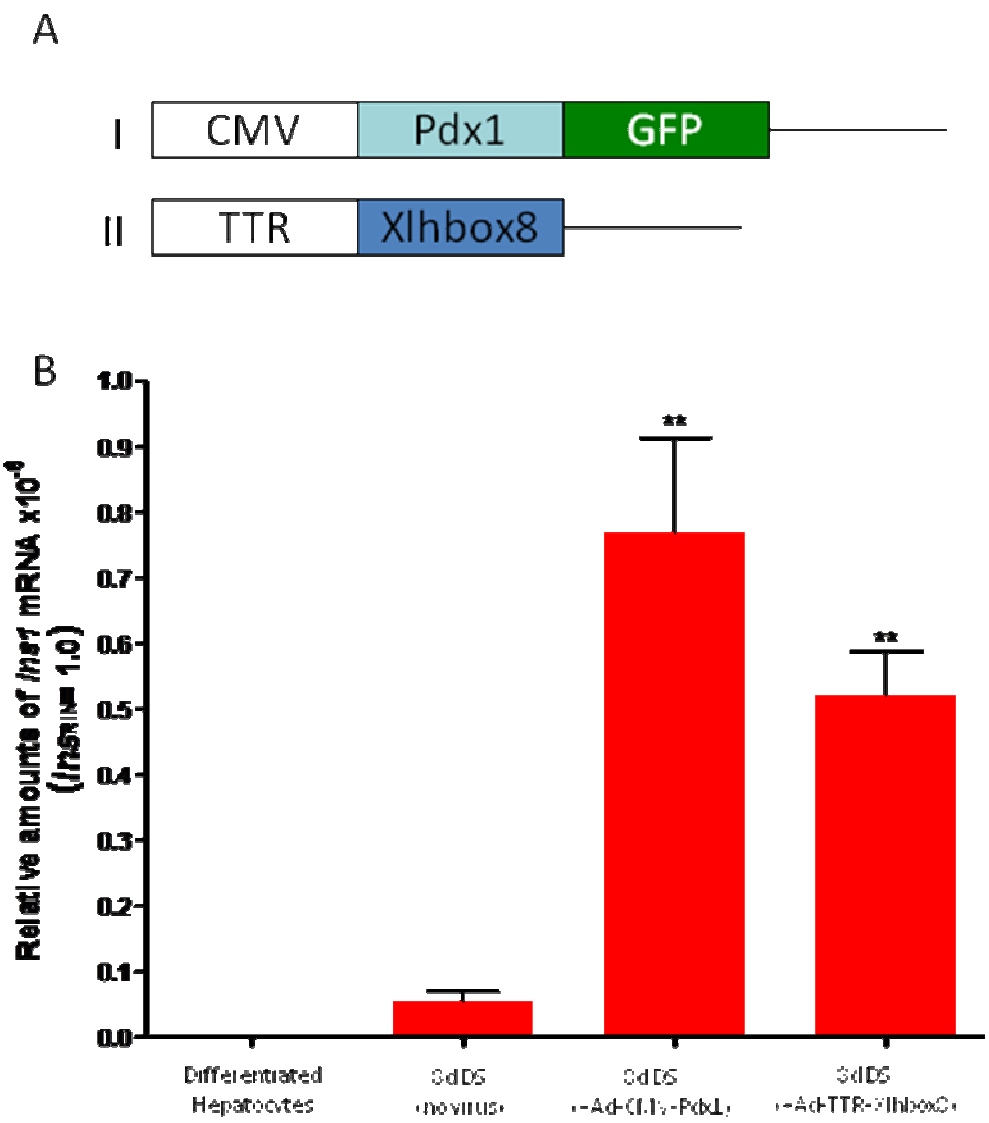


Figure 4.10. **Pdx1 induces *insulin* gene expression specifically in hepatocytes.** A shows diagrammatic representations of the Ad-Pdx1 (I) and Ad-Xlhbox8 (II) viral constructs used for hepatocytes transduction. B represents the relative amounts of *insulin* 1 mRNA (by real time RT-PCR) at 5 days after transduction with adenoviruses with Pdx1 driven off the ubiquitous CMV promoter and the Pdx1 homolog Xlhbox8 driven off the liver-specific TTR promoter. Since a comparative enhancement of *ins1* expression is seen with the TTR-Xlhbox8 construct, it indicates that the effect of Pdx1 (and its homolog) is on hepatocytes and not any other cell type. The viral infections were done at an M.O.I. of 10. * *p< 0.007, n=3.

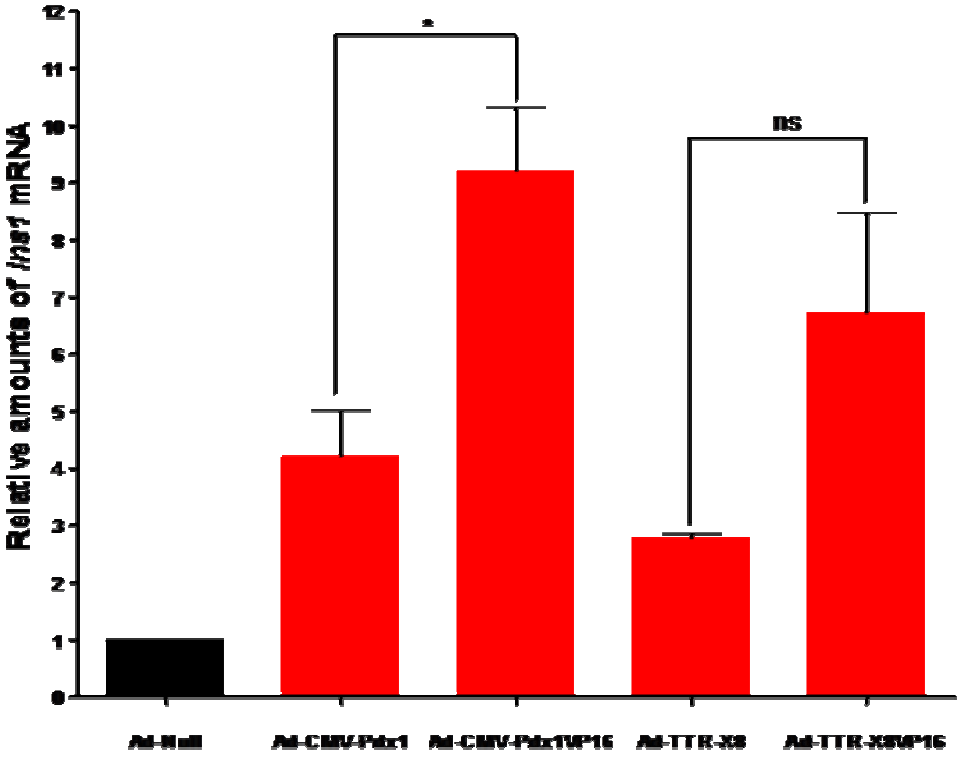


Figure 4.11. **The effect of the VP16-transactivating domain on the enhancement of pancreatic gene expression is of low or no significance.** The effects of the VP16 transactivation domain on the activity of Pdx1 and Xlhbox8 (X8) represented as the relative amount of *insulin 1* mRNA (by real time RT-PCR) of hepatocytes cultured under DS conditions, detected 5 days after infection with each of the viruses. The level of insulin in de-differentiated cells infected with the Ad-Null virus was normalised to 1. The viral infections were done at an M.O.I. of 10. *p=0.025, nsp=0.0875, n=3.

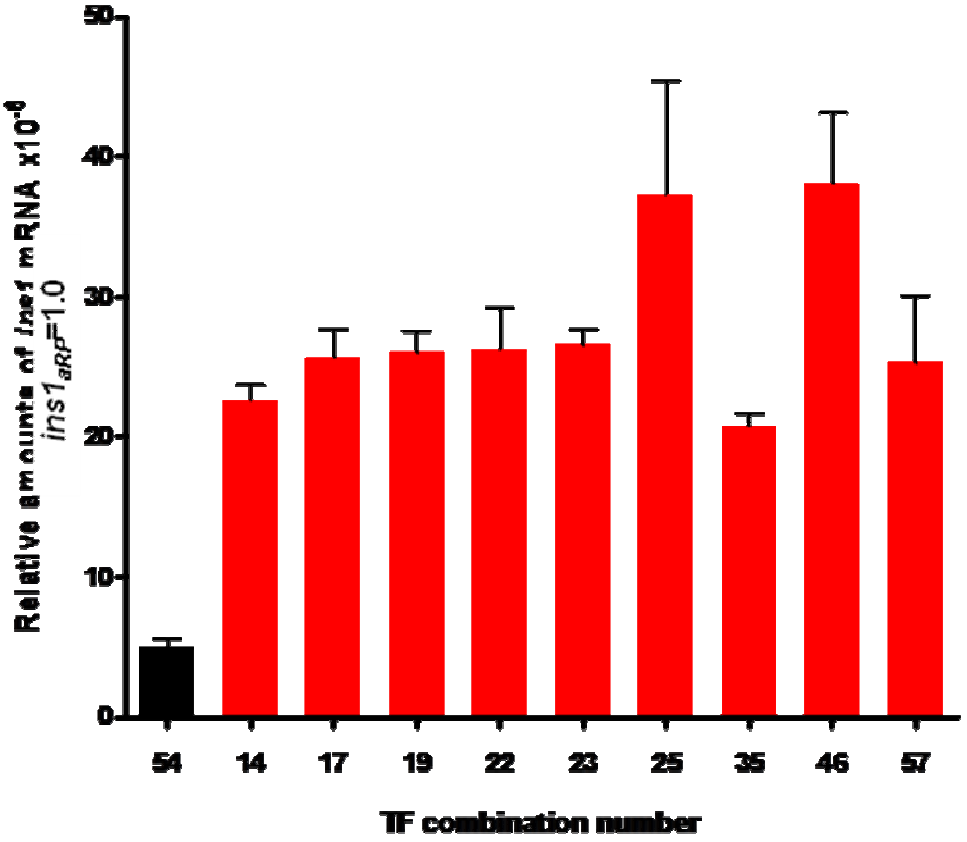


Figure 4.12. **Working out an optimal viral combination for hepato-pancreatic conversion.** Isolated rat hepatocytes were cultured in DS medium and on day 3, infected with different combinations of adenoviruses coding for key-regulatory pancreatic transcription factors. On day 8, total RNA was extracted from the cells and the relative amount of *insulin 1* was analysed by real time RT-PCR. The relative amount of *insulin 1* in hepatocytes transduced with the 9 high-scoring combinations of transcription factors compared with a low-scoring combination (54) is shown. The combinations were:-

54- Pdx1,Ngn3,Nkx6.1,Pax4,IA1,Sox9,p48
14- Pdx1,Ngn3,NeuroD,Nkx2.2,Pax4,IA1,Sox9
17- Pdx1,Ngn3,NeuroD,Nkx2.2,Pax6,IA1,Sox9
19- Pdx1,Ngn3,NeuroD,Nkx2.2,Pax6,Sox9,p48
22- Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,Sox9
23- Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,p48
25- Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,IA1,p48
35- Pdx1,Ngn3,NeuroD,Pax4,IA1,Sox9,p48
46- Pdx1,Ngn3,Nkx2.2,Pax4,Pax6,IA1,Sox9
57- Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1

The viruses were used at an individual M.O.I. of 5 with the total M.O.I. maintained at 50 using the Ad-Null virus. The results were normalised to the amount of *insulin 1* in total RNA extracted from the adult rat pancreas ($ins1_{pa}$) taken as 1. The difference in levels between the individual high-scoring combinations are not significant. n=3.

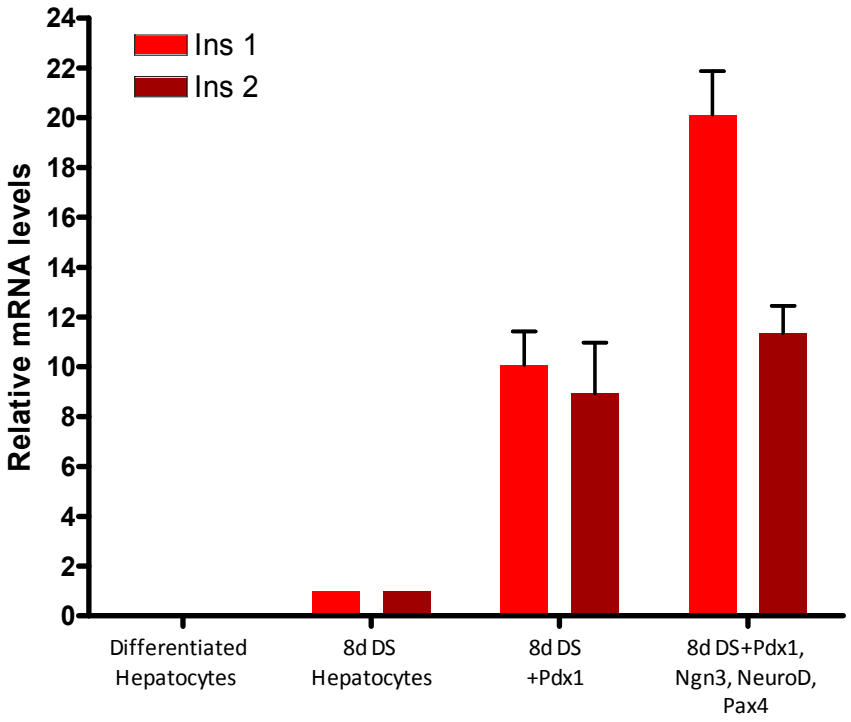


***Figure 4.13*. A combination of Pdx1, Neurogenin3, NeuroD and Pax4 enhances the levels of *insulin* expression in de-differentiated hepatocytes.** Rat hepatocytes were isolated and cultured in the DS medium, infected with the adenoviruses on day three and maintained for five more days. The relative amount of *insulin 1* and *insulin 2* in de-differentiated hepatocytes transduced with the combination of four transcription factors (Pdx1, Ngn3, NeuroD and Pax4; each with individual M.O.I. of 10) compared to those which were uninfected (normalised to 1) or infected with Pdx1 alone. n=3.

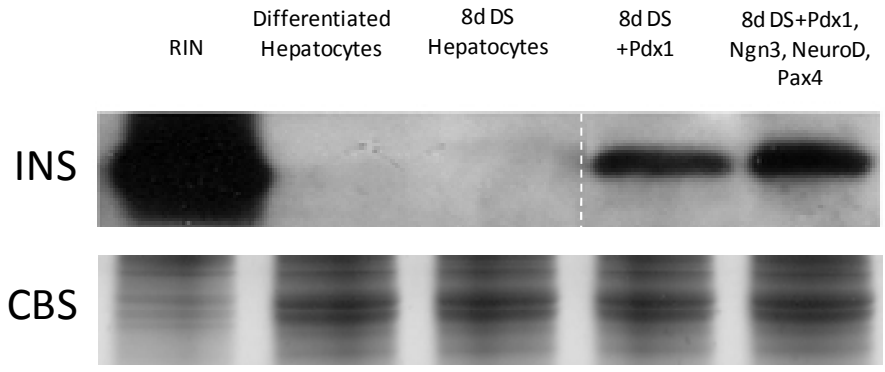


***Figure 4.14*. De-differentiated hepatocytes transduced with pancreatic transcription factors synthesise insulin protein.** Analysis for the levels of insulin protein (INS) in de-differentiated hepatocytes was done by Western blot analysis. The levels of insulin in de-differentiated hepatocytes are too low to be detected by Western blot analysis. However after Pdx1 infection and infection with the four viruses, the levels significantly increase. Protein from RIN cells were used as a positive control. A Coomassie Blue Stain (CBS) of total protein is shown to indicate equal loading.

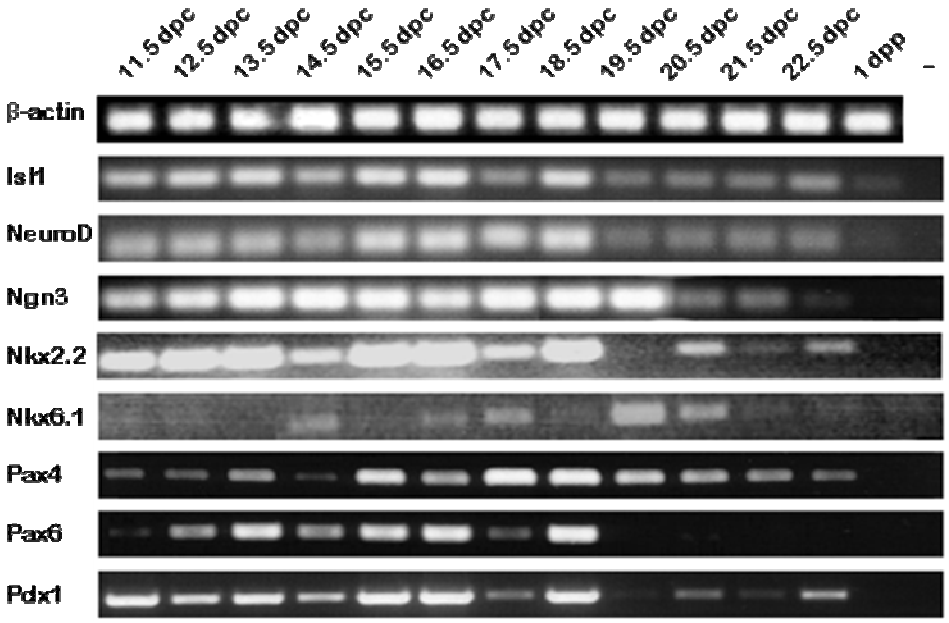


Figure 4.15. **The transcription factor expression profile during rat pancreatic organogenesis is similar to that during mouse development.** Analysis of the expression of pancreatic transcription factors during rat embryonic development was done by RT-PCR. Pancreata were isolated from rat embryos at different stages of development from 11.5 days post-coitus (dpc) to 1 days post partum (dpp). No independent positive controls were used as one or more of the embryonic developmental stages serves as a positive control for each gene.

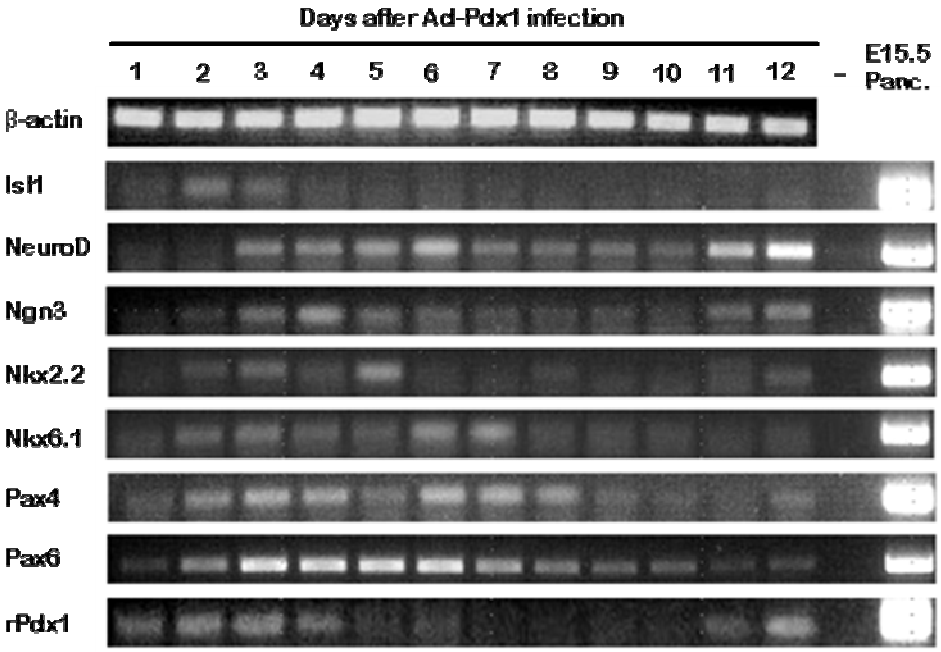


Figure 4.16. **Pancreatic transcription factors can be induced at low levels in de-differentiated hepatocytes transduced with Pdx1.** The expression of pancreatic transcription factors in de-differentiated hepatocytes over a course of 12 days after Pdx1 transduction was analysed by RT-PCR. The cells were cultured in DS medium throughout the experimental period and Pdx1 infections were carried out on day three after isolation. cDNA from stage E15.5 embryonic rat pancreas was used as the positive control.

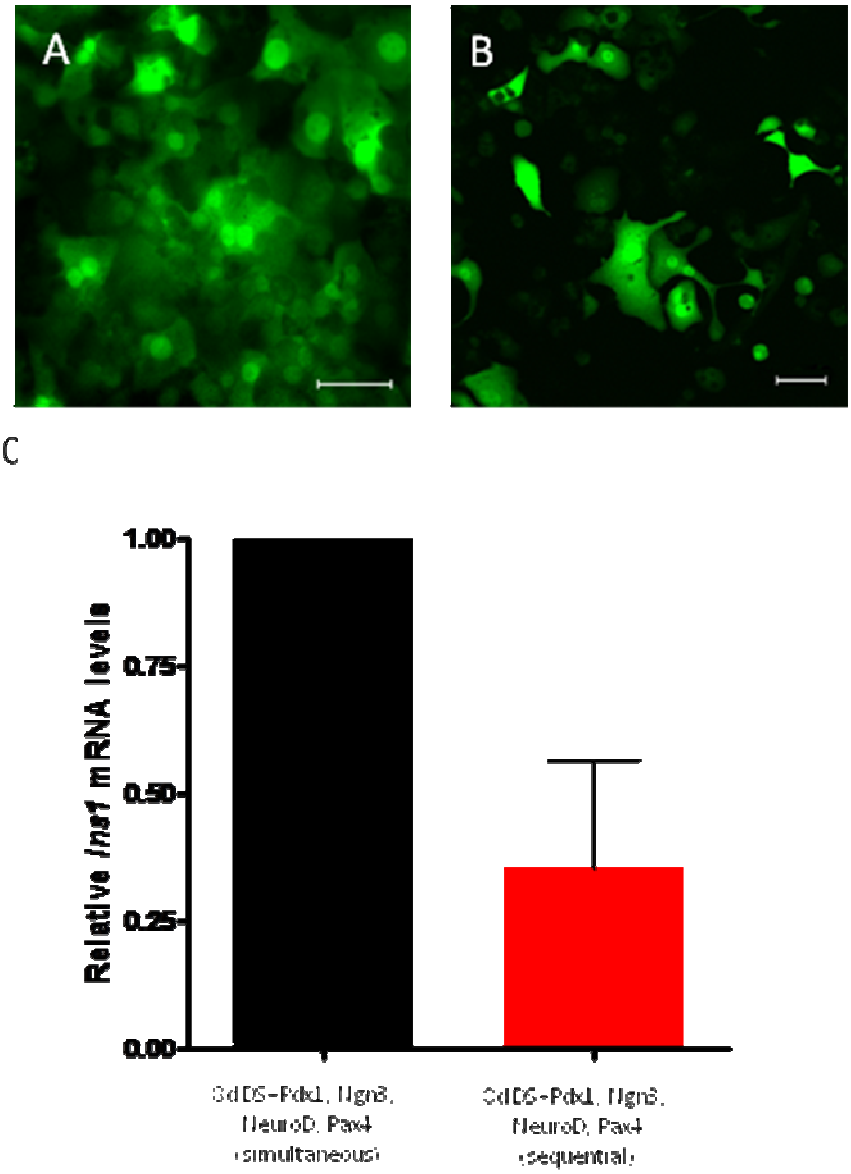


Figure 4.17. **Sequential viral infections result in high cell mortality and reduced levels of *insulin*.** De-differentiated hepatocytes that have been infected with the four viruses simultaneously (A) and sequentially (B) were compared. The number of GFP positive cells and the overall viability was much reduced after sequential infection. C- The reduction in the amount of *insulin 1* expression (seen by real time RT-PCR) in de-differentiated hepatocytes sequentially infected with four transcription factor coding viruses, compared to those infected simultaneous with the same combination could be due to high cell mortality or cells developing resistance to subsequent viral infections. The level of *insulin 1* in de-differentiated hepatocytes infected with the four adenoviruses was normalised to 1. n=3.

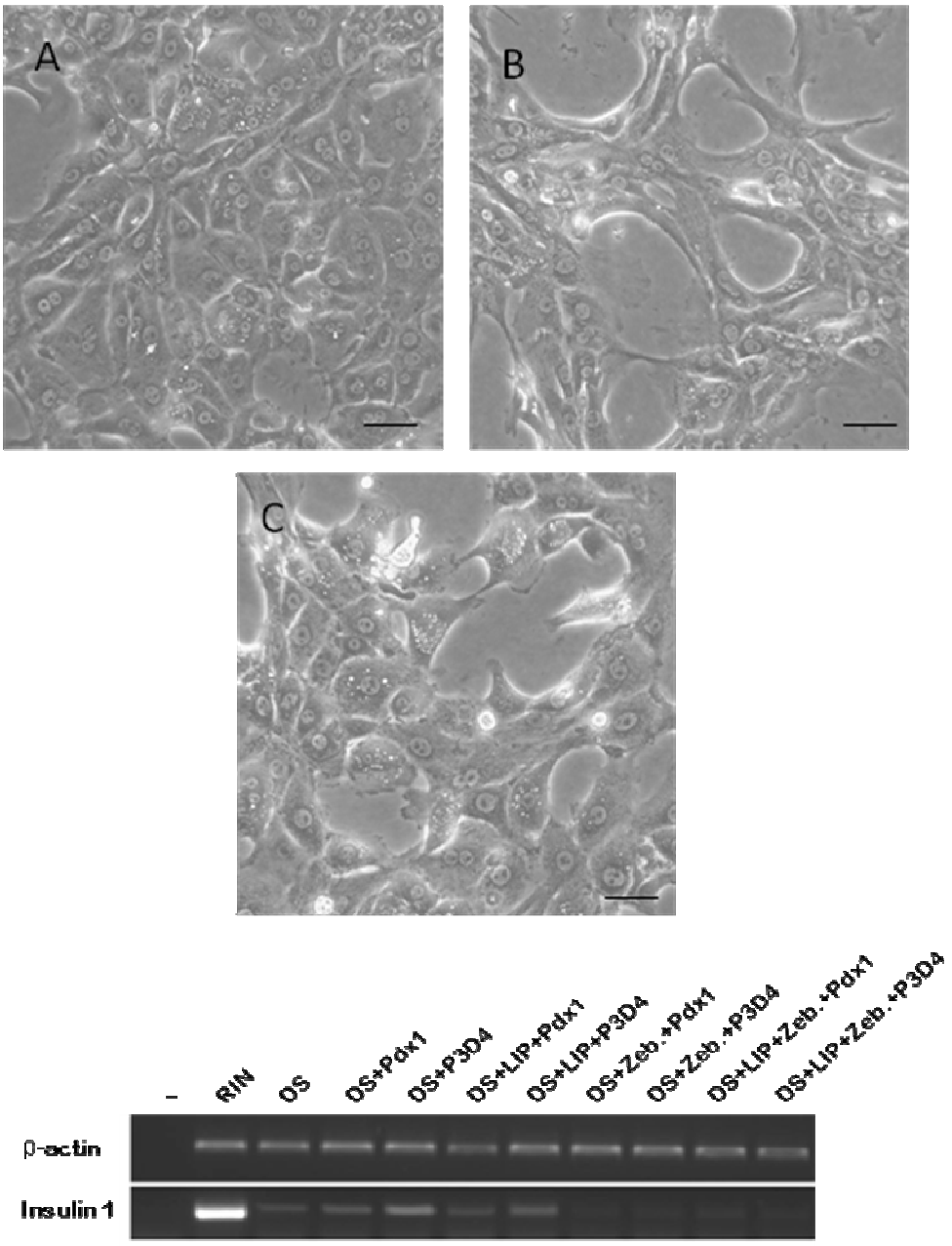
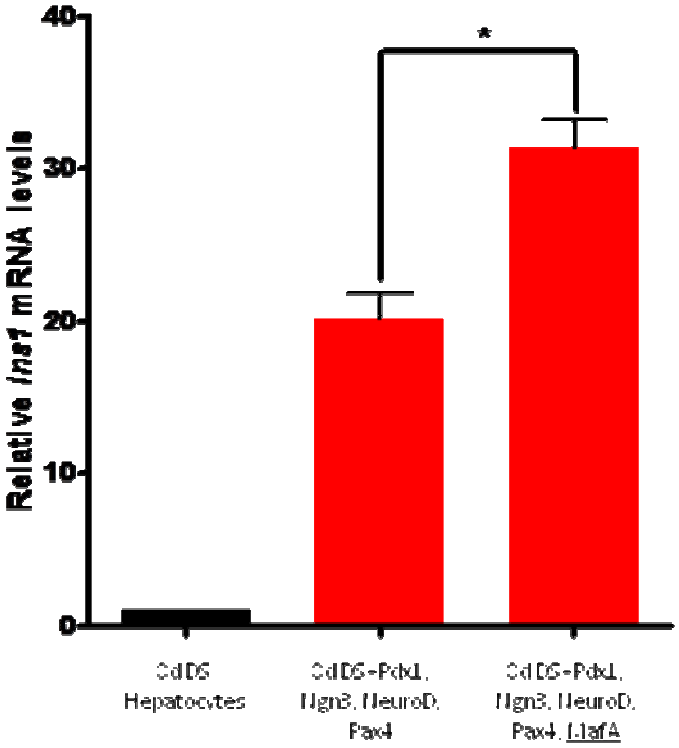


Figure 4.18. **Ad-LIP infections and DNA methylation inhibitor treatments have no positive effect on the levels of *insulin*.** A- hepatocytes cultured in the DS-medium at 3 days into culture. B- Hepatocytes infected with Ad-LIP show a more rapid change in morphology. C- Subsequent zebularine treatment however reduces the effect of LIP in terms of morphological changes and restores a more epithelial-like morphology. The scale bars represent 20μm. D- The levels of *insulin 1* expression analysed by RT-PCR were not affected by LIP transduction. However, zebularine (Zeb.) treatment reduced the amount of *insulin 1*.


40
30
20
10
0
Relative Ins1 mRNA levels
*
CdDS
Hepatocytes
CdDS+Pdx1,
Ngn3, NeuroD,
Pax4
CdDS+Pdx1,
Ngn3, NeuroD,
Pax4, MafA

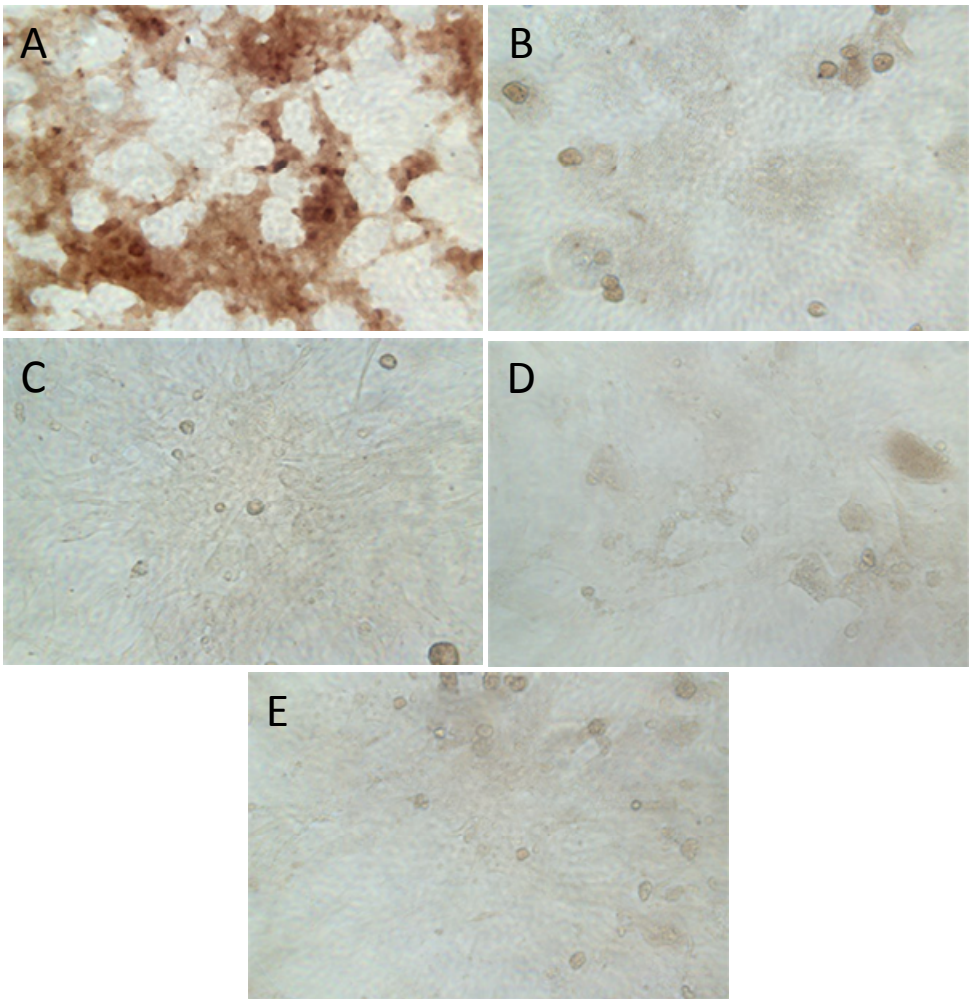


***Figure 4.20*. Immunodetectable levels of the insulin protein is not seen in de-differentiated hepatocytes even after multiple pancreatic transcription factor transductions.** Immunocytochemistry for insulin expression in RIN-5F cells (A), de-differentiated hepatocytes (B), de-differentiated hepatocytes transduced with Pdx1 (C), de-differentiated hepatocytes transduced with Pdx1, Ngn3, NeuroD and Pax4 (D) and de-differentiated hepatocytes transduced with Pdx1, Ngn3, NeuroD, Pax4 and MafA (E). No immuno-detectable insulin was seen by DAB staining in any of the samples except in the RIN-5F positive control. The scale bar represents 10µm.

DISCUSSION

One of the main problems with manipulating gene expression in adult hepatocytes maintained in primary culture is that it is difficult to transfect the cells with plasmid DNA constructs. Most of the transfection agents available commercially are targeted for immortal carcinoma cells and are often either incompatible with primary hepatocytes or the turnover of successful transformations is very low. Furthermore, transfection by electroporation of plasmid DNA requires intensive optimisation procedures each time so as not to cause extensive cell damage (Yao et al., 2002). However, the results confirm that adenoviral vectors are an excellent vehicle for gene delivery into primary hepatocytes with good efficiency and low cell mortality. In addition to their safely, adenoviruses are attractive vectors as they are capable of transducing non-dividing cells, which is important if infecting differentiated hepatocytes maintained in the KS medium. Furthermore, genes delivered by adenoviral vectors do not integrate into the host genome, thus their expression is short-lived (Thomas et al., 2003, McConnell and Imperiale, 2004). Therefore in theory, the use of adenoviral vectors would facilitate a 'hit and run' approach for molecular reprogramming, where reprogramming is initiated by the transduced gene, but then the cell could recruit its own molecular machinery of pancreatic transcription factors to maintain the newly acquired phenotype.

The results of the experiments with the over-expression of Pdx1 alone, shows that Pdx1 can promote a hepato-pancreatic conversion by enhancing the expression of pancreatic genes. Transformation of primary hepatocytes using adenovirally transduced Pdx1 is not a new area of research. Adenovirus mediated transformation of hepatocytes into β-like cells has been previously achieved both by *in vivo* (Ferber et al., 2000, Ber et al., 2003, Kojima et al., 2003, Koizumi et al., 2004, Kaneto et al., 2005a, Kaneto et al., 2005b, Imai et al., 2005, Shternhall-Ron et al., 2007) and *in vitro* (Sapir et al., 2005, Meivar-Levy et al., 2007) approaches. Compared to the *in vivo* approaches, *in vitro* transformation of cells has the added advantage of overcoming the narrow tropism of the viruses especially for organs such as

the liver. It also reduces vector toxicity by minimising systemic infections. When Pdx1 is directly introduced to the livers of rodents, it can trigger the expression of pancreatic exocrine genes and produce cells that secrete destructive enzymes such as amylase, trypsin, chymotrypsin and lipase (Kojima et al., 2003). Therefore, *in vitro* transformation of cells would also provide a greater quality control over the cells that can be selected especially if the transplantation of cells is desired. It has also been suggested that the induction of pancreatic genes by Pdx1 depends on an immune reaction to the adenoviral vector and is instead an artefact of viral infection (Wang et al., 2007). Although this may hold true *in vivo,* the results reported in this chapter suggest that this is not the case *in vitro*. In the *in vitro* hepatocyte culture system, immune effects are likely to be minimal, but Pdx1 does enhance pancreatic gene expression. Furthermore any immune effects should also be shown by the various control viruses, but none of the other adenoviruses; null, protein coding or transcription factor coding, had the same effect as the Pdx1 coding adenovirus.

The effect of Pdx1 has been proposed to be mediated by the suppression of C/EBPβ, which ultimately results in the loss of the hepatic phenotype (Meivar-Levy et al., 2007). This could be true for the cells cultured in the KS medium where no pancreatic gene expression or hepatic gene downregulation is seen prior to Pdx1 transduction. However, in the DS medium cells de-differentiate and C/EBPβ expression is downregulated even without Pdx1 transduction. In this case, Pdx1 transduction merely acts to enhance the expression of the already induced pancreatic genes. Similarly, the de-differentiation process in terms of loss of hepatic epithelial morphology can be speeded up by over-expressing LIP which antagonises the activity of endogenous C/EBPβ. However, no enhancement of pancreatic gene expression was seen following the over-expression of LIP. Although C/EBPβ itself might be important for maintenance of hepatic gene expression, these results support a C/EBPβ-independent mechanism by which Pdx1 enhances pancreatic gene expression in the already de-differentiated hepatocytes. Therefore de-differentiation synergises the reprogramming effects of Pdx1 although it is not a prerequisite for the

molecular reprogramming process (as pancreatic genes can be induced by Pdx1 in differentiated hepatocytes as well).

The mechanism by which de-differentiation potentiates Pdx1 action required some comment. A possible mechanism by which Pdx1 has a more augmented effect on cells cultured in DS medium as opposed to those in the KS medium could be the increased accessibility of Pdx1-target sites on pancreatic promoters by the changes in chromatin configuration that takes place during the de-differentiation process. The fact that the DNA demethylating agent, zebularine, did not enhance pancreatic gene expression does not rule out this possibility. The genome-wide effects of zebularine on global methylation patterns, including that of hepatic promoters could indeed have acted in favour of maintaining a hepatic phenotype, contrary to expectations. Therefore, if using DNA demethylating and histone acetylating agents for chromatin remodelling, a more targeted approach is necessary in the future.

Insulin was used as the read-out in the screen for the transcription factor combination. The three transcription factors that gave any enhanced *insulin* expression on their own, compared to the uninfected control were Pdx1, Neurogenin3 and NeuroD. Pdx1 is a member of the homeodomain-containing transcription factor family and it plays a crucial role in pancreogenesis as well as beta cell differentiation and function (Ohlsson et al., 1993, Jonsson et al., 1994, Holland et al., 2002). Neurogenin3 and NeuroD are both basic helix-loop-helix (bHLH) transcription factors that are downstream of Pdx1 in the endocrine developmental cascade and are essential for the differentiation of endocrine precursors and insulin gene expression in beta cells respectively (Schwitzgebel et al., 2000, Gradwohl et al., 2000, Naya et al., 1997, Naya et al., 1995). All of these transcription factors can directly or indirectly activate beta cell specific insulin gene expression. Pdx1 binds to the A-box enhancer element and NeuroD binds to the E-box region of the *insulin* promoter (German and Wang, 1994, Sharma and Stein, 1994, Cerf, 2006). However since the effects of adenoviral vectors are short-lived, any effect seen 5 days after transduction are presumably

effects of molecular reprogramming rather than direct promoter activation. Previously, Neurogenin3 and NeuroD have both been used separately with Pdx1-VP16 to produce stable endocrine cells in the liver (Kaneto et al., 2005b). The levels of *insulin* expression with each of the three transcription factors were different with Pdx1 being the strongest inducer of *insulin* expression followed by Neurogenin3. This could be so, because Pdx1 binding to the A3/4 sites can synergise transcription factor binding to the E-box (Glick et al., 2000, Ohneda et al., 2000b) and Neurogenin3 can in turn recruit NeuroD to the *insulin* promoter and also activate other transcription factors such as Pax4 (Naya et al., 1997, Smith et al., 2003, Cerf, 2006).

In the rest of the screen where different combinations of transcription factors were used, no single combination gave a comparatively higher reading. One explanation for this is the possible competition between the different transcription factors to common binding sites on the *insulin* promoter. However, in addition to Pdx1, Neurogenin3 and NeuroD, Pax4 was the other transcription factor that frequently occurred in combinations that produced high insulin expression. Pax4 is a late-stage paired box transcription factor, that promotes beta cell differentiation and maturation, and is expressed downstream of NeuroD (Sosa-Pineda et al., 1997). Pax4 has previously been used in combination with Pdx1-VP16 to transform the pancreatic precursor cell line (WB-1) to cells with a gene expression profile similar to that of rat insulinoma INS-1 cells (Tang et al., 2006). However, thus far a combination of Pdx1, Neurogenin3, NeuroD and Pax4 has not been used for the *in vitro* molecular reprogramming of any cell type into pancreatic beta-like cells. However, recently a combination of Pdx1, Neurogenin3 and MafA were used for the *in vivo* reprogramming of differentiated adult murine pancreatic exocrine cells into beta-like cells. The reprogrammed cells were identified as extra-islet insulin-positive cells and they closely resembled endogenous islet beta cells in size, shape, ultrastructure, gene expression profile and function (Zhou et al., 2008).

The results show that the combination of Pdx1, Neurogenin3, NeuroD and Pax4 was capable of producing higher levels of both *insulin 1* and *insulin 2* gene expression as well as yielding comparatively higher levels of insulin

protein. Unfortunately sequential infection of de-differentiated hepatocytes to mimic the development cascade of transcription factor expression was not possible due to high mortality of cells during sequential infections. One possible solution to this would be to design differentially inducible adeno- or lentiviruses that can be infected simultaneously but induced sequentially. The reason why MafA was chosen as the fifth transcription factor was because, like Pax4 it is a late-stage transcription factor during beta cell development and is important for beta cell functions such as glucose-stimulated insulin secretions (Kataoka et al., 2002, Matsuoka et al., 2004, Matsuoka et al., 2007, Zhao et al., 2001). There are three conserved *cis*-regulatory elements within the *insulin* promoter that are indispensable for proper beta cell specific insulin gene regulation. They are the E1 and A3 elements to which Pdx1 and NeuroD bind to respectively and the RIPE3b/C1 element to which MafA binds (Olbrot et al., 2002). Since neither, Pdx1, Neurogenin3, NeuroD nor Pax4 binds to the RIPE3b/C1 element, MafA would not need to compete with any other transcription factor for efficient *insulin* promoter binding thus making it an ideal candidate as the fifth transcription factor to further push the beta cell phenotype. However even with MafA, the levels of both insulin mRNA and protein expression was relatively low compared to RIN-5F cells as well as the adult rat pancreas.

Chapter FIVE

The Use of Betacellulin and other Extrinsic Factors to Promote the Beta Cell Phenotype

BACKGROUND

In addition to the transcription factors discussed in Chapter 4, a number of extrinsic signals play major roles in determining the fate adopted by the pancreatic epithelium. Such signals include growth factors, cytokines, hormones, circulating metabolites, nutrients, extracellular matrix components and membrane bound molecules, that interact with receptors and receptor complexes. The interactions are conveyed by intracellular signalling pathways to bring about changes in patterns of genes expression that ultimately controls the specification, proliferation, differentiation and programmed death of cells during development of the pancreas (Edlund, 2002). Therefore, major efforts have been implemented to identify extrinsic factors, as well as to produce synthetic molecules, that mimic the function of such factors found *in vivo,* in order to enhance beta cell differentiation, maturation and function (see Table 5.1). Much of this information has been garnered by *in vitro* studies using pancreatic cell lines as well as organ culture models.

The work described in this chapter comprises two parts. The first part will investigate the effects of the extracellular factor, betacellulin, on the differentiation of pancreatic cell types in an *in vitro* organ culture model. Betacellulin is a 32 kDa glycoprotein that has a widespread expression pattern. It has particularly high levels in the pancreas, liver and kidneys (Miyagawa et al., 1999). In the foetal pancreas, betacellulin has been immunohistochemically localised to the primitive duct cells and alpha cells and probably beta cells (Miyagawa et al., 1999). Although the physiological function of betacellulin is not definitely known, in various systems betacellulin has been shown to induce beta cell neogenesis and regeneration (Demeterco et al., 2000, Li et al., 2003). The second part of this chapter will focus on the effects of betacellulin and other extrinsic factors (see Table 5.1), on ability to enhance the pancreatic beta cell phenotype in reprogrammed hepatocytes. Analyses of the function of the beta-like cells generated by the over-expression of multiple key-regulatory pancreatic transcription factors and treatment with extracellular factors, will also be examined.

Table 5.1. Some extrinsic factors and their known effects on beta cell proliferation, differentiation and function (amended from Soria et al., 2001).

Factor	Effect of beta cells	References
Activin A	Induces the conversion of rat pancreatic cell line AR42J to insulin-secreting cells.	(Mashima et al., 1996)
Betacellulin	Facilitates beta cell differentiation in pancreatic buds.	(Huotari et al., 1998, Thowfeequ et al., 2007, Watada et al., 1996)
Epidermal Growth Factor (EGF)	Stimulates islet growth.	(Chatterjee et al., 1986, Verme and Hootman, 1990)
Exendin-4	An analogue of GLP1. Is 10-fold more potent than GLP1 (see GLP1 below).	(Zhou et al., 1999)
Glucagon like peptide I (GLP1)	Converts AR42J cells into endocrine hormone producing cells (insulin, glucagon and PP).	(Zhou et al., 1999)
FGF7 (KGF)	Induces the formation of islet-like cell clusters from human embryonic stem cells.	(Lumelsky et al., 2001, Schuldiner et al., 2000)
Gamma secretase inhibitors	Blocks Notch activation and releases beta cell progenitors from differentiation arrest.	(Murtaugh et al., 2003)
Glucose	At high concentrations: increases beta cell replication. At low concentrations: increases insulin content of cells.	(Bonner-Weir et al., 1989, Soria et al., 2000)
Growth Hormone	Increases beta cell mass during pregnancy.	(Billestrup and Nielsen, 1991)
Metformin	Improves beta cell function by increasing the nuclear localisation of Pdx1. Used as a first line anti-diabetic drug for type 2 diabetes patients.	(Richardson et al., 2006)
Nicotinamide	Increase islet regeneration after partial pancreatectomy. Increases insulin content both at the DNA level as well as protein level.	(Otonkoski et al., 1993, Sjoholm et al., 1994, Soria et al., 2001, Sugiyama et al., 1991)
PI3K inhibitors	Increases the number of insulin producing cells as PI3K acts as an inhibitor of differentiation.	(Ptasznik et al., 1997)
Prolactin	Promotes cell proliferation *in vitro*.	(Brelje et al., 1993, Moldrup et al., 1993)
Sodium butyrate	Inhibitor of histone deacetylases and a activator of gene expression. Increases insulin content.	(Bartholomeusz et al., 1989, Philippe et al., 1987)
Vascular Endothelial Growth Factor (VEGF)	Upregulated during pancreatic regeneration after duct ligation.	(Oberg-Welsh et al., 1997)

5.1 The expression of betacellulin and its receptors in the developing mouse pancreas.

Betacellulin is an epidermal growth factor family protein that can bind to and signals through both ErbB-1 and ErbB-4 receptors (Miyagawa et al., 1999, Sasada et al., 1993). These two receptors belong to the ErbB family of transmembrane receptor tyrosine kinases. This class of receptors also encompasses ErbB-2 or Neu and ErbB-3 that bind similar ligands to betacellulin and maintain inter-receptor interactions through ligand-induced homo- and hetero-dimerisation (Dunbar and Goddard, 2000).

Signalling through the ErbB receptors are known to be important for lineage determination during organogenesis (Huotari et al., 2002). In order to determine if betacellulin and its receptors were expressed in the developing pancreas, pancreata were isolated from E11.5 mouse embryos as described in Chapter 2 Section 2B.2. The epithelium and mesenchyme were separated from pancreata (15 in total). RNA was isolated from pooled pancreata and gene expression was analysed by RT-PCR (Fig. 5.1). Initially, the expression of the genes for the intermediate filament proteins *cytokeratin 19* (*CK19*) and *vimentin* was analysed. These were used as indicators of the purity of the mesenchymal and epithelial isolates respectively. Although the RNA isolated from the pancreatic epithelium contained considerable mesenchymal contamination (seen as *vimentin* expression), the RNA from the pancreatic mesenchyme did not have any significant epithelial contamination. *Btc,* the gene for betacellulin was expressed only in the pancreatic epithelium and not in the mesenchyme. The receptor genes *ErbB-1* and *ErbB-4* were both strongly expressed in the pancreatic epithelium but in a lower level in the mesenchyme. Erb-B1 seemed to be the predominant receptor type in the pancreatic mesenchyme. Despite some mesenchymal contamination in the epithelial isolate, the results were conclusive for the pattern of betacellulin expression being confined to the epithelium and its receptors being expressed in both the tissue types.

5.2 The effect of betacellulin on the branching morphogenesis of the developing pancreas.

Although the main interest was to determine the effects of betacellulin on beta cell differentiation, there were other changes in the pancreatic buds following culture with betacellulin. These changes are also worthy of comment. ErbB receptor ligands are known to act as strong *in vitro* branching morphogens (Huotari et al., 2002, Li et al., 2003, Sakurai et al., 1997). During the first few days of culture the epithelium became an extended branched structure, which can be observed under transmitted light conditions (Fig. 5.2A). Addition of betacellulin increased branching and dilation of the incipient pancreatic ducts (Fig. 5.2B). CK19, a cytoskeletal protein, is present in the incipient ductal cells of the pancreas as well as in developing acini (Bouwens et al., 1995). To confirm the increase in branching morphogenesis, control and betacellulin-treated cultures were immunostained with an anti-CK19 antibody. Betacellulin promoted branching morphogenesis based on the more extensive CK19 immunostaining (Fig. 5.3). The differences in branching between the untreated and betacellulin-treated buds were quantified by measuring the length along the CK19-positive branches of the pancreatic ductal network. The results indicated that a 3.5-fold increase in branching was induced by betacellulin (Table 5.2). This implies a possible role for betacellulin in proliferation of the incipient ducts. To test this idea, the cell-proliferation marker phosphohistone-3 (PH3) was co-stained with CK19 in control and betacellulin-treated samples. However, no co-localisation of PH3 with CK19-positive cells was observed at any of the time-points examined (days 2, 3, 5 and 9), suggesting that proliferation of the ductal cells may take place prior to them becoming CK19-positive (Fig. 5.4A-B). Instead, PH3-positive cells were mainly localised to the mesenchyme layer (Fig. 5.4C-D). Overall, betacellulin-treated samples contained increased numbers of PH3-positive cells compared with the controls (Table 5.2).

After 10 days of culture with betacellulin, pancreatic buds had a thicker and a wider area of mesenchyme in comparison with untreated buds which ties in with the increased mesenchymal proliferation (PH3-positive cells)

observation following betacellulin treatment. The total area occupied by the epithelium and the mesenchyme was measured using E-cadherin and smooth muscle actin as their respective markers. The mesenchymal:epithelial ratio for control and betacellulin-treated buds is shown in Table 5.2. The results indicate that there was more than a 1.5-fold increase in the mesenchymal:epithelial ratio after betacellulin treatment. However it is not clear if the expansion of the mesenchyme following betacellulin treatment was a direct effect mediated via the ErbB receptors in the mesenchyme or an indirect effect due to a more elaborate ductal network.

Table 5.2. Betacellulin regulates embryonic pancreatic development

	Control	**Betacellulin**	***n***
Mesenchymal:epithelial ratio	13.20±1.01	21.73±1.12[a]	3
Length of the entire ductal network ($\times 10^3$ μm)	2.40±0.25	8.43±0.53[b]	4
Total area of amylase-positive cells ($\times 10^3$ μm^2)	32.11±3.41	1.73±0.45[b]	4
Total area of insulin-positive cells ($\times 10^3$ μm^2)	72.75±8.26	235.60±29.34[a]	6
Area of insulin-positive cells/no. of SS cells (μm^2)	439.7±43.24	398.0±33.59[c]	4
Area of insulin-positive cells/no. of PP cells (μm^2)	578.8±50.47	1,026.0±132.00[a]	4
Area of insulin-positive cells/no. of ghrelin cells (μm^2)	3,490±215.80	1,937±224.50[a]	4
Total PH3-positive cell count	1,377±72.26	2,165±115.10[a]	4
Maximum diameter of beta cells (μm)	13.91±0.24	13.61±0.17[c]	50

[a] *p*=0.0286; [b] *p*=0.0079; [c] non-significant between control and betacellulin-treated

5.3 Betacellulin reduces the pancreatic acinar cell population.

Amylase-expressing cells were present in the control pancreatic organ cultures at day 7 and the numbers increased further by day 10. The amylase-expressing cells were distributed around the periphery of the branches and were distinct from the endocrine cell populations (Fig. 5.5A,C). In contrast, the betacellulin-treated pancreatic tissue either completely lacked or had reduced numbers of amylase-expressing cells (Fig. 5.5B,D). Under transmitted light, the zymogen granules, which store the exocrine enzymes, were clearly visible in the control (Fig. 5.6A) but not in the betacellulin-treated

(Fig. 5.6B). Overall, betacellulin treatment reduced the number of amylase-positive cells (measured as the total area covered by amylase-positive cells) by 18.5-fold (Table 5.2). Western blot analysis confirmed this observation and showed the virtual absence of the amylase protein in betacellulin-treated samples (see Fig. 5.11). Furthermore, the pro-apoptotic protein caspase-3 was observed in acinar tissue of betacellulin-treated samples (Fig. 5.7), whereas it was much more difficult to find caspase-3-positive cells in control buds. Therefore the reduction in amylase-positive cells may be mediated through apoptosis in betacellulin-treated samples. However, following the upregulation of activated caspase-3 in betacellulin-treated cultures, a concurrent decrease in the level of the transcription factor Ptf1a was also observed (Fig. 5.11). Since Ptf1a plays a vital role in early exocrine fate determination, the combined data suggest that the effect of betacellulin on the exocrine cells might in fact act at two different levels- before and after endocrine differentiation has taken place. On the other hand, hepatocyte nuclear factor (HNF) 4α, which is not known to play a pivotal role in either alpha or beta cell differentiation, was present at similar levels in both treated and untreated samples.

5.4 The effects of betacellulin on the pancreatic endocrine lineages.

One of the main aims of this chapter was to identify factors that promote beta cell differentiation. To address this point, the effect of betacellulin on insulin-expressing endocrine cells was investigated. In both betacellulin-treated and untreated samples, after 10 days of culture, insulin-positive beta cells outnumbered any other endocrine cell type. However, the distribution and organisation of the beta cells were noticeably different between treated and untreated samples. In the untreated pancreatic buds, there were greater numbers of beta cells clumped together in large islet-like structures (Fig. 5.8A). In contrast, the betacellulin-treated buds had smaller clumps of beta cells that were dispersed throughout the organ culture (Fig. 5.8B). Additionally there were many individual cells or small islet-like cell clusters positive for insulin in the inter-islet regions and in close proximity to the

ducts. Betacellulin induced an approximately three-fold increase in the number of beta cells (measured as the total area covered by insulin-positive cells) with no significant change in cell size (Table 5.2). This observation was further confirmed by western blotting for insulin protein (see Fig. 5.11). To test whether betacellulin increased the number of beta cells by increasing the proliferation of beta cells, the pancreatic buds were co-stained for insulin and the proliferation marker PH3. Betacellulin did not affect the proliferation of insulin-positive beta cells and only a few insulin-positive cells co-stained with PH3 (see Fig. 5.4A, B).

In control samples, the classic islet morphology was preserved with glucagon-expressing cells present in the periphery and these cells outnumbered all other endocrine cell types except beta cells by day 10 of culture (Fig. 5.8A). However, in the betacellulin-treated samples, the number of glucagon-positive cells was reduced to just a few cells in each pancreas (Fig. 5.8B). Although glucagon protein was not detectable on the western blot, presumably because of its small size, the amount of the 18 kDa proglucagon peptide from which glucagon is derived was much higher in the untreated than betacellulin-treated pancreatic samples (Fig. 5.11).

Since there was no evidence for changes of proliferative rate underlying the observed effects of betacellulin on the epithelial cell types it is more likely that the effects are due to an alteration in lineage determination of precursor cells. To examine this possibility, we determined the expression of pancreatic transcription factors by immunohistochemistry. By day 2 of culture, both control and betacellulin-treated samples showed high levels of Pdx1 (Fig. 5.9). Pdx1 is a homeodomain protein that is expressed in the entire pancreatic anlagen at early stages of development. Normally, Pdx1 expression declines during later embryonic stages in most of the pancreas but remains selectively expressed in the beta cells where it binds to and activates the *insulin* promoter. By day 5 of culture, the expression of Pdx1 was indeed limited to insulin-positive cells in the untreated samples. In comparison, Pdx1 expression in the betacellulin-treated samples was not strictly confined to such insulin-positive cell clusters. Pdx1-positive cells in betacellulin-treated samples outnumbered all of the differentiated endocrine

cells put together and the expression was more ubiquitous, extending to cell populations that might be acinar or ductal in nature (although this was not conclusively determined due to difficulty of co-staining acinar and ductal markers along with Pdx1). Pdx1 protein was also higher in betacellulin-treated samples compared with controls (see Fig. 5.11). Pdx1 expression is normally downregulated in non-beta cells during late embryonic development (Stoffers et al., 1999) and prolonged expression of Pdx1 in the exocrine portion of the pancreas has been shown to cause acinar-to-ductal metaplasia mediated through activation of the signal transducer and activator of transcription-3 (Stat3) (Miyatsuka et al., 2006). This is very similar to what was observed, in that an increase of developing ducts relative to developing amylase-positive cells was seen. Therefore it was of interest to find out if Stat3 was activated following betacellulin treatment. Previously, Stat3 has been shown to be activated in cells of human pancreatic cancers (Greten et al., 2002, Scholz et al., 2003) and in the metaplastic cells that form ductal cells (Miyatsuka et al., 2006). When pancreata were cultured with and without betacellulin for 7 days activated Stat3 (phosphorylated on Tyr705) was found in betacellulin-treated samples (Fig. 5.11). Phosphorylated Stat3 was absent in control samples at the time-point examined. A higher level of expression of the pro-endocrine transcription factor Sox9 (Fig. 5.11) was also seen following betacellulin treatment suggesting that endocrine progenitors may also contribute to the increases beta cell mass.

The effects of betacellulin on the pancreatic polypeptide producing PP cells, somatostatin-containing delta cells and the ghrelin-containing cells were also investigated (Fig. 5.10). All these cell types were found in both the control and betacellulin-treated samples, but in smaller numbers compared with beta and alpha cells. PP cells were observed at a greater frequency in the untreated cultures than in the betacellulin-treated cultures (Table 5.2). Normally they are found clustered in or near the islets. In the betacellulin-treated samples, clusters of PP cells were absent and they were found mostly individually, in pairs or as triplets (Fig. 5.10D). The frequency of occurrence of delta cells within islets was similar in both betacellulin-treated and untreated cultures (Table 5.2). The differences in cell numbers for delta

cells were relative to the size of the pancreatic buds with and without treatment. This observation was also confirmed by western blot analysis for the amounts of SS protein normalised to the total amount of protein (Fig. 5.11). Unlike PP cells, the delta cells did not cluster. Furthermore, as is typical in both treated and untreated samples, some of the SS-positive cells also co-expressed insulin (appears yellow on merged images; Fig. 5.10B). Ghrelin was the least abundant islet hormone in the pancreatic organ cultures (Fig. 5.10E, F). However, the number of cells expressing ghrelin was higher following betacellulin treatment (Table 5.2) and western blotting showed that the betacellulin-treated samples had considerably higher levels of ghrelin protein compared with the untreated samples (Fig. 5.11). Overall, betacellulin-treatment had an effect on most of the exocrine and endocrine cell types analysed; with a positive effect on beta cell differentiation as well as the numbers of ghrelin-positive cells and a negative effect on the numbers of alpha, acinar and PP cells.

5.5 Screen for extrinsic factors that facilitate hepatic to beta cell conversion.

For the induction of a beta cell phenotype in hepatocytes, de-differentiation and transcription factor over-expression alone was not sufficient to produce high levels of insulin expression. Cell differentiation and maturation can be expressed as functions of time, cell lineage and niche or milieu. Various extrinsic factors play an important role in providing this niche in which cells differentiate. Therefore it was necessary to resort to extrinsic factors to produce more as well as mature beta-like cells. The use of extrinsic factors for the *in vitro* differentiation of cells is not a new area of research. For example, interleukin-3 and interleukin-6 treatment can drive mouse ES cells into erythroid and lymphoid lineages respectively (Wiles and Keller, 1991). More recently, D'Amour *et al.,* (2006) has developed a five stage protocol for generating pancreatic-hormone expressing cells from human ES cells. By altering media conditions (serum content and media compositions) along with media supplementation with growth factors, the procedure mimics

normal pancreatic development by driving the cells through five identifiable endodermal intermediates en route to becoming hormone producing endocrine cells.

A screen was carried out for extrinsic factors (including growth factors and factors that modify gene expression) that have previously been reported to either promote beta cell differentiation, proliferation, maturation and survival, or that increase levels of insulin expression and secretion (see Table 5.1). The rationale for using the diabetogenic agents, streptozotocin and alloxan, was based on reports that, elevated levels of insulin transcript and insulin positive cells were observed in the livers of streptozotocin-induced diabetic mice and not in non-diabetic mice (Kojima et al., 2003). For these experiments, hepatocytes were isolated from rats and cultured under DS conditions to facilitate de-differentiation. The de-differentiated hepatocytes were then transduced with a combination of viruses encoding Pdx1, Neurogenin3, NeuroD, Pax4 and MafA on day 3 after isolation (as described in Chapter 4). The treatments were initiated on day 3 after isolation (except for γ-secretase inhibitors which were added into the medium soon after cell-attachment) and continued by supplementing the culture media with the relevant concentration of the extrinsic factors each time the media was changed daily. The treatments were followed for five days, before RNA isolation and analysis of *insulin 1* expression by RT-PCR.

Betacellulin, in accordance with the data above also increased the level of insulin expression in de-differentiated hepatocytes transduced with the combination of the five transcription factors (Fig. 5.12). On the first round of the experiments, similar high levels of *insulin 1* expression was seen with nicotinamide and exendin-4 treatments as well as with a combination treatment of Activin A and betacellulin. All the other factors screened did not give any notable increase in the levels of *insulin 1* expression compared to the untreated controls (Fig 5.12). The experiment was repeated for the four highest scoring treatments, however it is important to note that there was some variation in the response to the extrinsic factors. In the second and third rounds of the experiment, exendin-4 did not give notably high levels of *insulin 1* expression, but betacellulin, Activin A and nicotinamide did.

Ultimately, the highest level of *insulin 1* expression was reproducibly seen with a combination of 10ng/ml betacellulin, 10nM Activin A and 20mM nicotinamide (Fig. 5.13).

Addition of betacellulin to cultured embryonic pancreata enhanced the differentiation of some cells (e.g. beta cells) whereas it inhibited the production of others (e.g. alpha cells. The ability to enhance the differentiation of beta cells is instrumental in producing a homogenous population of beta-like cells for transplantation. Following de-differentiation and Pdx1 transduction alone, both pancreatic endocrine (*insulin, SUR1, Kir6.2*) and exocrine (*amylase)* markers were also induced. However, the inclusion of such exocrine enzyme secreting cell types, could lead to pancreatitis. When the expression of various endocrine hormone genes as well as exocrine genes were analysed after multiple viral transductions and extrinsic factor treatments, it was observed that the levels of the exocrine gene *amylase* was much reduced compared to untreated samples (Fig. 5.14). Although glucagon, somatostatin and pancreatic polypeptide expression still remained, it was much more diminished after the extrinsic factor treatments. Significantly, both multiple transcription factor transduction (Pdx1, Neurogenin3, NeuroD, Pax4 and MafA) and extrinsic factor treatments (betacellulin, Activin A and nicotinamide) enhanced the levels of insulin gene expression. The next step was to assess the maturity of the beta-like cells generated by determining the performance of the beta-like cells in different functional assays.

5.6 Analysis of pro-insulin processing in beta-like cells.

In functional beta cells, insulin hormone is produced from the pro-insulin precursor molecule by the action of proteolytic enzymes which include the prohormone convertases and the exoprotease, carboxypeptidase E. The type I (PC1/PC3) and type II (PC2) prohormone convertases are involved in the cleavage of the connecting peptide (C-peptide) from the pro-insulin molecule leaving the remaining A- and B-chains of the mature insulin molecule bound together by disulphide bonds. Both type I and type II

prohormone convertases were induced in hepatocytes following de-differentiation and further enhanced by Pdx1 and multiple transcription factor transduction (Fig 5.15A). The levels of *PC2* were generally lower than the levels of *PC1*/*3*. Analysis of the expression of these proteolytic enzyme genes provides a clue as to whether cells are capable of processing pro-insulin into its mature functional form. However, it does not serve as conclusive evidence to if pro-insulin processing takes place.

Once cleaved, the C-peptide is stored together with insulin in mature granules until it is released intraportally with insulin, on an equimolar basis. But unlike insulin which undergoes a large hepatic extraction (~50%), C-peptide can be detected in the peripheral circulation without appreciable extraction by the liver (Ferrannini and Cobelli, 1987). The level of C-peptide in the blood is hence often used as an indicator of how much insulin is made in the pancreas. Therefore the C-peptide content of cell lysates was measured using a Rat C-peptide enzyme-linked immunosorbant assay. De-differentiated hepatocytes transformed with the four different transcription factors, Pdx1, Neurogenin3, NeuroD and Pax4, showed more efficient insulin processing capabilities indicated by a 1.6-fold increase in the amount of C-peptide levels compared to those infected with just Ad-Pdx1 alone (Fig. 5.15B). A further 1.6-fold enhancement of the levels of C-peptide was obtained following the inclusion of MafA in the transcription factor combination and a further augmention by the treatments with the combination of extrinsic factors- betacellulin, Activin A and nicotinamide. In contrary to the stoichiometry of the proteolytic reaction, the levels of insulin and C-peptide were not detected at the expected equimolar concentrations. The levels of insulin were consistently higher than the C-peptide levels. C-peptide could be lost during the protein isolation protocol by acid-ethanol extraction. C-peptide is a much smaller protein (2.75kDa) compared to insulin (5.81kDa).

5.7 Assessing beta cell function by glucose-stimulated insulin secretion (GSIS).

De-differentiated hepatocytes express genes encoding the glucose sensing and insulin release machinery. If beta-like cells generated from hepatocytes are to serve as surrogate beta cells they need to be able to secrete insulin in a glucose sensitive manner. Therefore the percentage of stored insulin released by the reprogrammed hepatocytes, in response to stimulation by 25mM glucose was determined by measuring the levels of insulin in the cells and the media before and after a 1 hour period of stimulation with 25mM glucose. De-differentiated cells were able to respond to high glucose concentration by releasing more insulin into the medium. A somewhat higher constitutive insulin release was shown by de-differentiated cells after infection with Ad-Pdx1, and a further 2.8-fold increase was seen in response to the 25mM glucose (Fig. 5.16). The response was more regulated in de-differentiated cells after infection with adenoviruses coding for Pdx1, Neurogenin3, NeuroD and Pax4. These cells showed a minimal basal level of insulin release at 5.5mM glucose but a 9.2-fold increase following glucose stimulation with 25mM glucose. The levels of basal insulin secretion remained low at around 2.7% (of the total insulin content of the cell being released) in 5.5mM glucose even with MafA over-expression and extrinsic factor treatments combined. Although the amount of insulin in the cells was more with the four transcription factors plus MafA (see Fig. 5.15B), the percentage released in response to the higher glucose concentration was lower than the combination without MafA. This is indicative of a more regulated secretion and a more mature beta cell phenotype brought about by MafA over-expression. A slight increase in the percentage of stored insulin released in response to glucose stimulation was observed following the extrinsic factor treatments. However, this does not necessarily mean a loss of maturity or the ability to regulate insulin secretion, but instead highlights the fact that betacellulin, nicotinamide and possibly Activin A can stimulate insulin release (Soria et al., 2001). The ability to secrete insulin following external glucose changes of this magnitude is a key aspect of the beta cell phenotype.

5.8 Incretin responsiveness of beta-like cells.

In addition to glucose, other factors such as the gut hormone glucagon like peptide 1 (GLP1) can act as insulin secreatgogues. GLP1 is also considered to act as an incretin by which it augments insulin secretion. It is released into the circulatory system in response to ingestion of a meal (Ahren, 1998, Drucker, 2002). *In vivo*, the incretin effect is necessary to mediate the beta cell response to ingested glucose. It is therefore of interest to test the response of beta-like cells to incretins. We first determined, the expression of the GLP1 receptor (GLP1-R) by RT-PCR. *GLP1-R* was one of the pancreatic beta cell genes that was induced following de-differentiation (Fig. 5.17A). Similar to *insulin* and the other beta-specific cell genes, the level of *GLP1-R* expression was further increased by transcription factor over-expression as well as by the addition of extrinsic factor treatments. To test the responsiveness of the cells to incretins, the GLP1-R agonist and GLP1 homolog, exendin-4 (at a 10nM final concentration) was added in the presence of 5.5mM and 25mM glucose and left for 1 hour. Addition of exendin-4 increased the levels of insulin secretion under both high and low glucose conditions. However, the changes in insulin following exendin-4 addition were not statistically significant (5.17B). The insulinotropic properties of GLP1 are largely dose-dependent as well as glucose-dependent. Therefore more intricate adjustments to the incretin dose, length of stimulation and glucose concentration maybe necessary to achieve more overt GLP1 or exendin-4 responsiveness in reprogrammed hepatocytes.

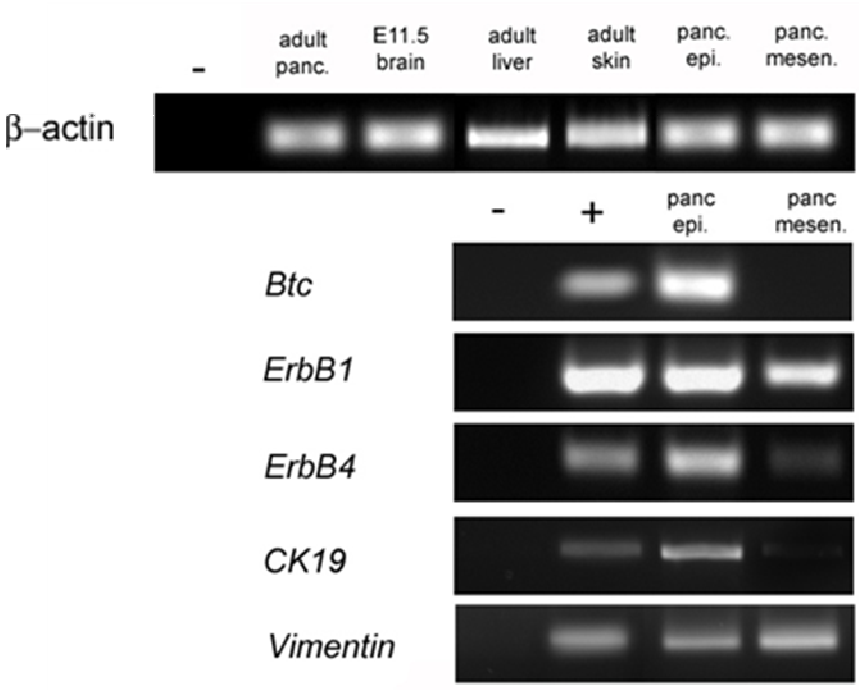


Figure 5.1. **The expression of *Btc* and *ErbB1* and *ErbB4* receptors in the epithelium and mesenchyme of E11.5d mouse dorsal pancreas.** Epithelium (panc epi.) and mesenchyme (panc mesen.) were separated as described previously (Chapter 2 Section 2B.2). Expression of markers of epithelium (*CK19*) and mesenchyme (*Vimentin*) are also shown to demonstrate efficient separation of the two tissues. *Btc* expression is seen preferentially in the pancreatic epithelium. cDNA from adult pancreas and E11.5d embryonic brain were used as positive controls (−) for *Btc* and the *ErbB* receptors, respectively, and cDNA from liver and skin were positive controls for the epithelium (*CK19*) and mesenchyme (*Vimentin*), respectively. The upper panel shows the beta-actin (BA) equal loading control for all the samples and the positive controls used. The − lane represents the water control (no cDNA).

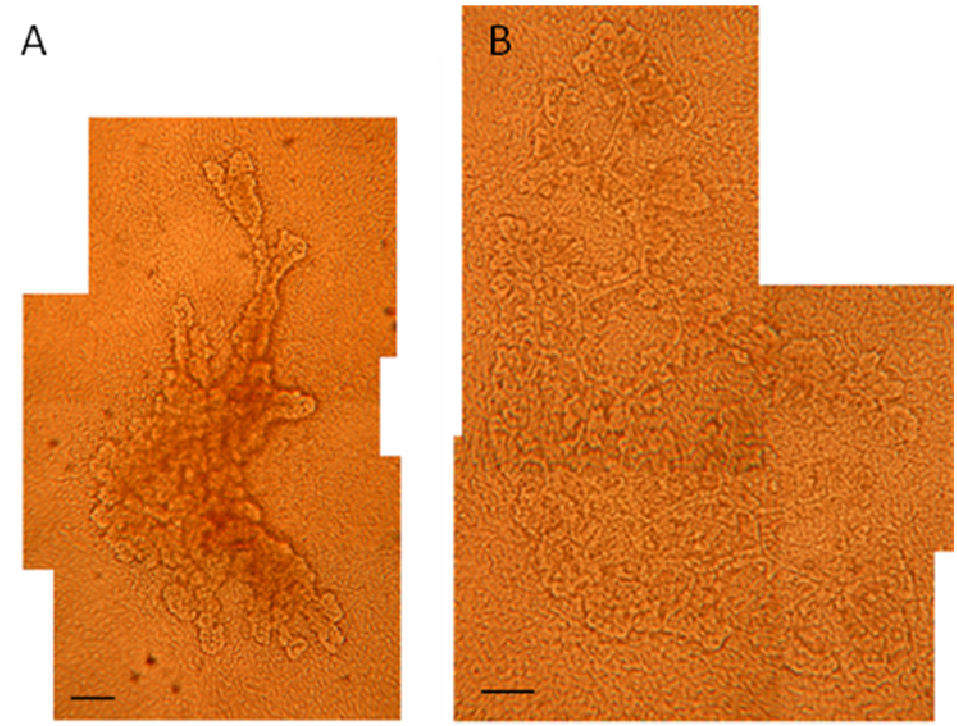


Figure 5.2. **The effect of betacellulin on branching morphogenesis of the developing embryonic pancreas.** Pancreatic buds were isolated from E11.5d embryos and cultured for 24 h to allow attachment. The medium was then replaced with fresh medium with and without betacellulin (10 ng/ml). Transmitted light images of control cultures (A) and betacellulin-treated cultures (B) 9 days after isolated, demonstrating the extensive branching seen following betacellulin treatment. The scale bars represent 100 µm.

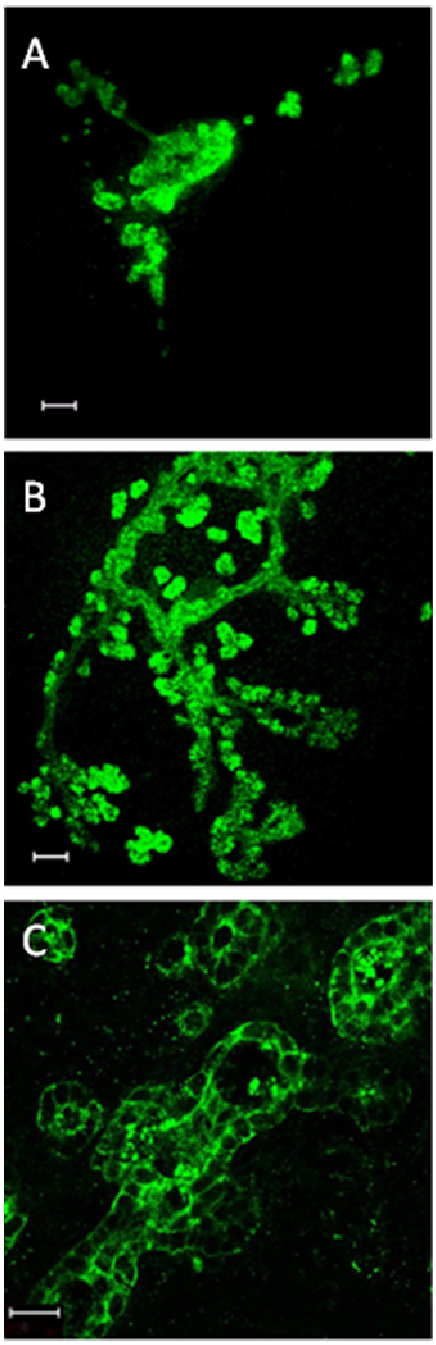


Figure 5.3. **The effect of betacellulin on branching morphogenesis of the developing embryonic pancreas.** Immunostaining for CK19 (green) in control cultures (A) and betacellulin-treated cultures (B) showing the elaborate branching network induced after treatments. A high magnification image of a betacellulin- treated sample (C) is shown with the CK19 positive cells are confined to the pancreatic ducts. The scale bars represent 100 µm (A and B) and 20 µm (C).

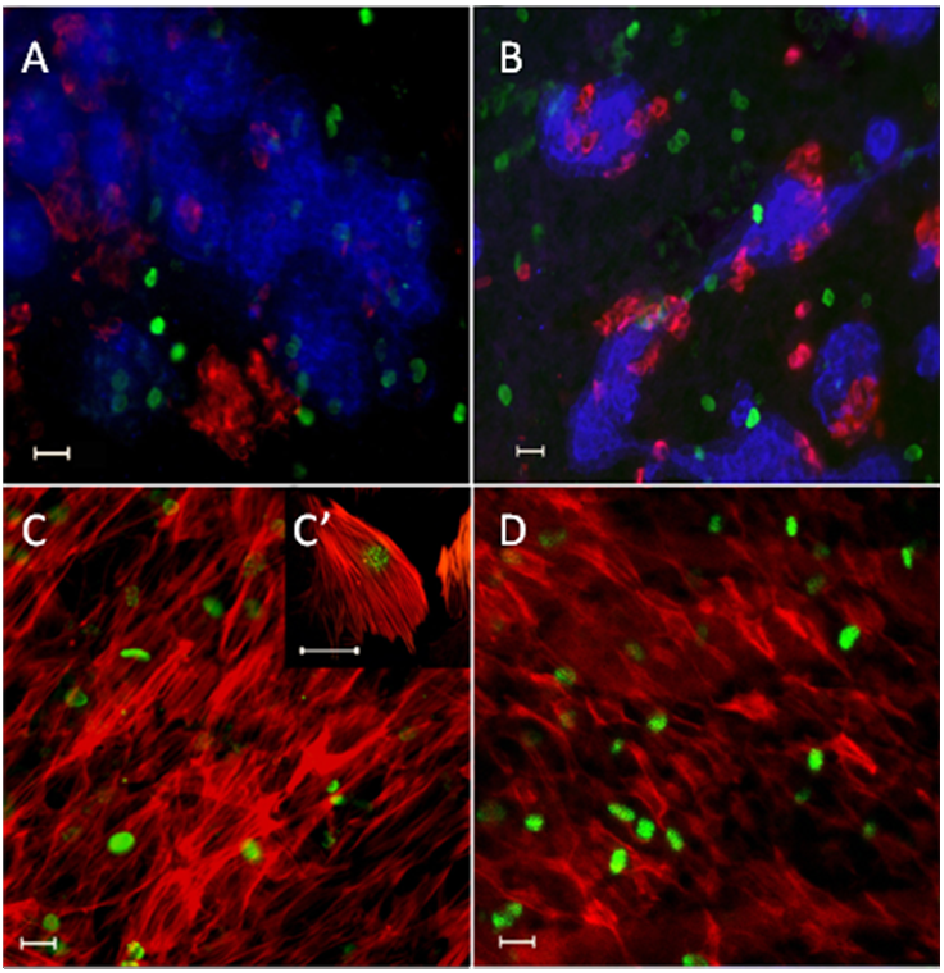


Figure 5.4. More PH3 positive (green) proliferating cells were seen in total in the betacellulin-treated samples (B and D) compared to the controls (A and C). (A and B)- however, increase in the number of insulin (red) positive beta cells was not due to an increase in cell division since there was little or no co-localisation of the proliferation marker PH3 (green) with insulin (red). Similarly, increased levels of co-localisation of PH3 was not seen with CK19 (blue). Instead, PH3 (green) tended to co-localise with the mesenchymal cells stained for smooth muscle actin (red) in both control (C) and betacellulin-treated tissue (D). C' shows the pattern of smooth muscle actin staining in a PH3 positive mesenchymal cell under high magnification. The scale bars represent 20 µm.

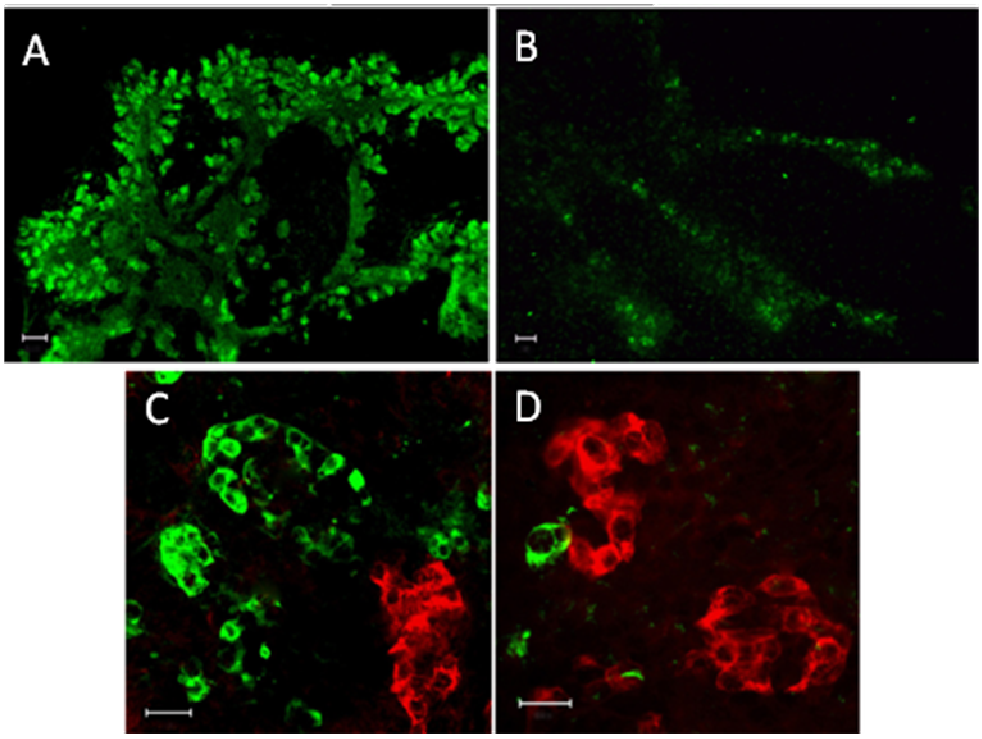


***Figure* 5.5. The effect of betacellulin on amylase expression in the embryonic pancreas.** Immunostaining for the exocrine marker amylase (green) in control (A) and betacellulin-treated (B) pancreatic buds showing the reduction in the number of amylase positive cells after betacellulin treatment. High magnification of dual immunostaining for amylase (green) and insulin (red) in control (C) and betacellulin-treated (D) cultures show that the amylase positive acini are lost after betacellulin treatments and what remains are individual amylase expression cells distributed in an unorganised manner in between the insulin positive islets. The scale bars represent 100 µm (A and B) and 20 µm (C and D).

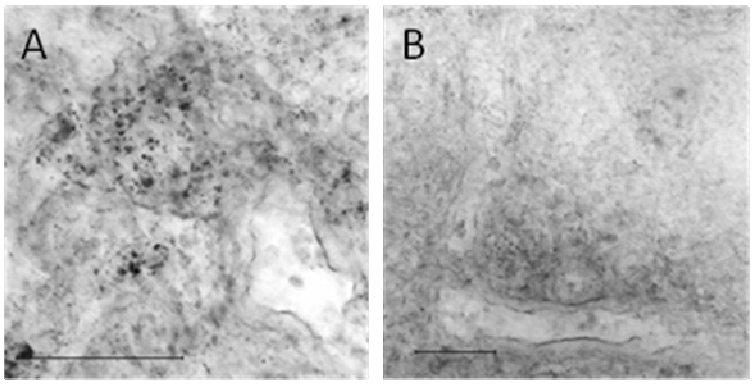


***Figure** 5.6.* **The effect of betacellulin on digestive enzyme storage in the pancreatic acniar cells.** Under transmitted light, at high magnification the dense zymogen granules (seen as black dots) are visible in the control cultures (A) but not in betacellulin-treated cultures (B). The scale bars represent 20µm.

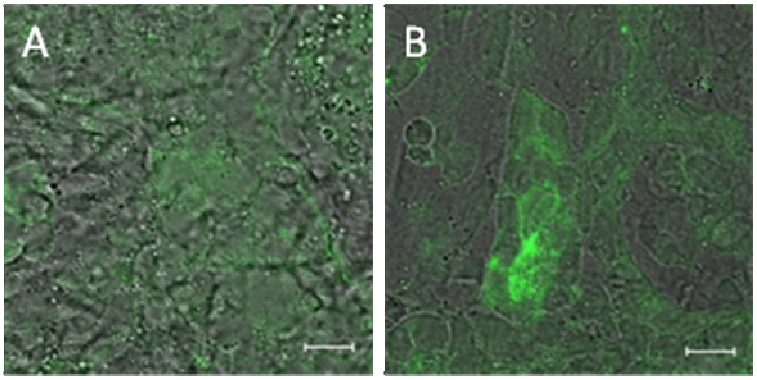


***Figure** 5.7.* **The effect of betacellulin on acinar cell survival and apoptosis.** The expression of activated caspase-3 is not clearly visible in the controls (A). However, activated caspase-3 (green) can be seen confined to what appears to be apoptotic bodies in individual cells in the betacellulin-treated samples (B). The scale bar represents 10µm.

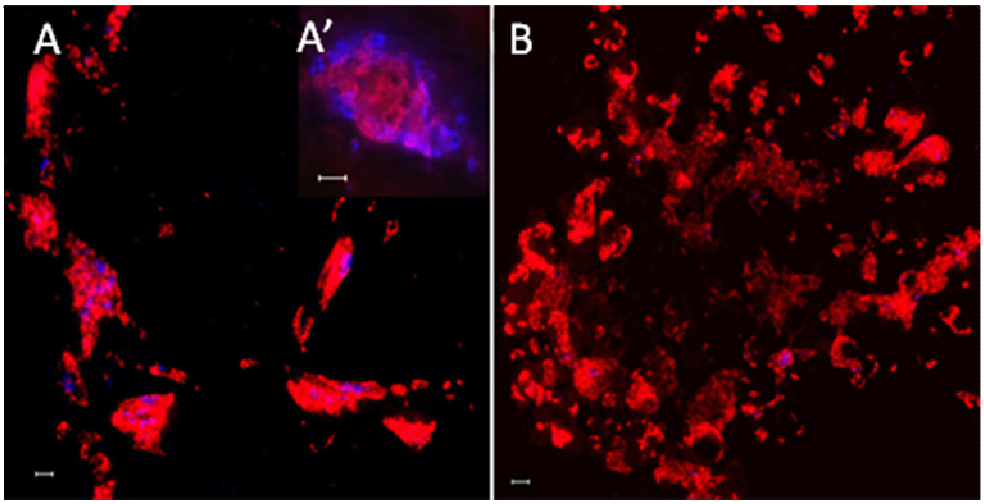


Figure 5.8. **The effect of betacellulin on insulin and glucagon expression in the embryonic pancreas.** Control (A) and betacellulin-treated (B) pancreatic buds after 9 days of treatment, immunostained for insulin (red) and glucagon (blue). Insulin-positive cells are more abundant in betacellulin-treated cultures compared with the controls. Treatment with betacellulin also resulted in a marked reduction in the number of glucagon-positive cells (blue). Additionally, the insulin positive cells were found individually in betcellulin treated samples whereas in the controls they were confined to well organised islets (A') with glucagon expressing cells in the periphery. The scale bars represent 100 µm (and 10µm in A').

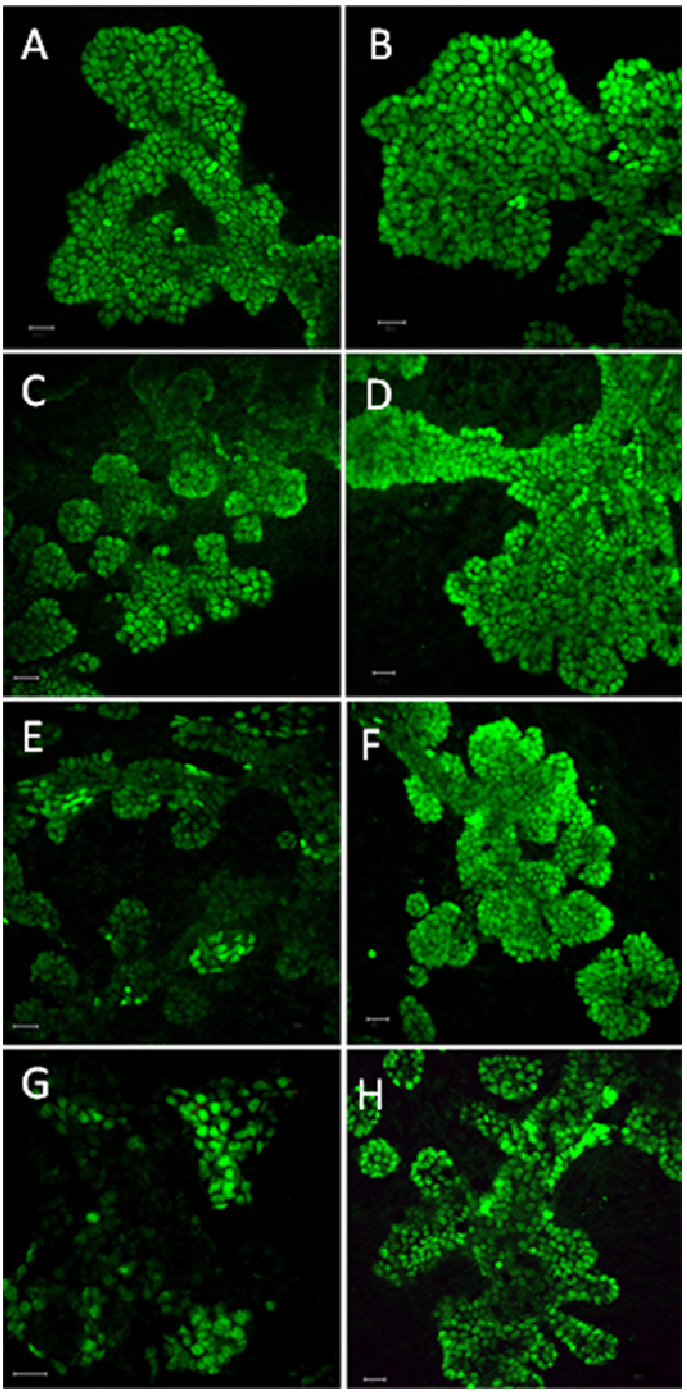


***Figure* 5.9. Betacellulin induces the expression of Pdx1 in pancreatic cultures.** Time course of expression of Pdx1 in control (A, C, E, G) and betacellulin-treated (B, D, F, H) cultures at 2 (A, B), 3 (C, D), 5 (E, F) and 7 (G, H) days into culture with and without treatment. Buds were then fixed and immunostained for Pdx1 (green). In control cultures, Pdx1 becomes restricted to islet-like structures as development proceeds whereas in betacellulin-treated cultures there is much sustained widespread expression of Pdx1 even at 7 days into culture (H). Scale bars represent 20 µm.

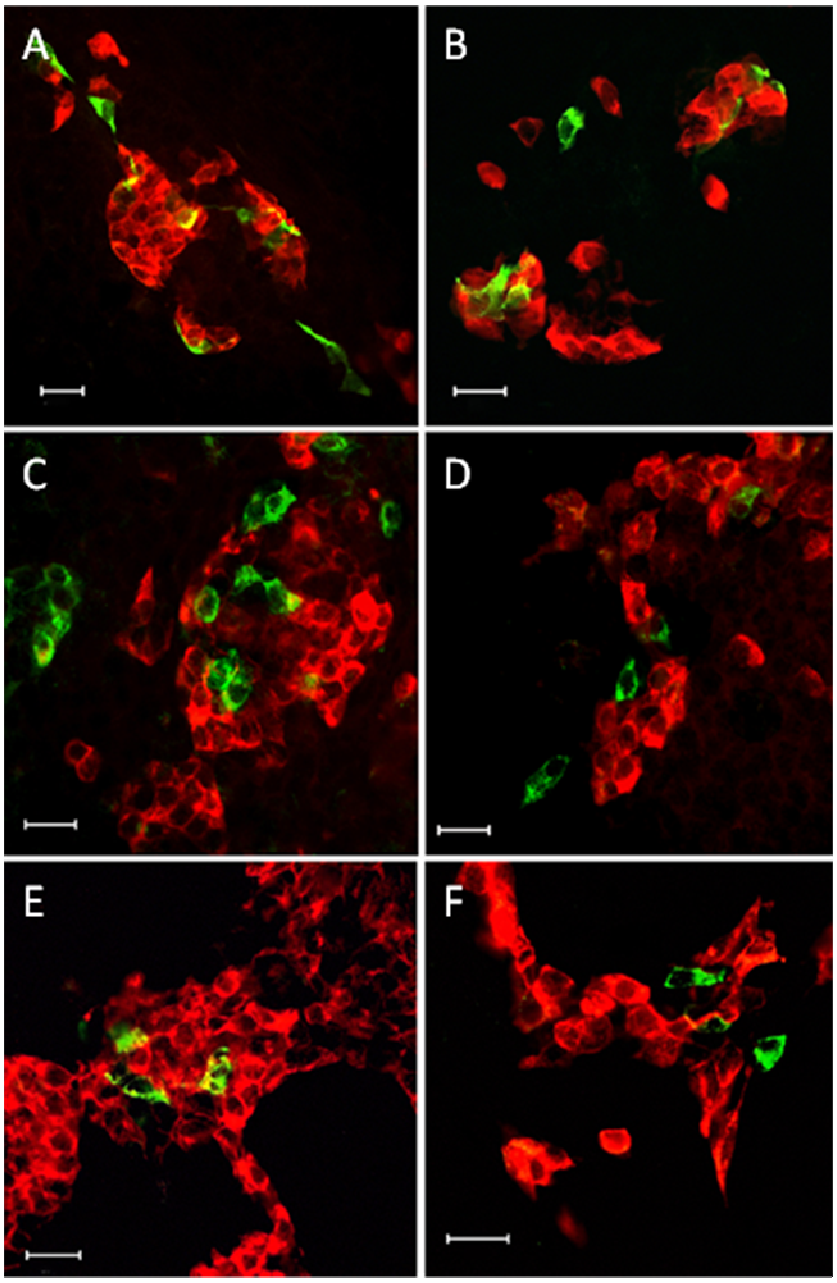


Figure 5.10. **The effect of betacellulin on the expression of the endocrine hormones, somatostatin (SS), pancreatic polypeptide (PP) and ghrelin in the embryonic pancreas.** E11.5d embryonic pancreatic buds were cultured with (B, D, F) or without (A, C, E) betacellulin for 10 days and then co-stained for SS (A, B), PP (C, D) or ghrelin (E, F) (green) and insulin (red). Note that since these cell types are rare and only found within islets, the pictures show high magnification images of only the areas with an abundance of insulin-positive cells for both controls and betacellulin-treated samples. The scale bars represent 20 μm.

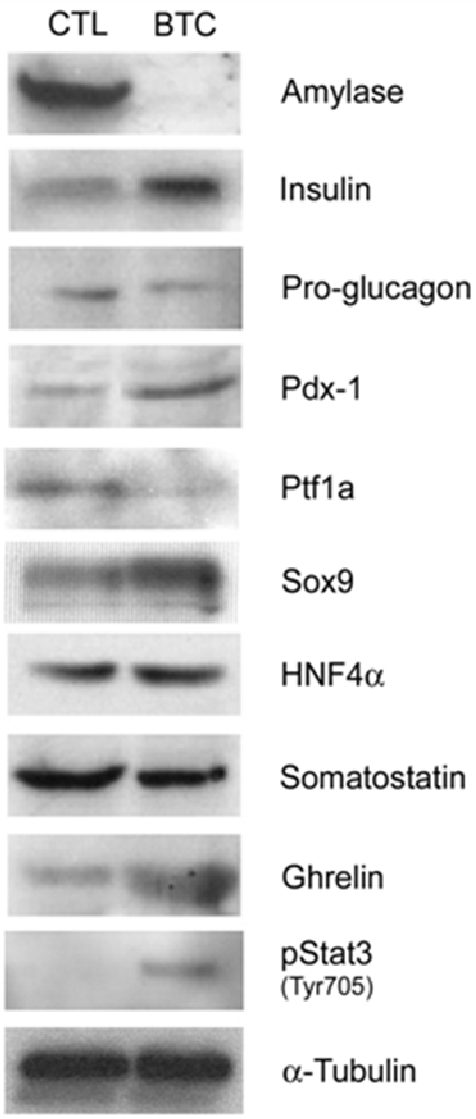


Figure 5.11. **The effects of betacellulin (BTC) treatment on the levels of pancreatic proteins.** Protein was isolated from pancreatic buds cultured with and without betacellulin for 7 days. Following BTC treatment, there was an increase in the levels of the pancreatic hormones insulin and ghrelin, the transcription factors Pdx1 and Sox9, and the phosphorylated form (Tyr705) of the signalling molecule Stat3 (pStat3). The levels of the enzyme amylase, the immature form of the pancreatic hormone glucagon and the exocrine promoting transcription factor Ptf1a were reduced. The levels of SS and HNF4α remained the same in both control (CTL) and BTC-treated samples. α-Tubulin was used as the loading control.

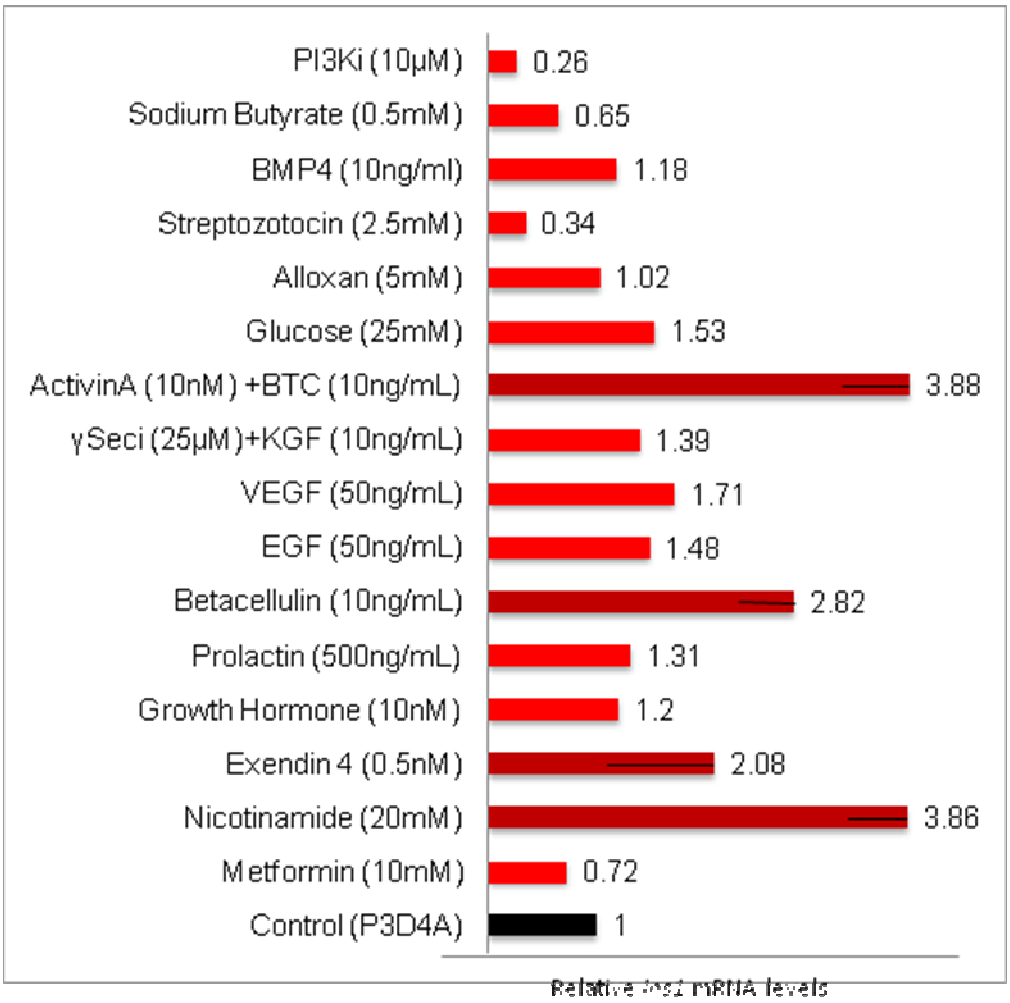


Figure 5.12. **Extrinsic factors can enhance the beta cell phenotype of de-differentiated hepatocytes transduced with pancreatic transcription factors.** The result of the screen for extrinsic factors that enhance the beta cell phenotype in reprogrammed hepatocytes. The relative amounts of *Ins1* mRNA was used as a read-out. The treatments were carried out on de-differentiated hepatocytes that had been transduced with Pdx1, Neurogenin3, NeuroD, Pax4 and MafA. Note that only the four highest scoring treatments (i.e. ActivinA and betacellulin in combination, betacellulin on its own, and, exendin-4 and nicotinamide on their own) were replicated (N=3) and the error bars are shown as –SEM below the mean.

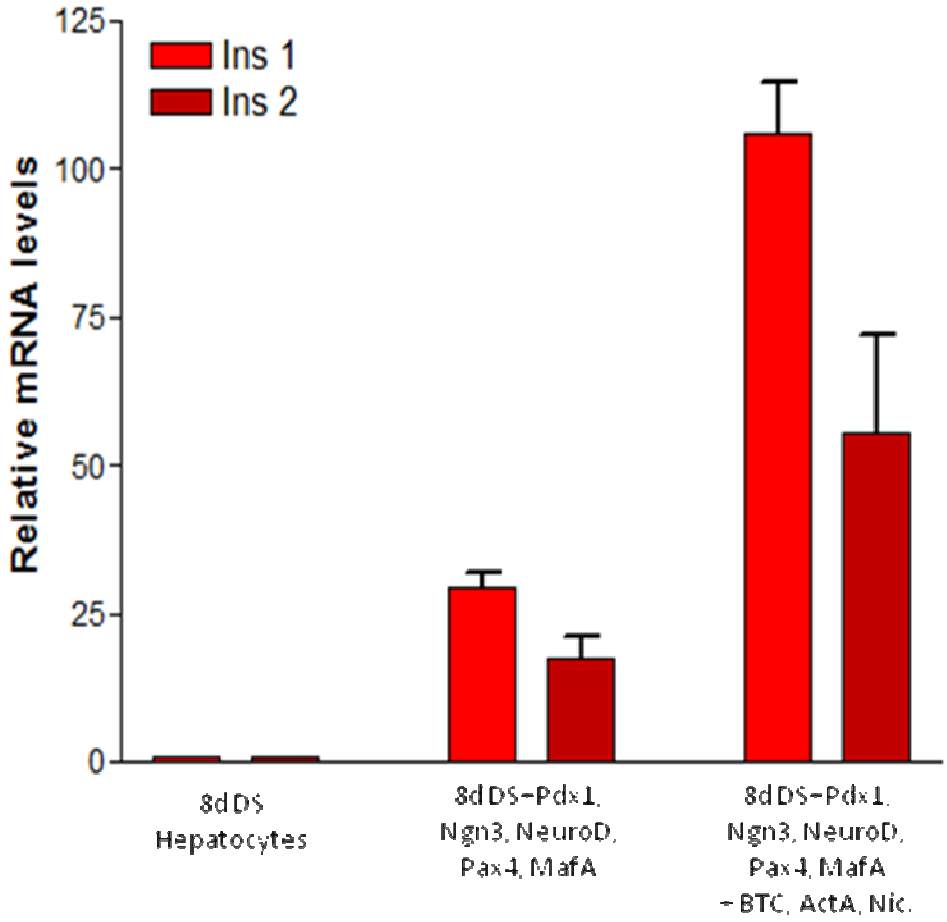


Figure 5.13. **Combinations of multiple extrinsic factors and transcription factors promote the beta cell phenotype of de-differentiated hepatocytes.** The effect of the combination of transcription factor transduction and extrinsic factor treatments on the levels of *ins1* and *ins2* expression in reprogrammed hepatocytes analysed by real time RT-PCR. Hepatocytes were cultured in DS medium and infected with the transcription factor coding adenoviruses on day three. The extrinsic factor treatments with 10ng/mL betacellulin (BTC), 10ng/mL ActivinA (ActA) and 20mM nicotinamide (Nic) were carried out daily for up to 8 days. The results are compared relative to the levels of *ins 1* expression (normalised to 1) in de-differentiated hepatocytes at day 8 without any transcription factor transduction and extrinsic factor treatment. n=3.

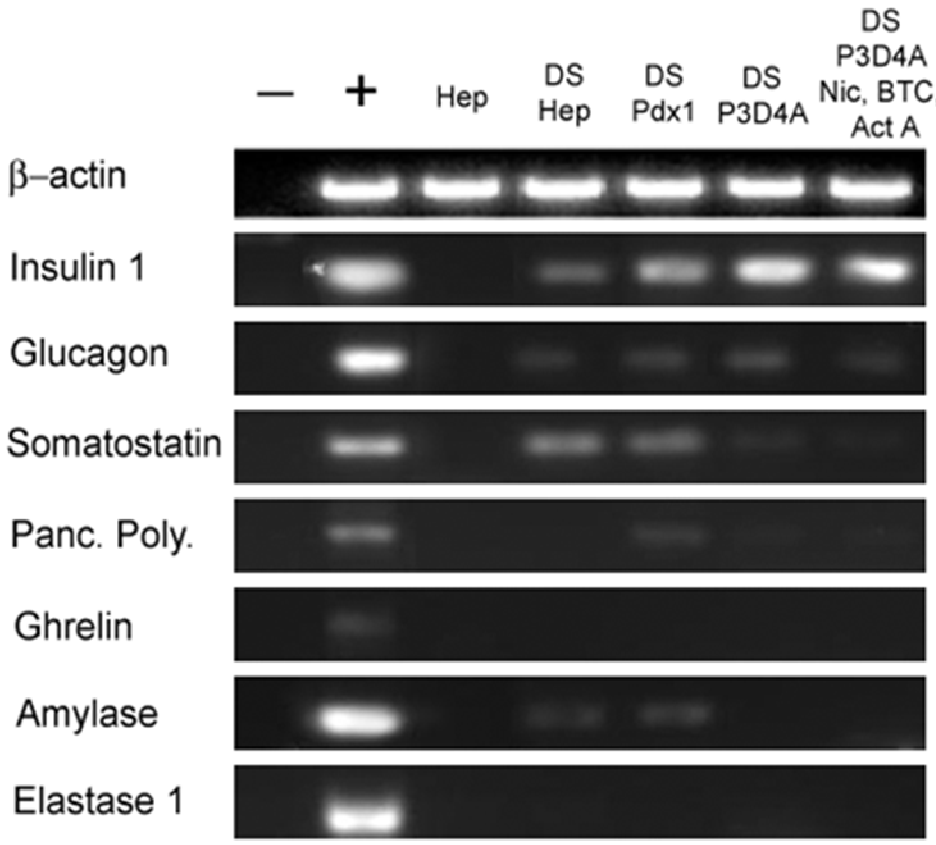


Figure 5.14. **The transcription factor and extrinsic factor combinations promote a beta cell phenotype in de-differentiated hepatocytes as opposed to any other pancreatic phenotype.** The expression of genes encoding endocrine hormones (*insulin1, glucagon, somatostatin, pancreatic polypeptide (Panc. Poly.)* and *ghrelin*) and exocrine enzymes (*amylase* and *elastase1*) in de-differentiated (DS) hepatocytes at different stages of reprogramming analysed by RT-PCR. cDNA from adult rat pancreas was used as the positive control. P3D4A- represents transduction of Pdx1, Neurogenin3, NeuroD, Pax4 and MafA; Nic, BTC, ActA represents treatment with nicotinamide, betacellulin and ActivinA.

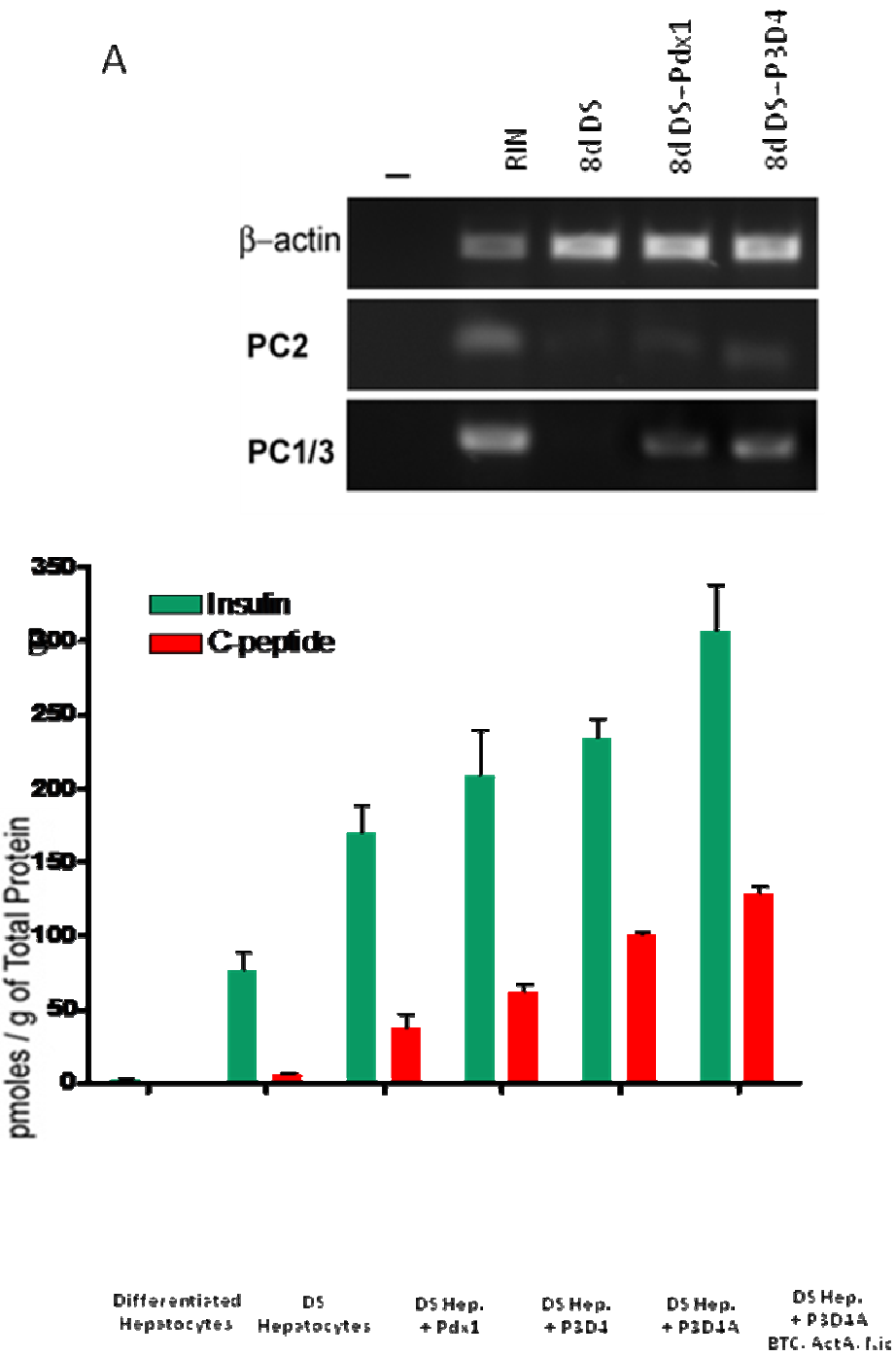


Figure 5.15. **Reprogrammed hepatocytes possess pro-insulin processing capabilities.** A- The induction of prohormone convertases 2 and 1/3 (*PC2* and *PC1/3*) expression in de-differentiated hepatocytes and its upregulation following multiple transcription factor transduction. cDNA from adult rat pancreas was used as the positive control. B- The levels of insulin protein (green) and C-peptide (red) in differentiated and de-differentiated (DS) hepatocytes with and without viral infection and/or extrinsic factor treatments. N=3.

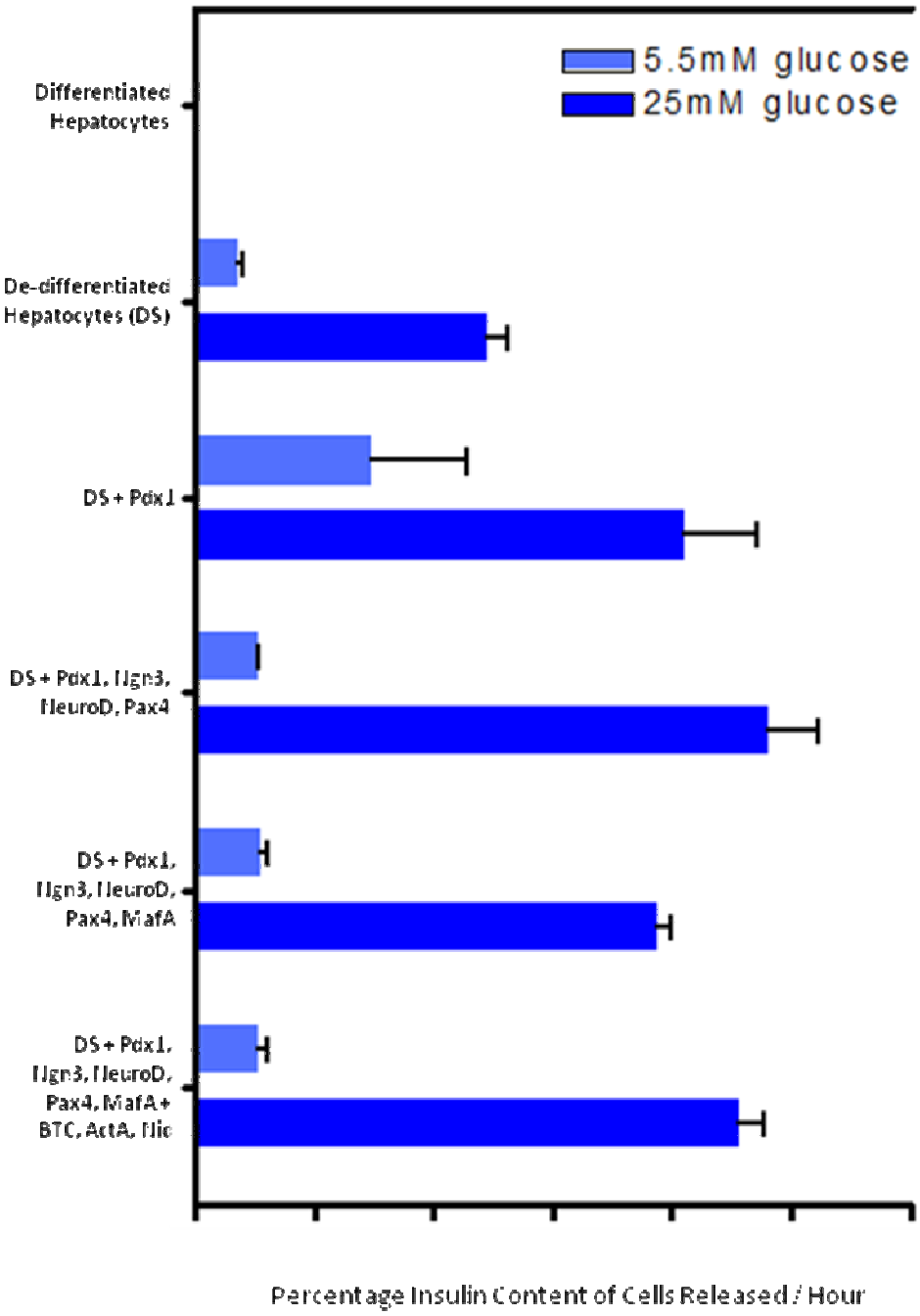


Figure 5.16. **Reprogrammed hepatocytes respond to changes in external glucose concentrations.** Secretion of insulin protein in differentiated and de-differentiated (DS) hepatocytes undergoing reprogramming analysed by insulin ELISA. There is a small effect of de-differentiation on glucose-stimulated insulin release, and a larger effect of Pdx1 and the other transcription factors as well as the extrinsic factor treatments on both constitutive insulin secretion at 5.5mM glucose and glucose-stimulated insulin secretion at 25mM glucose concentration after 1 hour of stimulation after 8 days of culture in DS medium.

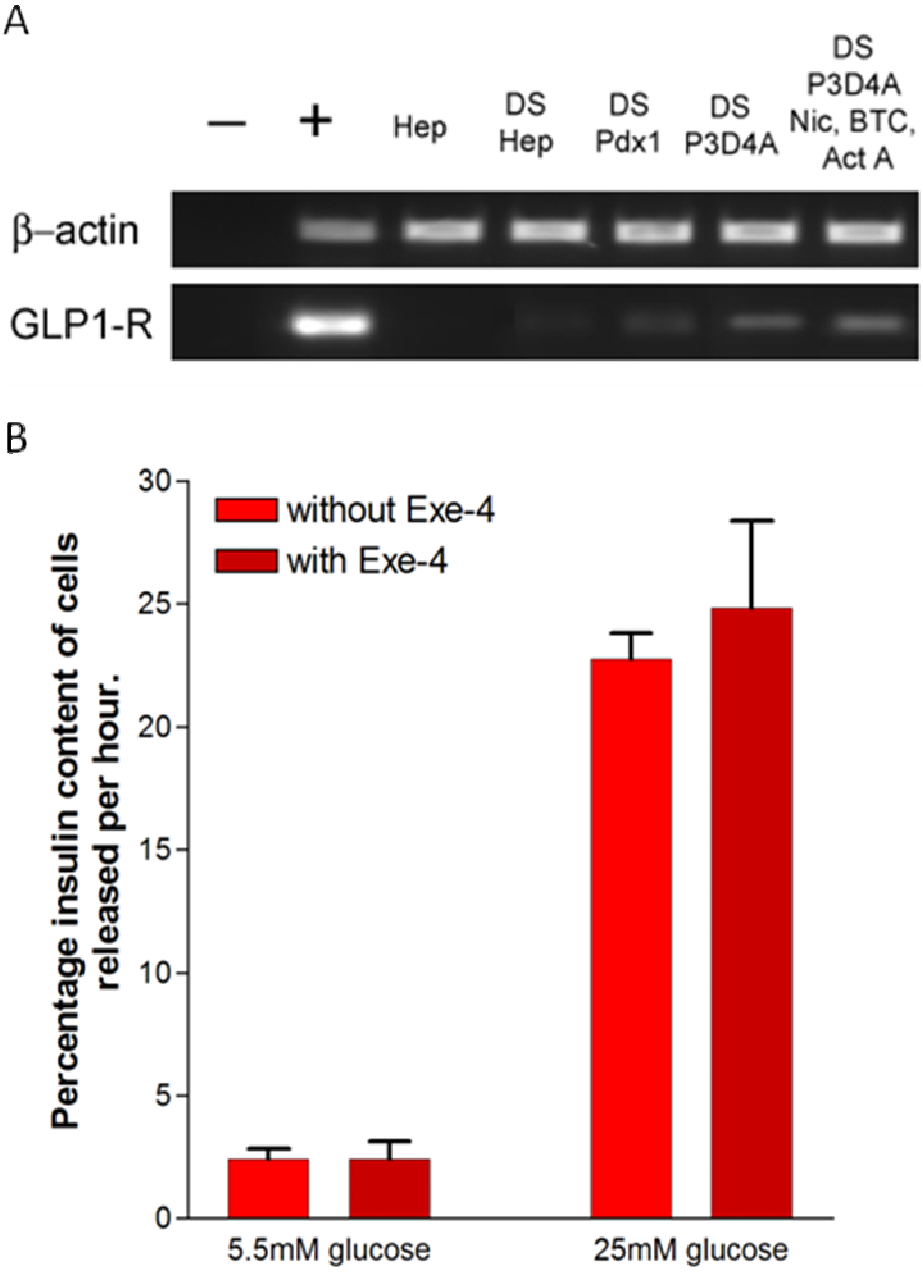


Figure 5.17. **Reprogrammed hepatocytes do not know any notable incretin responsiveness.** A- The expression of glucagon like peptide 1 receptor (GLP1-R) in differentiated and de-differentiated (DS) hepatocytes with and without transcription factor transduction and extrinsic factor treatments. cDNA from the adult rat pancreas was used as a positive control. B- The responsiveness of reprogrammed hepatocytes (transduced with Pdx1, Neurogenin3, NeuroD, Pax4 and MafA and then treated with betacellulin, ActivinA and nicotinamide) to the GLP1-R ligand exendin-4. The difference between percentage insulin content of cells released with and without exendin-4 in both high and low glucose are not statistically significant. n=3.

DISCUSSION

While the complex transcription factor networks constitute the intracellular signals that control beta cell differentiation, the extrinsic factors are a means of mimicking intercellular communication. The interplay between these intra- and intercellular components is necessary for cell specification, differentiation as well as the patterning of virtually homogenous progenitor cell populations into the well-organised, complex, functional organs. Most of these extrinsic factors influence some the major signalling pathways involved in pancreatic development, including the TGFβ, Notch, hedgehog, FGF and EGF pathways (Kim and Hebrok, 2001). Further knowledge of how these extrinsic factors govern normal pancreatic development would serve as a platform for their use in cellular reprogramming. Therefore the effect of betacellulin on the development of the embryonic pancreas was investigated in detail.

Addition of exogenous betacellulin induced multiple effects on embryonic pancreatic development. Betacellulin inhibited expression of amylase and glucagon, increased the expression of insulin and ghrelin and enhanced branching morphogenesis in an *in vitro* model of murine embryonic pancreas development. Betacellulin-null mice do not display any overt pancreatic defects, possibly due to functional redundancy and compensation by other EGFR ligands (Jackson et al., 2003). Pancreata of EGFR-deficient mice show impaired ductal branching, suggesting that this phenotype is mediated, in part, by the activation of a common EGFR downstream signalling pathway, possibly the MEK/ERK pathway (Miettinen et al., 2000, Rescan et al., 2005). To date, no study has examined the effects of betacellulin on branching morphogenesis in pancreatic explant cultures from E11.5d embryos. With CK19 as a marker of ductal cells, the results show that betacellulin increases branching morphogenesis. The mechanism of betacellulin action on ductal morphogenesis is probably mediated by increased differentiation from epithelial progenitors rather than by increased proliferation. This is reminiscent of the transdifferentiation of acinar cells to ductal cells that has previously been observed in culture (Rooman et al., 2000) and in transgenic

mice over-expressing *Pdx1* (Miyatsuka et al., 2006). Consistent with these studies an increase in the level of activated Stat3 is also seen. It is also probable that betacellulin is delaying or suppressing differentiation of the primordial duct-like epithelium into amylase-positive cells as a result of the continued expression of *Pdx1*. It is known that mesenchymal factors such as follistatin can regulate the proportion of exocrine and endocrine cell types (Miralles et al 1998). A reduction in the number of amylase-positive cells in the presence of mesenchymal proliferation was observed. Since the genes for *ErbB1* and *ErbB4* receptors are differentially expressed in both the mesenchyme and the epithelium, it is possible that the effect of betacellulin on both tissue types is independent rather than the change in amylase being brought about via a paracrine action emanating from the mesenchyme. Therefore the reduction in amylase-positive cells might also be mediated through apoptosis, since persistent upregulation of *Pdx*1 has previously been shown to destroy pancreatic acinar cells by the activation of the apoptotic pathways (Heller et al., 2001). It has also been observed that acinar apoptosis is associated with ductal proliferation after duct ligation (Abe and Watanabe, 1995). An upregulation of activated caspase-3 was seen, after betacellulin treatment and a concurrent decrease in the level of the transcription factor Ptf1a, which is necessary for early exocrine determination. One hypothesis to explain the pro-apoptotic action of betacellulin compared with other receptor tyrosine kinase ligands (e.g. EGF) is that it is acting through a different receptor or post-receptor events (Jones et al., 1999).

Both by immunofluorescence microscopy and western blotting, it was seen that betacellulin enhances the number of insulin-positive beta cells and the overall levels of insulin in the developing pancreas. Betacellulin is reported to regulate the proportion of beta cells in other systems (Demeterco et al., 2000, Huotari et al., 2002, Li et al., 2001). Three mechanisms might explain the increase in the number of beta cells. The additional beta cells could arise by: (1) the proliferation of existing beta cells; (2) the differentiation of endocrine precursor cells into beta cells rather than other endocrine cells; or (3) the conversion of exocrine and ductal cell types into beta cells. The first

possibility can be ruled out because if the extra beta cells did arise due to the proliferation of existing beta cells, larger islets would be seen and an increased number of cells that show co-localisation for PH3 and insulin. However this was not the case. Similarly, the third possibility is unlikely as exocrine cell apoptosis was widespread in betacellulin treated samples. Huotari *et al.,* (2002) described the modulation of endocrine lineages by different growth factors. In the absence of EGFR signalling, differentiation followed a default pathway towards alpha and PP cells. The reduction in the number of alpha and PP cells seen in the presence of betacellulin suggests that betacellulin might be influencing the fate choice made by the early endocrine precursor cells and shifting the balance towards a beta cell phenotype. All pancreatic endocrine cell types initially arise from common multipotent precursor cells that express Pdx1 but the continued expression of Pdx1 is only necessary for the development of beta cells and not other endocrine cell types (Jensen, 2004). The widespread late Pdx1 expression seen in the betacellulin-treated pancreata might therefore bias the commitment of endocrine precursors toward beta cells. Cell lineage studies have localised the endocrine progenitor cells close to the primitive ducts of the foetal pancreas (Ogata et al., 2004). The Pdx1 expression that is not restricted to the islets is seen in these regions after betacellulin treatment. During proliferation, duct cells have the potential to lose their ductal phenotype and revert back to multipotent cells (Bonner-Weir et al., 2000). This is achieved by the ductal cells expressing Pdx1, differentiating into beta cells, then migrating away from the ducts and clustering to form islets (Abe and Watanabe, 1995). The possibility of duct cell derived beta cells in this manner could explain the enhanced number of Pdx1-expressing cells in the ducts, the presence of islet-like cell clusters and the insulin-positive cells found in isolation. So, betacellulin is probably influencing beta cell formation both by increasing the proportion of endocrine cells and by increasing the proportion of endocrine precursors that become beta cells.

Collectively the changes seen may largely be brought about by prolonged ubiquitous expression of Pdx1. Elevated Pdx1 expression suppresses early differentiation of the endodermal pancreatic precursor tissue and enhances

branching and formation of beta cells. This makes betacellulin a hopeful candidate for stimulating beta cell regeneration *in vivo*, or increasing the proportion of beta cells *in vitro* in situations where beta cell differentiation is being sought with a view to transplantation.

In light of such evidence, betacellulin transduction has been used in combination with NeuroD to trigger formation of insulin-secreting cells in the liver and completely reverse diabetes in STZ-treated mice (Kojima et al., 2003). The result of treating reprogrammed hepatocytes with betacellulin was promising, giving a much more enhanced level of insulin expression compared to the other treatments. All the factors that were highlighted along with betacellulin in the screen (Activin A and nicotinamide) have all be shown to facilitate beta cell differentiation (eg:- Sjoholm et al., 1994, Mashima et al., 1996, D'Amour et al., 2006) although not in combination nor from reprogrammed hepatocytes. Furthermore, nicotinamide has also been shown to protect beta cells against necrosis thereby proving useful for intervention trials undertaken to prevent type I diabetes in high risk subjects (Hoorens and Pipeleers, 1999). Factors such as betacellulin and nicotinamide have effects on both undifferentiated progenitors in terms of differentiation and on differentiated mature beta cells in terms of insulin secretion. The inconsistency of the results with the other extrinsic factors (that affects either progenitors or mature beta cells) could be in part due to the heterogeneity of the treated cell population, where the proportions of cells at each stage of differentiation are not identical each time. Therefore, a more directed stepwise differentiation protocol and prior selection might be necessary to see an effect with the other extrinsic factors rather than treating a heterogeneous population.

The treatment with betacellulin, Activin A and nicotinamide following transcription factor transductions brings hepatocyte reprogramming a step closer to producing mature beta-like cells. Following the treatments and transcription factor over-expression, a propitious shift towards homogeneity and concurrent diminution of exocrine and ‘non-beta’ endocrine genes was observed. This is partly due the inclusion of transcription factors specific to mature beta cells such as MafA as well as the influence of betacellulin (and

possibly Activin A and nicotinamide) in directing beta cell differentiation at the expense of other cell types as discussed above for the organ cultures.

At the final stage of differentiation described here, the reprogrammed hepatocytes exhibited several properties of mature beta cells. The cells were capable of processing pro-insulin into mature insulin and were responsive to changes in external glucose concentrations. At a low external glucose concentration, the level of insulin secretion was present though minimal. However, this constitutive insulin secretion is paramount for beta cells to maintain their sensitivity to glucose as well as to amplify the glucose-stimulated insulin response at higher glucose concentrations (Srivastava and Goren, 2003). The percentage of insulin secreted in response to high glucose by reprogrammed hepatocytes was above ~15%. Although a direct comparison was not made with bone-fide beta cells or isolated islets, based on the literature, the percentage of stored insulin released by beta cells in response to glucose is only 2-4%. The reason for this difference could either be because the reprogrammed hepatocytes exhibit only low levels of stored insulin compared to beta cells or are still immature and lack some of the molecular machinery needed for efficient regulated insulin secretion. The latter reason could likewise explain the low response to incretins that was also observed.

In summary, the extrinsic factor treatments proved beneficial in pushing the beta-like cells generated from reprogrammed hepatocytes towards maturity. It was not sufficient to produce cells that exactly mimic beta cell function; nonetheless many of the criteria were fulfilled. Methods for further maturation will be needed and await elucidation. Although sequential transcription factor transduction of primary hepatocytes is not a reality at moment, such an approach may be redeemed in order to bring about a more directed differentiation towards mature beta-like cells using the transcription factors and extrinsic signals identified.

Chapter SIX

General Discussion

GENERAL DISCUSSION AND CONCLUSION

The molecular reprogramming of hepatocytes into insulin producing beta-like cells has challenged our view of the rigidity of the differentiated phenotype of cells. It has also brought us a step closer in producing extra-pancreatic surrogate beta cells for diabetes therapy. Overall the results reported in this thesis outline the stepwise development of a protocol for the generation of functional beta-like cells from hepatocytes by molecular reprogramming strategies. Firstly the phenomenon of de-differentiation, though long considered a hindrance for hepatocyte culture, in facts acts in favour of molecular reprogramming. De-differentiation of hepatocytes resulted in the induction of mature pancreatic and pancreatic progenitor-specific genes, while synergising the effects of the transduced Pdx1. However it can be semantically argued that this does not constitute a true transdifferentiation event as previously envisaged, as the change is mediated via a progenitor-like de-differentiated intermediate instead of being a direct conversion. Furthermore, it is imperative that the mechanism behind this spontaneous gene induction be elucidated. It would be important to ascertain the changes taking place at the chromatin level in terms of switching off the hepatic genes and dictating the transcriptional activation of the pancreatic genes. This would enable us to bring about similar changes in gene expression through a more defined protocol- encompassing more specific chromatin remodelling agents, siRNA gene-silencing techniques as well as selecting the particular components of the DS media that are preponderant in the de-differentiation process.

Although many approaches have been used in the past to generate beta-like cells from hepatocytes, those involving *in vitro* molecular reprogramming strategies are very limited. Furthermore, no other study has utilised a protocol integrating such a broad combination of transcription factors (Pdx1, Neurogenin3, NeuroD, Pax4 and MafA) along with a unique combination of extrinsic factors (betacellulin, nicotinamide and Activin A). However, it is important to note that the list of transcription factors screened while extensive was not exhaustive. It was based on the resources (in terms of

adenoviral constructs) available at the time. Of late, there has been tremendous progress in revealing the emerging role of novel transcription factors (such as FoxO1) in beta cell differentiation and function (Glauser and Schlegel, 2007). Similarly several novel extrinsic factors and small molecules have been implied in beta cell differentiation, while the role of others have turned out to be more disputable (reviewed in Oliver-Krasinski and Stoffers, 2008). Notwithstanding such emerging inconsistencies, the transcription factors and extrinsic factors used here have long been established as positive modulators of beta cell development and function.

It is possible to generate insulin producing cells that are capable of processing pro-insulin and secreting insulin in a glucose-responsive manner. Despite this, a significant challenge that remains to be overcome is the overall low levels of insulin protein that was present, which was only a fraction (approximately $1/10^4$) of the level in normal beta cells. A major part of continuing research should thus investigate means of intensifying the levels of insulin in the reprogrammed cells. Firstly, the extracellular factors during late-embryonic and neonatal beta cell development, of which we know little about, should be taken into consideration along with the indispensable importance of intercellular and epithelial-mesenchymal interactions. Secondly, the low levels of insulin could be due to the poor accessibility of the promoter targets for the transcription factors to have any effect. Therefore as already mentioned above, more efficient chromatin remodelling agents would be necessary to significantly improve the performance of the transcription factors themselves. Attaining immunodetectable levels of insulin would enable us to assess the efficiency of the reprogramming process in obtaining a potentially limitless source of stable and mature beta-like cells. This will in turn impinge on further improving the fidelity of the reprogramming protocol in a more systemic manner.

Parallel studies also need to be carried out in assessing the *in vivo* functionality of the cells generated, in their competence to rescue hyperglycaemia in rodent models of diabetes. Surrogate beta cells can be transplanted into the liver via the hepatic vein, under the kidney capsule or into the epididymal fat pads of alloxan or steptozotocin-induced

hyperglycaemic SCID or NOD/SCID mice. In light of recent evidence, it might not be necessary to delay transplantation until fully mature beta cells are obtainable by the reprogramming protocol. For instance, when deriving insulin-expressing cells from human ES cells, transplanting progenitor-like pancreatic endodermal cells enable them to mature *in vivo* into single-hormone expressing endocrine cells more effectively, compared to transplantation of cells from later stages of the differentiation protocol (D'Amour et al., 2006, Kroon et al., 2008). Therefore the transplantation of more immature, endocrine or islet progenitor-like cells derived from de-differentiated hepatocytes, into a diabetic environment could facilitate their differentiation into mature beta cells more efficiently under conditions otherwise irreproducible *in vitro*.

Beta-like cells generated from hepatocytes by molecular reprogramming might not be capable of recapitulating all the functions of *bone fide* beta cells. Yet it would be beneficial in generating *in vitro*, a quality controlled and carefully selected source of cells to complement the inadequate supply of islets and pancreata available for transplantation. Moreover, it serves as a general paradigm for treating type 1 diabetes by cell-based therapies utilising beta cells generated *in vitro*.

BIBLIOGRAPHY

ABE, K. & WATANABE, S. (1995) Apoptosis of mouse pancreatic acinar cells after duct ligation. *Arch Histol Cytol,* 58**,** 221-9.

ACKERMANN, A. M. & GANNON, M. (2007) Molecular regulation of pancreatic beta cell mass development, maintenance, and expansion. *J Mol Endocrinol,* 38**,** 193-206.

AHLGREN, U., PFAFF, S. L., JESSELL, T. M., EDLUND, T. & EDLUND, H. (1997) Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. *Nature,* 385**,** 257-60.

AHREN, B. (1998) Glucagon-like peptide-1 (GLP-1): a gut hormone of potential interest in the treatment of diabetes. *Bioessays,* 20**,** 642-51.

ALDER, H. & SCHMID, V. (1987) Cell cycles and in vitro transdifferentiation and regeneration of isolated, striated muscle of jellyfish. *Dev Biol,* 124**,** 358-69.

ARTERBURN, L. M., ZURLO, J., YAGER, J. D., OVERTON, R. M. & HEIFETZ, A. H. (1995) A morphological study of differentiated hepatocytes in vitro. *Hepatology,* 22**,** 175-87.

ATOUF, F., PARK, C. H., PECHHOLD, K., TA, M., CHOI, Y. & LUMELSKY, N. L. (2007) No evidence for mouse pancreatic beta cell epithelial-mesenchymal transition in vitro. *Diabetes,* 56**,** 699-702.

AUERBACH, O., STOUT, A. P., HAMMOND, E. C. & GARFINKEL, L. (1961) Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer. *N Engl J Med,* 265**,** 253-67.

BAEYENS, L., DE BREUCK, S., LARDON, J., MFOPOU, J. K., ROOMAN, I. & BOUWENS, L. (2005) In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. *Diabetologia,* 48**,** 49-57.

BAKER, T. K., CARFAGNA, M. A., GAO, H., DOW, E. R., LI, Q., SEARFOSS, G. H. & RYAN, T. P. (2001) Temporal gene expression analysis of monolayer cultured rat hepatocytes. *Chem Res Toxicol,* 14**,** 1218-31.

BARRETT, N. R. (1957) The lower esophagus lined by columnar epithelium. *Surgery,* 41**,** 881-94.

BARROW, J., HAY, C. W., FERGUSON, L. A., DOCHERTY, H. M. & DOCHERTY, K. (2006) Transcription factor cycling on the insulin promoter. *FEBS Lett,* 580**,** 711-5.

BARTHOLOMEUSZ, R. K., CAMPBELL, I. L. & HARRISON, L. C. (1989) Pancreatic islet A2B5- and 3G5-reactive gangliosides are markers of differentiation in rat insulinoma cells. *Endocrinology,* 124**,** 2680-5.

BEATTIE, G. M., MONTGOMERY, A. M., LOPEZ, A. D., HAO, E., PEREZ, B., JUST, M. L., LAKEY, J. R., HART, M. E. & HAYEK, A. (2002) A novel approach to increase human islet cell mass while preserving beta cell function. *Diabetes,* 51**,** 3435-9.

BECK, C. W. & SLACK, J. M. (1999) Gut specific expression using mammalian promoters in transgenic Xenopus laevis. *Mech Dev,* 88**,** 221-7.

BER, I., SHTERNHALL, K., PERL, S., OHANUNA, Z., GOLDBERG, I., BARSHACK, I., BENVENISTI-ZARUM, L., MEIVAR-LEVY, I. & FERBER, S. (2003) Functional,

persistent, and extended liver to pancreas transdifferentiation. *J Biol Chem,* 278**,** 31950-7.

BILLESTRUP, N. & NIELSEN, J. H. (1991) The stimulatory effect of growth hormone, prolactin, and placental lactogen on beta cell proliferation is not mediated by insulin-like growth factor-I. *Endocrinology,* 129**,** 883-8.

BILLESTRUP, N. & OTONKOSKI, T. (2008) Dedifferentiation for replication of human beta cells: a division between mice and men? *Diabetes,* 57**,** 1457-8.

BJERKVIG, R., TYSNES, B. B., ABOODY, K. S., NAJBAUER, J. & TERZIS, A. J. (2005) Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat Rev Cancer,* 5**,** 899-904.

BLYSZCZUK, P., ASBRAND, C., ROZZO, A., KANIA, G., ST-ONGE, L., RUPNIK, M. & WOBUS, A. M. (2004) Embryonic stem cells differentiate into insulin-producing cells without selection of nestin-expressing cells. *Int J Dev Biol,* 48**,** 1095-104.

BONNER-WEIR, S., DEERY, D., LEAHY, J. L. & WEIR, G. C. (1989) Compensatory growth of pancreatic beta cells in adult rats after short-term glucose infusion. *Diabetes,* 38**,** 49-53.

BONNER-WEIR, S., TANEJA, M., WEIR, G. C., TATARKIEWICZ, K., SONG, K. H., SHARMA, A. & O'NEIL, J. J. (2000) In vitro cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci U S A,* 97**,** 7999-8004.

BOUWENS, L., BRAET, F. & HEIMBERG, H. (1995) Identification of rat pancreatic duct cells by their expression of cytokeratins 7, 19, and 20 in vivo and after isolation and culture. *J Histochem Cytochem,* 43**,** 245-53.

BOUWENS, L. & ROOMAN, I. (2005) Regulation of pancreatic beta cell mass. *Physiol Rev,* 85**,** 1255-70.

BRELJE, T. C., SCHARP, D. W., LACY, P. E., OGREN, L., TALAMANTES, F., ROBERTSON, M., FRIESEN, H. G. & SORENSON, R. L. (1993) Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. *Endocrinology,* 132**,** 879-87.

BRISSOVA, M., SHIOTA, M., NICHOLSON, W. E., GANNON, M., KNOBEL, S. M., PISTON, D. W., WRIGHT, C. V. & POWERS, A. C. (2002) Reduction in pancreatic transcription factor PDX-1 impairs glucose-stimulated insulin secretion. *J Biol Chem,* 277**,** 11225-32.

BROCKES, J. P. & KUMAR, A. (2002) Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nat Rev Mol Cell Biol,* 3**,** 566-74.

BROWNLEE, M. (2001) Biochemistry and molecular cell biology of diabetic complications. *Nature,* 414**,** 813-20.

BRUBAKER, P. L. & DRUCKER, D. J. (2004) Minireview: Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. *Endocrinology,* 145**,** 2653-9.

BURKE, Z. D., SHEN, C. N., RALPHS, K. L. & TOSH, D. (2006) Characterization of liver function in transdifferentiated hepatocytes. *J Cell Physiol,* 206**,** 147-59.

BUTLER, A. E., JANSON, J., BONNER-WEIR, S., RITZEL, R., RIZZA, R. A. & BUTLER, P. C. (2003) Beta cell deficit and increased beta cell apoptosis in humans with type 2 diabetes. *Diabetes,* 52**,** 102-10.

CEREGHINI, S. (1996) Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J,* 10**,** 267-82.

CERF, M. E. (2006) Transcription factors regulating beta cell function. *Eur J Endocrinol,* 155**,** 671-9.

CHAKRABARTI, S. K. & MIRMIRA, R. G. (2003) Transcription factors direct the development and function of pancreatic beta cells. *Trends Endocrinol Metab,* 14**,** 78-84.

CHATTERJEE, A. K., SIERADZKI, J. & SCHATZ, H. (1986) Epidermal growth factor stimulates (pro-)insulin biosynthesis and 3H-thymidine incorporation in isolated pancreatic rat islets. *Horm Metab Res,* 18**,** 873-4.

CHEN, K. & RAJEWSKY, N. (2007) The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet,* 8**,** 93-103.

COSTA, R. H., KALINICHENKO, V. V., HOLTERMAN, A. X. & WANG, X. (2003) Transcription factors in liver development, differentiation, and regeneration. *Hepatology,* 38**,** 1331-47.

COSTA, R. H., KALINICHENKO, V. V. & LIM, L. (2001) Transcription factors in mouse lung development and function. *Am J Physiol Lung Cell Mol Physiol,* 280**,** L823-38.

COURT, F. G., WEMYSS-HOLDEN, S. A., DENNISON, A. R. & MADDERN, G. J. (2003) Bioartificial liver support devices: historical perspectives. *ANZ J Surg,* 73**,** 739-48.

CUNHA, D. A., CARNEIRO, E. M., ALVES MDE, C., JORGE, A. G., DE SOUSA, S. M., BOSCHERO, A. C., SAAD, M. J., VELLOSO, L. A. & ROCHA, E. M. (2005) Insulin secretion by rat lacrimal glands: effects of systemic and local variables. *Am J Physiol Endocrinol Metab,* 289**,** E768-75.

CUNHA, G. R. (1976) Stromal induction and specification of morphogenesis and cytodifferentiation of the epithelia of the Mullerian ducts and urogenital sinus during development of the uterus and vagina in mice. *J Exp Zool,* 196**,** 361-70.

D'AMOUR, K. A., AGULNICK, A. D., ELIAZER, S., KELLY, O. G., KROON, E. & BAETGE, E. E. (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol,* 23**,** 1534-41.

D'AMOUR, K. A., BANG, A. G., ELIAZER, S., KELLY, O. G., AGULNICK, A. D., SMART, N. G., MOORMAN, M. A., KROON, E., CARPENTER, M. K. & BAETGE, E. E. (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol,* 24**,** 1392-401.

DAHL, U., SJODIN, A. & SEMB, H. (1996) Cadherins regulate aggregation of pancreatic beta cells in vivo. *Development,* 122**,** 2895-902.

DAVIS, R. L., WEINTRAUB, H. & LASSAR, A. B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell,* 51**,** 987-1000.

DE LOTT, L. B., MORRISON, C., SUSTER, S., COHN, D. E. & FRANKEL, W. L. (2005) CDX2 is a useful marker of intestinal-type differentiation: a tissue microarray-based study of 629 tumors from various sites. *Arch Pathol Lab Med,* 129**,** 1100-5.

DEMETERCO, C., BEATTIE, G. M., DIB, S. A., LOPEZ, A. D. & HAYEK, A. (2000) A role for activin A and betacellulin in human fetal pancreatic cell differentiation and growth. *J Clin Endocrinol Metab,* 85**,** 3892-7.

DESCOMBES, P. & SCHIBLER, U. (1991) A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell,* 67**,** 569-79.

DEUTSCH, G., JUNG, J., ZHENG, M., LORA, J. & ZARET, K. S. (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. *Development,* 128**,** 871-81.

DEVASKAR, S. U., GIDDINGS, S. J., RAJAKUMAR, P. A., CARNAGHI, L. R., MENON, R. K. & ZAHM, D. S. (1994) Insulin gene expression and insulin synthesis in mammalian neuronal cells. *J Biol Chem,* 269**,** 8445-54.

DOCHERTY, H. M., HAY, C. W., FERGUSON, L. A., BARROW, J., DURWARD, E. & DOCHERTY, K. (2005) Relative contribution of PDX-1, MafA and E47/beta2 to the regulation of the human insulin promoter. *Biochem J,* 389**,** 813-20.

DOCHERTY, K. (2001) Growth and development of the islets of Langerhans: implications for the treatment of diabetes mellitus. *Curr Opin Pharmacol,* 1**,** 641-50.

DOCHERTY, K., BERNARDO, A. S. & VALLIER, L. (2007) Embryonic stem cell therapy for diabetes mellitus. *Semin Cell Dev Biol,* 18**,** 827-38.

DOCHERTY, K. & CLARK, A. R. (1994) Nutrient regulation of insulin gene expression. *FASEB J,* 8**,** 20-7.

DOR, Y., BROWN, J., MARTINEZ, O. I. & MELTON, D. A. (2004) Adult pancreatic beta cells are formed by self-duplication rather than stem-cell differentiation. *Nature,* 429**,** 41-6.

DRUCKER, D. J. (2002) Biological actions and therapeutic potential of the glucagon-like peptides. *Gastroenterology,* 122**,** 531-44.

DUNBAR, A. J. & GODDARD, C. (2000) Structure-function and biological role of betacellulin. *Int J Biochem Cell Biol,* 32**,** 805-15.

DUNNE, M. J. (2000) Ions, genes and insulin release: from basic science to clinical disease. Based on the 1998 R. D. Lawrence Lecture. *Diabet Med,* 17**,** 91-104.

EDLUND, H. (2002) Pancreatic organogenesis--developmental mechanisms and implications for therapy. *Nat Rev Genet,* 3**,** 524-32.

ELAUT, G., HENKENS, T., PAPELEU, P., SNYKERS, S., VINKEN, M., VANHAECKE, T. & ROGIERS, V. (2006) Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. *Curr Drug Metab,* 7**,** 629-60.

FAJANS, S. S., BELL, G. I. & POLONSKY, K. S. (2001) Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. *N Engl J Med,* 345**,** 971-80.

FERBER, S., HALKIN, A., COHEN, H., BER, I., EINAV, Y., GOLDBERG, I., BARSHACK, I., SEIJFFERS, R., KOPOLOVIC, J., KAISER, N. & KARASIK, A. (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. *Nat Med,* 6**,** 568-72.

FERRANNINI, E. & COBELLI, C. (1987) The kinetics of insulin in man. II. Role of the liver. *Diabetes Metab Rev,* 3**,** 365-97.

FERRIER, D. E., DEWAR, K., COOK, A., CHANG, J. L., HILL-FORCE, A. & AMEMIYA, C. (2005) The chordate ParaHox cluster. *Curr Biol,* 15**,** R820-2.

FINEGOOD, D. T., SCAGLIA, L. & BONNER-WEIR, S. (1995) Dynamics of beta cell mass in the growing rat pancreas. Estimation with a simple mathematical model. *Diabetes,* 44**,** 249-56.

FONDEVILA, C., BUSUTTIL, R. W. & KUPIEC-WEGLINSKI, J. W. (2003) Hepatic ischemia/reperfusion injury--a fresh look. *Exp Mol Pathol,* 74**,** 86-93.

FORINO, M., TORREGROSSA, R., CEOL, M., MURER, L., DELLA VELLA, M., DEL PRETE, D., D'ANGELO, A. & ANGLANI, F. (2006) TGFbeta1 induces epithelial-mesenchymal transition, but not myofibroblast transdifferentiation of human kidney tubular epithelial cells in primary culture. *Int J Exp Pathol,* 87**,** 197-208.

GAO, R., USTINOV, J., PULKKINEN, M. A., LUNDIN, K., KORSGREN, O. & OTONKOSKI, T. (2003) Characterization of endocrine progenitor cells and critical factors for their differentiation in human adult pancreatic cell culture. *Diabetes,* 52**,** 2007-15.

GEORGIA, S. & BHUSHAN, A. (2004) Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest,* 114**,** 963-8.

GERMAN, M. S. & WANG, J. (1994) The insulin gene contains multiple transcriptional elements that respond to glucose. *Mol Cell Biol,* 14**,** 4067-75.

GERSHENGORN, M. C., HARDIKAR, A. A., WEI, C., GERAS-RAAKA, E., MARCUS-SAMUELS, B. & RAAKA, B. M. (2004) Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. *Science,* 306**,** 2261-4.

GHOSH, S., THOROGOOD, P. & FERRETTI, P. (1994) Regenerative capability of upper and lower jaws in the newt. *Int J Dev Biol,* 38**,** 479-90.

GIERL, M. S., KAROULIAS, N., WENDE, H., STREHLE, M. & BIRCHMEIER, C. (2006) The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. *Genes Dev,* 20**,** 2465-78.

GILON, P., RAVIER, M. A., JONAS, J. C. & HENQUIN, J. C. (2002) Control mechanisms of the oscillations of insulin secretion in vitro and in vivo. *Diabetes,* 51 Suppl 1**,** S144-51.

GLAUSER, D. A. & SCHLEGEL, W. (2007) The emerging role of FOXO transcription factors in pancreatic beta cells. *J Endocrinol,* 193**,** 195-207.

GLICK, E., LESHKOWITZ, D. & WALKER, M. D. (2000) Transcription factor BETA2 acts cooperatively with E2A and PDX1 to activate the insulin gene promoter. *J Biol Chem,* 275**,** 2199-204.

GRADWOHL, G., DIERICH, A., LEMEUR, M. & GUILLEMOT, F. (2000) neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A,* 97**,** 1607-11.

GRAFI, G. (2004) How cells dedifferentiate: a lesson from plants. *Dev Biol,* 268**,** 1-6.

GRETEN, F. R., WEBER, C. K., GRETEN, T. F., SCHNEIDER, G., WAGNER, M., ADLER, G. & SCHMID, R. M. (2002) Stat3 and NF-kappaB activation prevents apoptosis in pancreatic carcinogenesis. *Gastroenterology,* 123**,** 2052-63.

GROGG, M. W., CALL, M. K., OKAMOTO, M., VERGARA, M. N., DEL RIO-TSONIS, K. & TSONIS, P. A. (2005) BMP inhibition-driven regulation of six-3 underlies induction of newt lens regeneration. *Nature,* 438**,** 858-62.

GUILLOUZO, A. (1986) IN GUILLOUZO, A. & GUGUEN-GUILLOUZO, C. (Eds.) *In Isolated and Cultured Hepatocytes.* London, John Libbey & Co.

HAGMAN, D. K., HAYS, L. B., PARAZZOLI, S. D. & POITOUT, V. (2005) Palmitate inhibits insulin gene expression by altering PDX-1 nuclear localization and reducing MafA expression in isolated rat islets of Langerhans. *J Biol Chem,* 280**,** 32413-8.

HALBAN, P. A. (1994) Proinsulin processing in the regulated and the constitutive secretory pathway. *Diabetologia,* 37 Suppl 2**,** S65-72.

HARPER, M. E., ULLRICH, A. & SAUNDERS, G. F. (1981) Localization of the human insulin gene to the distal end of the short arm of chromosome 11. *Proc Natl Acad Sci U S A,* 78**,** 4458-60.

HARRISON, K. A., THALER, J., PFAFF, S. L., GU, H. & KEHRL, J. H. (1999) Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice. *Nat Genet,* 23**,** 71-5.

HAY, C. W. & DOCHERTY, K. (2006) Comparative analysis of insulin gene promoters: implications for diabetes research. *Diabetes,* 55**,** 3201-13.

HAYHURST, G. P., LEE, Y. H., LAMBERT, G., WARD, J. M. & GONZALEZ, F. J. (2001) Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol,* 21**,** 1393-403.

HEBROK, M., KIM, S. K. & MELTON, D. A. (1998) Notochord repression of endodermal Sonic hedgehog permits pancreas development. *Genes Dev,* 12**,** 1705-13.

HEBROK, M., KIM, S. K., ST JACQUES, B., MCMAHON, A. P. & MELTON, D. A. (2000) Regulation of pancreas development by hedgehog signalling. *Development,* 127**,** 4905-13.

HEIMBERG, H., DE VOS, A., MOENS, K., QUARTIER, E., BOUWENS, L., PIPELEERS, D., VAN SCHAFTINGEN, E., MADSEN, O. & SCHUIT, F. (1996) The glucose sensor protein glucokinase is expressed in glucagon-producing alpha-cells. *Proc Natl Acad Sci U S A,* 93**,** 7036-41.

HELLER, R. S., STOFFERS, D. A., BOCK, T., SVENSTRUP, K., JENSEN, J., HORN, T., MILLER, C. P., HABENER, J. F., MADSEN, O. D. & SERUP, P. (2001) Improved glucose tolerance and acinar dysmorphogenesis by targeted expression of transcription factor PDX-1 to the exocrine pancreas. *Diabetes,* 50**,** 1553-61.

HERRERA, P. L. (2000) Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development,* 127**,** 2317-22.

HOLLAND, A. M., HALE, M. A., KAGAMI, H., HAMMER, R. E. & MACDONALD, R. J. (2002) Experimental control of pancreatic development and maintenance. *Proc Natl Acad Sci U S A,* 99**,** 12236-41.

HONDA, T., SAITOH, H., MASUKO, M., KATAGIRI-ABE, T., TOMINAGA, K., KOZAKAI, I., KOBAYASHI, K., KUMANISHI, T., WATANABE, Y. G., ODANI, S. & KUWANO, R. (2000) The coxsackievirus-adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. *Brain Res Mol Brain Res,* 77**,** 19-28.

HOORENS, A. & PIPELEERS, D. (1999) Nicotinamide protects human beta cells against chemically-induced necrosis, but not against cytokine-induced apoptosis. *Diabetologia,* 42**,** 55-9.

HORB, M. E., SHEN, C. N., TOSH, D. & SLACK, J. M. (2003) Experimental conversion of liver to pancreas. *Curr Biol,* 13**,** 105-15.

HORI, Y., RULIFSON, I. C., TSAI, B. C., HEIT, J. J., CAHOY, J. D. & KIM, S. K. (2002) Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proc Natl Acad Sci U S A,* 99**,** 16105-10.

HOWITT, J., ANDERSON, C. W. & FREIMUTH, P. (2003) Adenovirus interaction with its cellular receptor CAR. *Curr Top Microbiol Immunol,* 272**,** 331-64.

HUI, H., WRIGHT, C. & PERFETTI, R. (2001) Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells. *Diabetes,* 50**,** 785-96.

HUOTARI, M. A., MIETTINEN, P. J., PALGI, J., KOIVISTO, T., USTINOV, J., HARARI, D., YARDEN, Y. & OTONKOSKI, T. (2002) ErbB signalling regulates lineage determination of developing pancreatic islet cells in embryonic organ culture. *Endocrinology,* 143**,** 4437-46.

HUOTARI, M. A., PALGI, J. & OTONKOSKI, T. (1998) Growth factor-mediated proliferation and differentiation of insulin-producing INS-1 and RINm5F cells: identification of betacellulin as a novel beta cell mitogen. *Endocrinology,* 139**,** 1494-9.

HUTTON, J. C. (1994) Insulin secretory granule biogenesis and the proinsulin-processing endopeptidases. *Diabetologia,* 37 Suppl 2**,** S48-56.

IMAI, J., KATAGIRI, H., YAMADA, T., ISHIGAKI, Y., OGIHARA, T., UNO, K., HASEGAWA, Y., GAO, J., ISHIHARA, H., SASANO, H., MIZUGUCHI, H., ASANO, T. & OKA, Y. (2005) Constitutively active PDX1 induced efficient insulin production in adult murine liver. *Biochem Biophys Res Commun,* 326**,** 402-9.

JACKSON, L. F., QIU, T. H., SUNNARBORG, S. W., CHANG, A., ZHANG, C., PATTERSON, C. & LEE, D. C. (2003) Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signalling. *EMBO J,* 22**,** 2704-16.

JAENISCH, R. & BIRD, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet,* 33 Suppl**,** 245-54.

JENSEN, J. (2004) Gene regulatory factors in pancreatic development. *Dev Dyn,* 229**,** 176-200.

JOHNSON, J. D., AHMED, N. T., LUCIANI, D. S., HAN, Z., TRAN, H., FUJITA, J., MISLER, S., EDLUND, H. & POLONSKY, K. S. (2003) Increased islet apoptosis in Pdx1+/- mice. *J Clin Invest,* 111**,** 1147-60.

JONES, J. T., AKITA, R. W. & SLIWKOWSKI, M. X. (1999) Binding specificities and affinities of egf domains for ErbB receptors. *FEBS Lett,* 447**,** 227-31.

JONSSON, J., CARLSSON, L., EDLUND, T. & EDLUND, H. (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature,* 371**,** 606-9.

KANETO, H., MATSUOKA, T. A., NAKATANI, Y., MIYATSUKA, T., MATSUHISA, M., HORI, M. & YAMASAKI, Y. (2005a) A crucial role of MafA as a novel therapeutic target for diabetes. *J Biol Chem,* 280**,** 15047-52.

KANETO, H., NAKATANI, Y., MIYATSUKA, T., MATSUOKA, T. A., MATSUHISA, M., HORI, M. & YAMASAKI, Y. (2005b) PDX-1/VP16 fusion protein, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. *Diabetes,* 54**,** 1009-22.

KATAOKA, K., HAN, S. I., SHIODA, S., HIRAI, M., NISHIZAWA, M. & HANDA, H. (2002) MafA is a glucose-regulated and pancreatic beta cell-specific transcriptional activator for the insulin gene. *J Biol Chem,* 277**,** 49903-10.

KAWAGUCHI, Y., COOPER, B., GANNON, M., RAY, M., MACDONALD, R. J. & WRIGHT, C. V. (2002) The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet,* 32**,** 128-34.

KENNEDY, L. & BAYNES, J. W. (1984) Non-enzymatic glycosylation and the chronic complications of diabetes: an overview. *Diabetologia,* 26**,** 93-8.

KIEFER, J. C. (2007) Epigenetics in development. *Dev Dyn,* 236**,** 1144-56.

KIM, S. K. & HEBROK, M. (2001) Intercellular signals regulating pancreas development and function. *Genes Dev,* 15**,** 111-27.

KIM, S. K., HEBROK, M. & MELTON, D. A. (1997) Notochord to endoderm signalling is required for pancreas development. *Development,* 124**,** 4243-52.

KIM, S. K. & MACDONALD, R. J. (2002) Signalling and transcriptional control of pancreatic organogenesis. *Curr Opin Genet Dev,* 12**,** 540-7.

KING, G. L., SHIBA, T., OLIVER, J., INOGUCHI, T. & BURSELL, S. E. (1994) Cellular and molecular abnormalities in the vascular endothelium of diabetes mellitus. *Annu Rev Med,* 45**,** 179-88.

KITZMANN, M., CARNAC, G., VANDROMME, M., PRIMIG, M., LAMB, N. J. & FERNANDEZ, A. (1998) The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J Cell Biol,* 142**,** 1447-59.

KNIP, M. & AKERBLOM, H. K. (1999) Environmental factors in the pathogenesis of type 1 diabetes mellitus. *Exp Clin Endocrinol Diabetes,* 107 Suppl 3**,** S93-100.

KOIZUMI, M., DOI, R., TOYODA, E., TULACHAN, S. S., KAMI, K., MORI, T., ITO, D., KAWAGUCHI, Y., FUJIMOTO, K., GITTES, G. K. & IMAMURA, M. (2004) Hepatic regeneration and enforced PDX-1 expression accelerate transdifferentiation in liver. *Surgery,* 136**,** 449-57.

KOJIMA, H., FUJIMIYA, M., MATSUMURA, K., YOUNAN, P., IMAEDA, H., MAEDA, M. & CHAN, L. (2003) NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. *Nat Med,* 9**,** 596-603.

KRAPP, A., KNOFLER, M., LEDERMANN, B., BURKI, K., BERNEY, C., ZOERKLER, N., HAGENBUCHLE, O. & WELLAUER, P. K. (1998) The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. *Genes Dev,* 12**,** 3752-63.

KROON, E., MARTINSON, L. A., KADOYA, K., BANG, A. G., KELLY, O. G., ELIAZER, S., YOUNG, H., RICHARDSON, M., SMART, N. G., CUNNINGHAM, J., AGULNICK, A. D., D'AMOUR, K. A., CARPENTER, M. K. & BAETGE, E. E. (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol,* 26**,** 443-52.

KUSHNER, J. A., CIEMERYCH, M. A., SICINSKA, E., WARTSCHOW, L. M., TETA, M., LONG, S. Y., SICINSKI, P. & WHITE, M. F. (2005) Cyclins D2 and D1 are essential for postnatal pancreatic beta cell growth. *Mol Cell Biol,* 25**,** 3752-62.

LAMMERT, E., CLEAVER, O. & MELTON, D. (2001) Induction of pancreatic differentiation by signals from blood vessels. *Science,* 294**,** 564-7.

LANTZ, K. A. & KAESTNER, K. H. (2005) Winged-helix transcription factors and pancreatic development. *Clin Sci (Lond),* 108**,** 195-204.

LARDON, J., DE BREUCK, S., ROOMAN, I., VAN LOMMEL, L., KRUHOFFER, M., ORNTOFT, T., SCHUIT, F. & BOUWENS, L. (2004) Plasticity in the adult rat

pancreas: transdifferentiation of exocrine to hepatocyte-like cells in primary culture. *Hepatology,* 39**,** 1499-507.

LECHNER, A. & HABENER, J. F. (2003) Stem/progenitor cells derived from adult tissues: potential for the treatment of diabetes mellitus. *Am J Physiol Endocrinol Metab,* 284**,** E259-66.

LEE, R. H., SEO, M. J., REGER, R. L., SPEES, J. L., PULIN, A. A., OLSON, S. D. & PROCKOP, D. J. (2006) Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc Natl Acad Sci U S A,* 103**,** 17438-43.

LEKSTROM-HIMES, J. & XANTHOPOULOS, K. G. (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J Biol Chem,* 273**,** 28545-8.

LEVENSON, J. M. & SWEATT, J. D. (2005) Epigenetic mechanisms in memory formation. *Nat Rev Neurosci,* 6**,** 108-18.

LI, E. (2002) Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet,* 3**,** 662-73.

LI, L., SENO, M., YAMADA, H. & KOJIMA, I. (2001) Promotion of beta cell regeneration by betacellulin in ninety percent-pancreatectomized rats. *Endocrinology,* 142**,** 5379-85.

LI, L., SENO, M., YAMADA, H. & KOJIMA, I. (2003) Betacellulin improves glucose metabolism by promoting conversion of intraislet precursor cells to beta cells in streptozotocin-treated mice. *Am J Physiol Endocrinol Metab,* 285**,** E577-83.

LI, W. C., HORB, M. E., TOSH, D. & SLACK, J. M. (2005a) In vitro transdifferentiation of hepatoma cells into functional pancreatic cells. *Mech Dev,* 122**,** 835-47.

LI, W. C., RALPHS, K. L., SLACK, J. M. & TOSH, D. (2007) Keratinocyte serum-free medium maintains long-term liver gene expression and function in cultured rat hepatocytes by preventing the loss of liver-enriched transcription factors. *Int J Biochem Cell Biol,* 39**,** 541-54.

LI, W. C., YU, W. Y., QUINLAN, J. M., BURKE, Z. D. & TOSH, D. (2005b) The molecular basis of transdifferentiation. *J Cell Mol Med,* 9**,** 569-82.

LIMBERT, C., PATH, G., JAKOB, F. & SEUFERT, J. (2008) Beta cell replacement and regeneration: Strategies of cell-based therapy for type 1 diabetes mellitus. *Diabetes Res Clin Pract,* 79**,** 389-99.

LIU, Q. & MURUVE, D. A. (2003) Molecular basis of the inflammatory response to adenovirus vectors. *Gene Ther,* 10**,** 935-40.

LO, D. C., ALLEN, F. & BROCKES, J. P. (1993) Reversal of muscle differentiation during urodele limb regeneration. *Proc Natl Acad Sci U S A,* 90**,** 7230-4.

LUMELSKY, N., BLONDEL, O., LAENG, P., VELASCO, I., RAVIN, R. & MCKAY, R. (2001) Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science,* 292**,** 1389-94.

LUTTRINGER, O., THEIL, F. P., LAVE, T., WERNLI-KURATLI, K., GUENTERT, T. W. & DE SAIZIEU, A. (2002) Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes. *Biochem Pharmacol,* 64**,** 1637-50.

LYNN, F. C., SMITH, S. B., WILSON, M. E., YANG, K. Y., NEKREP, N. & GERMAN, M. S. (2007) Sox9 coordinates a transcriptional network in pancreatic progenitor cells. *Proc Natl Acad Sci U S A,* 104**,** 10500-5.

LYTTLE, B. M., LI, J., KRISHNAMURTHY, M., FELLOWS, F., WHEELER, M. B., GOODYER, C. G. & WANG, R. (2008) Transcription factor expression in the developing human fetal endocrine pancreas. *Diabetologia,* 51**,** 1169-80.

MACFARLANE, W. M., READ, M. L., GILLIGAN, M., BUJALSKA, I. & DOCHERTY, K. (1994) Glucose modulates the binding activity of the beta cell transcription factor IUF1 in a phosphorylation-dependent manner. *Biochem J,* 303 (Pt 2)**,** 625-31.

MASHIMA, H., OHNISHI, H., WAKABAYASHI, K., MINE, T., MIYAGAWA, J., HANAFUSA, T., SENO, M., YAMADA, H. & KOJIMA, I. (1996) Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. *J Clin Invest,* 97**,** 1647-54.

MATSUOKA, T. A., ARTNER, I., HENDERSON, E., MEANS, A., SANDER, M. & STEIN, R. (2004) The MafA transcription factor appears to be responsible for tissue-specific expression of insulin. *Proc Natl Acad Sci U S A,* 101**,** 2930-3.

MATSUOKA, T. A., KANETO, H., STEIN, R., MIYATSUKA, T., KAWAMORI, D., HENDERSON, E., KOJIMA, I., MATSUHISA, M., HORI, M. & YAMASAKI, Y. (2007) MafA regulates expression of genes important to islet beta cell function. *Mol Endocrinol,* 21**,** 2764-74.

MCCONNELL, M. J. & IMPERIALE, M. J. (2004) Biology of adenovirus and its use as a vector for gene therapy. *Hum Gene Ther,* 15**,** 1022-33.

MCKINNON, C. M. & DOCHERTY, K. (2001) Pancreatic duodenal homeobox-1, PDX-1, a major regulator of beta cell identity and function. *Diabetologia,* 44**,** 1203-14.

MEIVAR-LEVY, I., SAPIR, T., GEFEN-HALEVI, S., AVIV, V., BARSHACK, I., ONACA, N., MOR, E. & FERBER, S. (2007) Pancreatic and duodenal homeobox gene 1 induces hepatic dedifferentiation by suppressing the expression of CCAAT/enhancer-binding protein beta. *Hepatology,* 46**,** 898-905.

MIETTINEN, P. J., HUOTARI, M., KOIVISTO, T., USTINOV, J., PALGI, J., RASILAINEN, S., LEHTONEN, E., KESKI-OJA, J. & OTONKOSKI, T. (2000) Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors. *Development,* 127**,** 2617-27.

MIRALLES, F., CZERNICHOW, P. & SCHARFMANN, R. (1998) Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. *Development,* 125**,** 1017-24.

MITAKA, T. (1998) The current status of primary hepatocyte culture. *Int J Exp Pathol,* 79**,** 393-409.

MIYAGAWA, J., HANAFUSA, O., SASADA, R., YAMAMOTO, K., IGARASHI, K., YAMAMORI, K., SENO, M., TADA, H., NAMMO, T., LI, M., YAMAGATA, K., NAKAJIMA, H., NAMBA, M., KUWAJIMA, M. & MATSUZAWA, Y. (1999) Immunohistochemical localization of betacellulin, a new member of the EGF family, in normal human pancreas and islet tumor cells. *Endocr J,* 46**,** 755-64.

MIYATSUKA, T., KANETO, H., SHIRAIWA, T., MATSUOKA, T. A., YAMAMOTO, K., KATO, K., NAKAMURA, Y., AKIRA, S., TAKEDA, K., KAJIMOTO, Y., YAMASAKI, Y., SANDGREN, E. P., KAWAGUCHI, Y., WRIGHT, C. V. & FUJITANI, Y. (2006)

Persistent expression of PDX-1 in the pancreas causes acinar-to-ductal metaplasia through Stat3 activation. *Genes Dev,* 20**,** 1435-40.

MOATES, J. M., NANDA, S., CISSELL, M. A., TSAI, M. J. & STEIN, R. (2003) BETA2 activates transcription from the upstream glucokinase gene promoter in islet beta cells and gut endocrine cells. *Diabetes,* 52**,** 403-8.

MOLDRUP, A., PETERSEN, E. D. & NIELSEN, J. H. (1993) Effects of sex and pregnancy hormones on growth hormone and prolactin receptor gene expression in insulin-producing cells. *Endocrinology,* 133**,** 1165-72.

MURTAUGH, L. C., STANGER, B. Z., KWAN, K. M. & MELTON, D. A. (2003) Notch signalling controls multiple steps of pancreatic differentiation. *Proc Natl Acad Sci U S A,* 100**,** 14920-5.

NARUSHIMA, M., KOBAYASHI, N., OKITSU, T., TANAKA, Y., LI, S. A., CHEN, Y., MIKI, A., TANAKA, K., NAKAJI, S., TAKEI, K., GUTIERREZ, A. S., RIVAS-CARRILLO, J. D., NAVARRO-ALVAREZ, N., JUN, H. S., WESTERMAN, K. A., NOGUCHI, H., LAKEY, J. R., LEBOULCH, P., TANAKA, N. & YOON, J. W. (2005) A human beta cell line for transplantation therapy to control type 1 diabetes. *Nat Biotechnol,* 23**,** 1274-82.

NATHAN, D. M. (1996) The pathophysiology of diabetic complications: how much does the glucose hypothesis explain? *Ann Intern Med,* 124**,** 86-9.

NAYA, F. J., HUANG, H. P., QIU, Y., MUTOH, H., DEMAYO, F. J., LEITER, A. B. & TSAI, M. J. (1997) Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev,* 11**,** 2323-34.

NAYA, F. J., STELLRECHT, C. M. & TSAI, M. J. (1995) Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev,* 9**,** 1009-19.

NELSON, W. G., DEWEESE, T. L. & DEMARZO, A. M. (2002) The diet, prostate inflammation, and the development of prostate cancer. *Cancer Metastasis Rev,* 21**,** 3-16.

NIR, T., MELTON, D. A. & DOR, Y. (2007) Recovery from diabetes in mice by beta cell regeneration. *J Clin Invest,* 117**,** 2553-61.

O'HARA, C. M., EGAR, M. W. & CHERNOFF, E. A. (1992) Reorganization of the ependyma during axolotl spinal cord regeneration: changes in intermediate filament and fibronectin expression. *Dev Dyn,* 193**,** 103-15.

O'NEILL, K. E., EBERHARD, D. & TOSH, D. (2008) Origin of beta cells in regenerating pancreas. *Bioessays,* 30**,** 617-20.

OBERG-WELSH, C., SANDLER, S., ANDERSSON, A. & WELSH, M. (1997) Effects of vascular endothelial growth factor on pancreatic duct cell replication and the insulin production of fetal islet-like cell clusters in vitro. *Mol Cell Endocrinol,* 126**,** 125-32.

OBERPRILLER, J. O., OBERPRILLER, J. C., MATZ, D. G. & SOONPAA, M. H. (1995) Stimulation of proliferative events in the adult amphibian cardiac myocyte. *Ann N Y Acad Sci,* 752**,** 30-46.

ODELBERG, S. J. (2002) Inducing cellular dedifferentiation: a potential method for enhancing endogenous regeneration in mammals. *Semin Cell Dev Biol,* 13**,** 335-43.

ODELBERG, S. J., KOLLHOFF, A. & KEATING, M. T. (2000) Dedifferentiation of mammalian myotubes induced by msx1. *Cell,* 103**,** 1099-109.

OFFIELD, M. F., JETTON, T. L., LABOSKY, P. A., RAY, M., STEIN, R. W., MAGNUSON, M. A., HOGAN, B. L. & WRIGHT, C. V. (1996) PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development,* 122**,** 983-95.

OGATA, T., LI, L., YAMADA, S., YAMAMOTO, Y., TANAKA, Y., TAKEI, I., UMEZAWA, K. & KOJIMA, I. (2004) Promotion of beta cell differentiation by conophylline in fetal and neonatal rat pancreas. *Diabetes,* 53**,** 2596-602.

OHLSSON, H., KARLSSON, K. & EDLUND, T. (1993) IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J,* 12**,** 4251-9.

OHNEDA, K., EE, H. & GERMAN, M. (2000a) Regulation of insulin gene transcription. *Semin Cell Dev Biol,* 11**,** 227-33.

OHNEDA, K., MIRMIRA, R. G., WANG, J., JOHNSON, J. D. & GERMAN, M. S. (2000b) The homeodomain of PDX-1 mediates multiple protein-protein interactions in the formation of a transcriptional activation complex on the insulin promoter. *Mol Cell Biol,* 20**,** 900-11.

OKITA, K., NAKAGAWA, M., HYENJONG, H., ICHISAKA, T. & YAMANAKA, S. (2008) Generation of Mouse Induced Pluripotent Stem Cells Without Viral Vectors. *Science*.

OKUMURA, K., NAKAMURA, K., HISATOMI, Y., NAGANO, K., TANAKA, Y., TERADA, K., SUGIYAMA, T., UMEYAMA, K., MATSUMOTO, K., YAMAMOTO, T. & ENDO, F. (2003) Salivary gland progenitor cells induced by duct ligation differentiate into hepatic and pancreatic lineages. *Hepatology,* 38**,** 104-13.

OLBROT, M., RUD, J., MOSS, L. G. & SHARMA, A. (2002) Identification of beta cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. *Proc Natl Acad Sci U S A,* 99**,** 6737-42.

OLDSTONE, M. B., NERENBERG, M., SOUTHERN, P., PRICE, J. & LEWICKI, H. (1991) Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell,* 65**,** 319-31.

OLIVER-KRASINSKI, J. M. & STOFFERS, D. A. (2008) On the origin of the beta cell. *Genes Dev,* 22**,** 1998-2021.

OPAS, M. & DZIAK, E. (1998) Direct transdifferentiation in the vertebrate retina. *Int J Dev Biol,* 42**,** 199-206.

OSSIPOW, V., DESCOMBES, P. & SCHIBLER, U. (1993) CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc Natl Acad Sci U S A,* 90**,** 8219-23.

OTONKOSKI, T., BEATTIE, G. M., MALLY, M. I., RICORDI, C. & HAYEK, A. (1993) Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. *J Clin Invest,* 92**,** 1459-66.

OUZIEL-YAHALOM, L., ZALZMAN, M., ANKER-KITAI, L., KNOLLER, S., BAR, Y., GLANDT, M., HEROLD, K. & EFRAT, S. (2006) Expansion and redifferentiation of adult human pancreatic islet cells. *Biochem Biophys Res Commun,* 341**,** 291-8.

PAGAN, R., MARTIN, I., ALONSO, A., LLOBERA, M. & VILARO, S. (1996) Vimentin filaments follow the preexisting cytokeratin network during epithelial-

mesenchymal transition of cultured neonatal rat hepatocytes. *Exp Cell Res,* 222**,** 333-44.

PERCIVAL, A. C. & SLACK, J. M. W. S. (1999) Analysis of pancreatic development using a cell lineage label. *Exp Cell Res,* 247**,** 123-32.

PERTUSA, J. A., SANCHEZ-ANDRES, J. V., MARTIN, F. & SORIA, B. (1999) Effects of calcium buffering on glucose-induced insulin release in mouse pancreatic islets: an approximation to the calcium sensor. *J Physiol,* 520 Pt 2**,** 473-83.

PETERSEN, H. V., PESHAVARIA, M., PEDERSEN, A. A., PHILIPPE, J., STEIN, R., MADSEN, O. D. & SERUP, P. (1998) Glucose stimulates the activation domain potential of the PDX-1 homeodomain transcription factor. *FEBS Lett,* 431**,** 362-6.

PHELPS, C. J., KOIKE, C., VAUGHT, T. D., BOONE, J., WELLS, K. D., CHEN, S. H., BALL, S., SPECHT, S. M., POLEJAEVA, I. A., MONAHAN, J. A., JOBST, P. M., SHARMA, S. B., LAMBORN, A. E., GARST, A. S., MOORE, M., DEMETRIS, A. J., RUDERT, W. A., BOTTINO, R., BERTERA, S., TRUCCO, M., STARZL, T. E., DAI, Y. & AYARES, D. L. (2003) Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science,* 299**,** 411-4.

PHILIPPE, J. (1993) Somatostatin inhibits insulin-gene expression through a posttranscriptional mechanism in a hamster islet cell line. *Diabetes,* 42**,** 244-9.

PHILIPPE, J., DRUCKER, D. J., CHICK, W. L. & HABENER, J. F. (1987) Transcriptional regulation of genes encoding insulin, glucagon, and angiotensinogen by sodium butyrate in a rat islet cell line. *Mol Cell Biol,* 7**,** 560-3.

POITOUT, V., HAGMAN, D., STEIN, R., ARTNER, I., ROBERTSON, R. P. & HARMON, J. S. (2006) Regulation of the insulin gene by glucose and fatty acids. *J Nutr,* 136**,** 873-6.

PORTE, D., JR., BASKIN, D. G. & SCHWARTZ, M. W. (2005) Insulin signalling in the central nervous system: a critical role in metabolic homeostasis and disease from C. elegans to humans. *Diabetes,* 54**,** 1264-76.

POSTIC, C. & MAGNUSON, M. A. (2000) DNA excision in liver by an albumin-Cre transgene occurs progressively with age. *Genesis,* 26**,** 149-50.

PTASZNIK, A., BEATTIE, G. M., MALLY, M. I., CIRULLI, V., LOPEZ, A. & HAYEK, A. (1997) Phosphatidylinositol 3-kinase is a negative regulator of cellular differentiation. *J Cell Biol,* 137**,** 1127-36.

PUGLIESE, A., ZELLER, M., FERNANDEZ, A., JR., ZALCBERG, L. J., BARTLETT, R. J., RICORDI, C., PIETROPAOLO, M., EISENBARTH, G. S., BENNETT, S. T. & PATEL, D. D. (1997) The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. *Nat Genet,* 15**,** 293-7.

QIU, Y., GUO, M., HUANG, S. & STEIN, R. (2002) Insulin gene transcription is mediated by interactions between the p300 coactivator and PDX-1, BETA2, and E47. *Mol Cell Biol,* 22**,** 412-20.

QUINLAN, J. M., COLLEYPRIEST, B. J., FARRANT, M. & TOSH, D. (2007) Epithelial metaplasia and the development of cancer. *Biochim Biophys Acta,* 1776**,** 10-21.

RAO, M. S., SUBBARAO, V. & REDDY, J. K. (1986) Induction of hepatocytes in the pancreas of copper-depleted rats following copper repletion. *Cell Differ,* 18**,** 109-17.

RAWLINS, E. L. & HOGAN, B. L. (2006) Epithelial stem cells of the lung: privileged few or opportunities for many? *Development,* 133**,** 2455-65.

RESCAN, C., LE BRAS, S., LEFEBVRE, V. H., FRANDSEN, U., KLEIN, T., FOSCHI, M., PIPELEERS, D. G., SCHARFMANN, R., MADSEN, O. D. & HEIMBERG, H. (2005) EGF-induced proliferation of adult human pancreatic duct cells is mediated by the MEK/ERK cascade. *Lab Invest,* 85**,** 65-74.

RICHARDSON, H., CAMPBELL, S. C., SMITH, S. A. & MACFARLANE, W. M. (2006) Effects of rosiglitazone and metformin on pancreatic beta cell gene expression. *Diabetologia,* 49**,** 685-96.

RIDGWAY, D. M., WHITE, S. A., KIMBER, R. M. & NICHOLSON, M. L. (2005) Current practices of donor pancreas allocation in the UK: future implications for pancreas and islet transplantation. *Transpl Int,* 18**,** 828-34.

RODERIGO-MILNE, H., HAUGE-EVANS, A. C., PERSAUD, S. J. & JONES, P. M. (2002) Differential expression of insulin genes 1 and 2 in MIN6 cells and pseudoislets. *Biochem Biophys Res Commun,* 296**,** 589-95.

ROLLAND, F., WINDERICKX, J. & THEVELEIN, J. M. (2001) Glucose-sensing mechanisms in eukaryotic cells. *Trends Biochem Sci,* 26**,** 310-7.

ROOMAN, I., HEREMANS, Y., HEIMBERG, H. & BOUWENS, L. (2000) Modulation of rat pancreatic acinoductal transdifferentiation and expression of PDX-1 in vitro. *Diabetologia,* 43**,** 907-14.

ROSE, S. D., SWIFT, G. H., PEYTON, M. J., HAMMER, R. E. & MACDONALD, R. J. (2001) The role of PTF1-P48 in pancreatic acinar gene expression. *J Biol Chem,* 276**,** 44018-26.

ROSSI, J. M., DUNN, N. R., HOGAN, B. L. & ZARET, K. S. (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev,* 15**,** 1998-2009.

ROZEN, S. & SKALETSKY, H. J. (2000) Primer 3 on the WWW for general users and for biologist programmers. IN KRAWETZ, S. & MISENER, S. (Eds.) *Bioinformatics Methods and Protocols.* Totowa, NJ, Humana Press.

RYAN, E. A., PATY, B. W., SENIOR, P. A., BIGAM, D., ALFADHLI, E., KNETEMAN, N. M., LAKEY, J. R. & SHAPIRO, A. M. (2005) Five-year follow-up after clinical islet transplantation. *Diabetes,* 54**,** 2060-9.

SAKURAI, H., TSUKAMOTO, T., KJELSBERG, C. A., CANTLEY, L. G. & NIGAM, S. K. (1997) EGF receptor ligands are a large fraction of in vitro branching morphogens secreted by embryonic kidney. *Am J Physiol,* 273**,** F463-72.

SAMSON, S. L. & CHAN, L. (2006) Gene therapy for diabetes: reinventing the islet. *Trends Endocrinol Metab,* 17**,** 92-100.

SANDER, M., NEUBUSER, A., KALAMARAS, J., EE, H. C., MARTIN, G. R. & GERMAN, M. S. (1997) Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. *Genes Dev,* 11**,** 1662-73.

SANDER, M., SUSSEL, L., CONNERS, J., SCHEEL, D., KALAMARAS, J., DELA CRUZ, F., SCHWITZGEBEL, V., HAYES-JORDAN, A. & GERMAN, M. (2000) Homeobox

gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta cell formation in the pancreas. *Development,* 127**,** 5533-40.

SAPIR, T., SHTERNHALL, K., MEIVAR-LEVY, I., BLUMENFELD, T., COHEN, H., SKUTELSKY, E., EVENTOV-FRIEDMAN, S., BARSHACK, I., GOLDBERG, I., PRI-CHEN, S., BEN-DOR, L., POLAK-CHARCON, S., KARASIK, A., SHIMON, I., MOR, E. & FERBER, S. (2005) Cell-replacement therapy for diabetes: Generating functional insulin-producing tissue from adult human liver cells. *Proc Natl Acad Sci U S A,* 102**,** 7964-9.

SASADA, R., ONO, Y., TANIYAMA, Y., SHING, Y., FOLKMAN, J. & IGARASHI, K. (1993) Cloning and expression of cDNA encoding human betacellulin, a new member of the EGF family. *Biochem Biophys Res Commun,* 190**,** 1173-9.

SCHMID, V., ALDER, H., PLICKERT, G. & WEBER, C. (1988) Transdifferentiation from striated muscle of medusae in vitro. *Cell Differ Dev,* 25 Suppl**,** 137-46.

SCHOLZ, A., HEINZE, S., DETJEN, K. M., PETERS, M., WELZEL, M., HAUFF, P., SCHIRNER, M., WIEDENMANN, B. & ROSEWICZ, S. (2003) Activated signal transducer and activator of transcription 3 (STAT3) supports the malignant phenotype of human pancreatic cancer. *Gastroenterology,* 125**,** 891-905.

SCHULDINER, M., YANUKA, O., ITSKOVITZ-ELDOR, J., MELTON, D. A. & BENVENISTY, N. (2000) Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A,* 97**,** 11307-12.

SCHWITZGEBEL, V. M., SCHEEL, D. W., CONNERS, J. R., KALAMARAS, J., LEE, J. E., ANDERSON, D. J., SUSSEL, L., JOHNSON, J. D. & GERMAN, M. S. (2000) Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development,* 127**,** 3533-42.

SEABERG, R. M., SMUKLER, S. R., KIEFFER, T. J., ENIKOLOPOV, G., ASGHAR, Z., WHEELER, M. B., KORBUTT, G. & VAN DER KOOY, D. (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nat Biotechnol,* 22**,** 1115-24.

SERVITJA, J. M. & FERRER, J. (2004) Transcriptional networks controlling pancreatic development and beta cell function. *Diabetologia,* 47**,** 597-613.

SEYMOUR, P. A., FREUDE, K. K., TRAN, M. N., MAYES, E. E., JENSEN, J., KIST, R., SCHERER, G. & SANDER, M. (2007) SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc Natl Acad Sci U S A,* 104**,** 1865-70.

SHAPIRO, A. M., LAKEY, J. R., RYAN, E. A., KORBUTT, G. S., TOTH, E., WARNOCK, G. L., KNETEMAN, N. M. & RAJOTTE, R. V. (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med,* 343**,** 230-8.

SHARMA, A. & STEIN, R. (1994) Glucose-induced transcription of the insulin gene is mediated by factors required for beta cell-type-specific expression. *Mol Cell Biol,* 14**,** 871-9.

SHEN, C. N., SLACK, J. M. & TOSH, D. (2000) Molecular basis of transdifferentiation of pancreas to liver. *Nat Cell Biol,* 2**,** 879-87.

SHENK, T. (1996) Adenoviridae: The viruses and their replication. IN FIELDS, B. N., KNIPE, D. M. & HOWLEY, P. M. (Eds.) *Virology.* New York, Lippincott-Raven.

SHIAO, M. S., LIAO, B. Y., LONG, M. & YU, H. T. (2008) Adaptive evolution of the insulin two-gene system in mouse. *Genetics,* 178**,** 1683-91.

SHTERNHALL-RON, K., QUINTANA, F. J., PERL, S., MEIVAR-LEVY, I., BARSHACK, I., COHEN, I. R. & FERBER, S. (2007) Ectopic PDX-1 expression in liver ameliorates type 1 diabetes. *J Autoimmun,* 28**,** 134-42.

SJOHOLM, A., KORSGREN, O. & ANDERSSON, A. (1994) Polyamine requirements in nicotinamide-stimulated beta cell differentiation in fetal porcine islet-like cell clusters. *Endocrinology,* 135**,** 1559-65.

SKIPPER, M. & LEWIS, J. (2000) Getting to the guts of enteroendocrine differentiation. *Nat Genet,* 24**,** 3-4.

SLACK, J. M. (1995) Developmental biology of the pancreas. *Development,* 121**,** 1569-80.

SLACK, J. M. (2007) Metaplasia and transdifferentiation: from pure biology to the clinic. *Nat Rev Mol Cell Biol,* 8**,** 369-78.

SMITH, S. B., GASA, R., WATADA, H., WANG, J., GRIFFEN, S. C. & GERMAN, M. S. (2003) Neurogenin3 and hepatic nuclear factor 1 cooperate in activating pancreatic expression of Pax4. *J Biol Chem,* 278**,** 38254-9.

SORIA, B., ROCHE, E., BERNA, G., LEON-QUINTO, T., REIG, J. A. & MARTIN, F. (2000) Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes,* 49**,** 157-62.

SORIA, B., SKOUDY, A. & MARTIN, F. (2001) From stem cells to beta cells: new strategies in cell therapy of diabetes mellitus. *Diabetologia,* 44**,** 407-15.

SORIANO, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet,* 21**,** 70-1.

SOSA-PINEDA, B., CHOWDHURY, K., TORRES, M., OLIVER, G. & GRUSS, P. (1997) The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. *Nature,* 386**,** 399-402.

SPECHLER, S. J. (2002) Clinical practice. Barrett's Esophagus. *N Engl J Med,* 346**,** 836-42.

SRIVASTAVA, S. & GOREN, H. J. (2003) Insulin constitutively secreted by beta cells is necessary for glucose-stimulated insulin secretion. *Diabetes,* 52**,** 2049-56.

STEIN, D. T., ESSER, V., STEVENSON, B. E., LANE, K. E., WHITESIDE, J. H., DANIELS, M. B., CHEN, S. & MCGARRY, J. D. (1996) Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. *J Clin Invest,* 97**,** 2728-35.

STOFFERS, D. A., HELLER, R. S., MILLER, C. P. & HABENER, J. F. (1999) Developmental expression of the homeodomain protein IDX-1 in mice transgenic for an IDX-1 promoter/lacZ transcriptional reporter. *Endocrinology,* 140**,** 5374-81.

STRAHL, B. D. & ALLIS, C. D. (2000) The language of covalent histone modifications. *Nature,* 403**,** 41-5.

SU, A. I., GUIDOTTI, L. G., PEZACKI, J. P., CHISARI, F. V. & SCHULTZ, P. G. (2002) Gene expression during the priming phase of liver regeneration after partial hepatectomy in mice. *Proc Natl Acad Sci U S A,* 99**,** 11181-6.

SUAREZ-PINZON, W. L., LAKEY, J. R., BRAND, S. J. & RABINOVITCH, A. (2005) Combination therapy with epidermal growth factor and gastrin induces neogenesis of human islet {beta}-cells from pancreatic duct cells and an increase in functional {beta}-cell mass. *J Clin Endocrinol Metab,* 90**,** 3401-9.

SUGIYAMA, K., YONEMURA, Y. & OKAMOTO, H. (1991) Effects of poly (ADP-ribose) synthetase inhibitor on B-cells of a canine pancreas after massive pancreatectomy. *Int J Pancreatol,* 8**,** 85-95.

SUND, N. J., VATAMANIUK, M. Z., CASEY, M., ANG, S. L., MAGNUSON, M. A., STOFFERS, D. A., MATSCHINSKY, F. M. & KAESTNER, K. H. (2001) Tissue-specific deletion of Foxa2 in pancreatic beta cells results in hyperinsulinemic hypoglycemia. *Genes Dev,* 15**,** 1706-15.

SUSSEL, L., KALAMARAS, J., HARTIGAN-O'CONNOR, D. J., MENESES, J. J., PEDERSEN, R. A., RUBENSTEIN, J. L. & GERMAN, M. S. (1998) Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development,* 125**,** 2213-21.

SUZUKI, A., NAKAUCHI, H. & TANIGUCHI, H. (2004) Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting. *Diabetes,* 53**,** 2143-52.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 131**,** 861-72.

TAKAHASHI, K. & YAMANAKA, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126**,** 663-76.

TANG, D. Q., CAO, L. Z., BURKHARDT, B. R., XIA, C. Q., LITHERLAND, S. A., ATKINSON, M. A. & YANG, L. J. (2004) In vivo and in vitro characterization of insulin-producing cells obtained from murine bone marrow. *Diabetes,* 53**,** 1721-32.

TANG, D. Q., CAO, L. Z., CHOU, W., SHUN, L., FARAG, C., ATKINSON, M. A., LI, S. W., CHANG, L. J. & YANG, L. J. (2006) Role of Pax4 in Pdx1-VP16-mediated liver-to-endocrine pancreas transdifferentiation. *Lab Invest,* 86**,** 829-41.

TAYARAMMA, T., MA, B., ROHDE, M. & MAYER, H. (2006) Chromatin-remodeling factors allow differentiation of bone marrow cells into insulin-producing cells. *Stem Cells,* 24**,** 2858-67.

TETA, M., RANKIN, M. M., LONG, S. Y., STEIN, G. M. & KUSHNER, J. A. (2007) Growth and regeneration of adult beta cells does not involve specialized progenitors. *Dev Cell,* 12**,** 817-26.

THOMAS, C. E., EHRHARDT, A. & KAY, M. A. (2003) Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet,* 4**,** 346-58.

THOWFEEQU, S., RALPHS, K. L., YU, W. Y., SLACK, J. M. & TOSH, D. (2007) Betacellulin inhibits amylase and glucagon production and promotes beta cell differentiation in mouse embryonic pancreas. *Diabetologia,* 50**,** 1688-97.

TOSH, D., ALBERTI, G. M. & AGIUS, L. (1988) Glucagon regulation of gluconeogenesis and ketogenesis in periportal and perivenous rat hepatocytes. Heterogeneity of hormone action and of the mitochondrial redox state. *Biochem J,* 256**,** 197-204.

TOSH, D., SHEN, C. N. & SLACK, J. M. (2002) Differentiated properties of hepatocytes induced from pancreatic cells. *Hepatology,* 36**,** 534-43.

TRAUB, P. (1985) *Intermediate Filaments. A Review,* Heidelberg, Springer-Verlag.

TSONIS, P. A., MADHAVAN, M., TANCOUS, E. E. & DEL RIO-TSONIS, K. (2004) A newt's eye view of lens regeneration. *Int J Dev Biol,* 48**,** 975-80.

VALLEJO, G., MEAD, P. M., GAYNOR, D. H., DEVLIN, J. T. & ROBBINS, D. C. (1984) Characterization of immunoreactive insulin in human saliva: evidence against production in situ. *Diabetologia,* 27**,** 437-40.

VERME, T. B. & HOOTMAN, S. R. (1990) Regulation of pancreatic duct epithelial growth in vitro. *Am J Physiol,* 258**,** G833-40.

WANG, A. Y., EHRHARDT, A., XU, H. & KAY, M. A. (2007) Adenovirus transduction is required for the correction of diabetes using Pdx-1 or Neurogenin-3 in the liver. *Mol Ther,* 15**,** 255-63.

WANG, R. Y., SHEN, C. N., LIN, M. H., TOSH, D. & SHIH, C. (2005) Hepatocyte-like cells transdifferentiated from a pancreatic origin can support replication of hepatitis B virus. *J Virol,* 79**,** 13116-28.

WATADA, H. (2004) Neurogenin 3 is a key transcription factor for differentiation of the endocrine pancreas. *Endocr J,* 51**,** 255-64.

WATADA, H., KAJIMOTO, Y., MIYAGAWA, J., HANAFUSA, T., HAMAGUCHI, K., MATSUOKA, T., YAMAMOTO, K., MATSUZAWA, Y., KAWAMORI, R. & YAMASAKI, Y. (1996) PDX-1 induces insulin and glucokinase gene expressions in alphaTC1 clone 6 cells in the presence of betacellulin. *Diabetes,* 45**,** 1826-31.

WILD, S., ROGLIC, G., GREEN, A., SICREE, R. & KING, H. (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care,* 27**,** 1047-53.

WILES, M. V. & KELLER, G. (1991) Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development,* 111**,** 259-67.

WILLIAMS, L., ZHAO, J., MOROZOVA, N., LI, Y., AVIVI, Y. & GRAFI, G. (2003) Chromatin reorganization accompanying cellular dedifferentiation is associated with modifications of histone H3, redistribution of HP1, and activation of E2F-target genes. *Dev Dyn,* 228**,** 113-20.

WILSON, M. E., SCHEEL, D. & GERMAN, M. S. (2003) Gene expression cascades in pancreatic development. *Mech Dev,* 120**,** 65-80.

WOLF, H. K., BURCHETTE, J. L., JR., GARCIA, J. A. & MICHALOPOULOS, G. (1990) Exocrine pancreatic tissue in human liver: a metaplastic process? *Am J Surg Pathol,* 14**,** 590-5.

WOLFE-COOTE, S., LOUW, J., WOODROOF, C. & DU TOIT, D. F. (1996) The non-human primate endocrine pancreas: development, regeneration potential and metaplasia. *Cell Biol Int,* 20**,** 95-101.

WOLIN, S. L. & WALTER, P. (1988) Ribosome pausing and stacking during translation of a eukaryotic mRNA. *EMBO J,* 7**,** 3559-69.

XIE, H., YE, M., FENG, R. & GRAF, T. (2004) Stepwise reprogramming of B cells into macrophages. *Cell,* 117**,** 663-76.

XU, X., D'HOKER, J., STANGE, G., BONNE, S., DE LEU, N., XIAO, X., VAN DE CASTEELE, M., MELLITZER, G., LING, Z., PIPELEERS, D., BOUWENS, L., SCHARFMANN, R., GRADWOHL, G. & HEIMBERG, H. (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell,* 132**,** 197-207.

YAMAUCHI, K., TAKASU, N., ICHIKAWA, K., YAMADA, T. & AIZAWA, T. (1984) Effects of long-term treatment with thyroxine on pituitary TSH secretion and heart

action in patients with hypothyroidism. *Acta Endocrinol (Copenh),* 107**,** 218-24.

YANEY, G. C., KORCHAK, H. M. & CORKEY, B. E. (2000) Long-chain acyl CoA regulation of protein kinase C and fatty acid potentiation of glucose-stimulated insulin secretion in clonal beta cells. *Endocrinology,* 141**,** 1989-98.

YAO, Y. Q., ZHANG, D. F., HUANG, A. L., LUO, Y., ZHANG, D. Z., WANG, B., ZHOU, W. P., REN, H. & GUO, S. H. (2002) Effects of electroporation on primary rat hepatocytes in vitro. *World J Gastroenterol,* 8**,** 893-6.

YU, W. Y., SLACK, J. M. & TOSH, D. (2005) Conversion of columnar to stratified squamous epithelium in the developing mouse oesophagus. *Dev Biol,* 284**,** 157-70.

ZALZMAN, M., GUPTA, S., GIRI, R. K., BERKOVICH, I., SAPPAL, B. S., KARNIELI, O., ZERN, M. A., FLEISCHER, N. & EFRAT, S. (2003) Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells. *Proc Natl Acad Sci U S A,* 100**,** 7253-8.

ZAMBROWICZ, B. P., IMAMOTO, A., FIERING, S., HERZENBERG, L. A., KERR, W. G. & SORIANO, P. (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A,* 94**,** 3789-94.

ZARET, K. (1999) Developmental competence of the gut endoderm: genetic potentiation by GATA and HNF3/fork head proteins. *Dev Biol,* 209**,** 1-10.

ZHANG, C., MORIGUCHI, T., KAJIHARA, M., ESAKI, R., HARADA, A., SHIMOHATA, H., OISHI, H., HAMADA, M., MORITO, N., HASEGAWA, K., KUDO, T., ENGEL, J. D., YAMAMOTO, M. & TAKAHASHI, S. (2005) MafA is a key regulator of glucose-stimulated insulin secretion. *Mol Cell Biol,* 25**,** 4969-76.

ZHAO, J., MOROZOVA, N., WILLIAMS, L., LIBS, L., AVIVI, Y. & GRAFI, G. (2001) Two phases of chromatin decondensation during dedifferentiation of plant cells: distinction between competence for cell fate switch and a commitment for S phase. *J Biol Chem,* 276**,** 22772-8.

ZHOU, J., WANG, X., PINEYRO, M. A. & EGAN, J. M. (1999) Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. *Diabetes,* 48**,** 2358-66.

ZHOU, Q., BROWN, J., KANAREK, A., RAJAGOPAL, J. & MELTON, D. A. (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta cells. *Nature,* 455**,** 627-32.

ZHOU, Q., LAW, A. C., RAJAGOPAL, J., ANDERSON, W. J., GRAY, P. A. & MELTON, D. A. (2007) A multipotent progenitor domain guides pancreatic organogenesis. *Dev Cell,* 13**,** 103-14.

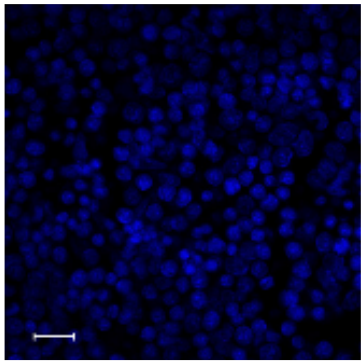
Appendices

Appendix A: List of all commercial suppliers of chemicals and antibodies.

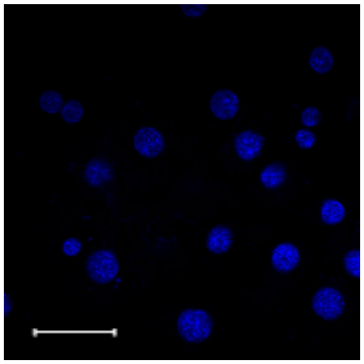
COMPANY	DISTRIBUTER LOCATION	INFORMATION
Abcam	Cambridge, UK	www.abcamplc.com
AbD Sero Tec	Oxford, UK	www.ab-direct.com
AbGene	Epsom, UK	www.abgene.com
Acris	Herford, Germany	www.acris-antibodies.com
Amersham Biosciences	Little Chalfont, UK	www.gelifesciences.com
Autogen-Bioclear	Calne, UK	www.autogenbioclear.com
Baxter Healthcare	Newbury, UK	www.baxterhealthcare.co.uk
BD Biosciences	Cowley, UK	www.bdbiosciences.com
Beckman	High Wycombe, UK	www.beckmancoulter.co.uk
Biogenesis	Poole, UK	www.biogenesis.co.uk
BioRad	Hemel Hempstead, UK	www.bio-rad.com
Calbiochem	San Diego, CA	www.calbiochem.com
Cell Signalling	Danvers, MA	www.cellsignal.com
Chemicon	Chandlers Ford	www.chemicon.com
Clontech Laboratories	Basingstoke, UK	www.clontech.com/clontech
Cypex	Dundee, UK	www.cypex.co.uk
DAKO	Ely, UK	www.dako.co.uk
Epitomics	Burlingame, CA	www.epitomics.com
Fisher Scientific	Loughborough, UK	www.fisher.co.uk
Gibco	*see Invitrogen*	*see Invitrogen*
Invitrogen	Paisley, UK	www.invitrogen.org.uk
Lab Vision	Newmarket, UK	www.labvision.com
Leica	Wetzler, Germany	www.leica-microsystems.com
Mercodia (Diagenics)	Milton Keynes, UK	www.diagenics.co.uk
Millipore	Bedford, UK	www.millipore.co.uk
MWG Primers	*see Invitrogen*	www.mwg-biotech.com
Phoenix Pharmaceuticals	Gloucester, UK	www.phoenixpeptide.com
Promega	Southampton, UK	www.promega.com/uk
R&D systems	Abingdon, UK	www.rndsystems.com
Roche	Mannheim, Germnay	www.roche.com
Santa Cruz Biotech.	Santa Cruz, CA	www.scbt.com
Sigma(-Aldrich)	Poole, UK	www.sigmaaldrich.com
Thermo Scientific	Winsford, UK	www.thermo.com
Transduction Labs	*see BD Biosciences*	*see BD Biosciences*
Upstate Biotech.	Lake Placid, NY	*see Chemicon*
Vector Laboratories	Peterborough, UK	www.vectorlabs.com
VWR International	Leighton Buzzard, UK	uk.vwr.com
Zymed	Cambridge, UK	www.zymed.com

Appendix B

- **No primary controls** were used to detect autoflorescence of secondary antibodies. Below are two examples. DAPI was used to stain the nuclei.



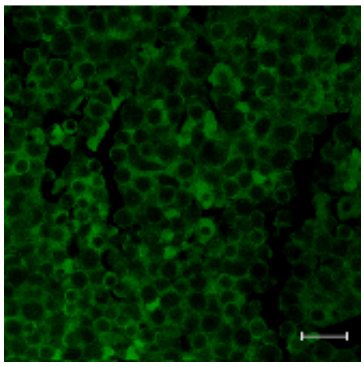
Antibody: *go* anti *rb* FITC
Tissue: rat liver section



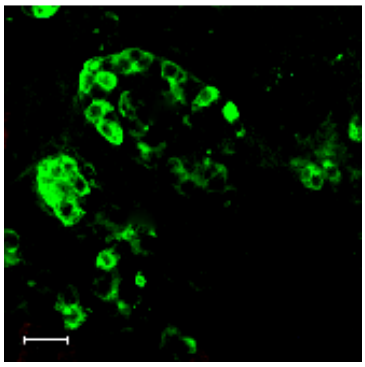
Antibody: *ho* anti *mo* Texas Red
Tissue: hepatocytes

NOTE: All scale bars represent 20µm.

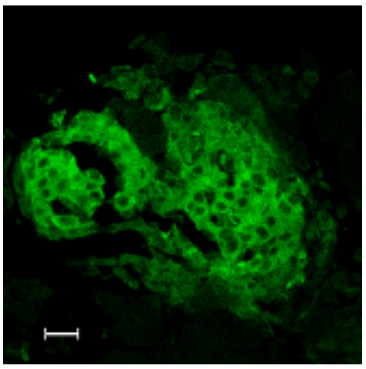
- **Positive controls** for the primary antibodies used. Scale bar indicates 20µm.



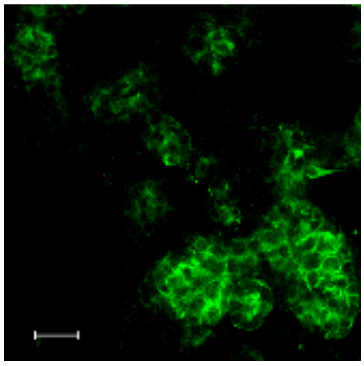
Antibody: *rb* anti-AFP
Tissue: foetal rat liver



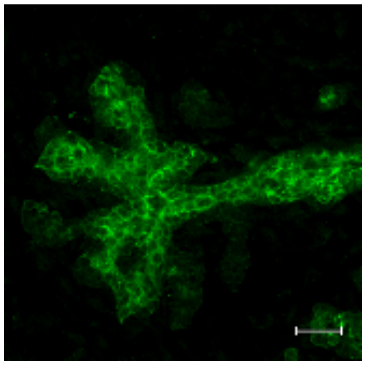
Antibody: *rb* anti-Amylase
Tissue: mouse panc. explant



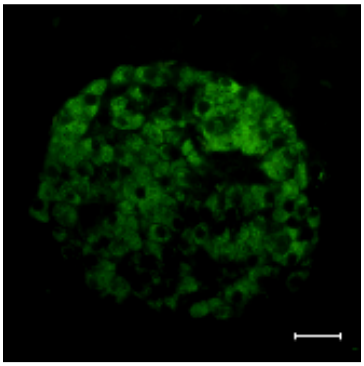
Antibody: *rb* anti-ChrA
Tissue: adult rat pancreas



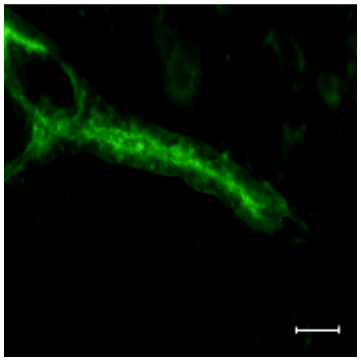
Antibody: *mo* anti-CK19
Tissue: mouse panc. explant



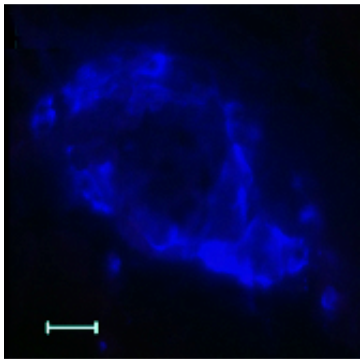
Antibody: *mo* anti-CK20
Tissue: mouse panc. explant



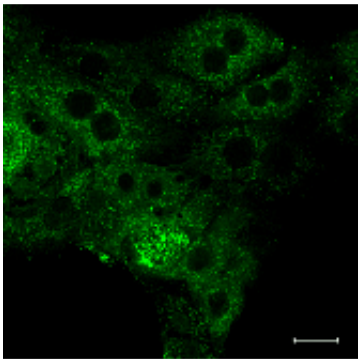
Antibody: *rb* anti-C-peptide
Tissue: adult rat pancreas



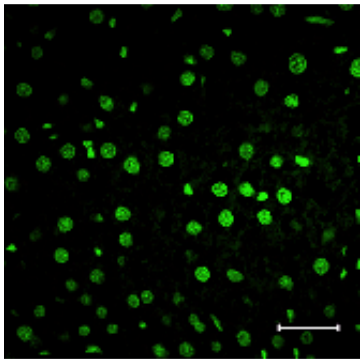
Antibody: DBA-FITC
Tissue: mouse panc. duct



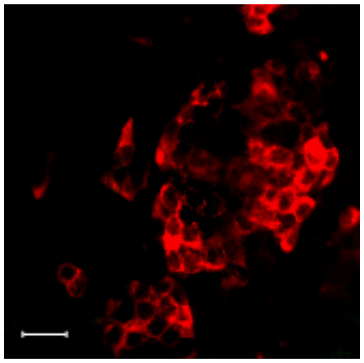
Antibody: *mo* anti-Glucagon
Tissue: mouse panc. explant



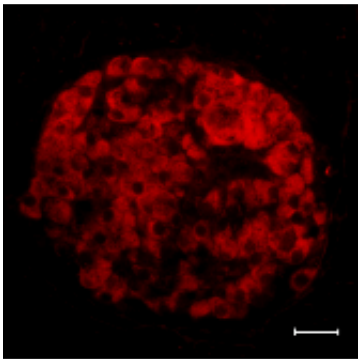
Antibody: *rb* anti-Glut2
Tissue: rat hepatocytes



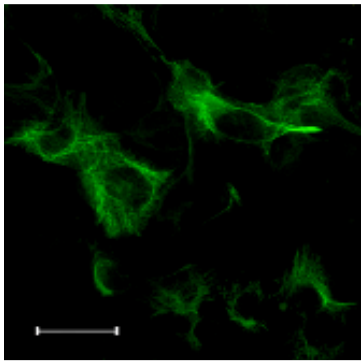
Antibody: *rb* anti-HNF4
Tissue: rat hepatocytes



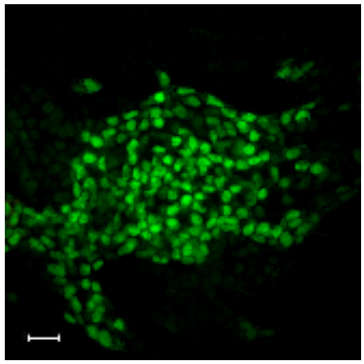
Antibody: *gp* anti-Insulin
Tissue: mouse panc. explant



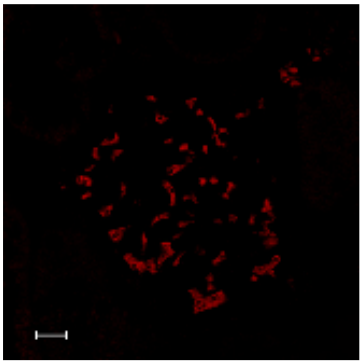
Antibody: *mo* anti-Insulin
Tissue: mouse pancreas



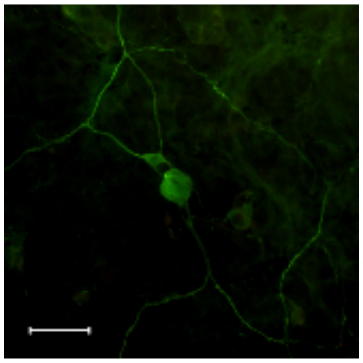
Antibody: *mo* anti-OV6
Tissue: Dex-treated B13 cells



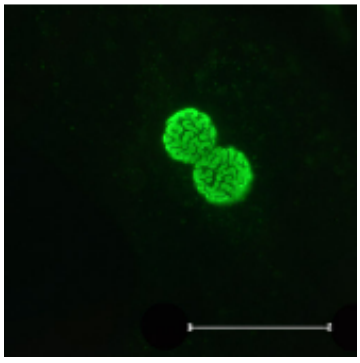
Antibody: *rb* anti-Pdx1
Tissue: mouse panc. explant



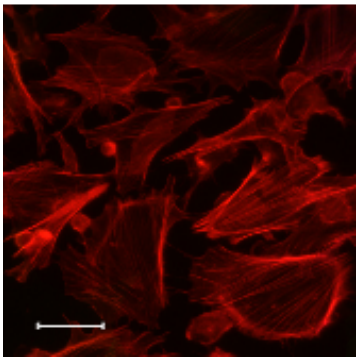
Antibody: *rt* anti-PECAM-1
Tissue: mouse glomerulus



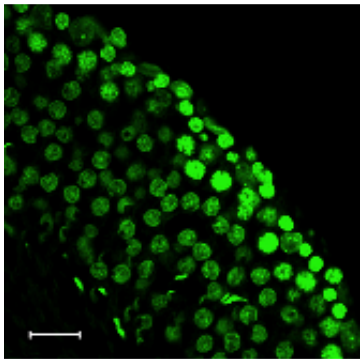
Antibody: *rb* anti-PGP9.5
Tissue: mouse panc. explant



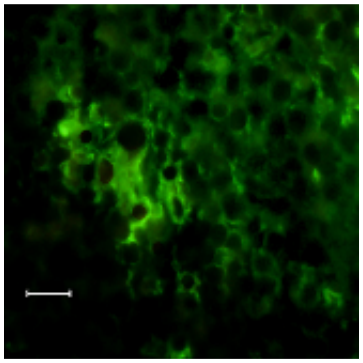
Antibody: *rb* anti-PH3
Tissue: proliferating hepatocytes



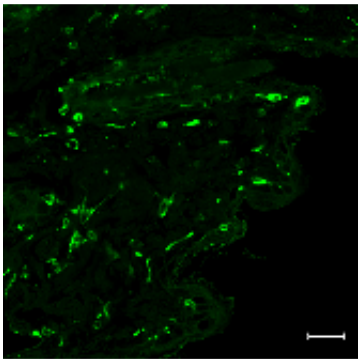
Antibody: *mo* anti-SMA-Cy3
Tissue: embryonic mesenchyme



Antibody: *rb* anti-Sox9
Tissue: rat testes



Antibody: *rb* anti-Thy1
Tissue: E11.5 mouse liver



Antibody: *mo* anti-Vimentin
Tissue: mouse skin section

KEY: ***ho***- horse; ***go***-goat; ***gp***-guinea pig; ***mo***- mouse; ***rb***- rabbit; ***rt***-rat.

Appendix C

Full list of data for the transcription factor combination experiments for all combinations of seven ($^{7}C_{10}$). The values shown are the $\Delta\Delta C_T$ values ($x10^{-6}$ normalised to *Ins1*$_{RIN}$=1.0). Highlighted are the 9 combinations that gave a value above the chosen threshold of 20.

1.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6	11.70
2.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,IA1	16.59
3.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,Sox9	9.53
4.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,p48	6.85
5.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax6,IA1	11.95
6.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax6,Sox9	6.43
7.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax6,p48	13.09
8.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,IA1,Sox9	8.01
9.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,IA1,p48	0.00
10.	Pdx1,Ngn3,NeuroD,Nkx2.2,Nkx6.1,Sox9,p48	0.00
11.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax4,Pax6,IA1	11.07
12.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax4,Pax6,Sox9	10.15
13.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax4,Pax6,p48	0.37
14.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax4,IA1,Sox9	22.31
15.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax4,IA1,p48	15.32
16.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax4,Sox9,p48	14.40
17.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax6,IA1,Sox9	24.93
18.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax6,IA1,p48	16.66
19.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax6,Sox9,p48	24.01
20.	Pdx1,Ngn3,NeuroD,Nkx2.2,IA1,Sox9,p48	12.11
21.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,IA1	10.67
22.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,Sox9	22.11
23.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,p48	24.79
24.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,IA1,Sox9	15.68
25.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,IA1,p48	200.45
26.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,Sox9,p48	5.88
27.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax6,IA1,Sox9	0.53
28.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax6,IA1,p48	4.19
29.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax6,Sox9,p48	2.42
30.	Pdx1,Ngn3,NeuroD,Nkx6.1,IA1,Sox9,p48	0.78
31.	Pdx1,Ngn3,NeuroD,Pax4,Pax6,IA1,Sox9	1.77
32.	Pdx1,Ngn3,NeuroD,Pax4,Pax6,IA1,p48	0.54
33.	Pdx1,Ngn3,NeuroD,Pax4,Pax6,Sox9,p48	2.30
34.	Pdx1,Ngn3,NeuroD,Pax4,IA1,Sox9,p48	0.30

35.	Pdx1,Ngn3,NeuroD,Pax6,IA1,Sox9,p48	20.15
36.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1	0.60
37.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax4,Pax6,Sox9	0.32
38.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax4,Pax6,p48	0.00
39.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax4,IA1,Sox9	0.39
40.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax4,IA1,p48	0.49
41.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax4,Sox9,p48	0.00
42.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax6,IA1,Sox9	0.16
43.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax6,IA1,p48	0.96
44.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,Pax6,Sox9,p48	1.40
45.	Pdx1,Ngn3,Nkx2.2,Nkx6.1,IA1,Sox9,p48	17.76
46.	Pdx1,Ngn3,Nkx2.2,Pax4,Pax6,IA1,Sox9	46.69
47.	Pdx1,Ngn3,Nkx2.2,Pax4,Pax6,IA1,p48	1.39
48.	Pdx1,Ngn3,Nkx2.2,Pax4,Pax6,Sox9,p48	6.10
49.	Pdx1,Ngn3,Nkx2.2,Pax4,IA1,Sox9,p48	1.17
50.	Pdx1,Ngn3,Nkx2.2,Pax6,IA1,Sox9,p48	2.53
51.	Pdx1,Ngn3,Nkx6.1,Pax4,Pax6,IA1,Sox9	0.37
52.	Pdx1,Ngn3,Nkx6.1,Pax4,Pax6,IA1,p48	0.81
53.	Pdx1,Ngn3,Nkx6.1,Pax4,Pax6,Sox9,p48	1.06
54.	Pdx1,Ngn3,Nkx6.1,Pax4,IA1,Sox9,p48	3.99
55.	Pdx1,Ngn3,Nkx6.1,Pax6,IA1,Sox9,p48	0.11
56.	Pdx1,Ngn3,Pax4,Pax6,IA1,Sox9,p48	1.98
57.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1	34.88
58.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,Sox9	15.65
59.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,p48	3.22
60.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax4,IA1,Sox9	2.48
61.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax4,IA1,p48	1.79
62.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax4,Sox9,p48	11.32
63.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax6,IA1,Sox9	17.97
64.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax6,IA1,p48	1.02
65.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax6,Sox9,p48	10.42
66.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,IA1,Sox9,p48	1.15
67.	Pdx1,NeuroD,Nkx2.2,Pax4,Pax6,IA1,Sox9	15.57
68.	Pdx1,NeuroD,Nkx2.2,Pax4,Pax6,IA1,p48	1.61
69.	Pdx1,NeuroD,Nkx2.2,Pax4,Pax6,Sox9,p48	8.08
70.	Pdx1,NeuroD,Nkx2.2,Pax4,IA1,Sox9,p48	2.60
71.	Pdx1,NeuroD,Nkx2.2,Pax6,IA1,Sox9,p48	2.98
72.	Pdx1,NeuroD,Nkx6.1,Pax4,Pax6,IA1,Sox9	9.52
73.	Pdx1,NeuroD,Nkx6.1,Pax4,Pax6,IA1,p48	4.27
74.	Pdx1,NeuroD,Nkx6.1,Pax4,Pax6,Sox9,p48	1.24
75.	Pdx1,NeuroD,Nkx6.1,Pax4,IA1,Sox9,p48	10.42
76.	Pdx1,NeuroD,Nkx6.1,Pax6,IA1,Sox9,p48	6.67

77.	Pdx1,NeuroD,Pax4,Pax6,IA1,Sox9,p48	2.73
78.	Pdx1,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1,Sox9	5.06
79.	Pdx1,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1,p48	4.07
80.	Pdx1,Nkx2.2,Nkx6.1,Pax4,Pax6,Sox9,p48	0.00
81.	Pdx1,Nkx2.2,Nkx6.1,Pax4,IA1,Sox9,p48	0.00
82.	Pdx1,Nkx2.2,Nkx6.1,Pax6,IA1,Sox9,p48	4.18
83.	Pdx1,Nkx2.2,Pax4,Pax6,IA1,Sox9,p48	0.00
84.	Pdx1,Nkx6.1,Pax4,Pax6,IA1,Sox9,p48	1.32
85.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1	5.85
86.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,Sox9	3.81
87.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,p48	4.66
88.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,IA1,Sox9	0.00
89.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,IA1,p48	2.86
90.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax4,Sox9,p48	0.81
91.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax6,IA1,Sox9	5.66
92.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax6,IA1,p48	1.10
93.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,Pax6,Sox9,p48	6.10
94.	Ngn3,NeuroD,Nkx2.2,Nkx6.1,IA1,Sox9,p48	1.69
95.	Ngn3,NeuroD,Nkx2.2,Pax4,Pax6,IA1,Sox9	0.00
96.	Ngn3,NeuroD,Nkx2.2,Pax4,Pax6,IA1,p48	2.22
97.	Ngn3,NeuroD,Nkx2.2,Pax4,Pax6,Sox9,p48	2.68
98.	Ngn3,NeuroD,Nkx2.2,Pax4,IA1,Sox9,p48	3.61
99.	Ngn3,NeuroD,Nkx2.2,Pax6,IA1,Sox9,p48	2.91
100.	Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,IA1,Sox9	2.52
101.	Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,IA1,p48	5.00
102.	Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,Sox9,p48	1.93
103.	Ngn3,NeuroD,Nkx6.1,Pax4,IA1,Sox9,p48	5.11
104.	Ngn3,NeuroD,Nkx6.1,Pax6,IA1,Sox9,p48	4.96
105.	Ngn3,NeuroD,Pax4,Pax6,IA1,Sox9,p48	3.11
106.	Ngn3,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1,Sox9	1.24
107.	Ngn3,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1,p48	2.01
108.	Ngn3,Nkx2.2,Nkx6.1,Pax4,Pax6,Sox9,p48	0.87
109.	Ngn3,Nkx2.2,Nkx6.1,Pax4,IA1,Sox9,p48	0.54
110.	Ngn3,Nkx2.2,Nkx6.1,Pax6,IA1,Sox9,p48	0.98
111.	Ngn3,Nkx2.2,Pax4,Pax6,IA1,Sox9,p48	1.04
112.	Ngn3,Nkx6.1,Pax4,Pax6,IA1,Sox9,p48	1.01
113.	NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1,Sox9	3.04
114.	NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1,p48	0.00
115.	NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,Sox9,p48	0.99
116.	NeuroD,Nkx2.2,Nkx6.1,Pax4,IA1,Sox9,p48	0.46
117.	NeuroD,Nkx2.2,Nkx6.1,Pax6,IA1,Sox9,p48	2.49
118.	NeuroD,Nkx2.2,Pax4,Pax6,IA1,Sox9,p48	1.76

119.	NeuroD,Nkx6.1,Pax4,Pax6,IA1,Sox9,p48	2.08
120.	Nkx2.2,Nkx6.1,Pax4,Pax6,IA1,Sox9,p48	0.00

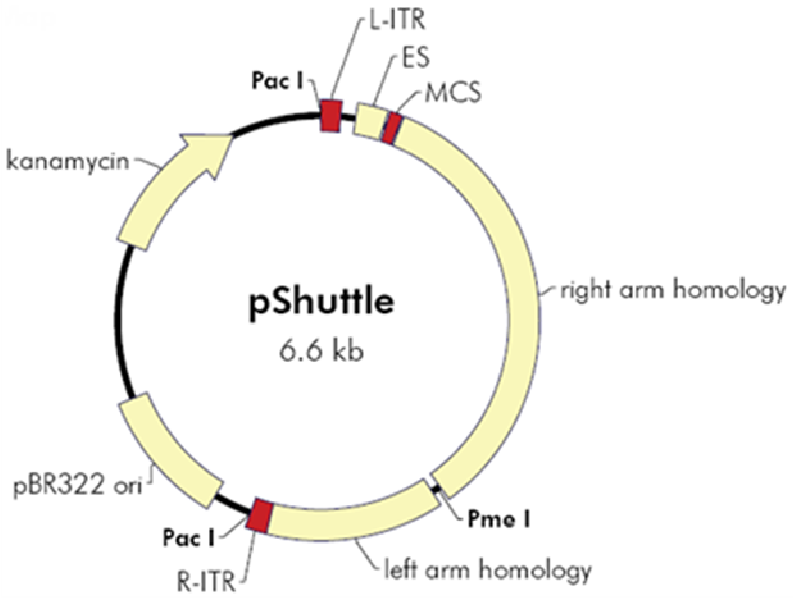
Repetition of the 9 combinations that gave a value above 20 along with a randomly chosen combination that gave a lower value (combination 54). Each value is a mean of a triplicate. *N1* and *N2* were two different PCR reactions from the same cDNA sample and *N3* was repeated with cDNA newly synthesised from RNA from a different experiment. †- indicates an anomalous result.

		N1	*N2*	*N3*
54.	Pdx1,Ngn3,Nkx6.1,Pax4,IA1,Sox9,p48	3.99	6.32	4.56
14.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax4,IA1,Sox9	22.31	24.72	20.93
17.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax6,IA1,Sox9	24.93	22.51	29.47
19.	Pdx1,Ngn3,NeuroD,Nkx2.2,Pax6,Sox9,p48	24.01	29.1	24.89
22.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,Sox9	22.11	32.06	24.64
23.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,Pax6,p48	24.79	28.6	26.31
25.	Pdx1,Ngn3,NeuroD,Nkx6.1,Pax4,IA1,p48	200.45†	30.44	28.01
35.	Pdx1,Ngn3,NeuroD,Pax4,IA1,Sox9,p48	20.15	22.51	19.64
46.	Pdx1,Ngn3,Nkx2.2,Pax4,Pax6,IA1,Sox9	46.69	38.26	29.4
57.	Pdx1,NeuroD,Nkx2.2,Nkx6.1,Pax4,Pax6,IA1	34.88	19.23	24.15

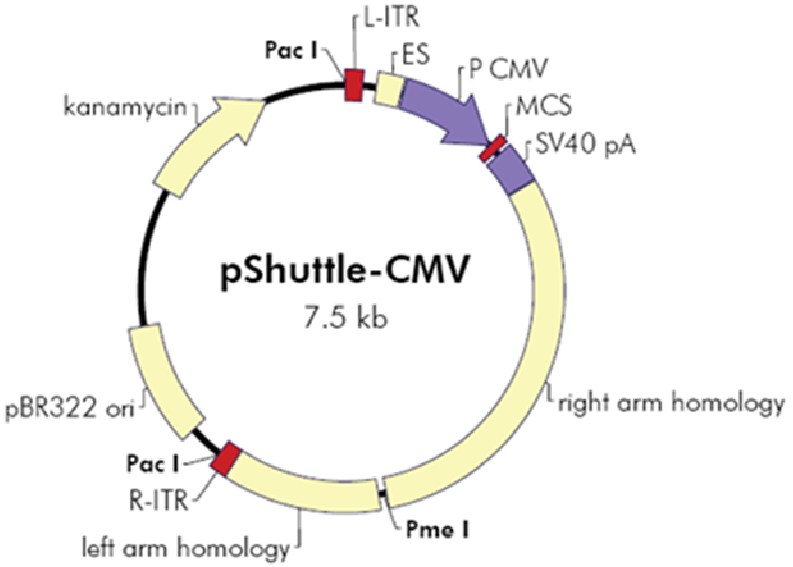
Appendix D : Vector maps for the adenoviral vectors used.

The genes of interest were initially cloned into the ***pShuttle*** vectors:

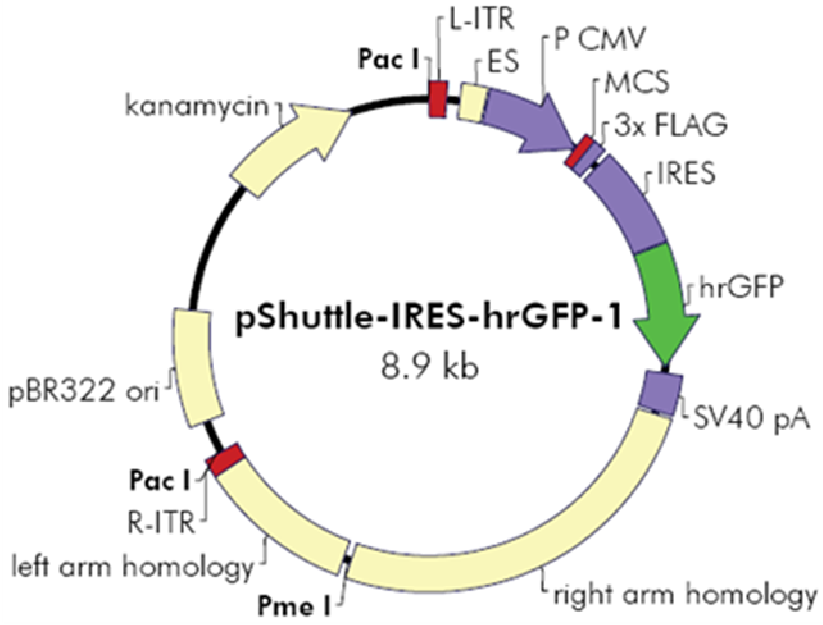
- For *Ad-Null*, *Ad-RSV-GFP*, *Ad-TTR-Xlhbox8* and *Ad-TTR-Xlhbox8VP16*.



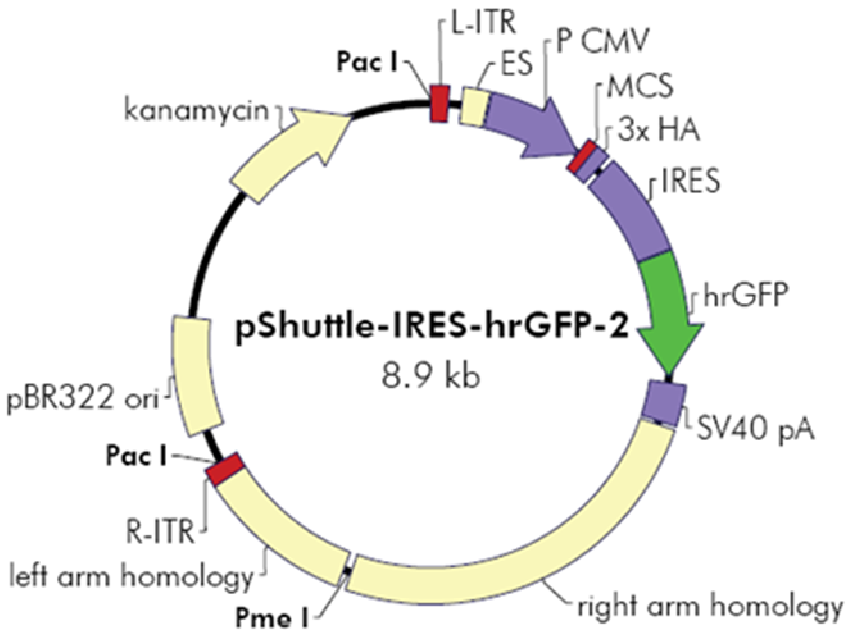
- For *Ad-CMV-Pax4*, *Ad-CMV-Pax6*, *Ad-CMV-NeuroD*, *Ad-CMV-Nkx2.2*, *Ad-CMV-p63* and *Ad-CMV-LIP*.



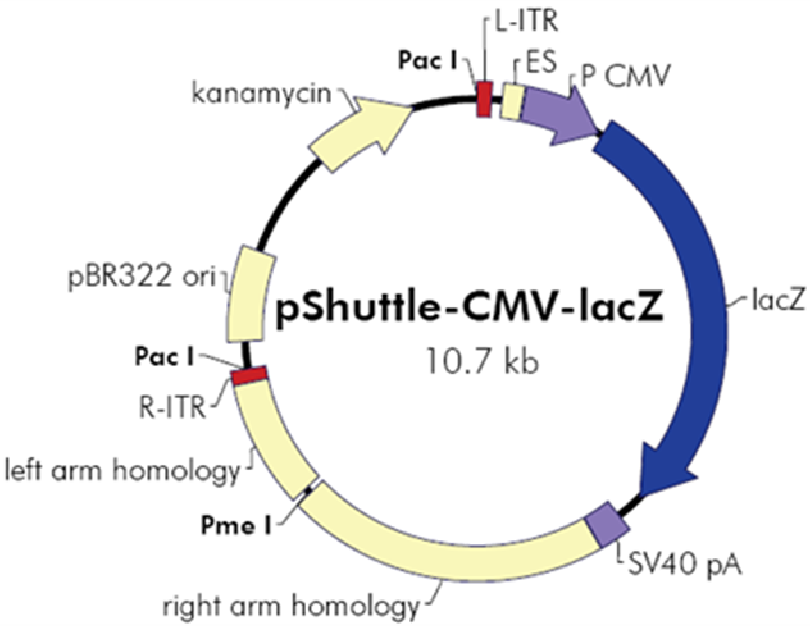
• For *Ad-CMV-Pdx1-GFP*, *Ad-CMV-Pdx1VP16-GFP*, *Ad-CMV-iA1-GFP*, *Ad-CMV-Sox9-GFP*, *Ad-CMV-p48-GFP* and *Ad-CMV-MafA-GFP*.



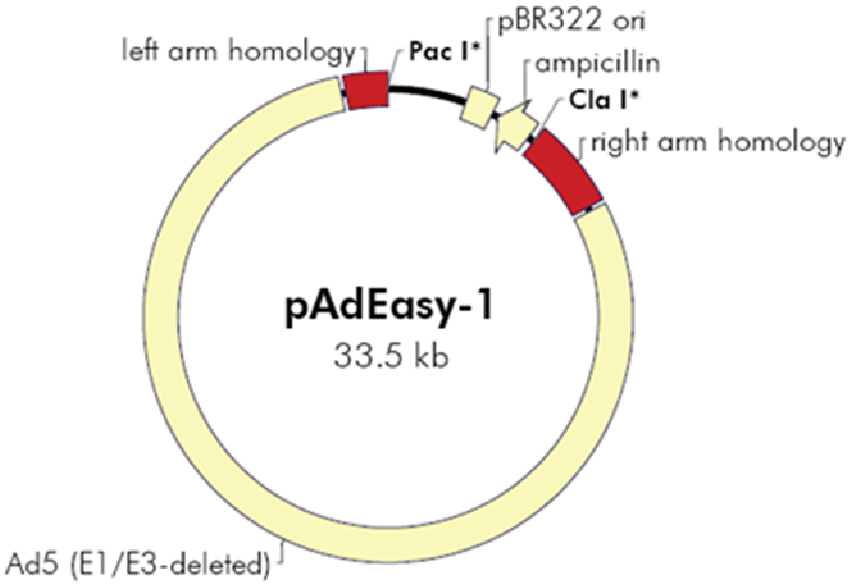
• For *Ad-CMV-HA-Ngn3-GFP* and *Ad-CMV-HA-Nkx6.1-GFP*.



• For *Ad-CMV-LAcZ*.



The ***pShuttle*** vectors were linearised with *Pme*I before transforming the pAdEasy competent cells containing the ***pAdEasy-1*** vector. Transformants were selected for kanamycin resistance. Successful recombinants were identified and produced in bulk in the recombinant-deficient XL10-Gold cells. Purified recombinant Ad plasmid DNA was then subjected to *Pac*I digestion before HEK293 transfection for adenovirus construction.



NOTE: The vector maps are from the AdEasy™ Adenoviral Vector Systems instruction manual.