# Reprogramming of Hepatic and Pancreatic Cells

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# Abstract

Cell therapy involving treatment of diseases with the body's own cells would benefit both liver diseases and Type 1 diabetes. Liver diseases are associated with a marked reduction in hepatocytes whilst Type 1 diabetes is characterized by the loss of functional insulin-producing β-cells. Treatment is currently achieved by whole organ liver (or hepatocyte) and islet transplantation methods respectively. However the major limitation to this approach is the shortage of organ donors, thus alternative sources of cells must be found. Potential sources with enormous therapeutic potential are existing cells in the liver and pancreas involved during the regeneration process. *In vivo* studies have shown progenitor oval cells differentiate into hepatocytes during liver regeneration and α-cells transdifferentiate into β-cells during pancreas regeneration. However neither can be fully exploited until the molecular mechanisms governing their proliferation and trans/differentiation are fully elucidated. Herein we characterise two *in vitro* cell models, a mouse adult oval cell line, known as BMOL-TAT1.1, and mouse adult pancreatic α-cell line, known as α-TC19 by RT-PCR and immunofluorescent staining. We found that under proliferating culture conditions BMOL-TAT1.1 were heterogenous consisting of two distinct cell types with different β-catenin signalling pathway activation. Inducible differentiation (dexamethasone) induced hepatic and non-hepatic markers in specific cell subtypes, indicating multi-potentiality. Ectopic expression of transcription factor HNF4α in homogenous small BMOL-TAT1.1 cells revealed no hepatic differentiation but potent expression of intestinal markers (Villin, ALPi, ApoAIV). HNF4α was identified as a candidate transcriptional regulator in α- to β-cell transdifferentiation, as ectopic expression in α-TC19 cells, suppressed glucagon and induced expression of several functionally important β-cell markers (GLUT2, GCK, insulin). The contribution of chromatin histone acetylation was also assessed, due to its importance in endocrine fate regulation. *In toto* these results have important implications for the development of potential therapies to treat liver diseases and Type 1 diabetes.

*For My Parents Who's Love and Support Taught Me To Take Everything In My Stride*

~

*.....And For My Husband Carl Who's Love and Support Always Pushes Me To Be My Best*

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# Abbreviations

| | | | |
|---|---|---|---|
| ***A*** | Absorbance | **CFTR** | Cystic Fibrosis Transmembrane Regulator |
| **A6** | A6 integrin | **CK7** | Cytokeratin 7 |
| **Ac:Me** | Acetone:Methanol | **CK14** | Cytokeratin 14 |
| **Ad** | Adenovirus | **CK19** | Cytokeratin 19 |
| **AFP** | Alpha- Fetoprotein | **CK20** | Cytokeratin 20 |
| **Akt** | Protein Kinase B | **cm** | Centimetre |
| **ALDPC** | Adult Liver Derived Progenitor Cell Population | **CMV** | Cytomegalovirus |
| **ALPi** | Alkaline Phosphatase Intestinal | **CV** | Central Vein |
| **AMCA** | Aminomethylcoumarin Acetate | **Cx43** | Connexin 43 |
| **APC** | Adenomatous Polyposis Coli | **DAB** | 3,3'-diaminobenzidine |
| **ApoAIV** | Apolipoprotein AIV | **DAPI** | 4,6-diamidino-2-phenylindole |
| **APTS** | (3-amino-propyl1) triethoxysilane | **DAPT** | N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester |
| **Arx** | Aristaless Related Homeobox | **DBA** | *Dolichos Biflorus* Agglutinin |
| **α-TC19** | Alpha TC1 clone 9 | **DEAE** | Diethylaminoethyl |
| **BD** | Bile Duct | **DEPC** | Diethylpyrocarbonate |
| **BME** | Basal Medium Eagle | **Dkk** | Dickkopfs |
| **BMOL** | Bi-potential Murine Oval Cell Line | **DMEM** | Dulbecco's Modified Eagle Medium |
| **BMP** | Bone Morphogenetic Protein | **DMF** | Dimethylformamide |
| **Bp** | Base Pairs | **DMSO** | Dimethyl Sulfoxide |
| **BSA** | Bovine Serum Albumin | **DNA** | Deoxyribonucleic Acid |
| ***c*** | Concentration | **dNTP** | Deoxyribonucleotide Triphosphate |
| **°C** | Degree Celsius | **DT** | Diphtheria Toxin A |
| **cAMP** | Cyclic Adenosine Monophosphate | **DTT** | Dithiothreitol |
| **CAR** | Coxsackie Adenovirus Receptor | ***ε*** | Extinction Coefficient |
| **CDE** | Choline Deficient Ethionine Supplemented | **EDTA** | Ethylene Diamine Tetraacetic Acid |
| **cDNA** | Complementary DNA | **EGF** | Epidermal Growth Factor |
| **C/EBPα** | CCAAT Enhancer Binding Protein Alpha | **EGTA** | Ethylene Glycol Tetraacetic Acid |
| **C/EBPβ** | CCAAT Enhancer Binding Protein Beta | **ELISA** | Enzyme Linked Immunosorbent Assay |

| | | | |
|---|---|---|---|
| **ESCs** | Embryonic Stem Cells | **HNF4α** | Hepatocyte Nuclear Factor 4 Alpha |
| **ERK** | Extracellular Regulated Kinase | **HNF6** | Hepatocyte Nuclear Factor 6 |
| **Fabp2** | Fatty Acid Binding Protein 2 | **HPCs** | Hepatic Progenitor Cells |
| **FACS** | Fluorescent Activated Cell Sorting | **hrGFP** | Humanized Renilla Green Fluorescent Protein |
| **FBS** | Fetal Bovine Serum | **HRP** | Horeradish Peroxidase |
| **FITC** | Fluorescein Isothiocyanate | **HSP90** | Hest-Shock Protein 90 |
| **Fwd** | Forward | **HT** | Hepatocyte Transplantation |
| **G** | Gauge | **IDX-1** | Islet Duodenal Homeobox-1 |
| **GaIN** | D-Galactosamine | **IGF-2** | Insulin Growth Factor – 2 |
| **GCi** | Guanylate Cyclase intestinal | **INS-1** | Rat Insulinoma Cell Line |
| **GCK** | Glucokinase | **IRES** | Internal Ribosome Entry Site |
| **GFP** | Green Fluorescent Protein | **ITS** | Insulin Transferrin Selenious Acid |
| **GGT** | Gamma-Glutamyl Transpeptidase | **IU** | Infection Units |
| **GLUT2** | Glucose Transporter 2 | **$K_3Fe(CN)_6$** | Potassium Ferricyanide |
| **GRE** | Glucocorticoid Response Element | **$K_4Fe(CN)_6$-$3H_2O$** | Potassium Ferrocyanide |
| **GSK-3β** | Glycogen Synthase Kinase 3 Beta | ***l*** | Light Path Length |
| **HAT** | Histone Acetyltransferase | ***lacZ*** | Lactose Operon Z |
| **HCC** | Hepatocellular Carcinoma | **LAP** | Liver-Enriched Activator Protein |
| **HCl** | Hydrochloric Acid | **LEF** | Lymphocyte Enhancer Factor |
| **HDAC** | Histone Deacetylase | **LETF** | Liver-Enriched Transcription Factors |
| **HDACi** | HDAC inhibitors | **LIP** | Liver-Inhibitor Protein |
| **HEK-293** | Human Embryonic Kidney 293 | **LRP** | Low Density Lipoprotein Related Protein |
| **HeLa** | Henrietta Lacks Cell Line | **LSM** | Laser Scanning Microscope |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid | **M** | Molar |
| **$H_2O$** | Water | **mAb** | Monoclonal Antibodies |
| **$H_2O_2$** | Hydrogen Peroxide | **MACS** | Magnetic Activated Cell Sorting |
| **HNF1α** | Hepatocyte Nuclear Factor 1 Alpha | **$M_2$-PK** | Muscle Pyruvate Kinase 2 |
| **HNF1β** | Hepatocyte Nuclear Factor 1 Beta | **Me** | Methanol |
| **HNF3α/ FoxA1** | Hepatocyte Nuclear Factor 3 Alpha/ Forkhead Box A1 | **MEM** | MOPS, EGTA, $MgSO_4$ |
| **HNF3β/ FoxA2** | Hepatocyte Nuclear Factor 3 Beta/ Forkhead Box A2 | **MEMFA** | MEM + 3.8% Formaldehyde |

| | | | |
|---|---|---|---|
| **mg** | Milligram | **PCR** | Polymerase Chain Reaction |
| **$MgCl_2$** | Magnesium Chloride | **PDGF** | Platelet Derived Growth Factor |
| **$MgSO_4$** | Magnesium Sulphate | **Pdx1** | Pancreatic Duodenal Homeobox 1 |
| **ml** | Millilitre | **PEPCK** | Phosphoenolpyruvate carboxykinase |
| **mm** | Millimetre | **PFA** | PBS + 4% Formaldehyde |
| **mM** | Millimolar | **PI-3K** | Phosphoinositide- 3 Kinase |
| **MOI** | Multiplicity Of Infection | **PKA** | Protein Kinase A |
| **MOPS** | 4-morpholinepropanesulfonic Acid | **PNA** | Peanut Agglutinin |
| **mRNA** | Messenger Ribonucleic Acid | **PP** | Pancreatic Polypeptide |
| **Muc2** | Mucin 2 | PP | Periportal |
| **Muc5ac** | Mucin 5, Subtypes A and C | **PV** | Perivenous |
| **NaCl** | Sodium Chloride | **Rev** | Reverse |
| **$NaH_2PO_4$-$H_2O$** | Monobasic Sodium Phosphate | **RIN** | Rat Insulinoma Cell Line |
| **$Na_2HPO_4$** | Dibasic Sodium Phosphate | **RIP** | Rat Insulin Promoter |
| **Ngn3** | Neurogenin 3 | **RNA** | Ribonucleic Acid |
| **Nkx2.2** | NK2 Homeobox 2 | **rpm** | Revolutions Per Minute |
| **ng** | Nanogram | **rRNA** | Ribosomal Ribonucleic Acid |
| **Ngn3** | Neurogenin 3 | **RSV** | *Respiratory Syncytial Virus* |
| **Nkx2.2** | NK2 Homeobox 2 | **RT** | Reverse Transcription |
| **Nkx6.1** | NK6 Homeobox 1 | **SDS** | Sodium Dodecyl Sulfate |
| **nm** | Nanometres | **sFRP** | Secreted Frizzled-Related Protein |
| **Oligo (dT)** | Oligodeoxythymidylic Acid | **SI** | Sucrase-Isomaltase |
| **OLT** | Orthotopic Whole-liver Transplantation | **SMA** | α-Smooth Muscle Actin |
| **OPN** | Osteopontin | **$SSTR_2$** | Somatostatin Receptor 2 |
| **OV6** | Oval Cell Marker | **SV40** | Simian Virus 40 |
| **P** | Phosphate | **TAE** | Tris Acetic Acid + Ethylenediaminetetraacetic Acid |
| **PAS** | Periodic Acid Schiff | **TAT** | Tyrosine Aminotransferase |
| **Pax4** | Paired Box Gene 4 | **TCF** | T Cell Factor |
| **Pax6** | Paired Box Gene 6 | **TFF3** | Trefoil Factor 3 |
| **PBS** | Phosphate Buffered Saline | **TGF-β1** | Transforming Growth Factor Beta 1 |

| | | | |
|---|---|---|---|
| **$T_M$** | Annealing Temperature | **V** | Volts |
| **Tris** | Tris(hydroxymethyl)aminomethane | **v/v** | Volume/ Volume |
| **U** | Units | **vWFC** | Von Willebrand Factor C |
| **µg** | Microgram | **WIF-1** | Wnt Inhibitory Factor-1 |
| **µl** | Microlitre | ***x g*** | Times Gravity (Relative Centrifugal Force) |
| **µm** | Micrometre | **X-gal** | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |
| **µM** | Micromolar | **%** | Percent |
| **UV** | Ultraviolet | | |

# Chapter 1 Introduction

## 1.1 Transdifferentiation definitions and explanations

Transdifferentiation (also referred to as reprogramming) belongs to a wider class of cell-type switches termed 'metaplasias', and is defined as the stable conversion of one cellular phenotype of an already differentiated cell to another type of normal differentiated cell (Okada, 1986, Slack and Tosh, 2001). A differentiated cell is characterised by a distinct phenotype that demarcates it from other cells, either by functional, morphological or biochemical differences. *Eguchi and Kodama* defined two important experimental criteria required to define a conversion as transdifferentiation. First the two differentiated states must be clearly defined by biochemical and morphological characterisation, and secondly the cell lineage relationship between the two cell types must be established (Eguchi and Kodama, 1993). Transdifferentiation is a consequence of a change in expression of master regulatory transcription factors (i.e. master switch genes) whose normal function is to distinguish the two tissues or cell types in normal development (Li, et al., 2005b). The conversion of one differentiated cell type to another has been observed to occur by direct transdifferentiation or indirectly involving de-differentiation to a transitional cell state expressing a mixture of markers and transcription factors found in the progenitor and mature forms of both cell types, this confers multi-potentiality (Figure 1.1).

One of the best documented examples of transdifferentiation is the experimental conversion of pancreas to liver (Shen, et al., 2003). This reflects the close developmental relationship between the two tissues as they arise from adjacent regions of the anterior foregut endoderm, hence the tissues express common transcription factors during early stages of embryonic development (Wells and Melton, 1999) but during later development in response to tissue-specific inductive signals (e.g. FGF and BMP signalling in the liver) key master regulatory transcription factors are differentially expressed in each region, thus inducing the differentiation to the specific cell types.

The process of transdifferentiation is important to study for a number of reasons. Firstly understanding the molecular basis of the conversions will extend our knowledge of the normal developmental mechanisms. Secondly, some types of transdifferentiation which occur naturally predispose to neoplasia and are important in human pathology and the development of various cancers (Figure 1.1), for example Barrett's oesophagus is a

switch from stratified squamous epithelium to columnar epithelium. Barrett's oesophagus is important because it is the only known precursor to oesophageal adenocarcinoma (Hameeteman, et al., 1989). Thirdly, understanding the molecular rules for cell or tissue-type conversions will improve our ability to reprogramme cells for the purpose of therapeutic transplantation (Sangan and Tosh, 2010b).

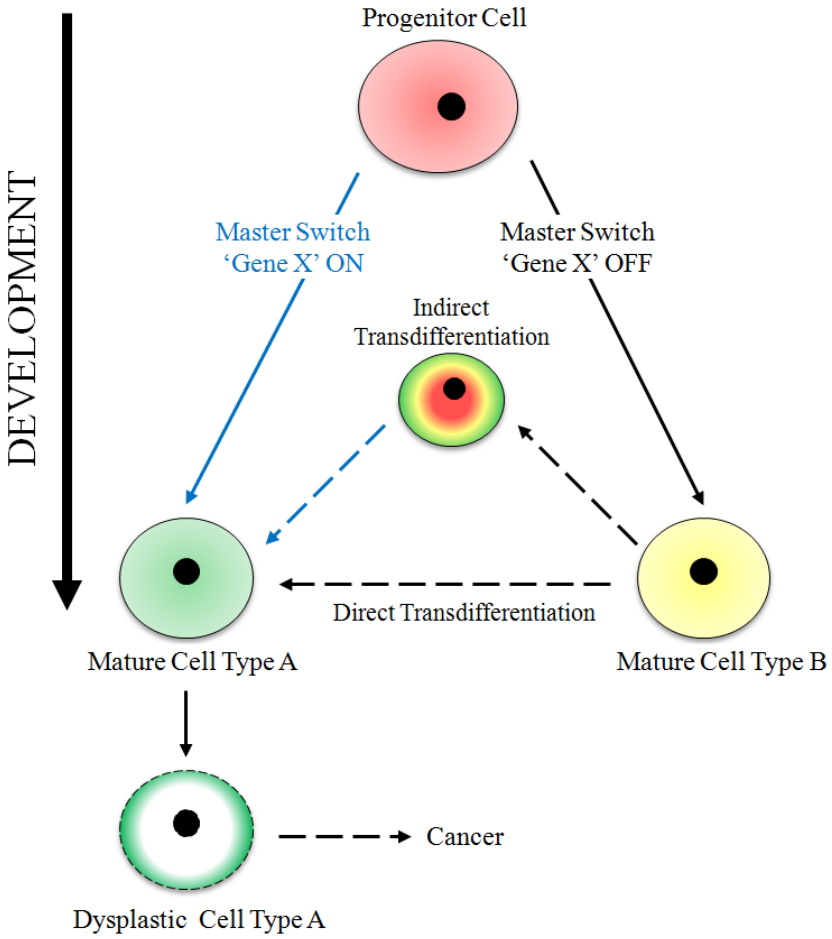



**Figure 1.1: Schematic representation of the relationship between differentiation during development, transdifferentiation and disease.** Adapted from (Eberhard and Tosh, 2008).

## 1.2 Transdifferentiation and regeneration

Many organisms are capable of regenerating parts of their body that have been lost due to injury. The origin of the cells that are involved in regeneration is of considerable debate. It is possible that regeneration arises by transdifferentiation of existing cells. It is equally possible that a reserve of undifferentiated cells, or in other words adult stem or progenitor cells exist, which, when subjected to appropriate cues can be activated to give rise to a whole array of cell types (Slack, 2003, Thowfeequ, et al., 2007).

## 1.3 The liver anatomy and physiology

The liver represents the largest organ in the body and is specialized to perform a wide range of tissue-specific metabolic functions including: gluconeogenesis, detoxification, plasma protein synthesis, bile acid formation and drug metabolism.

The functional units of the mature liver architecture are termed hepatic lobules (Kiernan, 1833) and these units assume a roughly hexagonal shape comprising of hepatocytes arranged in one cell thick plates radiating out from the central vein towards the portal triad (composed of a branch of the hepatic artery, portal vein and bile duct), with intervening sinusoids (Figure 1.2A,B). The lobule can be divided into two zones, the periportal zone surrounding the portal triad and the perivenous zone surrounding the central vein. Periportal and perivenous hepatocytes display the remarkable phenomenon of functional heterogeneity, as differences in ultra-structure and activities of key rate-limiting enzymes results in differences in cellular functions running along gradients from one zone to the other (Gebhardt, 1992) (Figure 1.2C). For instance rate-limiting enzymes for pathways such as oxidative metabolism (succinate dehydrogenase), amino acid metabolism (serine dehydratase) and gluconeogenesis (phosphoenolpyruvate carboxykinase, glucose-6-phosphatase) are higher in the periportal zone whereas activities of other enzymes such as in glycolysis (glucokinase), fatty acid synthesis (acetyl-CoA carboxylase) and xenobiotic metabolism (cytochrome P450) are higher in the perivenous zone (Jungermann and Kietzmann, 1996).

## 1.4 Cell types present in the adult liver

The pre-dominant parenchymal cells of the mature liver are the hepatocytes which constitute approximately 80% of the total hepatic cell volume (Blouin, et al., 1977). The remaining cell volume consists intra-hepatic ducts which include cholangiocytes (biliary epithelial cells) and the hepatic sinusoid which is lined by four different cell types, each with specific phenotypic characteristics, function and topography, including sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and pit cells (Zaret, 2002, Zhao and Duncan, 2005).

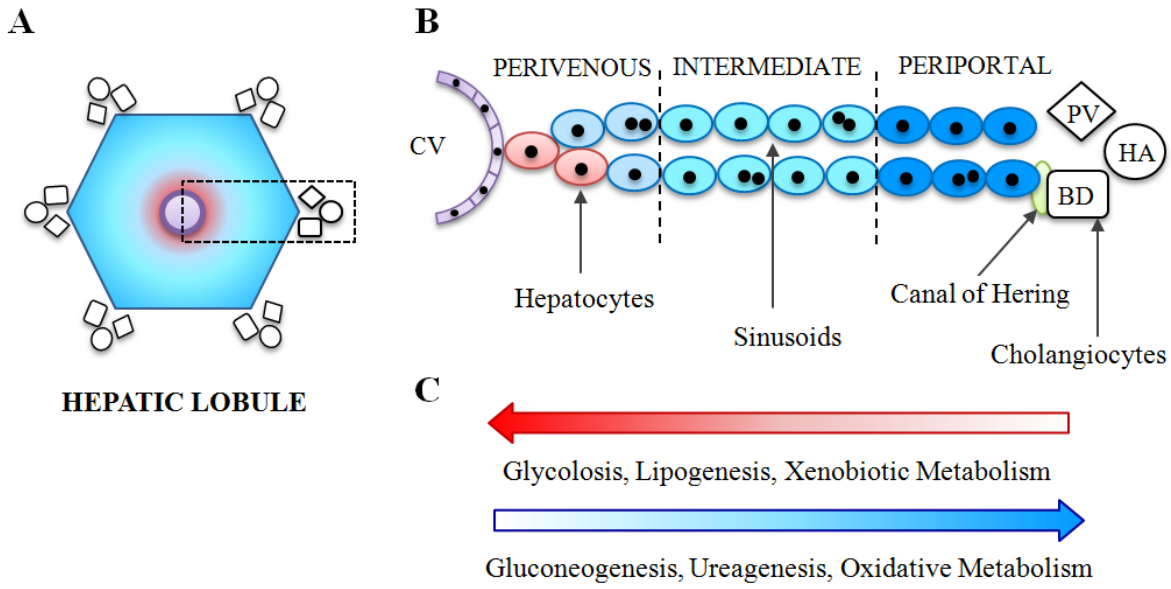



**Figure 1.2: Lobular Structure of the Liver and Hepatocyte Heterogeneity.** Schematic representation of the hexagonal hepatic lobule **(A)** and enlargement of a region labelled with micro-anatomical detail **(B)**. Blood flows through the sinusoids between one cell thick hepatocyte plates, from the hepatic artery (HA) and portal vein (PV) towards the central vein (CV). The Canal of Hering, which is the junction between terminal bile duct (cholangiocyte, BD) and hepatic plates is where oval cells are believed to originate from. **(C)** Hepatocytes exhibit gradients in metabolic functions.

### 1.4.1 Hepatocytes

Hepatocytes are characterised by a cuboidal epithelial cell morphology, ranging in size from 20-30µm. Hepatocytes can be, mono- or bi-nucleated and are rich in organelles such as endoplasmic reticulum and mitochondria, in order to facilitate their enormous metabolic activity. Hepatocytes are polarised, and although lack a basement membrane, have three functionally specialised membrane domains. The basolateral domain consists of numerous surface microvilli to facilitate the flow of molecules between the sinusoidal blood, as it faces the space of Disse, which is the perisinusoidal space between the endothelium lining the sinusoids and the hepatocytes (Grisham, et al., 1975). The lateral intercellular membrane domain connects the basolateral to the apical domain. The apical domain forms channels known as canaliculi with the apical domain of opposing hepatocytes. The canaliculi via site specific transport systems allow the bile secreted from the apical membrane of hepatocytes to be directed towards the periportal zone of the lobule so that it can be collected in the intra-hepatic bile duct and subsequently delivered to the gall bladder for storage before being transported to the intestine via the extra-hepatic bile duct.

### 1.4.2 Cholangiocytes

Cholangiocytes, also known as bile duct (or biliary epithelial) cells constitute approximately 3-5% of the total hepatic cell population (Tavoloni, 1987). Cholangiocytes line the small and large intra-hepatic ducts making up the biliary tree, with their primary function regulating, modifying and transporting secreted canalicular bile. Research has shown cholangiocytes are morphologically and functionally heterogeneous, not only in their proliferative response to injury (Kanno, et al., 2000) but also differing in their bile (bicarbonate and water) secretion in response to a number of factors. Gastrointestinal hormone secretin interacts with its own receptor thus increasing cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) activity, which activates chloride channel cystic fibrosis transmembrane regulator (CFTR), leading to $Cl_2/HCO_3$ exchanger activation and subsequent secretion of bicarbonate in water (Kanno, et al., 2001). On the other hand, gastrointestinal hormone somatostatin interacts with somatostatin receptor 2 ($SSTR_2$) to inhibit secretin-stimulated bile secretion via inhibition of exocytic vesicle insertion into cholangiocyte apical membranes (Tietz, et al., 1995). Numerous studies in rats have also shown certain bile acids enter cholangiocytes through sodium-dependent apical bile acid transporters in order to modify secretin-stimulated bile secretion (Lazaridis, et al., 1997). The expression of different proteins (e.g. receptors and transporters) important in regulating cholangiocyte function and conferring heterogeneity is dependent on the cholangiocyte's location within the intra-hepatic duct. Studies have shown that the secretin receptor and somatostatin receptor ($SSTR_2$) are solely expressed by the progressively larger and columnar cholangiocytes in the extra-hepatic duct, hence the smaller cuboidal cholangiocytes in the small intra-hepatic duct do not participate in hormone-regulated ductal secretion (Alpini, et al., 1997).

### 1.4.3 Sinusoidal endothelial cells

Blood from the hepatic artery and portal vein mix before entering the hepatic sinusoids, which are the blood vessels radiating out between the hepatocyte plates. The sinusoidal wall is lined with highly specialised sinusoidal endothelial cells with important filtration functions due to the presence of small fenestrations, which are small open pores lacking a diaphragm and a basal lamina underneath the endothelium. This allows rapid diffusion of selective nutrients, lipids and lipoproteins between the blood and the

hepatocyte surface required for liver metabolism. Sinusoidal endothelial cells also display a pronounced endocytotic capacity, this function is reflected by the presence of numerous endocytotic vesicles and by the effective uptake of a wide variety of substances (e.g. glycoproteins, extracellular matrix components and immune complexes) from the blood by receptor-mediated endocytosis (Kmiec, 2001, Smedsrod, et al., 1994).

### 1.4.4 Kupffer cells

Kupffer cells were named after German anatomist Karl Wilhelm von Kupffer when they were first described 1876. Kupffer cells are specialised macrophages and hence possess a pronounced endocytotic and phagocytic capacity. In the liver they are intra-sinusoidally located, pre-dominantly in the periportal zone where the blood enters, in order to clear dysfunctional erythrocytes and pathogens (Arii and Imamura, 2000). Kupffer cells are also potent mediators of the inflammatory response by the secretion of a variety of bioactive factors and thus play an important part in immune defence (Bouwens, et al., 1992).

### 1.4.5 Hepatic stellate cells

Hepatic stellate cells (also referred to as Ito cells or fat-storing cells) are present in the perisinusoidal space of Disse, extending well-developed long processes around the sinusoidal endothelial cells (Sato, et al., 2003). In the healthy liver, hepatic stellate cells are quiescent and the major site of vitamin A-rich lipid storage and possibly the predominant hepatic cell type in producing extracellular matrix components (Bouwens, et al., 1992, Friedman and Arthur, 1989, Gressner, 1995, Wake, 1971). When the liver is damaged, hepatic stellate cells proliferate and become activated, which involves loss of vitamin A and morphological changes to adopt a myofibroblastic-like phenotype with well-developed stress fibres of actin cytoskeleton. For instance the activated state of hepatic stellate cells can be distinguished by the expression of α-smooth muscle actin (Schmittgraff, et al., 1991). In addition to producing extracellular matrix components, activated hepatic stellate cells also secrete transforming growth factor beta 1 (TGF-β1) and platelet derived growth factor (PDGF), the best characterised fibrogenic and proliferative cytokines, in order to facilitate liver regeneration, including both parenchymal cell proliferation and extracellular matrix remodelling (i.e. collagen synthesis) (Sato, et al.,

2003). However there is now substantial evidence that exists showing hepatic stellate cells as the major matrix producing cell in the process of liver fibrosis (Moreira, 2007). Cytokines TGF-β1 and PDGF are postulated to be the key mediators in fibrogenesis, as both have been shown to be increased in experimental and human hepatic fibrosis (Friedman, 2000, Pinzani, et al., 1998).

### 1.4.6 Pit cells

Pit cells represent a liver associated population of large granular lymphocytes i.e. liver-specific natural killer cells present in the hepatic sinusoidal lumen (Kaneda, et al., 1983). Therefore pit cells perform important anti-tumorigenic and anti-viral activity within the liver (Bouwens and Wisse, 1992). It has also been reported that liver tumour cell killing is synergistically enhanced when pit cells attack tumour cells together with Kupffer cells (Wisse, et al., 1997).

## 1.5 Liver disease and current treatments

Liver disease is a broad term encompassing various specific diseases involving acute, mechanical, chemical or immune-related injury to the liver (e.g. cancer, cirrhosis and hepatitis). At present for patients presenting with liver failure (i.e. the liver is incapable of regeneration and repair) the only curative treatment option is orthotopic whole-liver transplantation (OLT) which has become an almost routine procedure with one-year survival rates exceeding 80% (Neuberger, 2000). This approach although successful possesses some serious limitations, primarily the availability of suitable donor livers. In the USA, an estimated 17,000 patients were on the waiting list for liver transplantation surgery during 2002, from which less than 30% of these received transplants (Knight, et al., 2005). Various investigations have therefore been stimulated to develop potential alternatives to OLT. One area of research has focused on developing hepatocyte transplantation (HT), which involves transfer of healthy adult hepatocytes to the patient via intraperitoneal or intrasplenic injection or directly via portal vein infusion which is much less invasive then OLT (Knight, et al., 2005). Because of the considerable regenerative capacity of the liver, a moderate size graft can, in principle, expand in size and colonise a significant fraction of a damaged liver. Such approaches have been used to re-establish

liver function in a modest number of cases, especially for patients with fulminant hepatic failure and hereditary liver disease (Galvao, et al., 2006). Unfortunately HT exhibits similar limitations to OLT, as currently the only source of hepatocytes for transplantation are those obtained from donor liver tissue deemed unsuitable for OLT, hence cells are of poor quality. Other problems related to utilising freshly isolated hepatocytes are that they are unable to readily proliferate in culture, are difficult to freeze down and store (cryoperserve), and rapidly lose hepatic function (i.e. de-differentiate) when maintained *in vitro* for more than one week, due to changes in environmental conditions (Padgham, et al., 1993). There is also the problem of allo-immunity which exists for all organ transplantation and cell therapy (Chidgey, et al., 2008). Much of the cost and complexity of organ transplantation arises because of the need for long term immunosuppression and the continual monitoring for rejection and treatment of opportunistic infections that is necessary as a result (Sangan and Tosh, 2010b). Consequently, some researchers have begun exploring alternative sources of donor cells which are capable of functioning as adult hepatocytes *in vivo* but are void of the limitations associated with OLT and HT. Alternatives with therapeutic potential include: embryonic and adult stem cells, conditionally immortalised adult hepatocytes, fetal hepatocytes, differentiated hepatic progenitor cells (HPCs) and transdifferentiated non-liver progenitor cells. In addition, generated hepatocytes would be suitable not only for transplantation but also bio-artificial liver systems, which are devices which can be connected to the patient's bloodstream in order to take over liver function for a period allowing the patient's own liver to recover from the damage it has sustained (Allen, et al., 2001, van de Kerkhove, et al., 2004). Despite fibrotic and cirrohotic livers being suboptimal for HT as described above, these livers provide a rich source HPCs, as HPCs have been shown to proliferate readily under chronic conditions. Therefore the molecular mechanisms governing the proliferation and differentiation of adult-derived HPCs towards functional hepatocytes need to be fully elucidated.

## 1.6 Oval cells: progenitor cell mediated regeneration

Progenitor cells (also known as transit amplifying cells) are defined as any rapidly dividing cells with the capacity to differentiate, but in contrast to *bona fide* stem cells are more specific to certain cell lineages (i.e. in a further stage of cell differentiation) and do not possess the ability to indefinitely self-renew (Smith, 2006). Progenitor cells have the potential to generate more than one differentiated cell type (e.g. hepatocytes) but cannot be serially transplanted (Potten, et al., 1997, Shafritz and Oertel, 2010). In the supporting literature a variety of nomenclatures exist to describe hepatic progenitor cells (HPCs), the term oval cells is utilised primarily in animal studies, whereas the term "intermediate hepatobillary cells" refers to human hepatic progenitor cells (Roskams, et al., 2004). Additional terms utilised for HPCs include: ductular progenitor cells, atypical ductular cells or peri-ductular liver progenitor cells. This thesis will utilise the nomenclature oval cells.

### 1.6.1 History and features of oval cells

During liver regeneration oval cells are essential at forming a 'second line of defence'. Although the liver has enormous potential to regenerate by replication of remaining healthy hepatocytes, numerous studies have exposed that when replacement of lost viable hepatic mass by remaining hepatocytes is partially or completely precluded, a distinct population of oval cells are induced to proliferate and differentiate towards hepatocytes (Knight, et al., 2005, Akhurst, et al., 2001). This property can be observed in several studies which show that oval cell numbers induced in pathologies are proportional to the progression and severity of the underlying liver disease (Lowes, et al., 1999).. A diverse range of protocols have been established in order to induce oval cell proliferation as detailed in Table 1.1 (Dolle, et al., 2010). Lineage tracing experiments in mice expressing tamoxifen-inducible Cre recombinase under control of the HPC marker Osteopontin (OPN) regulatory region, have also confirmed the capacity of HPCs to differentiate into functional hepatocytes *in vivo* and contribute to liver regeneration in response to several forms of chronic liver injury (Espanol-Suner, et al., 2012).

| Method (Abbreviation) | Model |
|---|---|
| Phenobarbital | mouse |
| 2-acetylamino-fluorene (AAF)<br>Allyl alcohol (AA) | mouse, rat |
| 2-acetylamino-fluorene (AAF)<br>Carbone tetrachloride ($CCl_4$) | rat |
| 2-acetylamino-fluorene (AAF)<br>Allyl alcohol (AA) | rat |
| Choline-deficient diet (CD)<br>2-acetylamino-fluorene (AAF) | rat |
| **Choline-deficient ethionine-supplemented diet (CDE)** | mouse, rat |
| **3-diethoxycarbonyl-1,4-dihydrocollidine (DDC)** | mouse, rat |
| **N-acetyl-p-aminophenol (APAP)** | mouse |
| Allyl alcohol (AA) | mouse, rat |
| Carbone tetrachloride ($CCl_4$) | mouse |
| Diethylnitrosamine (DEN) | mouse, rat |
| Retrorsine<br>Carbone tetrachloride ($CCl_4$) | rat |
| Retrorsine<br>Allyl alcohol (AA) | rat |
| Retrorsine<br>Partial hepatectomy (PH) | rat |
| 1,4-bis[N.N'-di(ethylene)phosphamide]piperazine (DIPIN)<br>Partial hepatectomy (PH) | mouse, rat |
| D-Galactosamine (GaIN)<br>Partial hepatectomy (PH) | mouse, rat |
| Lasiocarpine<br>Partial hepatectomy (PH) | rat |
| Long term ethanol | mouse, rat |

**Table 1.1: Experimental models for oval cell induction.**
Most frequently utilised in rodents are highlighted in bold**.** See (Dolle, et al., 2010).

The first formal description of oval cells was in 1956 when it was suggested that they be termed "oval" due to their distinctive ovoid shape in cross section (Knight, et al., 2005Farber, 1956). They are substantially smaller than adult hepatocytes, ranging in size from 7-10μm, with scant organelle poor cytoplasm, and a distinctive ovoid nucleus. Oval cells are scarce in the healthy liver, but upon stimulation from the Canals of Hering (a space lying between the terminal branch of the bile duct and the first row of adjacent hepatocytes in the periportal region of the liver near the portal triad) proliferate across the hepatic lobule infiltrating the liver parenchyma (Akhurst, et al., 2001, Factor, et al., 1994, Knight, et al., 2005).

| Marker | Oval Cells | Hepatocytes | Cholangiocytes | Representative Reference |
|---|---|---|---|---|
| OV6 | + | -- | + | (Dunsford et al., 1989) |
| A6 Antigen | + | -- | + | (Engelhardt et al., 1990) |
| AFP | + | Fetal | + | (Kuhlmann and Peschke, 2006) |
| Transferrin | + | + | -- | (Fiorino et al., 1998) |
| $M_2$-PK | + | Fetal | + | (Tian et al., 1997) |
| E-cadherin | + | + | + | (Tirnitz-Parker et al., 2007) |
| CK7 | + | -- | + | (Libbrecht and Roskams, 2002) |
| CK8 | + | + | + | (Golding et al., 1995) |
| CK14 | +/-- | -- | -- | (Bisgaard et al., 1994) |
| CK18 | + | + | -- | (Golding et al., 1995) |
| CK19 | + | -- | + | (Libbrecht and Roskams, 2002) |
| OPN | + | -- | + | (Espanol-Suner, et al., 2012) |

**Table 1.2: Markers of oval cells**

Oval cells are a heterogeneous population expressing an array of phenotypic markers in common with cholangiocytes, fetal and adult hepatocytes, as detailed in Table 1.2 (Dorrell, et al., 2008). They have multi-potency capability to not only differentiate into hepatic lineages (i.e. hepatocytes and cholangiocytes) but also some non-hepatic lineages, such as intestinal and pancreatic cell types as illustrated in Figure 1.3 (Leite, et al., 2007, Tatematsu, et al., 1985, Yang, et al., 2002).

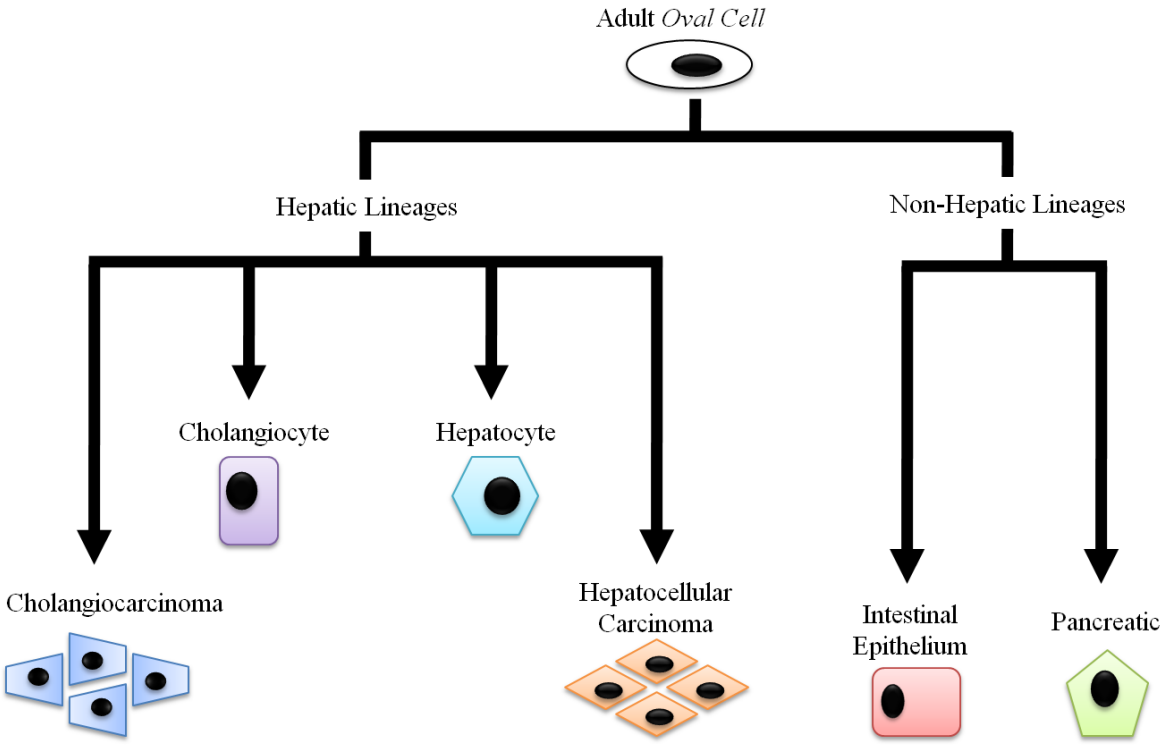



**Figure 1.3: Multi-potent capability of oval cells.**
Schematic representation of oval cell differentiation into hepatic or non-hepatic lineages

## 1.7 Oval cells and cancer

Figure 1.3 draws attention to the problem associated with oval cell differentiation during liver regeneration, in that these metaplasias (i.e. the irreversible conversion of one cellular phenotype of an already differentiated cell to another type of normal differentiated cell) result in cell types which can act as pre-cursors to neoplasia and thus carcinogenic.

### 1.7.1 Hepatocellular carcinoma and cholangiocarcinoma

To emphasis the potential for oval cells to be carcinogenic, studies have shown a correlation between oval cell appearance and the early stages of hepatocellular carcinoma (HCC) (Alison and Lovell, 2005, Hacker, et al., 1992), and cholangiocarcinoma (Roskams, 2006a). Research has also suggested that cholangiocarcinoma originates from HPCs (Komuta, et al., 2008, Nomoto, et al., 2006). For instance it has been shown that transformation and transplantation by subcutaneous injection of the rat oval cell lines OC/CDE 6 and OC/CDE 22 gave rise to cholangiocellular carcinoma (Steinberg, et al., 1994). Similarly the chemical transformation and transplantation by subcutaneous injection of the first available rat oval cell line WB-FB344, extensively characterised by *Grisham et al.,* led to the formation of HCC (Tsao and Grisham, 1987). Also it has been demonstrated that the common option of intestinal metaplasia for oval cells is related to cholangiofibrosis and subsequent cholangiocarcinoma in livers of rats exposed to the carcinogen 2-acetylaminofluorene (AAF) (Barut and Sarraf, 2009, Tatematsu, et al., 1985).

### 1.7.2 The maturation arrest hypothesis

There are at least two possible cellular lineages of cancer during HCC formation. Tumours may arise by de-differentiation of adult hepatocytes or by the maturation arrest of a progenitor/ stem cell like population (Sell, 1993). Therefore if maintained in a prolonged proliferative state, oval cells in the liver are likely candidates for transformation and subsequent progression of HCC and cholangiocarcinoma. This theory originates from the concept of maturation arrest or blocked ontogeny attributed to Van Rensselaer Potter (Potter, 1978). Potter in 1978 hypothesised that hepatic tumours are due to an accumulation of stem/ progenitor cells (i.e. oval cells) which are blocked from terminally differentiating, hence display an immature phenotype, partially differentiated towards

either hepatocytes or cholangiocytes and are unable to undergo apoptosis. Studies supporting the idea of maturation arrest show the presence of these arrested transitional progenitor cells in both HCC and cholangiocarcinoma (Libbrecht, et al., 2000, Shachaf, et al., 2004).

## 1.8 Transcriptional regulation of hepatocyte differentiation

The step-wise acquisition of the hepatocyte phenotype is associated with the sequential expression of various hepatocyte-specific genes which encode key functional proteins (Costa, et al., 2003); and the temporal and tissue-specific expression of all these genes is orchestrated by the synergistic binding of certain combinations of liver-enriched transcription factors (LETFs) (Darlington, 1999, Lemaigre and Zaret, 2004). Numerous investigations have focused on elucidating the roles of specific groups of LETFs during normal hepatocyte differentiation. Figure 1.4 provides a simplified schematic representation of the transcriptional networks operating during liver development. LETFs can be subdivided into two main groups.

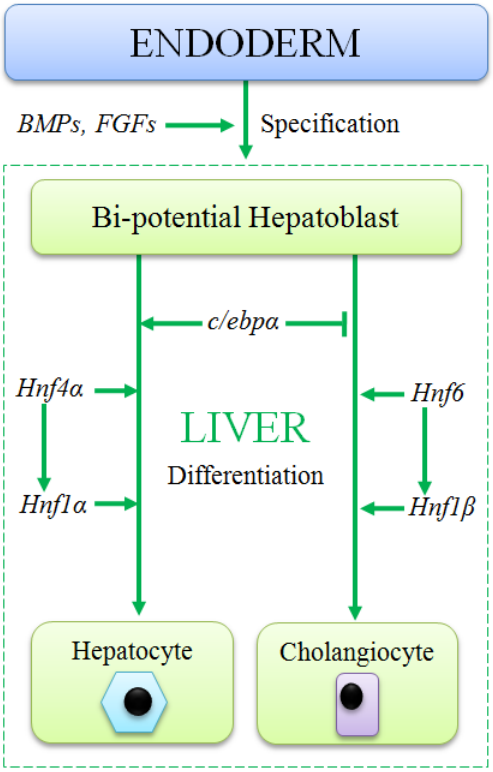



**Figure 1.4: Transcriptional cascade during liver development.** Summary of the important regulatory roles of hepatocyte nuclear factors (HNFs) and CCAAT/enhancer binding protein alpha (C/EBPα) in hepatocyte and cholangiocyte differentiation.

### 1.8.1 CCAAT/enhancer binding proteins (C/EBPs)

The CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors comprising six members (C/EBP α, β, γ, δ, ε, ζ), each capable of interacting with CCAAT motifs present in numerous gene promoters (Lekstrom-Himes and Xanthopoulos, 1998, Westmacott, et al., 2006). C/EBPs have pivotal roles in numerous cellular processes, for example control of growth and differentiation and also immune and inflammatory responses (e.g. regulation of acute phase proteins during initial response to injury) (Kurash, et al., 2004, Ramji and Foka, 2002). C/EBPα and C/EBPβ have been shown to be expressed at high levels in the liver, adipose and lung tissue. Both have been suggested as important master regulators of liver development and differentiation, which via cooperation with other LETFs activate several liver specific genes with high specificity (Diehl, 1998, Takiguchi, 1998). C/EBPα can activate the albumin promoter or phosphoenolpyruvate carboxykinase (PEPCK) promoter synergistically with HNF1α (Wu, et al., 1994, Yanukakashles, et al., 1994), whilst cooperation of C/EBPβ and HNF4α is essential for activation of the ornithine transcarbamylase enhancer (Nishiyori, et al., 1994). C/EBPα expression is rapidly induced during liver injury once the regeneration via differentiation of oval cells is nearly complete (Dabeva, et al., 1995). This relates to the paradigm that C/EBPα may have a role in terminal differentiation of hepatocytes through regulation of promoter and enhancer sequences of several important liver genes involved in hepatic glycogen synthesis, gluconeogenesis (e.g. tyrosine aminotransferase (TAT)) and lipid homeostasis. Furthermore, contrasting to increased C/EBPα expression being fundamental in hepatocyte differentiation, *Yamasaki et al.*, have postulated that the absence of C/EBPα in cholangiocytes has an indirect effect of inducing the expression of the genes HNF6 and HNF1β (Yamasaki, et al., 2006). Studies with targeted inactivation of HNF6 and HNF1β genes demonstrate that both have important regulatory roles in cholangiocyte differentiation, and that HNF6 acts upstream of HNF1β (Clotman, et al., 2002, Coffinier, et al., 2002). Therefore C/EBPα expression could be important in the decision of bi-potential precursors to differentiate towards hepatocytes or cholangiocytes (Figure 1.4).

### 1.8.2 Hepatocyte nuclear factors (HNFs)

The Hepatocyte nuclear factors (HNFs) encompass four families HNF1, HNF3 (FoxA), HNF4 and HNF6. HNF1, is a member of the POU homeobox gene family and

includes HNF1α and HNF1β which are involved in hepatocyte and cholangiocyte differentiation respectively (Figure 1.4). The HNF3 (FoxA) gene subfamily is composed of three proteins (HNF3α, β, γ) which are also named Forkhead Box (Fox) A1, A2, A3 respectively and play critical roles in organ formation and tissue specification. HNF4 belongs to the nuclear steroid-thyroid receptor super-family (Sladek, et al., 1990) and consists of members HNF4α, β, γ and many splice variants, however unlike HNF4α and β, HNF4γ is not expressed in the liver. The final family HNF6, has been established as an important factor in liver cholangiocyte differentiation (Figure 1.4) (Nagaki and Moriwaki, 2008).

There is conflicting evidence for the role of HNF4α during differentiation of oval cells to hepatocytes. *Dabeva et al.,* investigated oval cell differentiation *in vivo* by analysing the expression of liver-enriched transcription factors (including: HNF1α; FoxA1, 2, 3; HNF4α; C/EBPα, β, δ) following non-carcinogenic D-galactosamine (GaIN) induced liver injury in rats. *Dabeva and colleagues* suggested that because HNF4α and HNF1α are controlled by a higher order locus it is possible that in the oval cell differentiation program, when HNF1α is already expressed activation of high HNF4α levels are not required (Dabeva, et al., 1995). However the normal developmental hepatocyte differentiation program is suspected to be similar to that of oval cells, as developing liver cells (hepatoblasts) may correspond to immature progenitor cells, which are maintained after birth and constitute a minor subpopulation in the adult liver giving rise to the oval cells during liver injury (Lemire, et al., 1991). In normal development HNF4α may act upstream in a cascade to activate other hepatocyte transcription factors and thus there is no compensation by other factors, as many nuclear hormone receptors, such as HNF4α are involved in chromatin remodelling via interaction with transcriptional co-activators (Li, et al., 2000). In addition, from a morphological perspective, development of the normal liver architecture is crucial for correct liver function. HNF4α has been shown to be essential in regulating epithelial morphogenesis and functional differentiation of hepatocytes (Figure 1.5) (Parviz, et al., 2003).

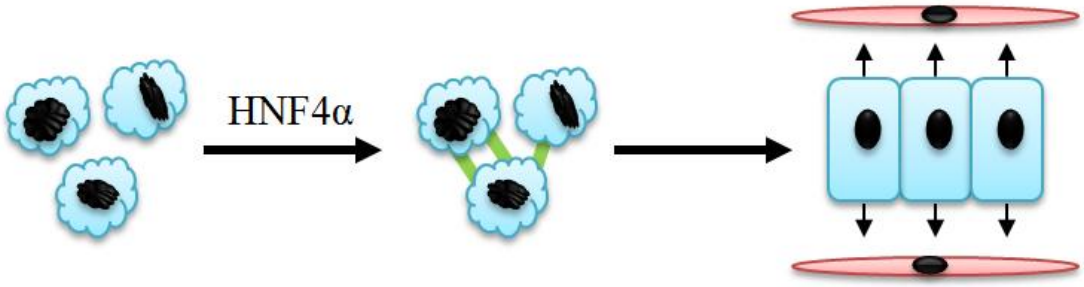



**Figure 1.5: HNF4α is essential for maintenance of hepatic architecture.** A schematic model showing that increases in HNF4α expression drives differentiation of hepatoblasts to hepatocytes. In addition, HNF4α is necessary for expression of cell adhesion molecules and junctional proteins that allow the hepatic cells to form a polarized epithelium.

*Suetsugu et al.*, demonstrated that over-expression of HNF4α *in vitro* induced a mature gene expression pattern and liver function in fetal hepatic progenitor cells, which when transplanted into mice with liver fibrosis improved survival (Suetsugu, et al., 2008). This study highlights that although *in vitro* work is beneficial to identifying key factors important for oval cell hepatocyte differentiation, further complementary *in vivo* work is ultimately required, as other extracellular environmental factors may influence oval cell differentiation. It is hypothesised that the adult hepatic progenitor cell population is located within a specialised environment, in which the extracellular matrix component laminin maintains the cells in a progenitor/ biliary undifferentiated state until proliferation in response to injury induces cells to leave the laminin region, and differentiate into a hepatocyte phenotype due to the effect of other matrix components (e.g. fibronectin) (Lorenzini, et al., 2010). A review by *Erker and Grompe* emphasises the importance of extracellular factors secreted by surrounding non-parenchymal cells types, such as inflammatory, Kupffer and hepatic stellate cells in stimulating oval cell proliferation and differentiation (Erker and Grompe, 2008). Indeed studies have shown differentiation of oval cells into mature hepatocytes to be induced by hepatic stellate cells (Chen, et al., 2009), and recently it has been shown during hepatocyte regeneration, Wnt3a expression by Kupffer cells (macrophages) following engulfment of hepatocyte debris induces canonical Wnt signalling in nearby oval cells which maintains Numb (a cell fate determinant) expression and promotes their hepatocyte cell fate specification (Boulter, et al., 2012).

## 1.9 The pancreas: physiological function and anatomy

The pancreas is a glandular organ divided into two separate functional units the exocrine and endocrine pancreas, which regulate the distinct physiological processes digestion and glucose metabolism respectively (Bardeesy and DePinho, 2002). Within the exocrine compartment there are acinar cells which constitute the bulk of the mature pancreatic tissue, and primarily produce digestive enzymes such as amylase, serine proteases (elastase, chymotrypsin and trypsin), lipases, and procarboxypeptidases. The acinar cells are organised into small grape like clusters located at the smallest termini of the highly branched ductal network (Figure 1.6B). These ducts add mucous and bicarbonate to the digestive enzymes released from the acinar cells into the ductal lumen, hence facilitating their delivery to the duodenum (Figure 1.6A). The endocrine pancreas on the other hand which is vital for regulation of blood glucose consists compact highly vascularised and highly innervated structures known as the islets of Langerhans which are embedded within acinar tissue and collectively constitute less than 2% of the pancreatic mass (Figure 1.6C).

## 1.10 Cell types in the adult endocrine pancreas

The endocrine islets of Langerhans are a heterogeneous cell population comprising five specialized cell types: alpha (α), beta (β), delta (δ), epsilon (ε), and pancreatic polypeptide (PP) cells, which produce the hormones glucagon, insulin, somatostatin, ghrelin, and PP, respectively. This diverse range of islet hormones is vital for control of blood glucose homeostasis.

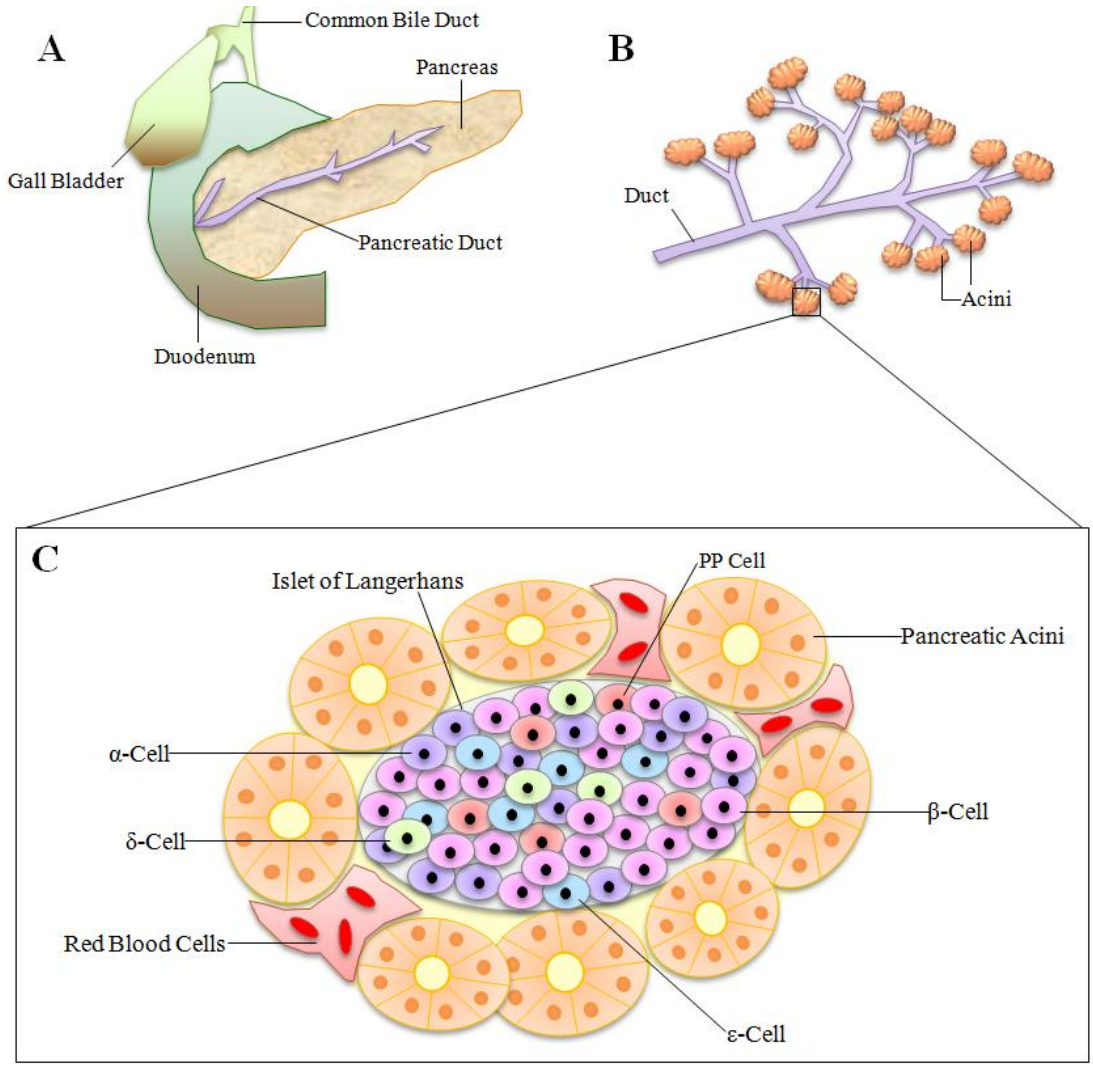



**Figure 1.6: The pancreas functional anatomy. (A)** The gross anatomy of the pancreas. **(B)** The exocrine pancreas. **(C)** The endocrine pancreas, islet of Langerhans embedded in the exocrine tissue.

### 1.10.1 β-cell: insulin

The β-cells are the most prominent cell type in the islet, segregating to the islet core, and constitute 50-80% of the total islet depending on the species (Brissova, et al., 2005, Cabrera, et al., 2006, Murtaugh, 2007). β-cells are approximately 9-15μm in size and are unique in their function, as in response to hyperglycaemia (i.e. increase in blood glucose concentrations after a meal) (Figure 1.7) the hormone insulin is transcribed, translated, processed (Figure 1.8) and secreted (Figure 1.9). Insulin secretion into the bloodstream simulates glucose transport and uptake into most of the body's cells (including muscle and adipocytes) and inhibits liver gluconeogenesis.

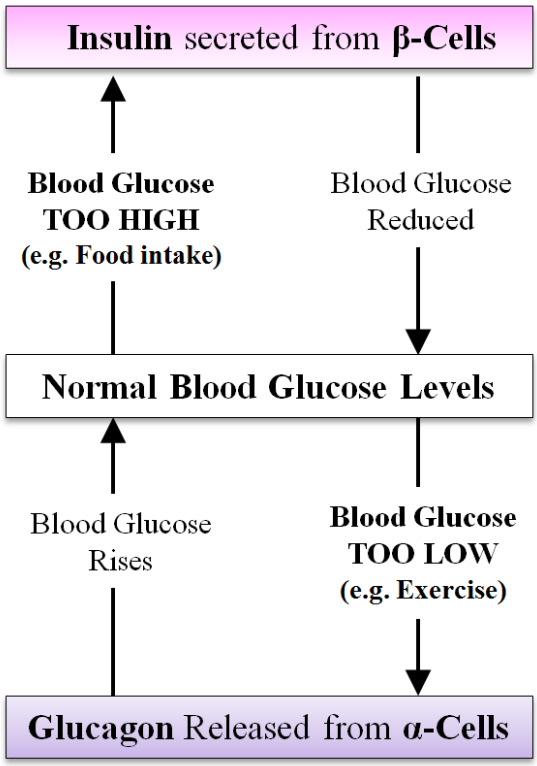



**Figure 1.7: Control of blood glucose homeostasis.**
The basic principle of how α- cells and β-cells regulate blood glucose levels.

Most mammals contain one copy of the insulin gene, for example humans possess one insulin gene located on chromosome 11 at position p5.15 (Harper, et al., 1981). Few species however, including the rodents (rat and mice) have two non-allelic-variants of the insulin gene, denoted insulin1 and insulin2, of which insulin2 corresponds to the single copy seen in humans. It is postulated insulin1 was retroposed from the partially processed insulin2 mRNA (Hay and Docherty, 2006, Shiao, et al., 2008). Control of the insulin gene expression is largely exerted at the transcriptional level through well-defined elements located within the promoter region (Chakrabarti and Mirmira, 2003).

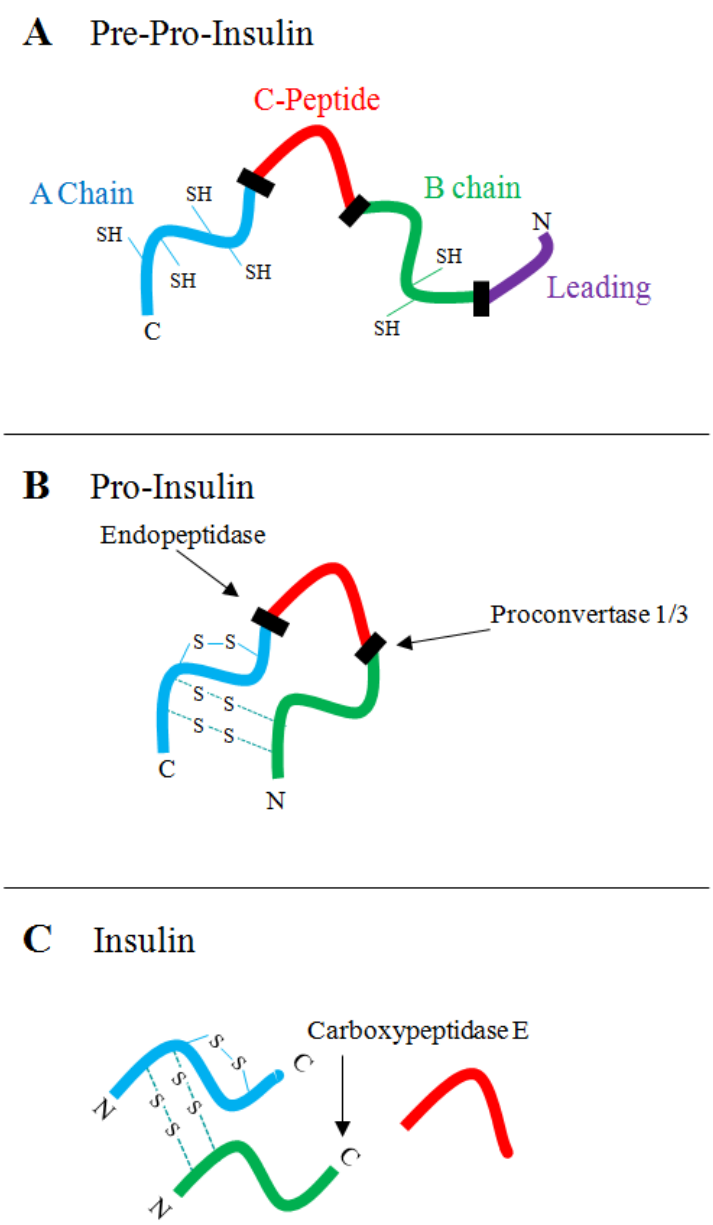



**Figure 1.8: Biosynthesis and enzymatic processing of insulin.** Following ribosome association with the rough ER, the leading region of synthesised pre-pro-insulin **(A)** is cleaved to produce pro-insulin **(B)**. Once pro-insulin is packaged within the secretory granules, C-peptide is removed from pro-insulin via a multi-step proteolytic process to produce insulin **(C)**.

The biosynthesis of insulin from a large precursor known as pre-pro-insulin was first observed in 1967 and is shown diagrammatically in Figure 1.8 (Steiner, et al., 1967). Pre-pro-insulin bound to the ribosome is initially synthesised and cleaved to pro-insulin following the association of the ribosome with the rough endoplasmic reticulum (ER). In the ER pro-insulin undergoes post-translational proteolysis prior to transport to the Golgi apparatus where it is packaged in β-cell characteristic small dense secretory granules close to the cell membrane. Pro-insulin contains distinct carboxy-terminal A and amino-terminal B chains linked together by an important connecting peptide known as C-peptide. C-

peptide is essential for correct synthesis of insulin, as it links the A- and B- chains of insulin in a manner allowing correct assembly, folding and inter-chain disulphide bond formation. Once in the secretory granule, C-peptide is removed from pro-insulin via a multi-step proteolytic process involving the enzymes prohormone convertase, endopeptidase and carboxypeptidase E (Halban, 1994). This results in insulin's A and B chains adopting an appropriate conformation for effective interaction with the insulin receptor. Equimolar amounts of C-peptide and insulin are then subsequently stored in the granules until required to be released by exocytosis into the bloodstream (Wahren, et al., 2000). C-peptide has been considered for a long time to be biologically inert, however research now indicates that C-peptide is biologically active, as it has been shown to bind specifically to G protein coupled receptors on human cell membranes, and also ameliorate Type 1 diabetes-induced microvascular complications (nephropathy and neuropathy) (Ishii, et al., 2012, Rigler, et al., 1999, Wahren and Jornvall, 2003).

In order to perform their function β-cells must express a range of genes/ proteins which enable the β-cell to sense changes in extracellular glucose concentrations and then release the necessary amount of stored insulin. Glucose transporter 2 (GLUT2) is a transmembrane carrier protein found on the cell membrane of pancreatic β-cells, that enables the facilitated movement of glucose from the bloodstream into the cell, due to its high affinity for glucose (Figure 1.9A). Glycolysis then takes place inside the cell, where the islet-specific homolog of glucokinase (GCK) is the major glucose phosphorylating enzyme, hence due to its high flux control coefficient on glucose metabolism acts as the rate-limiting step (Figure 1.9B). GLUT2 and GCK are frequently regarded as the indirect 'glucose sensors' in β-cells responsible for triggering insulin secretion (Arden, et al., 2004), as the increase in the ATP:ADP ratio as a result of glycolysis inhibits the hetero-octomeric ATP-dependent transmembrane potassium ($K^+$) channel which causes membrane depolarisation (Dunne, 2000). Consequently, voltage-gated calcium ($Ca^{2+}$) channels are activated and opened leading to the influx of calcium, in addition to the inositol 1,4,5-triphosphate (IP3) mediated release of ER intracellular calcium stores, which causes a significant increase in intracellular calcium concentrations leading to exocytosis of the stored insulin secretory granules (Pertusa, et al., 1999).

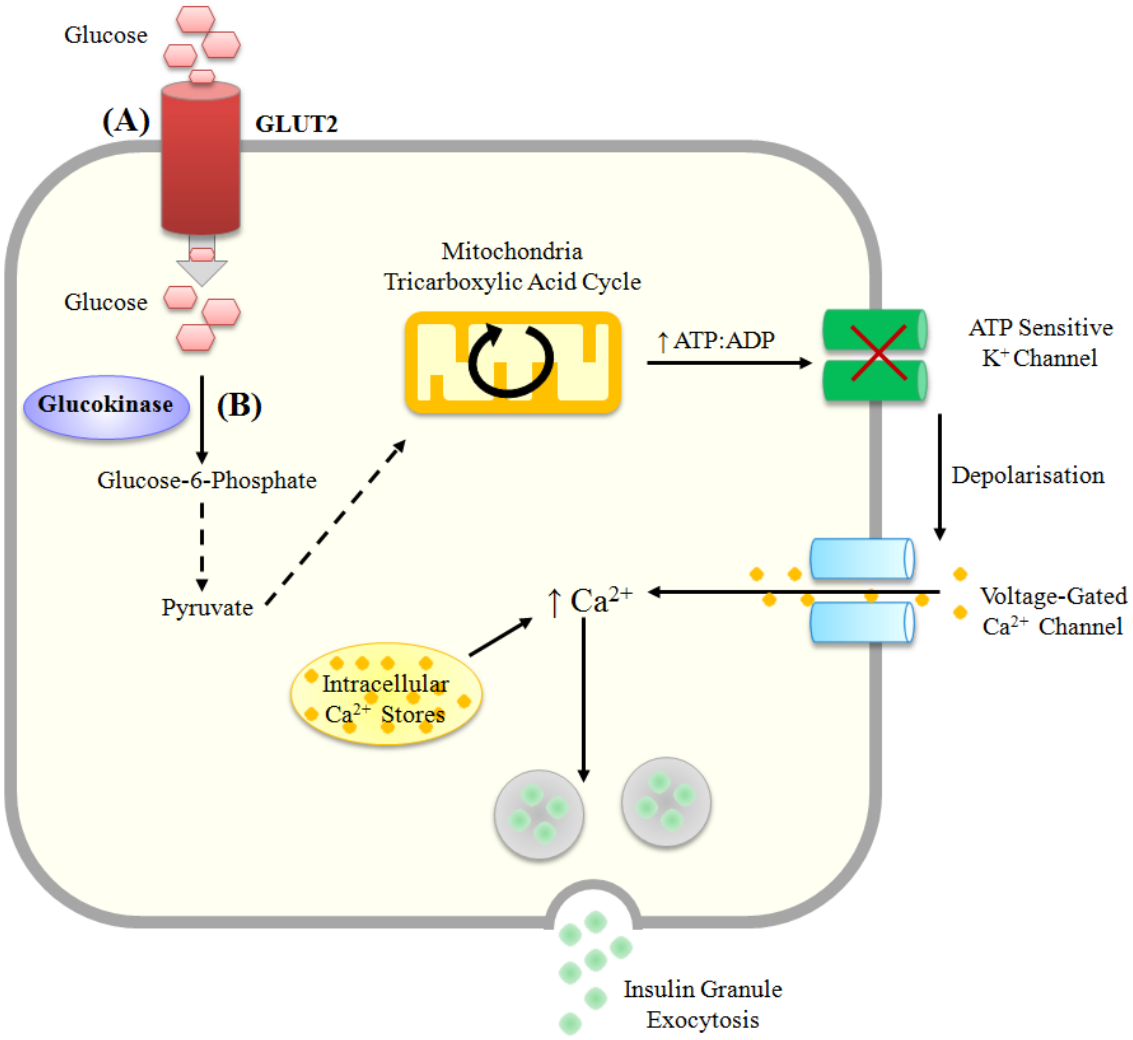



**Figure 1.9: Glucose stimulated insulin secretion in the β-cell.** Indirect glucose sensors **(A)** Glucose Transporter 2 (GLUT2), which facilitates the movement of glucose into the β-cell and **(B)** Glucokinase (GCK) which determines the rate limiting step of glycolysis.

## 1.10.2 α-cell: glucagon

Glucagon producing α-cells are the next most common cell type constituting approximately 15-20% of the islet (Edlund, 2002). Insulin and glucagon are counter-regulatory hormones whose opposing actions ensure normoglycaemia, as shown in Figure 1.7 (Goke, 2008). Glucagon is released from α-cells during periods of hypoglycaemia (e.g. after exercise or fasting), stimulating hepatic gluconeogenesis and glycogenolysis, and mobilisation of glucose from peripheral tissues in order to raise blood glucose levels. Glucagon is synthesised from pre-pro-glucagon via the action of enzyme prohormone convertase 2. Glucagons' secretion is not only regulated by the direct effect of blood glucose concentrations but also by additional regulators including intra-islet paracrine signals, including insulin from β-cells (Diao, et al., 2005, Gerich, et al., 1975, Greenbaum,

et al., 1991, Maruyama, et al., 1984, Ravier and Rutter, 2005, Stagner and Samols, 1986) and somatostatin from δ-cells (Gerich, et al., 1975, Gromada, et al., 2007).

### 1.10.3 δ cell: somatostatin

Islet δ-cells constitute 5-10% of the islet endocrine cells, and secrete the hormone somatostatin in response to increased extracellular glucose. This is similar to β-cells, however δ-cells respond to a lower threshold concentration of glucose. Somatostatin receptors have been identified on both α- and β-cells, and studies have shown exogenous somatostatin can inhibit insulin and glucagon secretion, consistent with a paracrine role for somatostatin in regulating α- and β-cell function (Hauge-Evans, et al., 2009).

### 1.10.4 PP-cell: PP

PP-producing-cells are very few in number (i.e. only 3-5% of the islet), approximately 140nm in size and identifiable by their polygonal shape and scant granules and organelles. Data suggests that PP is released in response to ingestion of food and that it plays an important role in the reduction of appetite, although the mechanism of PP remains to be fully elucidated (Batterham, et al., 2003).

### 1.10.5 ε-cell: ghrelin

ε-cells have only recently been described to be present sparingly (<1%) in the pancreatic islets (Prado, et al., 2004). ε-cells release the gastric hormone ghrelin, an acylated 28 amino acid peptide, postulated to be involved in down-regulation of glucose-induced insulin release (Dezaki, et al., 2010), enhancement of appetite, metabolic regulation and energy balance (Wierup, et al., 2002).

## 1.11 Diabetes mellitus and current treatments

Diabetes mellitus is the most common disorder of the endocrine pancreas and is associated with failure to produce and/or use insulin effectively, resulting in chronic hyperglycaemia. Diabetes mellitus affects approximately 180 million people world-wide

(World Health Organisation) and can be broadly classified as either Type 1, caused by the insufficient production of insulin or Type 2, caused by diminished response of the body to insulin. There are also more specific cases of diabetes for example gestational diabetes seen in women during pregnancy, and maturity onset diabetes of the young (MODY) syndromes, which encompass several hereditary conditions which unlike polygenic recessive Type 1 and Type 2 diabetes are caused by mutations in a single autosomal dominant gene (HNF4α, GCK, HNF1α, Pdx1, HNF1β and NeuroD) leading to progressive impairment in β-cell insulin function and eventually diabetes (Fajans, et al., 2001).

Type 1 diabetes is a serious metabolic disorder characterized by the auto-immune destruction of functional insulin producing β-cells, hence for the purposes of this thesis will be discussed in more detail. In Type 1 diabetes, islets contain mostly hyperplastic α-cells, producing an unregulated and inappropriate amount of glucagon. In the absence of the opposing action of insulin, unrestricted hepatic gluconeogenesis and glycogenolysis occurs, resulting in high blood glucose levels and potentially dangerous episodes of hyperglycaemia, which can contribute to complications such as diabetic ketoacidosis (i.e. production of acidic ketone bodies), cardiopathy, nephropathy, neuropathy and retinopathy.

At present the major method in order to manage Type 1 diabetes is to monitor blood glucose levels carefully and administer regular exogenous insulin, and although this allows a measure of control of blood glucose levels, patients are not void of the diabetes associated complications (Samson and Chan, 2006). The ultimate objective of therapy for Type 1 diabetes is therefore to replace the functioning β-cell component of the body. This can be achieved by an existing method of cell therapy: islet transplantation using the Edmonton protocol, in which islets are isolated from an organ donor and grafted into the liver of the patient via the portal vein. Islet transplantation can produce some improvement in diabetic patients but the major limitation of this approach as with most transplantation procedures is the scarcity of suitable organ donors and the 3-year organ survival rates of 70-80% (Shapiro, et al., 2006, Shapiro, et al., 2001). Consequently alternative strategies to prevent/cure diabetes are under investigation including: (1) prevention of β-cell destruction (2) stimulation of β-cell differentiation and regeneration (**1.12.1**) (3) ectopic production of insulin by substitute cells via the exploration of methods of recreating β-cells from other cell types (Barbu and Welsh, 2007).

## 1.12 Potential sources of β-cells

Four possible sources have been considered for obtaining β-cells. (1) From the other pancreatic exocrine and endocrine cell types by provoking neogenesis. *Zhou et al.,* showed the *in vivo* reprogramming of adult pancreatic acinar cells into insulin secreting pancreatic β-cells via adenovirus mediated expression of pancreatic transcription factors Pdx1, Ngn3 and MafA. Lineage analysis utilising double heterozygous Cpa1CreER-R26R mice to permanently label mature exocrine cells with β-galactosidase confirmed the exocrine origin of the nascent insulin positive cells (Zhou, et al., 2008). Also it has been shown that an incretin hormone glucagon-like peptide-1 can differentiate rat (ARIP) and human (PANC-1) pancreatic ductal epithelial cell lines into insulin-secreting cells, although this effect requires the expression of the β-cell differentiation factor islet duodenal homeobox-1 (IDX-1) (Hui, et al., 2001). (2) From human embryonic stem cells (ESCs), by recapitulating the sequence of developmental events that normally leads to β-cell differentiation. *Lumelsky et al.,* described the first culture-based protocol for the *in vitro* generation of pancreatic cells from mouse ESCs using a five-step protocol that was reported to generate insulin-expressing cells by selecting for nestin-positive progenitor cells (Lumelsky, et al., 2001). However the reliability of this strategy is questioned as the insulin expression may reflect uptake of insulin from the culture medium rather than the *de novo* synthesis associated with insulin gene expression. Therefore alternative studies have shown successful generation of insulin-expressing cells from mouse ESCs via over-expression of key β-cell transcription factors Pdx1, Pax4 and Nkx2.2 (Blyszczuk, et al., 2003, Miyazaki, et al., 2004, Shiroi, et al., 2005) or via exposure to extracellular factors and manipulation of signalling pathways (reviewed by (Tsaniras and Jones, 2010)). (3) From other endodermal tissue types, particularly those of the liver, by transdifferentiation. *Ferber et al.,* utilised a first-generation adenoviral vector containing *Pdx1* to infect streptozotocin-induced diabetic mice. The results showed that pancreatic genes insulin and prohormone convertase 1/3 (i.e. a functional marker for insulin maturation) were induced, and the levels of blood glucose were returned to normal levels (Ferber, et al., 2000). Moreover transdifferentiation of human HepG2 (hepatoma) cells to pancreatic cells following introduction of an activated version of the pancreatic transcription factor *Pdx1* (XlHbox8-VP16) has also been characterised, showing that during transdifferentiation the hepatic phenotype is suppressed and the *Pdx1* transgene is only required temporarily to activate pancreatic differentiation (Li, et al., 2005a). (4) From existing β-cells, by provoking multiplication.

### 1.12.1 Normal β-cell regeneration

β-cell regeneration is widely thought to rely on self-duplication as demonstrated in various models of partial β-cell ablation (Cano, et al., 2008, Nir, et al., 2007, Wang, et al., 1996). This stems from an organisms needs to be able to control its β-cell mass in accordance with its insulin requirements, and although the rate of β-cell replication in the adult pancreas is generally low (2-3% per day) it can undergo dynamic shifts to compensate increased insulin demand, for example during pregnancy (Ackermann and Gannon, 2007, Bouwens and Rooman, 2005, Finegood, et al., 1995). The regeneration of β-cells in the pancreas has been described in animal experiments and in human pathology, induced in response to various experimental treatments, for example following pancreatic duct ligation (O'Neill, et al., 2008, Xu, et al., 2008), streptozotocin-induced diabetes (Wang, et al., 1996) or following administration of exendin-4 (Xu, et al., 1999). The phenomenon has also been observed in diabetic patients following autoimmune suppression (Herold and Taylor, 2003). In some of these cases (e.g. following pancreatic duct ligation) the source of the regenerating β-cells has been characterised as originating from neurogenin-3-positive progenitor cells residing in the pancreatic ducts via neogenesis.

### 1.12.2 α-cells as a potential source of β-cell regeneration

Studies have recently successfully demonstrated the potential for reprogramming α-cells to β-cells (Collombat, et al., 2009b, Thorel, et al., 2010). *Thorel et al.,* set out to examine the role of β-cell regeneration following total or near total β-cell ablation, in an experimental model recapitulating Type 1 diabetes. In order to accomplish near-total specific β-cell ablation, transgenic mice were generated bearing a transgene containing the rat insulin promoter (RIP) and the diphtheria toxin receptor (DTR) coding sequence. This approach was unique and an improvement from other examples of β-cell regeneration, because previous studies have relied on less severe models of β-cell ablation such as pancreatectomy, in which damage is caused to more than one cell type. The overall aim of the study was to establish whether disparate mechanisms of β-cell regeneration, for example from other pre-existing endocrine cell types such as α-cells, were associated with increased β-cell ablation severity. Therefore by placing the DTR downstream of the RIP, diphtheria toxin A (DT) administration mediated >99% specific β-cell ablation. Analysis following β-cell regeneration in parallel with α-cell labelling experiments revealed the

appearance of bi-hormonal cells expressing both insulin and glucagon and nascent β-cells were confirmed to have originated from the transdifferentiation of the remaining α-cells by parallel cell lineage experiments (Sangan and Tosh, 2010a, Thorel, et al., 2010). The challenge however remains to identify and validate the 'master switch transcription factors' required for reprogramming of α-cells to β-cells.

## 1.13 Transcriptional regulation of β-cell differentiation

Candidate transcription factors which may possess a critical role in the molecular mechanisms responsible for reprogramming α-cells to β-cells may include transcription factors known to be important in β-cell differentiation and function during normal development (Figure 1.10). Pancreatic development is a highly coordinated process involving morphogenesis and differentiation, orchestrated by extracellular signals and transcription factor interactions.

### 1.13.1 Hepatocyte nuclear factor 4 α (HNF4α)

Hepatocyte nuclear factor 4α (HNF4α) (which as previously mentioned is important in liver development), has also been identified as a key transcription factor required for regulation of many genes and pathways responsible for the maintenance and proliferation of adult β-cells (Gupta, et al., 2005, Wollheim, et al., 2000). The importance of HNF4α for β-cell function is also highlighted as Maturity-Onset Diabetes of the Young (MODY), an autosomal dominantly inherited form of Type 2 diabetes, which results from mutations in at least six different genes, one of which is HNF4α (MODY1) (Gupta, et al., 2007).

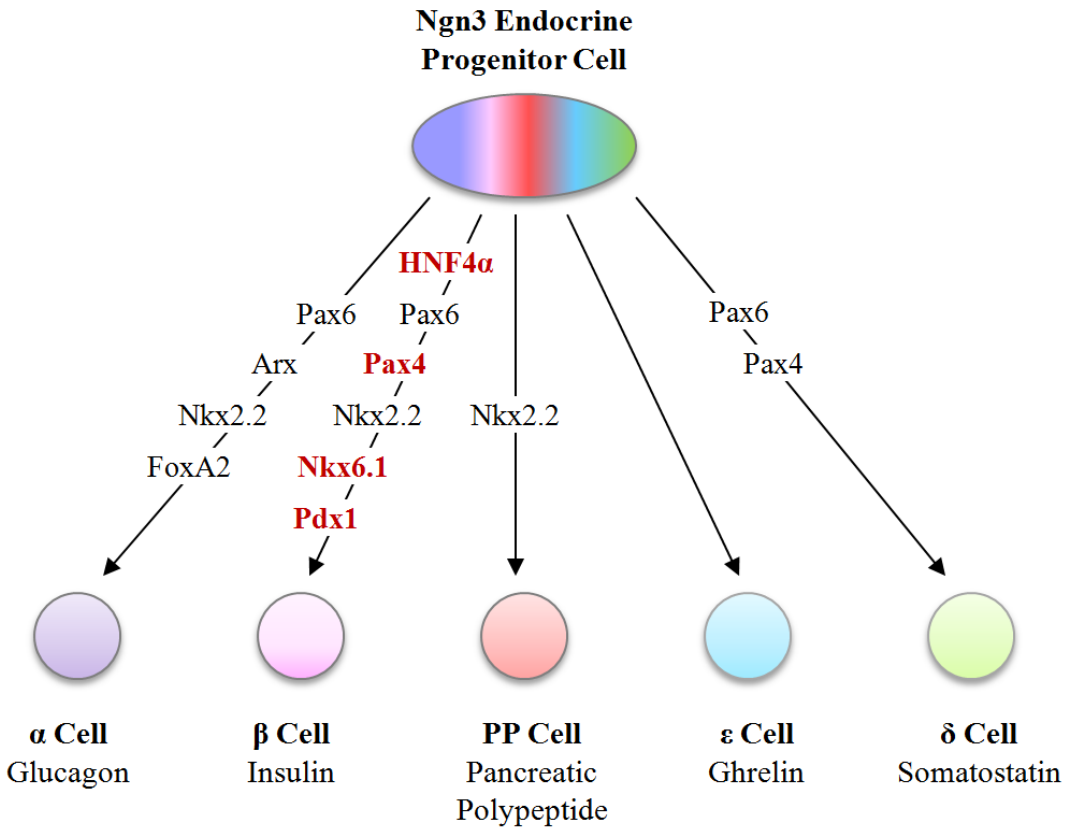



**Figure 1.10: Summary of transcription factors important in endocrine islet-subtype specification.** Highlighted in *red* are candidates for transdifferentiating α-cells to β-cells.

### 1.13.2 Paired box gene 4 (Pax4)

Paired box gene 4 (Pax4) is a member of the paired box family of transcription factors known to be important in β-cell differentiation. *Collombat et al.*, showed that the conditional ectopic expression of Pax4 in embryonic endocrine progenitor cells, as well as mature α-cells induces their conversion into β-cells *in vivo* (Collombat, et al., 2009a). Pax4 presumably induces the conversion from α-cells to β-cells by repressing the transcription factor Aristaless related homeobox (Arx). Indeed a previous study has shown that deleting Arx in mouse pancreas induces a switch from an α-cell to a β-cell fate (Collombat, et al., 2003), whilst conversely in the absence of Pax4, the opposite phenotype is observed (SosaPineda, et al., 1997), suggesting a reciprocal relationship exists between Arx and Pax4 transcription factors in pancreas development determining α- or β-cell fate.

### 1.13.3 Pancreatic duodenal homeobox 1 (Pdx1)

Pancreatic duodenal homeobox 1 (Pdx1) is believed to be a master switch gene for the formation of the pancreas, as homozygous Pdx1 knockout mice lack a pancreas (Jonsson, et al., 1994). During pancreas development Pdx1 is normally initially expressed in the pre-pancreatic endoderm at embryonic stage 8.5-9.0 expanding over the entire epithelial but prior to being progressively restricted to endocrine β-cells (Slack, 1995). A number of investigations have demonstrated the utility of Pdx1 (either alone or in combination) to induce the conversion of pancreatic acinar cells (Zhou, et al., 2008) or hepatocytes to β-cells (Ferber, et al., 2000, Li, et al., 2005a). Furthermore, Pdx1 also has the potential to suppress the α-cell phenotype and enhance the β-cell phenotype as it has been shown to bind directly to the glucagon and insulin promoters, respectively (Chakrabarti, et al., 2002b, Ritz-Laser, et al., 2003).

### 1.13.4 NK6 homeobox 1(Nkx6.1)

The homeodomain transcription factor Nkx6.1 is thought to be important in pancreas development, lying downstream of another NK-homeodomain transcription factor Nkx2.2 in the major pathway of β-cell differentiation. This is consistent with its restricted expression to β-cells and that disruption of *Nkx6.1* in mice results in loss of β-cell precursors and blockade of β-cell neogeneis (Jensen, et al., 1996, Sander, et al., 2000). Therefore Nkx6.1 may also be involved in the switch from α- to β-cells, since it suppresses glucagon gene expression (independently of Pdx1) and can induce glucose-stimulated insulin secretion in islet β-cells (Schisler, et al., 2005).

## 1.14 Thesis aims

This thesis consists of two distinct sections. The research contained in the first section originates from the fact that the therapeutic potential of hepatic progenitor cells (oval cells) cannot be harnessed until the factors and signalling pathways governing their proliferation and trans/differentiation are better understood. This thesis will address this issue directly through studies utilising an *in vitro* model based on an adult liver derived oval cell line, specialised growth and differentiation mediums (containing dexamethasone) previously demonstrated to maintain oval cell proliferation and induce hepatocyte differentiation

respectively (Tirnitz-Parker, et al., 2007), and adenovirus vectors containing important transcription factor in hepatocyte differentiation (i.e. HNF4α and C/EBPα).

Thesis aims for this section will include:

(1) Characterisation of the murine oval cell line (BMOL-TAT1.1) and investigation into oval cell heterogeneity during proliferation.
(2) Optimisation of an inducible hepatic differentiation protocol
(3) Investigation into the effect of the optimised inducible hepatic differentiation protocol on hepatic and non-hepatic BMOL-TAT1.1 differentiation.
(4) Investigation into the effect of ectopic expression of hepatocyte important transcription factors (HNF4α and C/EBPα) in a homogenous population of BMOL-TAT1.1 cells negative for these transcription factors.

The second section of this thesis is based on the recent study showing that following extreme β-cell ablation (i.e. resembling Type 1 diabetes), the remaining α-cells in the pancreas are involved in β-cell regeneration (Thorel, et al., 2010). The therapeutic potential of pancreatic α-cells as a source of β-cells cannot be harnessed until the 'master gene transcription factors' responsible for reprogramming α-cells into β-cells are identified. Whilst there is evidence for the role of Pax4 in converting α-cells into β-cells *in vivo* (Collombat, et al., 2009), this thesis is novel as it will utilise an *in vitro* adult α-cell model and an adenovirus ectopic expression strategy, in order to perform 'proof-of principle' studies aimed to address the fact that alternative players may exist in the form of HNF4α and Pdx1. Additionally the contribution of chromatin modification, in particular histone acetylation will also be assessed in the *in vitro* adult α-cell model as chromatin modification is involved in endocrine fate regulation, and hence may facilitate β-cell differentiation.

Thesis aims for this section will include:

(1) Characterisation of the pancreatic alpha cell line (α-TC19).
(2) Investigation into the reprogramming potential of α-TC19 cells into β-cells via the ectopic expression of β-cell specific transcription factors (e.g. HNF4α) or histone deacetylase inhibitior induced chromatin modification (i.e. histone hyperacteylation).

# Chapter 2 Materials and Method

## 2.1 General laboratory chemicals

General laboratory chemicals were of analytical research grade and from a range of manufacturers. Most chemicals came from Sigma or Fisher Scientific. For specialist reagents supplier specific information is provided.

## 2.2 Solutions and buffers

**Water ($H_2O$):** All general solutions were prepared with double-deionised water obtained from a Milli-Q Biocel System (Millipore). For molecular techniques involving RNA, diethylpyrocarbonate (DEPC) treated cross-linked water was utilised.

**0.1M Phosphate Buffer pH 7.3 (X-gal Staining):** 3.74g monobasic sodium phosphate ($NaH_2PO_4$-$H_2O$) and 10.35g dibasic sodium phosphate ($Na_2HPO_4$) were dissolved in 1 litre of Milli-Q $H_2O$ and then stored at 4°C indefinitely.

**Fixation Buffer (X-gal Staining):** 0.1M Phosphate Buffer (pH 7.3) was supplemented with 5mM ethylene glycol tetraacetic acid (EGTA), 2mM $MgCl_2$ and 0.2% glutaraldahyde. The solution was stored at 4°C for up to 4 months.

**Wash Buffer (X-gal Staining):** 0.1M Phosphate Buffer (pH 7.3) was supplemented with 2mM magnesium chloride ($MgCl_2$) and kept at 4°C indefinitely.

**X-gal Staining Solution (X-gal Staining):** 0.1M phosphate buffer (pH 7.3) was supplemented with 2mM $MgCl_2$, 5mM potassium ferrocyanide ($K_4Fe(CN)_6$-$3H_2O$) and 5mM potassium ferricyanide ($K_3Fe(CN)_6$). The X-gal staining solution was wrapped in foil as the solution is light sensitive and stored at 4°C indefinitely. Prior to use 1mg/ml X-gal was added (a stock solution of 40mg/ml was prepared in dimethylformamide (DMF) and stored at -20°C).

**RNA 5X Loading Buffer 1ml (RNA Integrity Analysis):** Loading buffer was made up by adding 1.6µl bromophenol blue, 8µl 500mM ethylene diamine tetraacetic acid (EDTA), 72µl 37% formaldehyde, 200µl glycerol, 308µl formamide, 400µl 10X 4-morpholinepropanesulfonic acid (MOPS) buffer, 10.4µl Milli-Q $H_2O$.

**Running Buffer (RNA Integrity Analysis):** 440ml Milli-Q $H_2O$ was supplemented with 50ml 10x MOPS and 10ml 37% formaldehyde.

**2% Blocking Buffer (Immunofluorescent Staining):** 10% blocking buffer stock was prepared by dissolving blocking buffer (Roche) in maleic acid buffer (100mM maleic acid, 150mM sodium chloride (NaCl), pH 7.5). The blocking buffer was then autoclaved and stored at -20°C. Prior to use, the 2% blocking buffer was made by diluting the 10% stock in phosphate buffered saline (PBS). The 2% blocking buffer was stored at 4°C.

**4% PFA Fixative (Immunofluorescent Staining):** A 4% PFA stock solution was prepared by dissolving 20g paraformaldehyde crystals in 500ml PBS (pH 7.3-7.4) and heating to 60°C. The 4% PFA solution was stored at -20°C (long term) or at 4°C (short term).

**Acetone: Methanol Fixative (Immunofluorescent Staining):** 1:1 v/v and stored at -20°C prior to use.

**MEMFA Fixative (Immunofluorescent Staining):** A 10X MEM stock solution was prepared (0.15M MOPS pH7.4, 2mM EGTA, and 1mM $MgSO_{4)}$. Prior to use 3.7% formaldehyde was added to 1X MEM stock solution and was stored at 4°C (short term).

## 2.3 Cell culture reagents and media

**BMOL-TAT1.1 Proliferation Medium (cell line passaging and maintainence (2.8.3)):** Williams' E medium (Sigma) was supplemented with 2.5µg/ml fungizone, 10U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 5% fetal bovine serum (FBS).

**BMOL-TAT1.1 Growth Medium (inducible differentiation protocol (2.8.6)):** Williams' E medium (Sigma) was supplemented with 2.5µg/ml fungizone, 10U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 5% FBS, 30ng/ml insulin growth factor-2 (IGF-2), 20ng/ml epidermal growth factor (EGF), and 10µg/ml human insulin.

**BMOL-TAT1.1 Differentiation Medium (inducible differentiation protocol (2.8.6)):** Williams' E medium (Sigma) was supplemented with 2.5µg/ml fungizone, 10U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 5% FBS, 20ng/ml EGF, 6.25µg/ml (each) insulin transferrin selenious acid+ (ITS+), 10mM nicotinamide, and 0.1µM dexamethasone.

**BMOL-TAT1.1 Freezing Medium:** FBS was supplemented with 10% dimethyl sulfoxide (DMSO).

**α-TC19 Medium:** Dulbecco's Modified Eagle Medium (DMEM D5546; Sigma) was supplemented with 2.5µg/ml fungizone, 10U/ml penicillin, 100µg/ml streptomycin, 4mM L-glutamine, 10% Heat-Inactivated FBS, 15mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3g/l glucose, 0.02% bovine serum albumin (BSA) and 1x MEM non-essential amino acids.

**α-TC19 Freezing Medium:** α-TC19 Medium was supplemented with 10% DMSO .

**Embryonic Culture Medium:** Basal Medium Eagle (BME) (Sigma) was supplemented with 10U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 20µg/ml gentamicin, 2.5µg/ml fungizone and 10% FBS.

**HEK-293 Medium:** Dulbecco's Modified Eagle Medium (DMEM 41966; Gibco) was supplemented with 2.5µg/ml fungizone, 10U/ml penicillin, 100µg/ml streptomycin and 10% Heat-Inactivated FBS.

## 2.4 Extracellular factors and additional compounds

| | Supplier Details | CONCENTRATION | |
|---|---|---|---|
| | | Stock | Working |
| **GENERAL CELL CULTURE** | | | |
| DMSO | Sigma | 100% | 10% |
| FBS | Gibco | 100% | 5% or 10% |
| FBS (Heat-Inactivated) | Sigma | 100% | 10% |
| Fungizone | Gibco | 250µg/ml | 2.5µg/ml |
| L-glutamine | Sigma | 200mM | 2mM or 4mM |
| Penicillin | Sigma | 5000U/ml | 10U/ml |
| Streptomycin | Sigma | 5mg | 100µg/ml |
| Gentamicin | Gibco | 10mg/ml | 20µg/ml |
| **BMOL-TAT1.1 CELL LINE SPECIFIC** | | | |
| Dexamethasone | Sigma | 1mM | 0.1µM |
| EGF | Sigma | 1mg/ml | 20ng/ml |
| IGF-2 | R&D Systems | 100µg/ml | 30ng/ml |
| Insulin (human) | Sigma | 10mg/ml | 10µg/ml |
| ITS+ | BD Biosciences | 625µg/ml | 6.25µg/ml |
| Nicotinamide | Sigma | 2.5M | 10mM |

| **α-TC19 CELL LINE SPECIFIC** | | | |
|---|---|---|---|
| BSA | Sigma | 50mg/ml | 0.02% |
| Glucose | Sigma | 450g/l | 3g/l |
| HEPES | Sigma | 1M | 15mM |
| MEM non-essential amino acids | Sigma | 100x | 1x |
| **ADENOVIRAL INFECTION** | | | |
| DEAE-dextran | Sigma | 1mg/ml | 5μg/ml |
| **HDAC INHIBITORS** | | | |
| Trichostatin A (TSA) | Sigma (T1952) | 5μM | 2, 5, 15 nM |
| Sodium Butyrate (NaB) | Sigma (B5887) | 100mM | 0.1, 0.3, 0.5 mM |
| Valporic Acid (VPA) | Sigma (P4543) | 200mM | 0.1, 0.3, 0.5 mM |

**Table 2.1: Extracellular factors and additional compounds added to cell culture medium**

## 2.5 Antibodies for immunofluorescent staining

| Primary Antibody | Species | Supplier Details | Dilution | Fixation | Antigen Retrieval |
|---|---|---|---|---|---|
| A6 | rat | Kind Gift: Dr. Factor, Bethesda, MD, USA | 1:50 | Ac:Me | Citrate |
| Beta-Catenin | mouse | BD Transduction Laboratories | 1:50 | PFA | EDTA |
| C/EBPα | rabbit | Santa Cruz Biotechnology | 1:50 | PFA | Citrate |
| C/EBPβ | mouse | Santa Cruz Biotechnology | 1:100 | PFA | EDTA |
| Cytokeratin 7 | mouse | Abcam | 1:50 | Ac:Me | Citrate |
| Cytokeratin 20 | mouse | Dako | 1:50 | Ac:Me | Citrate |
| E-Cadherin | mouse | BD Transduction Laboratories | 1:100 | PFA | ---- |
| FoxA2 | goat | Santa Cruz Biotechnology | 1:100 | PFA | ---- |
| Glucagon | mouse | Sigma | 1:300 | PFA | ---- |
| GLUT2 | rabbit | Biogenesis | 1:100 | PFA | Citrate |
| HNF4α | rabbit | Santa Cruz Biotechnology | 1:50 | PFA | Citrate |
| Insulin | guinea-pig | Sigma | 1:300 | PFA | ---- |
| OV6 | mouse | Dr. Sells Ordway Research Institute, NY, USA. | 1:1000 | Ac:Me | Citrate |
| Sox 9 | rabbit | Chemicon | 1:100 | PFA | ---- |
| Villin | mouse | Abcam | 1:100 | Ac:Me | ---- |
| Vimentin | mouse | Sigma | 1:100 | PFA | ---- |

**Table 2.2 List of primary antibodies.** All the antibodies were diluted in 2% Blocking Buffer (Roche).

| Secondary Antibody | Species | Supplier Details | Dilution |
|---|---|---|---|
| Anti-guinea-pig FITC-conjugated IgG | goat | Vector Laboratories | 1:200 |
| Anti-mouse FITC-conjugated IgG | horse | Vector Laboratories | 1:200 |
| Anti-mouse Texas Red-conjugated IgG | horse | Vector Laboratories | 1:200 |
| Anti-rabbit AMCA-conjugated IgG | goat | Vector Laboratories | 1:200 |
| Anti-rabbit FITC-conjugated IgG | goat | Vector Laboratories | 1:200 |
| Anti-rabbit Texas Red-conjugated IgG | goat | Vector Laboratories | 1:200 |
| Anti-rat FITC-conjugated IgG | rabbit | Vector Laboratories | 1:200 |

**Table 2.3 List of secondary antibodies.** All the antibodies were diluted in 2% Blocking Buffer (Roche).

| Lectin | Conjugate | Supplier Details | Dilution | Fixation |
|---|---|---|---|---|
| *Dolichos Biflorus* Agglutinin (DBA) | Fluorescein | Vector Laboratories | 1:100 | PFA |
| Peanut Agglutinin (PNA) | Rhodamine | Vector Laboratories | 1:100 | PFA |

**Table 2.4 Lectin Conjugated Markers.** All lectins were diluted in 2% Blocking Buffer (Roche).

## 2.6 Primers for polymerase chain reaction

Polymerase chain reaction (PCR) primers were designed using the online Primer3$^{TM}$ software (Rozen and Skaletsky, 2000) and Premier Biosoft Net Primer$^{TM}$ software (www.premierbiosoft.com), and purchased from Eurofins MWG Operon. Primer forward and reverse sequences, annealing temperatures ($T_M$), product sizes and number of RT-PCR cycles utilised are listed in Table 2.5.

| Gene | Species | Primer Sequences (5'-3') | $T_M$ (°C) | Product Size (bp) | Cycles |
|---|---|---|---|---|---|
| AFP | mouse | Fwd: GGAGGCTATGCATCACCAGT<br>Rev: CCGAGAAATCTGCAGTGACA | 58 | 207 | 33 |
| Albumin | mouse | Fwd: GCAGAGGCTGACAAGGAAAG<br>Rev: TTCTGCAAAGTGAGCATTGG | 58 | 183 | 33 |
| ALPi | mouse | Fwd: TGGATGCTGCCAAGAAGCTGC<br>Rev: AGAGATAGGCGGTTGCTGTGC | 56 | 243 | 35 |
| Amylase | mouse | Fwd: GGGAGGACTGCTATTGTCCA<br>Rev: CATTGTTGCACCTTGTCACC | 56 | 241 | 35 |

| | | | | | |
|---|---|---|---|---|---|
| ApoA-IVi | mouse | Fwd: GGTGTGGGATTACTTTACCC<br>Rev: CTTCACCCTCTCAGTTTCCT | 56 | 208 | 35 |
| Arx | mouse | Fwd: GCTCTCCTCCTACTGCATCG<br>Rev: GTGCAGCTCAGCCTCGAA | 56 | 172 | 33 |
| β-actin | mouse | Fwd: AAGAGCTATGAGCTGCCTGA<br>Rev: TACGGATGTCAACGTCACAC | 56 | 160 | 25 |
| C/EBPα | mouse | Fwd:<br>GCCAAGAAGTCGGTGGACAAGAAC<br>Rev: CGGTCATTGTCACTGGTCAACTCC | 60 | 149 | 35 |
| C/EBPβ | mouse | Fwd: ACAAGCTGAGCGACGAGTAC<br>Rev: ACAGCTGCTCCACCTTCTTC | 56 | 159 | 30 |
| CK7 | mouse | Fwd: GCAGGATGTGGTGGAAGATT<br>Rev: CGTGAAGGGTCTTGAGGAAG | 58 | 182 | 30 |
| CK14 | mouse | Fwd: TCTTCAGCAAGACAGAGGAG<br>Rev: GGAGAATTGAGAGGATGAGG | 56 | 398 | 35 |
| CK19 | mouse | Fwd: ACCCTCCCGAGATTACAACC<br>Rev: AGAGTCAGCTCATCCAGCAC | 58 | 219 | 33 |
| CK20 | mouse | Fwd: CGCATCTCTGTCTCCAAAGC<br>Rev: ACATTGTTGCCCAGCTGC | 56 | 538 | 33 |
| Connexin 43 | mouse | Fwd: GAGGGAAGTACCCAACAGCA<br>Rev: CCCAGGAGCAGGATTCTG | 56 | 267 | 35 |
| E-Cadherin | mouse | Fwd: TCGTTCTCCACTCTCACAT<br>Rev: GCTGGACCGAGAGAGTTA | 58 | 380 | 33 |
| Fabp2 | mouse | Fwd: AAGTAGACCGGAACGAGAAC<br>Rev: GTCTGCTAGACTGTAGGGAAAG | 56 | 203 | 35 |
| FoxA1 (HNF3α) | mouse | Fwd: AGTCTCCAGCGTCTTCATCT<br>Rev: GTCTGGAATACACACCTTGG | 56 | 351 | 33 |
| FoxA2 (HNF3β) | mouse | Fwd: CAAGTGTGAGAAGCAACTGG<br>Rev: GATAGAGAAGGGGTGGTTGA | 56 | 373 | 30 |
| GCi | mouse | Fwd: GACTGGACATAGTGCGAAAG<br>Rev: GTGTCGAGGTACATCTGGAA | 56 | 223 | 35 |
| GCK | mouse | Fwd: TATGAAGACCGCCAATGTGA<br>Rev: CACTGAGCTCTCATCCACCA | 58 | 204 | 35 |
| GGT | mouse | Fwd: GCTCATGAATGCCCACAGTA<br>Rev: CCAGCTCATAACCACGGATT | 58 | 203 | 33 |
| Glucagon | mouse | Fwd: GCACATTCACCAGCGACTAC<br>Rev: CTGGTGGCAAGATTGTCCAG | 56 | 328 | 23 |
| GLUT2 | mouse | Fwd: TGGACGAAGTGTATCAGGAC<br>Rev: CCTGACTTCCTCTTCCAACT | 57 | 298 | 35 |
| HNF1α | mouse | Fwd: ACGTCCGCAAGCAGCGAG<br>Rev: TACACTCTTCCACCAAGGTC | 55 | 213 | 30 |
| HNF1β | mouse | Fwd:<br>TTGAAATTCCAAGAGTGACTTGCTC<br>Rev: CTTTAATGGGAGGCTTCCTGAGATG | 56 | 279 | 33 |
| HNF4α | mouse | Fwd:<br>CTCTTCTGATTATAAGCTGAGGATG<br>Rev: CACAGGAAGGTGCAGATTGATCTG | 58 | 377 | 30 |
| HNF4α | human | Fwd: GAAATGCTTCCGGGCTGGC<br>Rev: CTGCAGCTCCTGGAAGGGC | 56 | 487 | 25 |
| HNF6 | mouse | Fwd: GCAATGGAAGTAATTCAGGGCAG<br>Rev: CATGAAGAAGTTGCTGACAGTGC | 56 | 461 | 30 |
| Insulin 1 | mouse | Fwd: TAGTGACCAGCTATAATCAGAG<br>Rev: ACGCCAAGGTCTGAAGGTCC | 56 | 289 | 35 |
| Insulin 2 | mouse | Fwd: CCCTGCTGGCCCTGCTCTT<br>Rev: AGGTCTGAAGGTCACCTGCT | 58 | 278 | 27 |
| Mucin2 | mouse | Fwd: GCAGTATCAGGCCTGTGGC<br>Rev: CACAATCTCGGTCTTCACTTCG | 56 | 430 | 35 |
| Mucin5ac | mouse | Fwd: GTGCAGGGCTCAGTTCTTTC<br>Rev: TGGTCTGTTTTCGTGCTG | 56 | 224 | 35 |
| Nkx2.2 | mouse | Fwd: TCTACGACAGCAGCGACAAC | 56 | 220 | 33 |

| | | | | | |
|---|---|---|---|---|---|
| | | Rev: GCTTTGGAGAAGAGCACTCG | | | |
| Nkx6.1 | mouse | Fwd: ACCTTTGGGCTCACATAACC<br>Rev: GCGCTGGGGCTAAAGTAGAG | 56 | 360 | 35 |
| Osteopontin | mouse | Fwd: GAATGCTGTGTCCTCTGAAG<br>Rev: TTCATGTGAGAGGTGAGGTC | 56 | 366 | 33 |
| Pax4 | mouse | Fwd: ACCCTGTGACATTTCACGGAG<br>Rev: GTACTCGATTGATAGAGGAC | 56 | 266 | 35 |
| Pax6 | mouse | Fwd: ACCAACGATAACATACCCAG<br>Rev: CTGAAGTCGCATCTGAGCTT | 56 | 279 | 35 |
| Pdx1 | mouse | Fwd: CCACCCCAGTTTACAAGCTC<br>Rev: TGTAGGCAGTACGGGTCCTC | 58 | 325 | 30 |
| PP | mouse | Fwd: TACTGCTGCCTCTCCCTGTT<br>Rev: CCAGGAAGTCCACCTGTGTT | 56 | 224 | 35 |
| SMA | mouse | Fwd: GTATTGTGCTGGACTCTGGA<br>Rev: AAGATGGCTGGAAGAGAGTC | 56 | 348 | 35 |
| Somatostatin | mouse | Fwd: CCGTCAGTTTCTGCAGAAGT<br>Rev: CAGGGGCAAGTTGAGCATCG | 56 | 356 | 35 |
| Sox9 | mouse | Fwd: CGCCTTGAAGATAGCATTAGGA<br>Rev: CAAGAACAAGCCAGCCGTCA | 58 | 322 | 30 |
| TAT | mouse | Fwd: CGTAATCCAGACGAATGTCAA<br>Rev: AGATGGGGCATAGCCATTGTA | 58 | 325 | 33 |
| TFF3 | mouse | Fwd: AGATTACGTTGGCCTGTCTCC<br>Rev: TCAGATCAGCCTTGTGTTGGC | 56 | 341 | 35 |
| Villin | mouse | Fwd: TATGATATCCACTACTGGATTGGC<br>Rev: GCTTGAGTGCAGCCTTAGCG | 54 | 586 | 30 |
| Vimentin | mouse | Fwd: AATGCTTCTCTGGCACGTCT<br>Rev: AGCCACGCTTTCATACTGCT | 56 | 207 | 33 |

**Table 2.5 Primer sequences**

## 2.7 Adenovirus reagents

Adenovirus reagent titres and source from which they were provided are listed in Table 2.6.

| Adenoviral Construct | Titre (IU/ml) | Source |
|---|---|---|
| Ad-RSV-GFP | $2 \times 10^{10}$ | Emma Regardsoe, University of Oxford, Oxford, UK |
| Ad-CMV-HNF4α | $1.2 \times 10^{11}$ | Ramiro Jover, Unit of Exp Hepatology, Valencia, Spain |
| Ad-CMV-*lacZ* | $7.74 \times 10^{10}$ | Andrew Byrnes, University of Oxford, Oxford, UK |
| Ad-Null | $6.7 \times 10^{10}$ | Harry Heimberg, Vrije Universiteit, Brussels, Belgium |
| Ad-CMV-C/EBPα | $5.44 \times 10^{11}$ | Vector Biolabs, Philadelphia, USA |
| Ad-CMV-Pax4 | $2.4 \times 10^{10}$ | Harry Heimberg, Vrije Universiteit, Brussels, Belgium |
| Ad-CMV-Pdx1 | $6 \times 10^{10}$ | Harry Heimberg, Vrije Universiteit, Brussels, Belgium |

**Table 2.6: Adenoviral reagents**

## 2.8 Cell culture techniques

### 2.8.1 BMOL-TAT1.1 cell line

BMOL-TAT1.1 cells were obtained from Professor George Yeoh, University of Western Australia, Perth, Australia. Primary cultures of oval cells were isolated from TAT-GRE *lacZ* transgenic mice subjected to a choline-deficient, ethionine (CDE)-supplemented diet. The BMOL-TAT1.1 cell line was subsequently clonally derived, undergoing spontaneous immortalisation following prolonged maintenance in culture (Tirnitz-Parker, et al., 2007). BMOL-TAT1.1 cells were serially passaged by the originators and survived cryopreservation and subsequent thawing.

### 2.8.2 α-TC19 cell line

α-TC19 cells were obtained from Professor Peter Jones, Kings College, London and were originally purchased from ATCC® (CRL-2350$^{TM}$). α-TC19 is a pancreatic alpha-cell line cloned (clone 9) from the α-TC1 cell line which was derived from an adenoma created in transgenic mice expressing the SV40 large T antigen oncogene under the control of the rat pre-pro-glucagon promoter.

### 2.8.3 Cell line passaging and maintenance

Prior to splitting BMOL-TAT1.1 cells, all medium and reagents were pre-warmed to 37°C. For a T-75 flask at approximately 80% confluency, the Proliferation Medium (2.3) was aspirated off and cells washed with 12ml autoclaved PBS. Removal of the PBS was followed by the addition of 5ml 0.05% trypsin-EDTA solution (Gibco) and incubation at 37°C for 5minutes in order to detach the cells from the flask. An equal volume of BMOL-TAT1.1 Proliferation Medium was added to neutralise the trypsin activity. The cells were then centrifuged at 1000rpm for 4 minutes in a MSE Mistral 1000 centrifuge (MSE UK Ltd). The supernatant was then removed and the cell pellet re-suspended in 1ml fresh medium and 20µl (split 1:50) of the total suspension was plated out on a new T-75 flask containing 12ml fresh Proliferation Medium. All cells were incubated at 37°C in an atmosphere of 5% (v/v) $CO_2$ in a humidified incubator.

Prior to splitting α-TC19 cells, all medium and reagents were pre-warmed to 37°C. For a T-75 flask at approximately 80% confluency, the culture medium was aspirated off and the cells incubated in 5ml cell dissociation buffer (Gibco) at room temperature for 2 minutes with gentle movement followed by a further 5minutes stationary in order to gently detach all the cells from the flask. An equal volume of α-TC19 Medium was added and the cells were then centrifuged at 125*xg* for 9 minutes in a MSE Mistral 1000 centrifuge (MSE UK Ltd). The supernatant was then removed and the cell pellet re-suspended in 4ml fresh medium and 1ml (split 1:4) of the total suspension was plated out on a new T-75 flask containing 12ml fresh medium. All cells were incubated at 37°C in an atmosphere of 5% (v/v) $CO_2$ in a humidified incubator.

### 2.8.4 Cell line storage and revival

BMOL-TAT1.1 cells and α-TC19 cells were washed and detached as detailed above. After the centrifugation step, the cell pellet was re-suspended in 1ml of the appropriate freezing medium (2.3) and transferred into cryovials and stored in a Nalgene™ Cryo 1°C Freezing Container (Nalgene® Labware, USA) at -80°C overnight. The next day cryovials were transferred to liquid nitrogen for long term storage.

Cells were revived from storage in liquid nitrogen by removal of the cryovial and rapidly thawing at 37°C. The BMOL-TAT1.1 cells and α-TC19 cells were then seeded in a T-75 flask and cultured at 37°C in an atmosphere of 5% (v/v) $CO_2$ in a humidified incubator. The medium was replaced with fresh medium the following day and changed every two/three days thereafter.

### 2.8.5 Culture of embryonic mouse pancreas, liver and intestine

Isolated pancreatic buds, liver buds and intestine from embryonic stage E11.5 were kindly provided by Gabriela Miron and used for positive controls for immunofluorescent staining. The embryonic organs were cultured on ‘subbed’ coverslips. Glass coverslips were rinsed in 95% ethanol/ 0.1% acetic acid, and allowed to dry. The coverslips were then immersed in 2% (3-amino-propyl1) triethoxysilane (APTS) (Sigma) in acetone for 10 minutes followed by rinsing in acetone and then Milli-Q $H_2O$. Following drying at 37°C the coverslips were sterilised by baking at 180°C for 3 hours. Individual coverslips were

placed into the well of a 4-well tissue culture plate (Thermo Scientific, Nunc). 40µl of 50µg/ml fibronectin (Invitrogen) was then placed on the centre of the 'subbed' coverslip and dried in a class II tissue culture hood. A sterile cloning ring was then placed at the centre of the dried fibronectin. The required tissue was transferred to the centre of the sterile cloning ring and supplemented with 500µl of Embryonic Culture Medium (2.3) and incubated at 37°C in an atmosphere of 5% (v/v) $CO_2$ in a humidified incubator for 24 hours. Once the tissue was attached the cloning ring was removed and the Embryonic Culture Medium replaced. The culture was then incubated for the desired duration (approximately 7 days) with the Embryonic Culture Medium changed every 2 days.

### 2.8.6 Inducible differentiation of BMOL-TAT1.1 cells

In order to investigate oval cell differentiation, BMOL-TAT1.1cells were grown in the different culture conditions as listed in Table 2.7. These conditions were modified from a previously published study utilising the BMOL-TAT1.1 cell line and the specialised 'growth' and 'differentiation' medium (2.3) (Tirnitz-Parker, et al., 2007).

| CONDITION | BMOL-TAT1.1 MEDIUM | |
|---|---|---|
| **1** | 6 days Growth Medium | |
| **2** | 17 days Growth Medium | |
| **3** | 10 days Growth Medium | 7 days Differentiation Medium |
| **4** | 7 days Growth Medium | 10 days Differentiation Medium |
| **5** | 3 days Growth Medium | 14 days Differentiation Medium |
| **6** | 17 days Differentiation Medium | |
| **7** | 3 days Growth Medium | 14 days ------ Medium |
| **8** | 3 days Growth Medium | 14 days 20ng/ml EGF |
| **9** | 3 days Growth Medium | 14 days 0.1µM Dexamethasone |
| **10** | 3 days Growth Medium | 14 days 10mM Nicotinamide |
| **11** | 3 days Growth Medium | 14 days 6.25µg/ml ITS |

**Table 2.7: BMOL-TAT1.1 culture conditions**

## 2.9 Molecular biology techniques

### 2.9.1 Adenoviral construct preparation

All adenoviral constructs were prepared using the AdEasy™ Adenoviral Vector System (Stratagene) (Appendix D). Amplification of adenoviral constructs was performed in HEK-293 cells. Four T-75 flasks were infected with 10µl, 1µl, 0.1µl and 0.01µl of concentrated adenovirus in HEK-293 Medium (2.3) to prepare a pre-stock. When 50% of the HEK-293 cells demonstrated cytopathic effect (usually 2-4 days) the cells were detached from the flask by gently tapping and transferred to a 15ml tube. A cell pellet was obtained by centrifugation at 1000rpm for 4 minutes and re-suspended in 1ml HEK-293 Medium. The HEK-293 cells were lysed with 4 freeze/ thaw cycles in a dry ice/ ethanol bath and 37°C water bath. The appropriate quantity of pre-stock (usually 125µl, 12.5µl or 2µl) which caused 50% cytopathic effect of HEK-293 cells in a T-175 flask, was utilised to infect 10 x T-175 flasks. The cells were harvested as described before and centrifuged at 2000rpm for 10 minutes and the supernatant discarded. All 10 pellets were pooled and re-suspended in 5ml 100mM Tris HCl pH 8.0, subjected to 4 freeze/ thaw cycles in a dry ice/ ethanol bath and 37°C water bath in order to release all the virus particles from the cells, and finally centrifuged at 2000rpm for 5 minutes to remove the cell debris. To the supernatant containing the virus, 0.6 volumes of Caesium Chloride supersaturated 100mM Tris HCl pH 8.0 was added, mixed gently and transferred into 2 Beckman centrifuge tubes (Beckman 342412). Following a centrifuge at 65000rpm at 22°C for 4 hours in a Beckman Ultracentrifuge (LL-TB003) using a Beckman Vti90 rotor, the virus particle band was removed with a 25G needle and 2ml syringe. The collected virus was added to a new Beckman centrifuge tube, which the remainder was filled with balance solution (1 volume 100mM Tris HCl pH 8.0: 0.6 volumes of Caesium Chloride supersaturated 100mM Tris HCl pH 8.0) and re-centrifuged at 65000rpm at 22°C over night. The resultant virus particle band was removed with a 25G needle and 1ml syringe and dialysed in a Gamma Irradiated Slide-A-Lyzer™ Dialysis Cassette (Thermo Scientific) against a buffer containing 10mM Tris HCl pH7.5, 1mM $MgCl_2$ and 135mM NaCl for 6 hours and 12 hours at 4°C. The virus was then filtered at 0.22µm, aliquoted and stored at -80°C. The adenovirus utilised are recorded in Table 2.6.

### 2.9.2 Adenoviral titre

Adenoviral constructs were titred using the Adeno-X™ Rapid Titer Kit (BD Biosciences) according to the manufacturer's guidelines. HEK-293 cells were seeded onto a 12 well tissue culture plate and grown to 70% confluence. To each well 100µl of 6 serial dilutions ranging from $10^2$ to $10^7$, or purified virus was added and incubated for 48 hours. The cells were fixed with 1ml per well of methanol at -20°C for 10 minutes after removal of the media. The cells were washed three times with PBS, blocked with 2% Blocking Buffer (Roche, Germany) for 1 hour at room temperature and incubated with mouse anti-Hexon primary antibody (1:1000 in 2% Blocking Buffer) at 37°C for 1 hour. After three PBS washes, the cells were incubated with rat anti-mouse secondary antibody (HRP conjugate, 1:500 in 2% Blocking Buffer) at 37°C for 1 hour. The peroxidase was developed using DAB Peroxidase Substrate Kit (Vector Laboratories) according to manufacturer's guidelines. Two drops of Buffer pH7.5, 4 drops of DAB substrate reagent and 2 drops of $H_2O_2$ were added to 5ml Milli-Q $H_2O$. To each well 500µl of this solution was added and incubated for 10 minutes at room temperature, each well was then quenched with water. Using an inverted Leica DMIRB microscope the mean number of positive cells in a 20x field was calculated from 4 separate fields. The Infectious Units (IU) per ml titre was calculated by:

$$\text{IU/ml} = \frac{\text{(Positive Cell per 20x field) x 5730}}{\text{Dilution Factor}}$$

### 2.9.3 Adenoviral infection

All cells were plated onto 35mm culture dishes at a density of approximately 1 x $10^5$. Specific Multiplicity of Infections (MOI) volumes i.e. number of virus particles per individual cell were calculated utilising the appropriate adenovirus titre (Table 2.6). Adenoviral infection of BMOL-TAT1.1 cells was carried out at a range of MOIs (50-300), with virus incubation at 37°C for 1 hour or overnight. Adenoviral infection of α-TC19 cells was carried out at 37°C a range of MOIs (50-300), with overnight virus incubation in the presence of 5µg/ml diethylaminoethyl (DEAE)-dextran to enhance infectivity. For multiple simultaneous infections the individual MOI for each virus was kept constant, using the Ad-Null virus to moderate the total MOI. Cells were maintained after infection for a maximum of 7 days before transmitted light images were collected on the Leica

DMIRB microscope using SPOT$^{TM}$ software and further analysis by immunofluorescent staining (2.10.1) and RT-PCR (2.9.4).

## 2.9.4 Semi-quantitative RT- PCR

### 2.9.4.1 RNA isolation, quantification and purity analysis

Total cellular RNA was extracted from cultured cells at the appropriate time point using 1ml TRI® reagent (Sigma) per 35mm dish according to the manufacturer's guidelines. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. After homogenization, the lysate was collected in and RNase-free eppendorf, and then 200µl of chloroform (Sigma) was added. Covering the sample, it was shaken vigorously for 15 seconds and allowed to stand for 15 minutes at room temperature. It was then centrifuged at 12100x*g* for 15 minutes at 4°C. The aqueous phase was transferred to a fresh RNase-free eppendorf; 500µl of isopropanol (Sigma) was added, the sample vortexed and then allowed to stand for 10 minutes at room temperature. It was then centrifuged at 12100x*g* for 10 minutes at 4°C. The supernatant was removed and the RNA pellet washed with 75% ethanol and centrifuged at 7600x*g* for 5minutes at 4°C. The RNA pellet was dried for 5-10 minutes, re-suspended in DEPC-treated water and incubated at 60°C for 10 minutes.

An aliquot of re-suspended RNA was diluted in water and quantified using spectrophotmetery at 260nm. The RNA concentrations (Appendix B) were calculated using the Beer-Lambert Law [$A=\varepsilon\ c\ l$; where $A$ is the absorbance at a particular wavelength, $\varepsilon$ is the extinction coefficient, $c$ is the concentration and $l$ is the light path length (1cm)] therefore:

$$\text{RNA Concentration (µg/ml)} = A_{260}\,\varepsilon\,l \times \textit{dilution factor}$$

Also samples with an $A_{260}/A_{280}$ ratio of below 2.0 and $A_{260}$ value below 1.0 were considered to be without significant contamination (Appendix B) and hence stored at -80°C.

### 2.9.4.2 RNA integrity analysis

RNA samples were analysed by electrophoresis on a 2% agarose gel (with Milli-Q $H_2O$) supplemented with 0.02% ethidium bromide, 2% formaldehyde, and 1X MOPS. Samples were loaded with RNA 5X Loading Buffer (2.2), electrophoresed at 110V in RNA Running Buffer and visualised using UV light. Gel images were captured using Alphaimager™ 3400 (Alpha Innotech Corporation, San Leandro, CA, USA). RNA specific gel electrophoresis was utilised to confirm all RNA isolated was intact, as RNA quality is a critical determinant for the success of many downstream applications. This was deduced from the crisp 28S and 18S bands; and 28S:18S ratio of approximately 2.0 as shown in Figure 2.1.

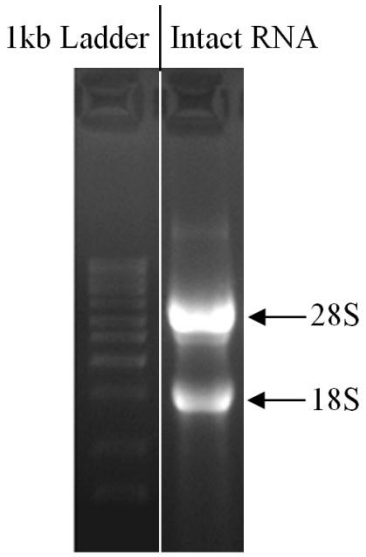



**Figure 2.1: RNA integrity analysis.**

### 2.9.4.3 RNA DNase treatment

Contaminating genomic DNA was removed using DNase (Ambion) according to the manufacturer's guidelines. 5µg of RNA was added to a 25.23µl reaction consisting of cross-linked Milli-Q water with, 3µl 10X DNase Buffer and 1µl DNase and incubated at 37°C for 30 minutes. In order to stop the DNase reaction 0.77µl 200mM EDTA was added and samples then incubated at 75°C for 10 minutes. Samples were then stored at -80°C.

### 2.9.4.4 Reverse transcription (RT)

Messenger RNA (mRNA) was isolated from total RNA by using SuperScript™ First Strand Synthesis System (Invitrogen) for RT-PCR. mRNA was isolated by incubation of Oligo (dT) oligonucleotides and 10mM dNTP with 1.5µg extracted total DNase-treated RNA at 65°C for 5 minutes. First strand complementary DNA (cDNA) was synthesized by incubation with SuperScript™ II Reverse Transcriptase (Invitrogen), 0.1M dithiothreitol (DTT) and 5X RT Buffer at 42°C for 52 minutes followed by inactivation at 70°C for 15 minutes. All cDNA samples were stored at -20°C. In parallel the RT reaction was performed with DNase treated RNA but without the Reverse Transcriptase enzyme, in order to serve as a 'RT no template control', thus confirming that the samples were void of genomic contamination.

### 2.9.4.5 Gene specific polymerase chain reaction (PCR)

Each PCR reaction contained 1µl of cDNA (50ng of total RNA), 10µl of 2X Reddy Mix™ PCR Master Mix Kit (Thermo Scientific), 0.5µl of each forward and reverse primers, and 8µl sterile cross-linked Milli-Q $H_2O$, as according to the manufacturer's guidelines. PCRs were performed in a TC-412 Techne Thermal Cycler (Jencons Pls) using the following conditions: denaturing at 94°C for 5 minutes, followed by 25-35 cycles of denaturing at 94°C for 1 minute, amplification at the appropriate primer specific annealing temperature for 1 minute, and elongation at 72°C for 1 minute. This was followed by a final extension step of 72°C for 10 minutes. The primer sequences, annealing temperatures, product sizes and cycle number utilised for PCR reactions are detailed in Table 2.5. PCR products were routinely analysed by electrophoresis on a 2% agarose gel supplemented with 0.02% ethidium bromide, in 1X tris acetic acid + ethylenediaminetetraacetic acid (TAE) Running Buffer. Samples were loaded, electrophoresed at 110V and visualised using UV light. Gel images were captured using Alphaimager™ 3400 (Alpha Innotech Corporation, San Leandro, CA, USA). All PCR results included were repeated at least twice for confirmation.

## 2.10 Staining techniques

### 2.10.1 Immunofluorescent staining

#### 2.10.1.1 Fixation of cultures

Cell lines (BMOL-TAT1.1 and α-TC19) and embryonic cultures were cultured on appropriate coverslips until the desired time point for the specific experiment. Coverslips were rinsed twice with PBS to remove excess medium. The fixation conditions were dependent on the specific antigen being detected (Table 2.2), hence the cell lines were either fixed with 4% PFA at room temperature for 25 minutes, and the embryonic cultures with MEMFA Fixative (2.2) at room temperature for 30 minutes or Acetone-Methanol (1:1 v/v) at -20°C for 7 minutes. The coverslips were then rinsed twice with PBS and kept at 4°C prior to staining.

#### 2.10.1.2 Fluorescent immuno-cytochemistry

PFA and MEMFA fixed samples were permeabilised with 0.1% (v/v) Triton X-100 (Sigma) or 1% (v/v) Triton X-100 (Sigma) respectively at room temperature for 20 minutes. Samples that were fixed in Acetone-Methanol (1:1 v/v) did not require permeabilisation. Also antigen retrieval was performed for certain antigens (Table 2.2), either incubated in 1X Citrate Buffer (Lab Vision Corporation) at 37°C for 1 hour or incubated in ethylene diamine tetraacetic acid (EDTA) Buffer at 37°C for 30 minutes, before blocking. Cells were incubated in 2% Blocking Buffer (Roche) for 45 minutes. The coverslips were then incubated with the appropriate primary antibody overnight at 4°C. The following day, coverslips were washed 3 x 10 minutes with PBS. The fluorescently-conjugated secondary antibodies (Table 2.3) were added to the coverslips for 1.5 hours and then washed 3 x 10 minutes with PBS. For detection of two (glucagon and insulin) or three (glucagon, insulin and HNF4α) antigens, the primary antibodies were added simultaneously, and the secondary antibodies were added sequentially using the same protocol to prevent cross reactivity. For detection of lectins DBA and PNA (Table 2.4), the fluorescently labelled lectins were added simultaneously as primary antibodies and no secondary antibodies were required. The cells were finally incubated with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) at room temperature for 5 minutes with light protection. DAPI was dissolved in PBS at 500μg/ml and used at a 1:1000 dilution.

Coverslips were mounted on non-subbed slides (Fisher Brand®) with GelMount™ Aqueous Mounting Medium (Sigma). For all antibodies utilised, positive and negative controls (i.e. no primary antibody) were performed in parallel (Appendix C1).

### 2.10.2 X-gal staining for beta-galactosidase

BMOL-TAT1.1 cells were analysed for *lacZ* transgene expression by performing X-gal staining. Cells were placed in Fixation Buffer (2.2) for 15 minutes at room temperature. After fixation, 2 x 5 minute washes with Wash Buffer (2.2) were performed. Cells were then immersed in X-gal Staining Solution (2.2) at 37°C overnight with light protection. The next day, cells were washed twice with Wash Buffer and mounted onto non-subbed slides (Fisher Brand®) using GelMount™ Aqueous Mounting Medium (Sigma). BMOL-TAT1.1 cells un-infected and infected with Ad-CMV-LacZ served as a negative and positive control respectively for the X-gal staining protocol (Appendix C2).

### 2.10.3 Periodic acid schiff (PAS) staining

Cells cultured on coverslips were initially fixed with 4% PFA at room temperature for 25 minutes. The coverslips were washed for 3 minutes under running tap water and then immersed in 1% Periodic Acid (Sigma) diluted in Milli-Q $H_2O$. The coverslips were washed for 3 minutes under running tap water and then incubated in Schiff Reagent (Sigma) with light protection for 2-6 minutes until the stain developed. The coverslips were then washed for 10 minutes under running tap water in order to reduce non-specific background staining. Finally coverslips were mounted on non-subbed microscope slides (Fisher Brand®) with GelMount™ Aqueous Mounting Medium (Sigma). Primary hepatocytes serve as a positive control (Appendix C3).

## 2.11 Image processing

Fluorescent, X-gal and PAS stained cells were analysed under a Leica DMRB microscope (10x, 20x, 40x, 63xoil objective lens) with images taken with a digital SPOT camera using NIS Elements software or under a LSM510META Zeiss Confocal Laser Scanning Microscope (LSM) (60xoil objective lens) using LSM Image Browser software.

Transmitted light images of cells in culture were analysed under an inverted Leica DMIRB microscope using (10x, 20x, 40x objective lens) with images taken with RT Colour SPOT camera using SPOT™ software. All images captured were colour balanced, contrast-enhanced and labelled using Adobe Photoshop Version CS.3 and Microsoft Office PowerPoint.

## 2.12 Measurements and statistics

Measurements of BMOL-TAT1.1 cell sizes (diameter) were performed using measurement tools on LSM Image Browser software. All numerical values are presented as mean ± standard deviation. The significance of the observed difference between the two distinctive BMOL-TAT1.1 cell types was tested by a Students t-test.

# Chapter 3 Characterisation of the oval cell line BMOL-TAT1.1 and optimisation of inducible differentiation

## 3.1 Introduction

### 3.1.1 BMOL-TAT1.1 cell line

The therapeutic potential of hepatic progenitor cells (oval cells) cannot be harnessed until the mechanisms governing their proliferation and trans/differentiation are better understood. In order to fully elucidate the factors and signalling pathways governing oval cell proliferation and trans/differentiation, it is beneficial to utilise an *in vitro* model. Initially oval cell lines were generated primarily from rats (Lazaro, et al., 1998, Pack, et al., 1993, Yin, et al., 2002), but investigators became keen to generate mouse oval cell lines instead. *Richards et al.*, produced one of the first mouse oval cell lines from TgN73/Rpw transgenic mice (Richards, et al., 1997). This shift from rat to mouse was due to the additional research benefits provided by mice, 99% of mouse genes have an equivalent in humans and they are the most amenable to genetic manipulation with many extensive knockout and over-expression mouse strains available, thus making them ideal for studying the function of human genes. Prior to this research commencing *Tirnitz-Parker et al.*, generated a novel immortalised, non-transformed, clonal murine adult oval cell line, defined as Bi-potential Murine Oval Liver – Tyrosine Aminotransferase (BMOL-TAT1.1) (Tirnitz-Parker, et al., 2007). This cell line was derived from highly enriched oval cell cultures, obtained from the liver of choline-deficient, ethionine-supplemented (CDE) diet fed 4 week old NMRI/Han-TAT-GRE *lacZ* mice (Akhurst, et al., 2001). The BMOL-TAT1.1 cell line was chosen for this research as it possesses the major advantage over other available adult oval cell lines as hepatic differentiation can be simply followed by X-gal staining analysis, as expression of the *lacZ*-transgene, is driven by the promoter element from TAT, a marker of mature adult hepatocytes (Montoliu, et al., 1995).

### 3.1.2 Inducible *in vitro* oval cell proliferation

In order to investigate oval cell proliferation, the BMOL-TAT1.1 cell line was cultured in a previously published specialised ‘growth medium’ (2.3), supplemented with

IGFII, insulin and EGF (Tirnitz-Parker, et al., 2007). Control of cell proliferation is crucial for the oval cell response during liver regeneration. A central role in regulation of proliferation is played by extracellular growth factors, which are secreted polypeptides interacting with specific high affinity plasma membrane anchored receptors in a paracrine or autocrine manner. One of the most strongly implicated signalling systems in liver regeneration, responsible for rebuilding liver mass and also restoration of glucose homeostasis is the insulin-like growth factor (IGF) receptor and their binding proteins, such as insulin growth factor II (IGF-II) and insulin (Liu, et al., 1999). IGF-II a 67 amino acid polypeptide is structurally related to pro-insulin and binds with high affinity to two structurally unrelated type 1 and type 2 IGF receptors. Oval cells have been shown to be heterogeneous for IGFII expression and IGFII shown to mediate stimulation of oval cell proliferation (Zhang, et al., 1997). Epidermal growth factor (EGF) is a member of the EGF family and binds the EGF receptor (EGFR) triggering a tyrosine kinase signalling system. EGF has been suggested to have a fundamental role in liver regeneration. EGF and EGFR-mediated autocrine signalling has been observed to elicit potent mitogenic activity in mouse oval cells in a dose responsive manner *in vitro* (Isfort, et al., 1997, Martinez-Palacian, et al., 2012, Michalopoulos and Khan, 2005). Furthermore *in vivo* infusion with EGF amplifies liver progenitor expansion following 2-acetylamino-fluorene induced liver injury and decreases cell apoptosis (Nagy, et al., 1996).

### 3.1.3 Inducible *in vitro* oval cell hepatocyte differentiation

In order to investigate oval cell hepatic differentiation, the BMOL-TAT1.1 cell line was cultured in a previously published specialised ‘differentiation medium’ (2.3), designed to enhance hepatocyte differentiation (Tirnitz-Parker, et al., 2007). The ‘differentiation medium’ is supplemented with EGF, dexamethasone, a synthetic glucocorticoid which has been reported to induce differentiation of liver progenitor cells (Yeoh, et al., 1979) and two other supplements, insulin-transferrin-selenium (ITS) and nicotinamide, both known to promote hepatocyte differentiation (Auth, et al., 2005, Inoue, et al., 1989).

### 3.1.4 Experimental Aims

The first phase of this research was to utilise the growth medium in order to perform a detailed characterisation of the proliferating BMOL-TAT1.1 cell line in terms of

morphology and protein expression of markers specific for oval cells, morphology and liver-enriched transcription factors. As well as address the potential role of the Wnt/β-catenin signalling pathway during BMOL-TAT1.1 proliferation via analysis of β-catenin cellular localisation. Secondly culturing of BMOL-TAT1.1 cells in different culture conditions, with variances in specialised medium and culture time was performed in order to optimise the *in vitro* hepatocytic differentiation protocol.

## 3.2 Results

### 3.2.1 Characterisation of the heterogeneous BMOL-TAT1.1 cell line

BMOL-TAT1.1 cell cultures were maintained in growth medium for 6 days to mimic proliferation, and then subsequently their morphology and protein expression were analysed in detail. Proliferating BMOL-TAT1.1 cells comprised monolayers of cells exhibiting two distinct morphological phenotypes (Figure 3.1). Immunofluorescent staining of the heterogeneous cell population for two commonly used oval cell markers, A6 and OV6 (Crosby, et al., 1998, Wang, et al., 2002b), confirmed their derivation from oval cells (Figure 3.2). It was illustrated that within proliferative colonies, centrally located cells with very high nuclear: cytoplasmic ratio and irregular shaped nuclei maintained a relatively small size, and were OV6 positive and A6 negative, with a mean cell diameter of 19.41μm (i.e. OV6 positive cells) (Figure 3.2). The peripheral cells contrastingly with abundant cytoplasm and regular shaped nuclei possessed a significantly larger flattened cuboidal morphology, and were OV6 negative and A6 positive with a mean cell diameter of 39.37μm (i.e. OV6 negative cells) (Figure 3.2).

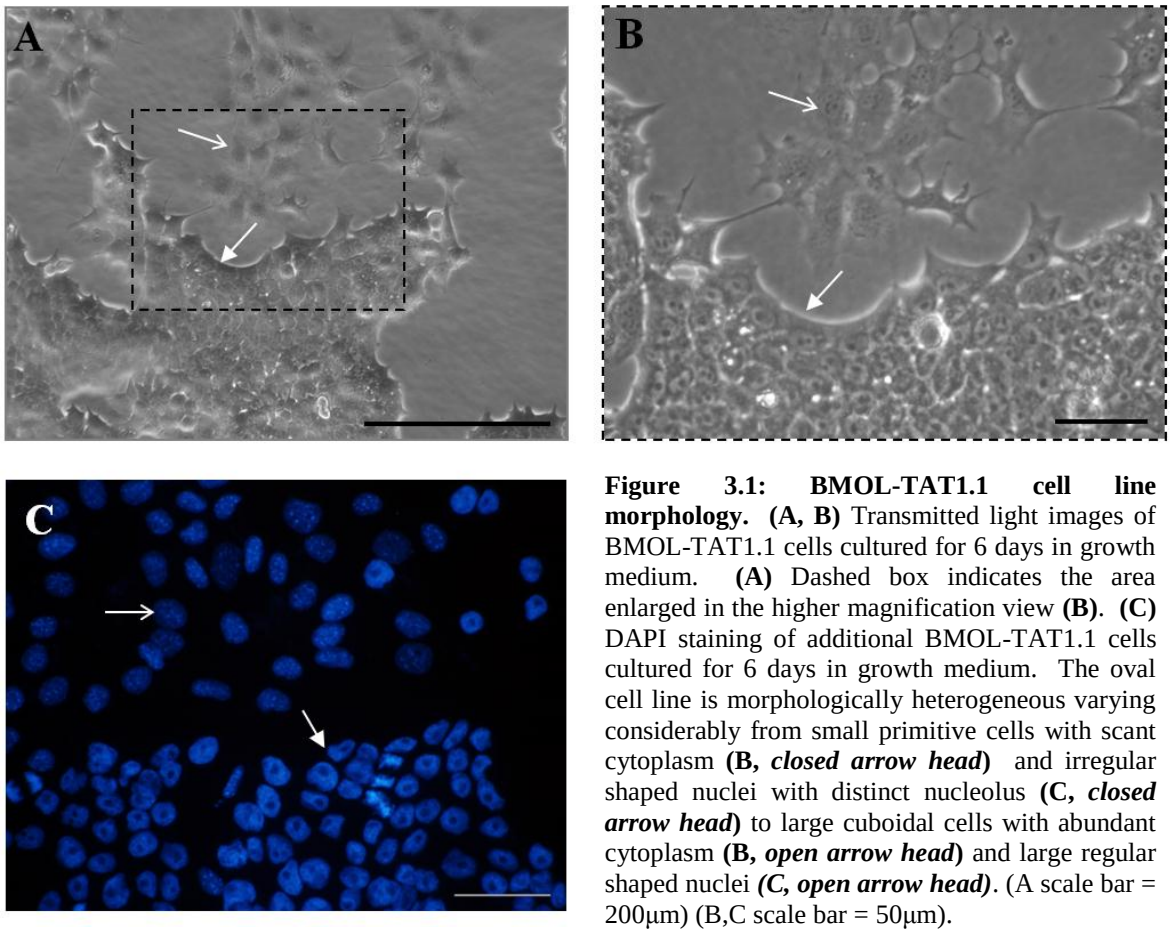
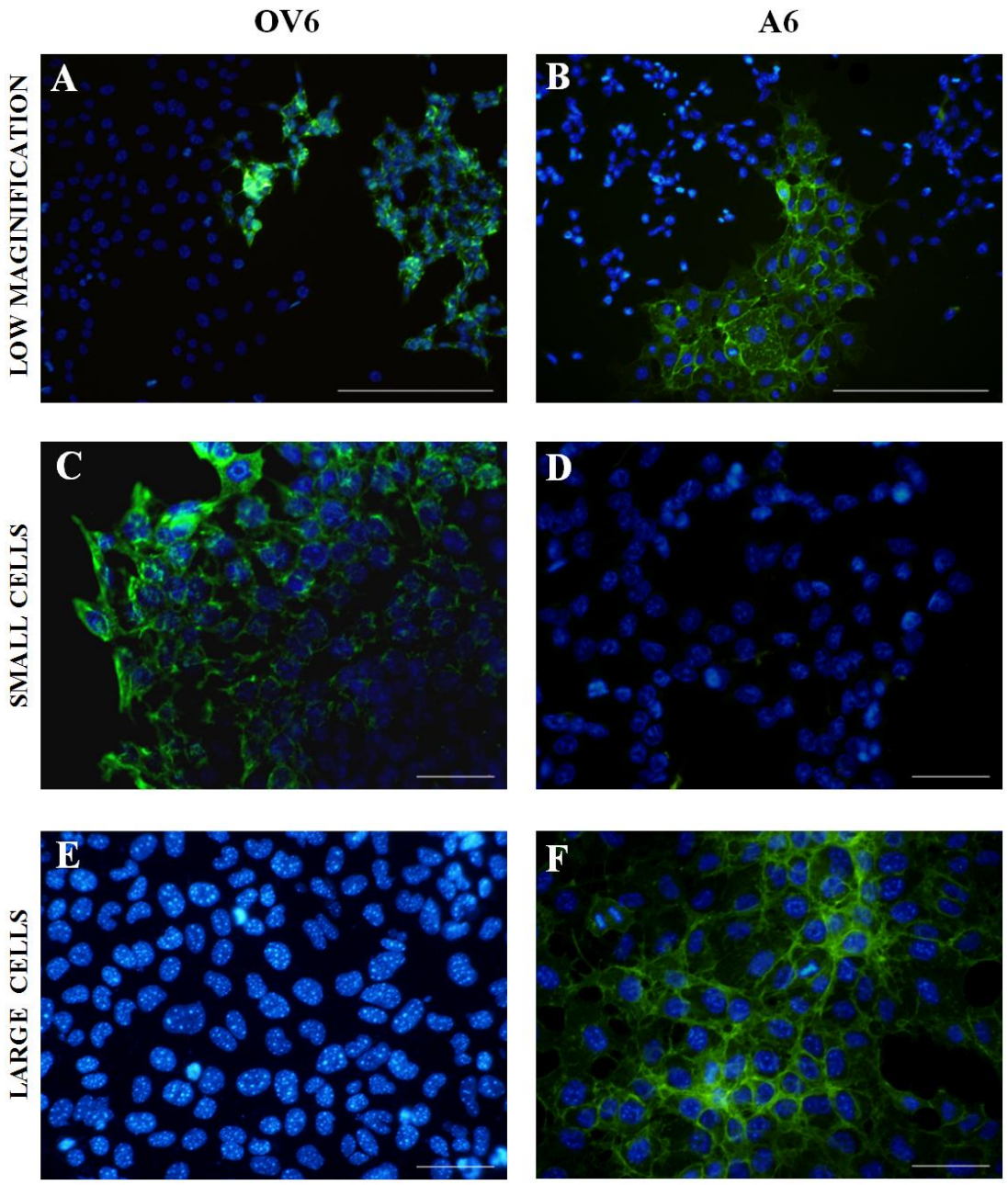



**Figure 3.1: BMOL-TAT1.1 cell line morphology. (A, B)** Transmitted light images of BMOL-TAT1.1 cells cultured for 6 days in growth medium. **(A)** Dashed box indicates the area enlarged in the higher magnification view **(B)**. **(C)** DAPI staining of additional BMOL-TAT1.1 cells cultured for 6 days in growth medium. The oval cell line is morphologically heterogeneous varying considerably from small primitive cells with scant cytoplasm **(B, *closed arrow head*)** and irregular shaped nuclei with distinct nucleolus **(C, *closed arrow head*)** to large cuboidal cells with abundant cytoplasm **(B, *open arrow head*)** and large regular shaped nuclei ***(C, open arrow head)***. (A scale bar = 200μm) (B,C scale bar = 50μm).



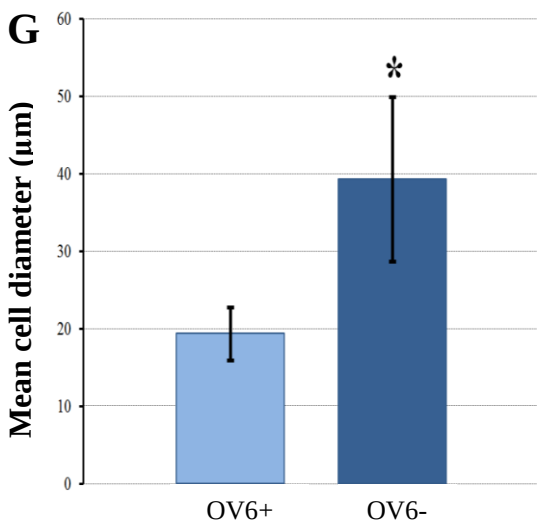




**Figure 3.2: BMOL-TAT1.1 cell line heterogeneity.** Immunofluorescent staining for OV6 **(A, C, E, *green*)** and A6 **(B, D, F, *green*)** expression in BMOL-TAT1.1 cells cultured for 6 days in growth medium. DAPI staining **(*blue*)** is included to distinguish the two different cell types based on their nuclear morphology. The small cells are OV6 positive **(C)**, A6 negative **(D)** whilst in contrast the large cells are OV6 negative **(E)** and A6 positive **(F)**. **(G)** Mean cell diameter for OV6+ and OV6- BMOL-TAT1.1 cells were calculated from 100 cells from 3 independent experiments, with all numerical results expressed as the mean ± standard deviation. The significant difference in cell size was evaluated by a Students t-test * ($p<0.05$). (A, B scale bar = 200µm) (C-F scale bar = 50µm).

Immunofluoresecent staining revealed the larger flattened cells were positive for the epithelial cell marker E-cadherin, with E-cadherin localised primarily to the plasma membrane but also partially in the cytoplasm adjacent to the nucleus (Figure 3.3A). Contrastingly the smaller cells were negative for E-cadherin but positive for the mesenchymal/ ductal marker Vimentin (Ko, et al., 2004) (Figure 3.3B).

We determined the expression of different liver-enriched transcription factors during BMOL-TAT1.1 cell proliferation. Both large and small cell types were C/EBPα negative (Figure 3.4A), but C/EBPβ (Figure 3.4D,G) and FoxA2 (Figure 3.5G) positive. However there was differential expression of HNF4α and Sox9, with the large cells appearing HNF4α positive and weakly Sox9 positive, whilst the small cells were HNF4α negative and strongly Sox9 positive (Figure 3.5A,D).

All immunofluorescent staining negative results obtained were confirmed by repetition of the staining protocol with various different conditions (i.e. antibody dilution, fixation, antigen retrieval methods) and obtained when performed simultaneously with a positive control under identical conditions (Appendix C)

In the liver, there is accumulating evidence that Wnt/β-catenin signalling plays a central role in various aspects of hepatic biology, including liver development, regeneration, growth and oncogenesis. During liver development, β-catenin critically regulates oval cell proliferation, with its activation being determined and identified by its cellular localisation. Analysis of β-catenin expression in the heterogeneous BMOL-TAT1.1 cell line revealed β-catenin to be activated and translocated in the cytoplasm and nuclei of the small cells whilst inactivated and bound to the plasma membrane in the large cells (Figure 3.6).

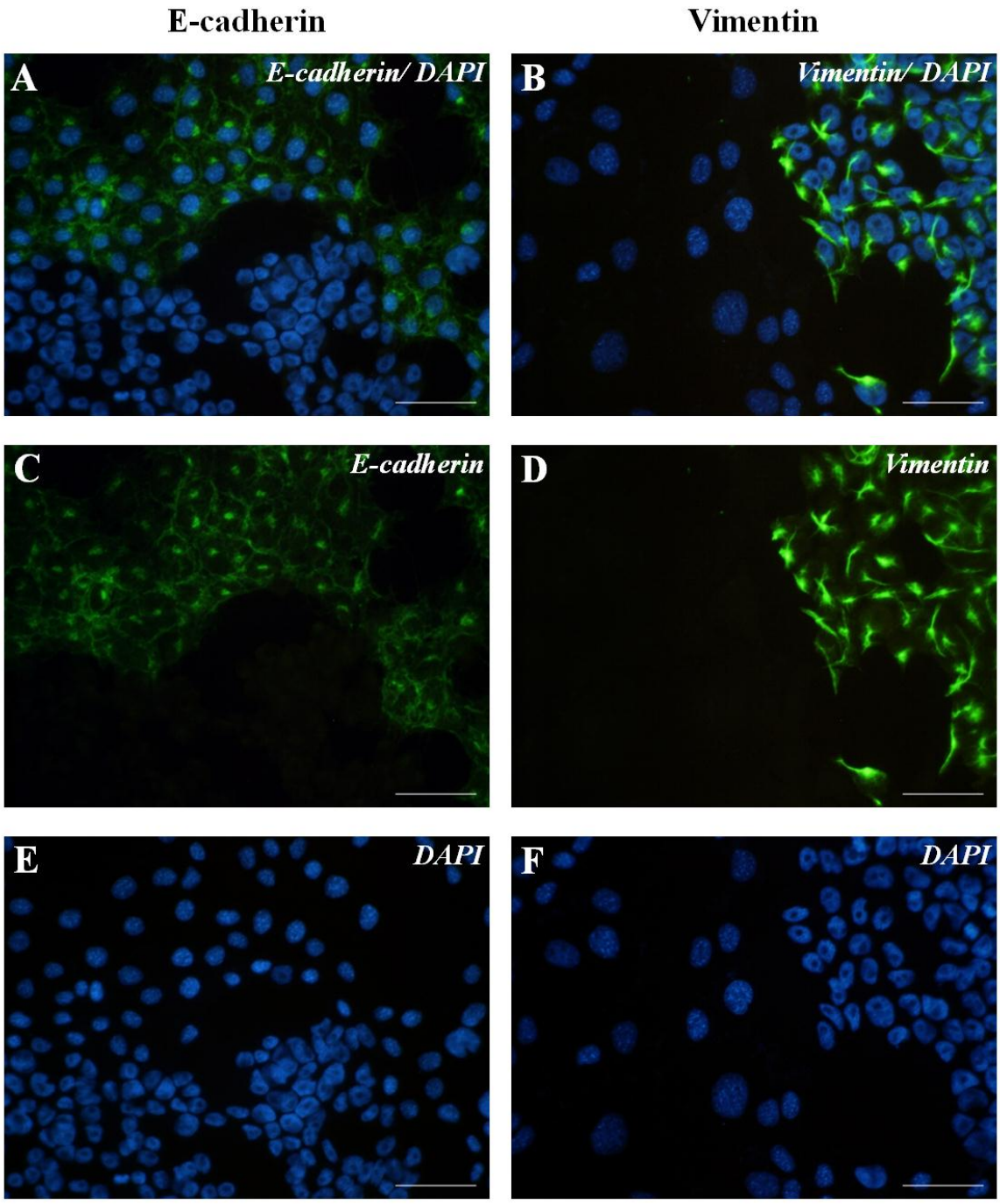



**Figure 3.3: BMOL-TAT1.1 cell line expression of morphological markers.** Immunofluorescent staining to identify E-cadherin **(C, *green*)** and Vimentin **(D, *green*)** expression in BMOL-TAT1.1 cells cultured for 6 days in growth medium. DAPI staining **(E, F, *blue*)** is included to distinguish the two different cell types based on their nuclear morphology. Images **A** and **B** are overlays of **C, E** and **D, F** respectively. The large cells are E-cadherin positive and Vimentin negative, whilst the small cells are E-cadherin negative and Vimentin positive. (scale bar = 50μm).

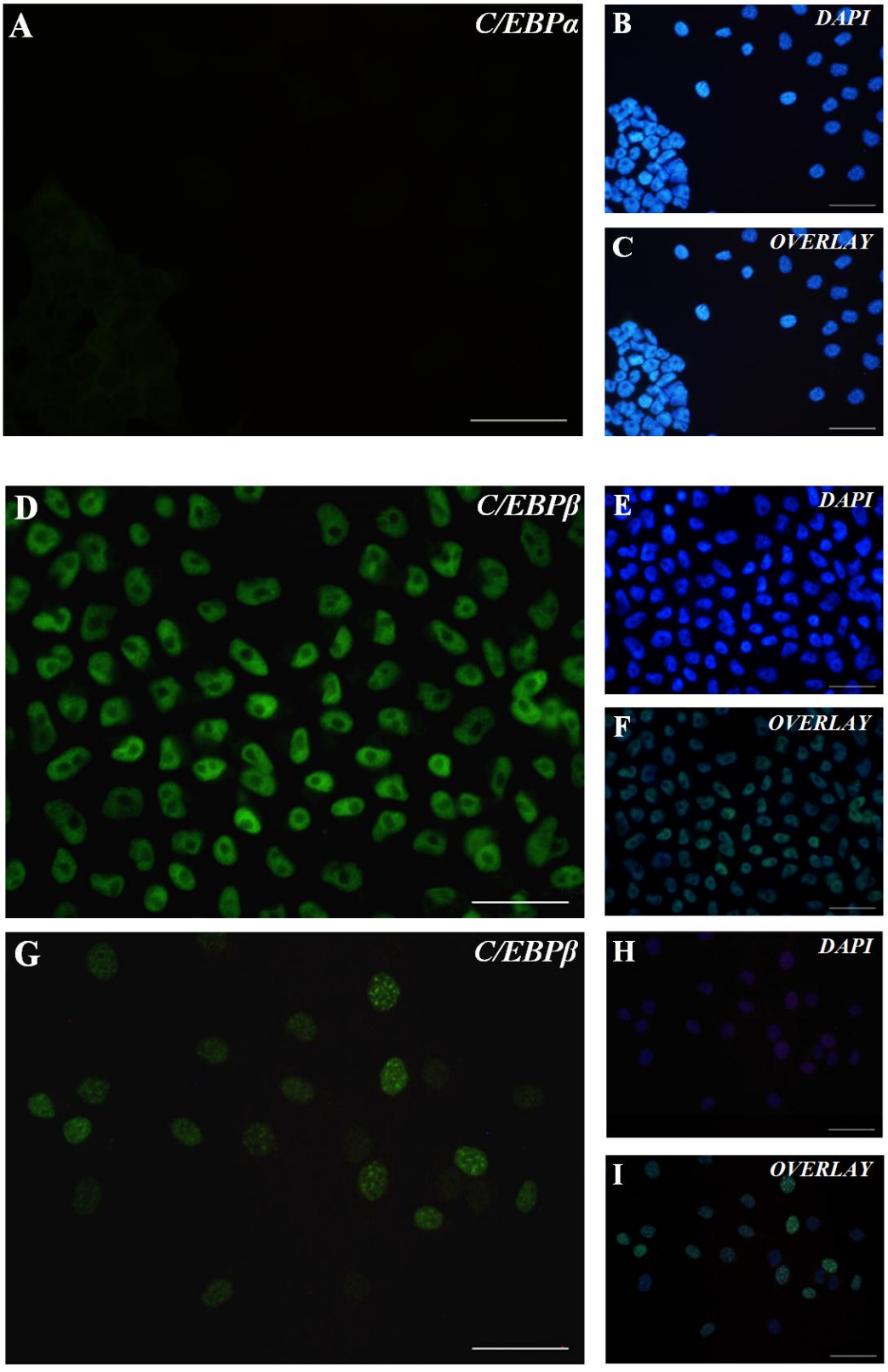



**Figure 3.4: Expression of C/EBP transcription factors in BMOL-TAT1.1 cells.** Immunofluorescent staining to identify C/EBPα **(A, *green*)** and C/EBPβ **(D and G, *green*)** expression in BMOL-TAT1.1 cells cultured for 6 days in growth medium. DAPI staining **(B, E, H, *blue*)** is included to distinguish the two different cell types based on their nuclear morphology. All cells are negative for C/EBPα expression **(A)** but positive for C/EBPβ expression both in the small cells **(D)** and large cells **(G)**. For all antibodies utilised, positive controls were performed in parallel and are included in *Appendix C1*. Images **C**, **F** and **I** are overlays of **A, B; D, E;** and **G, H** respectively. (scale bar = 50μm).

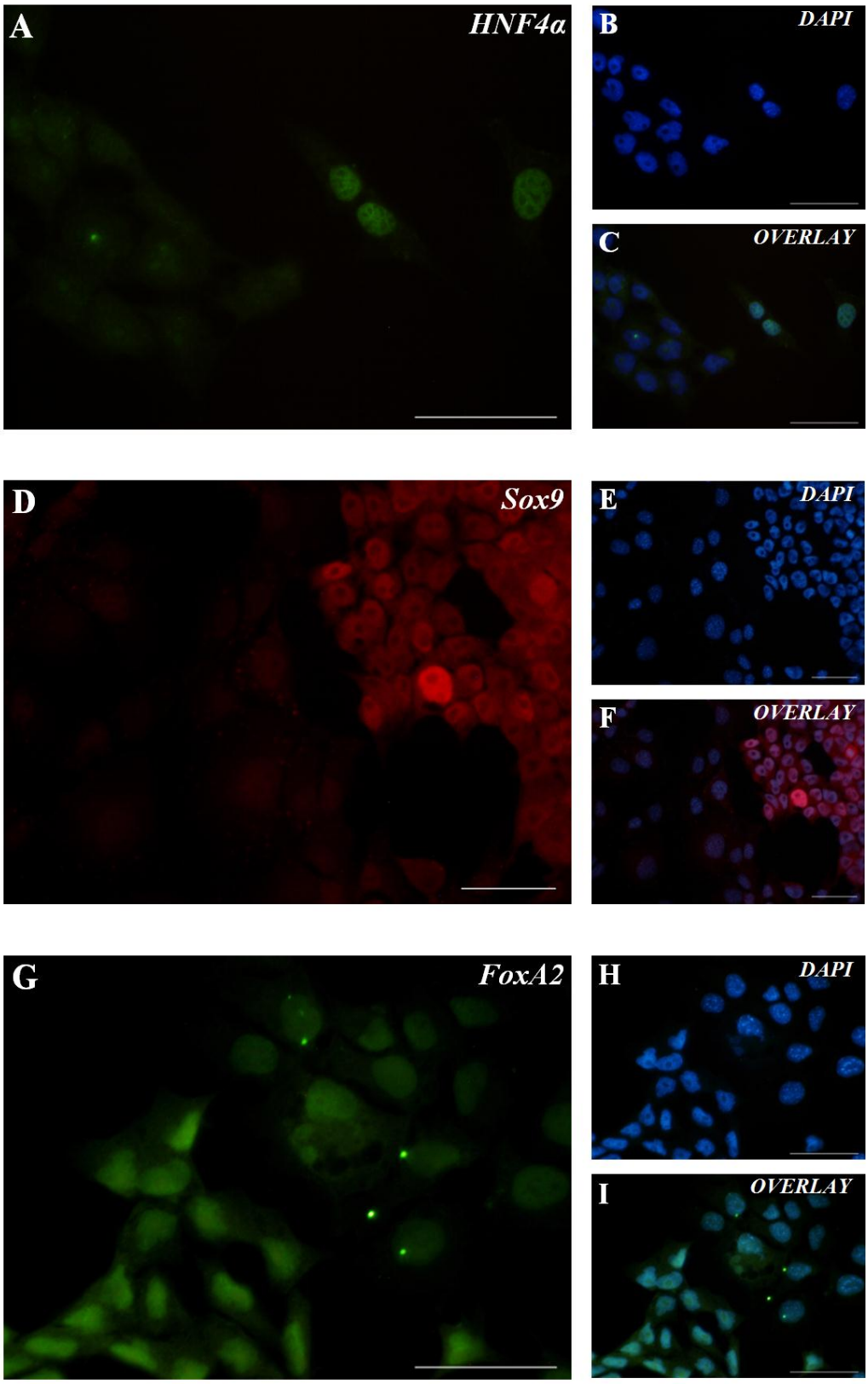



**Figure 3.5: BMOL-TAT1.1 cell line expression of transcription factors.** Immunofluorescent staining to identify HNF4α **(A, *green*)**, Sox9 **(D, *red*)** and FoxA2 **(G, *green*)** expression in BMOL-TAT1.1 cells cultured for 6 days in growth medium. DAPI staining **(B, E, H, *blue*)** is included to distinguish the two different cell types based on their nuclear morphology. Both cell types express FoxA2, whilst the large cells are HNF4α positive, Sox9 weakly positive and the small cells HNF4α negative, Sox9 positive. Images **C**, **F** and **I** are overlays of **A, B; D, E;** and **G, H** respectively. (scale bar = 50μm).

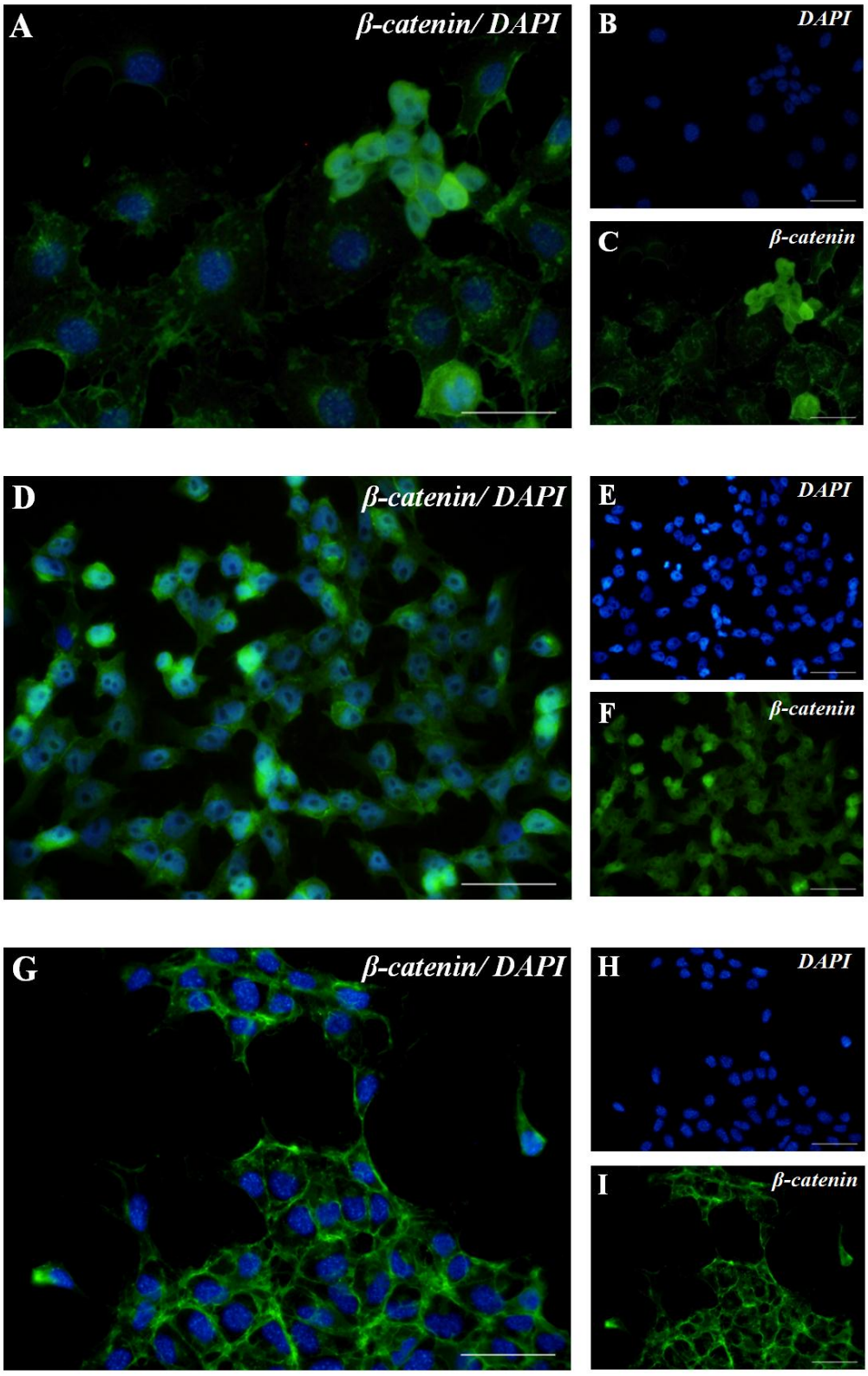



**Figure 3.6: Expression of β-catenin in BMOL-TAT1.1 cells cultured under proliferating conditions.** Immunofluorescent staining to identify β-catenin **(*green*)** expression in BMOL-TAT1.1 cells cultured for 6 days in growth medium. DAPI staining **(B, E, H, *blue*)** is included to distinguish the two different cell types based on their nuclear morphology. Both small and large cells express β-catenin, however its localisation differs as seen in the mixture of small and large cells **(A).** In the small cells β-catenin is located mainly in the nucleus with some minor membrane localisation **(D)**. However in the large cells β–catenin is only membrane bound and completely absent from the nucleus **(G)**. Images **A**, **D** and **G** are overlays of **B, C; E, F;** and **H, I** respectively. (scale bar = 50μm).

### 3.2.2 Optimisation of inducible differentiation of BMOL-TAT1.1 cells

In order to investigate the mechanisms governing oval cell hepatocytic differentiation it was essential to set up a model of differentiation that could be compared to proliferating oval cells. Therefore in order to assess which culture conditions could optimally enhance hepatocyte differentiation, BMOL-TAT1.1 cells were cultured for a total of 17 days under different culture conditions by varying the duration of exposure to growth and differentiation medium. The conditions tested are outlined in Table 2.7.

Cell proliferation under all conditions examined was accompanied by initial formation of a monolayer cell population (Figure 3.7A) prior to the establishment of spherical or ridge-like structures on top of the monolayer (Figure 3.7E). Due to oval cells' characteristic high proliferative rate, a correlation was observed between increasing the length of time cultured in growth medium, and an over-confluent cell population with large areas of detachment of cells from the culture dish. This observation was also obtained when the experiment was repeated several times and also when repeated with reduced cell seeding density and medium glutamine levels reduced from 2mM to 2µM.

The differentiation of cells towards a mature hepatocyte phenotype in the above culture conditions was subsequently analysed by X-gal staining, in order to detect cells which expressed the *lacZ* transgene under the promoter element of TAT (an adult hepatocyte marker). *Condition 1*, a 6 day growth control culture at approximately 50% confluency was negative for X-gal (Figure 3.8A). All of the other *Conditions 2-6* possessed X-gal positive cells (Figure 3.8B-F), with the most abundant expression in *Condition 5* (Figure 3.8E) and *Condition 6* (Figure 3.8F), which were cultured in 'differentiation medium' for the longest period of time. X-gal staining appeared punctuate in the cells and primarily in the cells on the periphery of the ridge-like structures between the two different cell types. However these X-gal positive cells did not exhibit an altered morphology, hence did not possess the typical hepatocyte morphology.

In order to validate that the X-gal staining observed was indicative of TAT expression, RNA was isolated and purified for *Conditions1-6* and semi-quantitative RT-PCR performed to determine TAT expression levels. In *Conditions 1 and 2* where cells were cultured solely in growth medium TAT expression was barely detectable although some cells may have potentially spontaneously differentiated. In contrast for *Conditions 3-6* which included culture in 'differentiation medium' TAT was highly expressed, especially

in *Condition 5* (Figure 3.8H). To simplify future analysis *Condition 5* (3G14D) was taken as the optimum model for oval cell hepatocytic differentiation to be compared against the control *Condition 1* (6G) and *Condition 2* (17G) which represented a model of oval cell proliferation. Analysis of *Condition 2* (17G) and *Condition 5* (3G14D) revealed the growth medium (i.e. EGF, IGF-II, insulin) to preferentially enhance proliferation of the small cells (Figure 3.7B). In contrast when cultured in differentiation medium (EGF, dexamethasone, nicotinamide, ITS) BMOL-TAT1.1 cells showed less proliferation maintaining a larger proportion of large cells in the culture (Figure 3.7E *black asterisk*).

Glycogen storage is another characteristic indicator or hepatocytes, and cultures containing X-gal stained positive BMOL-TAT1.1 cells deduced to be expressing TAT also contained Period Acid Schiff (PAS) positive cells (Figure 3.9). PAS is a technique used to detect glycogen and other polysaccharides PAS staining works via the periodic acid oxidising the diol functional groups in glucose and other sugars, creating aldehydes that react with the schiff reagent to give the pinkish colour.

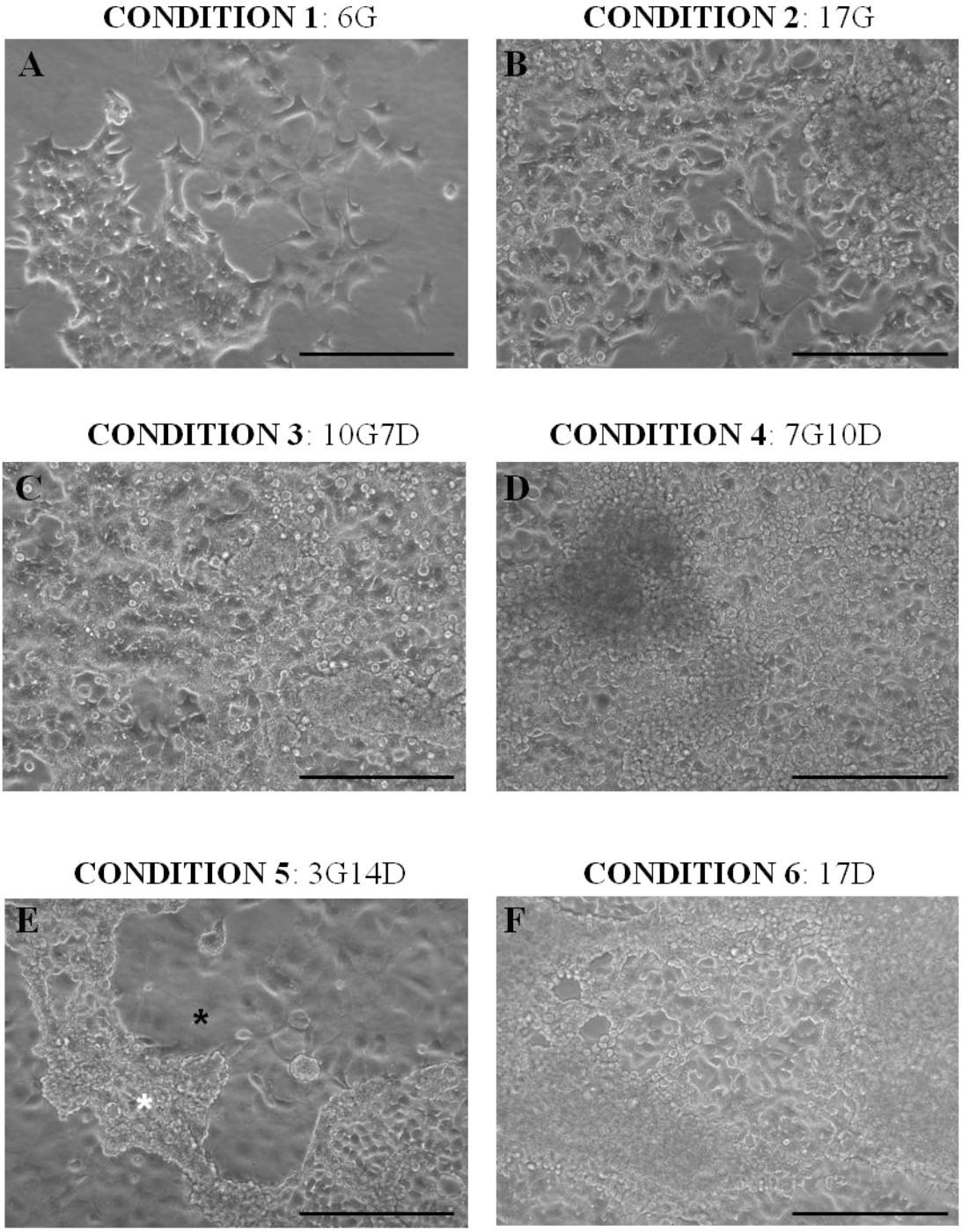



**Figure 3.7: BMOL-TAT1.1 cell line in different culture conditions.** Transmitted light images of BMOL-TAT1.1 cells cultured for 6 days in growth medium **(*Condition 1*, A)** or up to 17 days in specific culture conditions as detailed **(*Conditions 2-6*, B-F)**. Cells initially proliferated as a monolayer **(A; E, *black asterisk*)**, then formed spherical or ridge-like structures on top **(E, *white asterisk*)**. *Abbreviations*: D, Differentiation Medium; G, Growth Medium. (scale bar = 200µm).

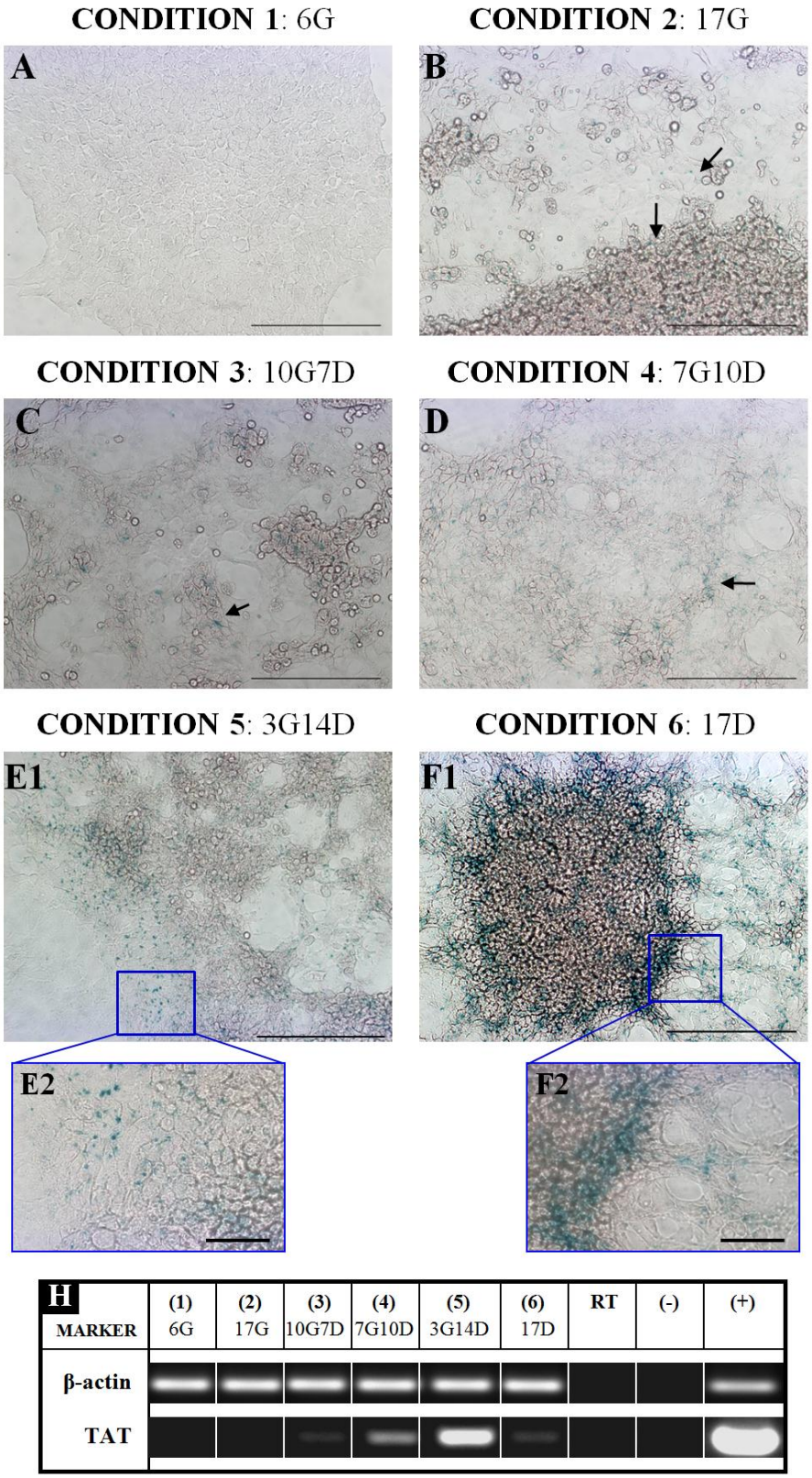



**Figure 3.8: X-gal staining representing TAT expression in different BMOL-TAT1.1 culture conditions. (A-F)** X-gal staining of BMOL-TAT1.1 cells cultured for 17 days in specific culture conditions as indicated. Black arrows identify areas of X-gal staining. *Conditions 5* **(E1)** *and 6* **(F1)** exhibited the most X-gal staining, with the blue boxes indicating the region enlarged in higher magnification views **(E2, F2)**. This is supported by analysis of TAT expression by RT-PCR **(H)**. *Abbreviations*: D, Differentiation Medium; G, Growth Medium; TAT, tyrosine aminotransferase; RT, no RT control; (-) negative control; (+), positive control. (A-D,E1,F1 scale bar = 200µm) (E2, F2 scale bar = 50µm).

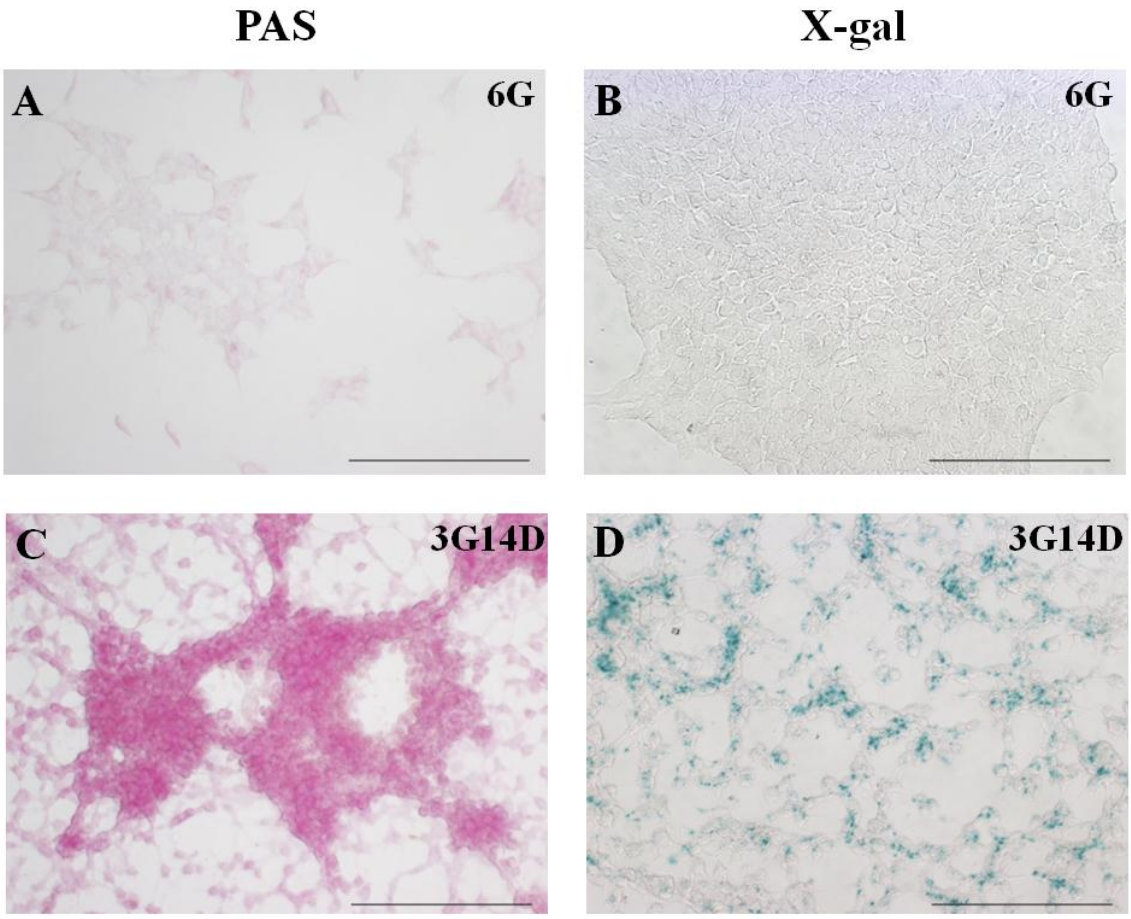



**Figure 3.9: Periodic acid schiff (PAS) and X-gal staining of BMOL-TAT1.1 cells cultured in differentiation medium.** PAS staining **(A, C, *pink*)** and X-gal staining **(B, D, *blue*)** expression in BMOL-TAT1.1 cells cultured for either 6 days in growth medium **(***Condition 1*, **6G**; **A, B)** or 3days in growth medium followed by 14 days in differentiation medium **(***Condition 5*, **3G14D**; **C, D)**. Staining revealed culturing in differentiation medium generated PAS positive cells and X-gal positive cells. Staining is predominantly in the ridge like structures where the cells are very dense. (scale bar = 200µm).

## 3.3 Discussion

### 3.3.1 BMOL-TAT1.1 cell line heterogeneity and the potential role of EGFR and β-catenin signalling

The preliminary conclusion for this chapter is that the proliferating BMOL-TAT1.1 cell line is heterogeneous possessing two distinctive morphological and antigenic phenotypes, denoted here as small and large BMOL-TAT1.1 cells. *Radaeva and Steinberg* observed a similar morphological heterogeneity in two different oval cell lines OC/CDE 6 and OC/CDE 22, with Transmission Electron Microscopy revealing small primitive elongated cells with a high nuclear: cytoplasmic ratio and larger cuboidal cells with abundant cytoplasm packed with organelles (Radaeva and Steinberg, 1995). In the present study, the significantly larger flattened cells with abundant cytoplasm are positive for the A6 antigen (Figure 3.2B), E-cadherin (Figure 3.3A) and HNF4α (Figure 3.5A), whereas the smaller cells with higher nuclear: cytoplasmic ratio which are positive for OV6 (Figure 3.2A), Vimentin (Figure 3.3B) and Sox9 (Figure 3.5D), markers commonly associated with the cholangiocyte lineage. This is consistent with the findings of *Ishikawa et al.*, showing large A6 positive hepatocyte-like cells express HNF4α, an important transcription factor in hepatocytic differentiation, whereas smaller ductular oval cells are HNF4α negative (Ishikawa, et al., 2012).

Another striking difference between the two cell types is the localisation of β-catenin expression. β-catenin is a multifunctional protein found in three cell compartments: (i) the plasma membrane (ii) cytoplasm and (iii) nucleus. The Wnt genes encode a large family of secreted glycoproteins that signal at the cell surface via at least two receptors: Frizzled, a seven-pass transmembrane domain-containing serpentine protein and the Low-density-lipoprotein-related protein (LRP) receptor. Transduction of the Wnt signal can be mediated through the canonical Wnt pathway dependent upon activation of β-catenin. The Wnt–Frizzled–LRP complex leads to the recruitment and phosphorylation of the cytoplasmic protein Dishevelled, a key transducer of the Wnt signal. Subsequently, Glycogen Synthase Kinase 3 Beta (GSK3β) and Axin, two proteins that form part of a complex with the tumour suppressor protein Adenomatous Polyposis Coli (APC) that normally directs the phosphorylation and ubiquitination of β-catenin, are inhibited and degraded. In the absence of the APC protein complex, dephosphorylated β-catenin is free to translocate to the nucleus where it can mediate transcription of Wnt target genes through

interaction with T-cell factor (TCF) and Lymphocyte Enhancer Factor (LEF) transcription factors (Burke and Tosh, 2006, Miller, et al., 1999). EGF Receptor (EGFR) signalling has been demonstrated to cross-talk with the canonical Wnt/ β-catenin pathway resulting in the accumulation of β-catenin in the nucleus (Hu and Li, 2010). Stimulation of the EGFR receptor by EGF family ligands on the plasma membrane activates the Phosphoinositide- 3 Kinase (PI-3K)/ Protein Kinase B (Akt) pathway, causing phosphorylation and deactivation of GSK3β thus inhibiting the β-catenin destruction complex. For characterisation of the BMOL-TAT1.1 cell line, cells were maintained in a specialised 'growth medium' (2.3), supplemented with EGF in order to allow oval cell proliferation (Tirntiz-Parker, et al., 2007).

β-catenin signalling has been proposed as a key pathway in biliary lineage determination, for example it has been shown that biliary differentiation is promoted by the Wnt/ β-catenin pathway in experiments utilising *in vitro* explants (Hussain, et al., 2004, Micsenyi, et al., 2004). Also studies have shown inactivation of APC in hepatoblasts which results in ectopic β-catenin activation leads to commitment to an incomplete biliary fate and thus loss of hepatocytes, potentially through preventing hepatocyte differentiation as indicated by the strong and early repression of HNF4α and E-cadherin (Decaens, et al., 2008). E-cadherin is an important transmembrane epithelial cell adhesion molecule whose cytoplasmic domain has the potent ability to recruit and sequester β-catenin to the plasma membrane to form mutually exclusive complexes (Aberle, et al., 1994, Kemler, 1993, Orsulic, et al., 1999), with the knockdown of E-cadherin resulting in β-catenin activation and accumulation in the nucleus (Wang, et al., 2010). Reduced membranous expression of E-cadherin and β-catenin (which has a dominant role in driving liver progenitor cells towards the cholangiocyte lineage (Hazan and Norton, 1998)) has been correlated with tumourigenesis and progression of many epithelial malignancies (Gu and Choi, 2012, Lim and Lee, 2002). More specifically Wnt/ β-catenin signalling has been shown to contribute to the activation of normal and tumorigenic liver progenitor cells as *Yang and colleagues* identified a subpopulation of less differentiated progenitor-like cells in hepatocellular carcinoma (HCC) cell lines and primary HCC tissues, which were defined by expression of the hepatic progenitor marker OV6 and endowed with endogenously active β-catenin. These OV6-positive HCC cells possess a greater ability to form a tumour *in vivo* and show a substantial resistance to standard chemotherapy compared with OV6 negative cells (Yang, et al., 2008).

In support of the theory that ectopic β-catenin activation leads to commitment to an incomplete biliary fate, it is hypothesised that culturing in 'growth medium' (i.e. EGF) will have stimulated EGFR signalling and thus β-catenin activation in the BMOL-TAT1.1 cell line. Therefore in the small BMOL-TAT1.1 cells associated with β-catenin accumulation in the nucleus (Figure 3.6A,D) is the expression of biliary associated markers Sox9 and Vimentin (Figure 3.3B; Figure 3.5D). Vimentin, an intermediate filament protein primarily of mesenchymal cells is observed in proliferating biliary epithelial cells but not in the mature bile duct (Haruna, et al., 1996, Milani, et al., 1989). Studies have demonstrated that subpopulations of oval cells express Vimentin and that the commitment of oval cells to the cholangiocyte lineage may involve the transient expression of Vimentin (Haruna, et al., 1996, Yovchev, et al., 2008). Also the Vimentin promoter has been identified as a target of the β-catenin/ TCF pathway (Gilles, et al., 2003).

Sox9, a member of the sry-related high mobility group transcription factors is known to have a pivotal role in embryonic intra-hepatic bile duct formation (Antoniou, et al., 2009). Cell tracking experiments have also suggested that in the adult Sox9 marks the oval cell population which contributes to the physiological regenerative process following liver injury, as Sox9 is expressed in the intra-hepatic bile duct and Canal of Hering, which is the area in the liver where oval cells are considered to originate from (Furuyama, et al., 2011). Recent results from our laboratories show in the normal mouse adult liver Sox9 expression is restricted to cholangiocytes. Utilising a mutant mouse strain, with *loxP*-flanked ('floxed') *Apc*, and Cre-mediated excision/deletion of the floxed *Apc*; ectopic β-catenin activation results in an increase in Sox9 expression throughout the entire mouse adult liver as revealed by immuno-histochemistry and RT-PCR analysis (Personal communication Sheng Wen Yeh and David Tosh).

*In toto* the supporting literature is consistent with BMOL-TAT1.1 cell line characterisation data. The larger cells with inactivated membrane-bound β-catenin are positive for A6, HNF4α and E-cadherin represent a more hepatocyte phenotype, whereas the smaller cells with activated nuclear β-catenin are positive for OV6, Sox9 and Vimentin, but negative for HNF4α and E-cadherin represent a more cholangiocyte cell phenotype. Figure 3.10 shows a schematic summary of the postulated role of β-catenin in the BMOL-TAT1.1 cell line.

Proliferating BMOL-TAT1.1 cells express the phenotypic repertoire of both hepatocytes and cholangiocytes, hence can be considered to be an *in vitro* model of bi-

potent oval cells. The advantage of a heterogeneous culture is not only a closer analogy to an *in vivo* system, but it also serves as a starting point to dissect the mechanisms and specific cell types involved in oval cell differentiation to other cell types. Consequently the next stage of this chapter addressed optimising the protocol for the inducible differentiation of BMOL-TAT1.1 cells.

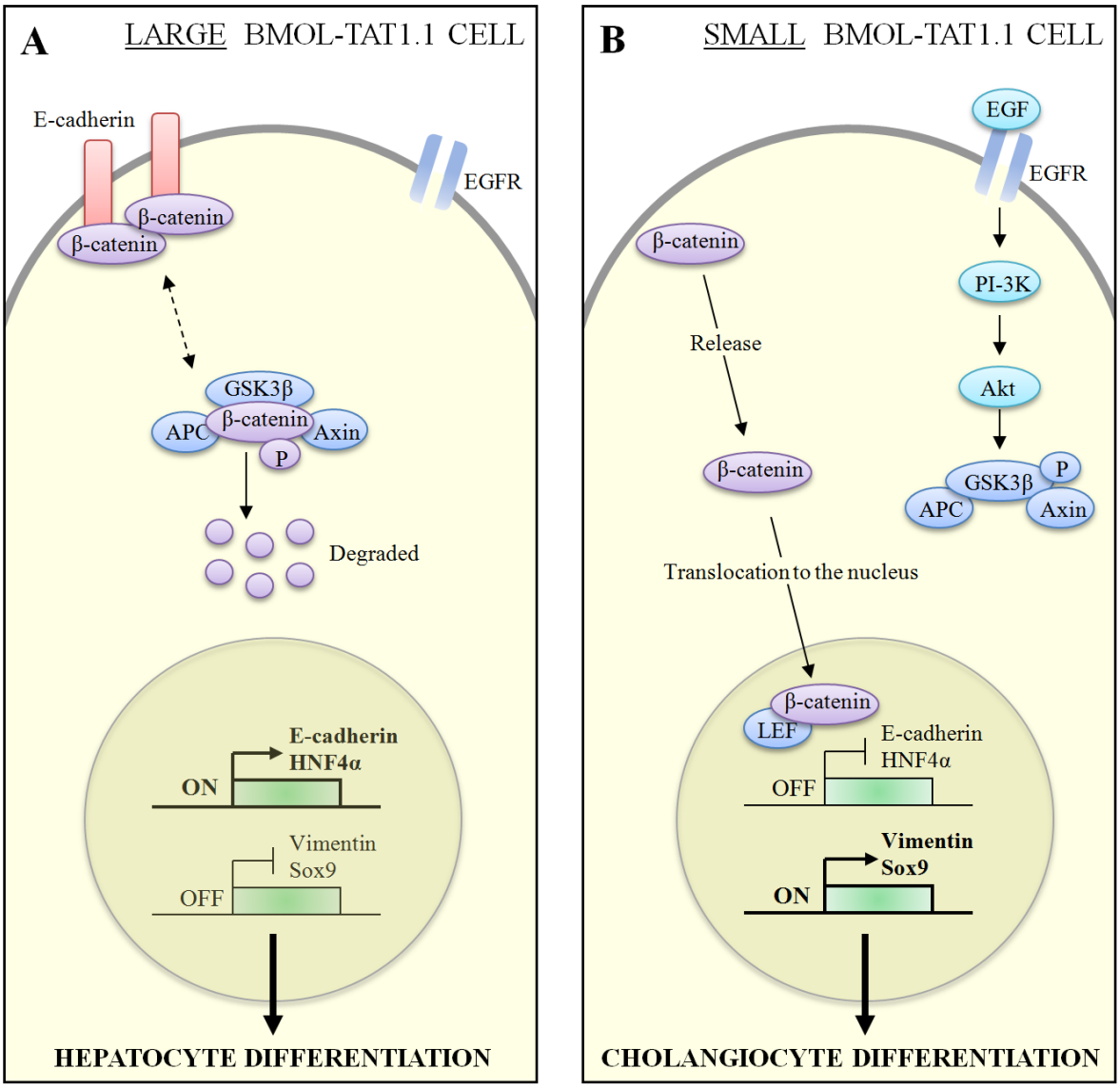



**Figure 3.10: Simplified view of the potential role of EGF activation of the Wnt/ β-catenin pathway in BMOL-TAT1.1 during oval cell proliferation. (A)** Large BMOL-TAT1.1 cells before EGF stimulation of the Wnt/β-catenin pathway. β-catenin remains bound at the plasma membrane to E-cadherin and the cytoplasmic β-catenin is kept low through the destruction complex (GSK-3β, Axin, APC) which phosphorylates β-catenin triggering ubiquitination and degradation by the proteosome. **(B)** Small BMOL-TAT1.1 cells following EGF stimulation of the Wnt/β-catenin pathway. EGFR activation prevents β-catenin phosphorylation via activation of the PI-3K/Akt pathway inhibiting GSK-3β, thus β-catenin is translocated to the nucleus where it binds to LEF transcription factor, activating cholangiocyte genes such as Vimentin and Sox 9 and repressing hepatocyte genes such as E-cadherin and HNF4α. *Abbreviations*: Akt, protein kinase B ;APC, adenomatous polyposis coli ; EGFR. EGFR receptor; GSK3β, glycogen synthase kinase 3 beta ; LEF, lymphocyte enhancer factor; P, phosphate; PI-3K, phosphoinositide- 3 kinase.

### 3.3.2 LETF expression during BMOL-TAT1.1 cell line proliferation

All BMOL-TAT1.1 cells were positive for the LETFs FoxA2 (Figure 3.5G) and C/EBPβ (Figure 3.4D,G), but C/EBPα was revealed to be barely detectable at the protein level during oval cell proliferation (Figure 3.4A). The expression of FoxA2 is expected as it is critical for the initiation of liver specification, with expression persisting into adult hepatocyte and cholangiocytes (Besnard, et al., 2004, Kaestner, et al., 1994). It is postulated that C/EBPα expression is induced during liver injury only once the regeneration via differentiation of oval cells is nearly complete (Dabeva, et al., 1995), relating to the paradigm that C/EBPα may have a role in terminal differentiation. Also in liver regeneration during the early proliferation stage decreased C/EBPα levels are exhibited, whilst C/EBPβ levels are significantly increased, suggesting an anti-proliferative role of C/EBPα (Flodby, et al., 1993, Ramji and Foka, 2002). Although C/EBPα is a transcription factor, its ability to negatively regulate proliferation does not require its DNA-binding activity, but instead is mediated via protein-protein interactions. C/EBPα has been shown to interact with several proteins involved in control of cell-cycle progression. For example C/EBPα, mediates postnatal inhibition of hepatocyte proliferation via binding and stabilising p21 (Cdk inhibitor) or interfering with Cdk2 and Cdk4 function (Johnson, 2005, Wang, et al., 2001). Therefore in the present study it is postulated that induction of the transcription factor C/EBPα may inhibit oval cell proliferation and stimulate hepatocyte differentiation.

### 3.3.3 Optimal BMOL-TAT1.1 cell line inducible differentiation

The optimal differentiation protocol was determined as the condition producing the maximum number of X-gal positive cells indicative of TAT expression (an adult hepatocyte marker), validated by the maximum level of TAT mRNA expression (Figure 3.8). *Condition 5* involving a short culture period (3 days) in growth medium to allow proliferation, followed by a longer culture period (14 days) in differentiation medium was confirmed as optimal (Figure 3.8E,H) based on the conditions tested. Hepatocyte differentiation in *Condition 5* was also supported by PAS staining, which identified glycogen storage in the BMOL-TAT1.1 cells, a characteristic functional feature of mature hepatocytes (Figure 3.9A). Oval cell proliferation was observed when cells were cultured in growth medium containing EGF and insulin. Indeed previous studies have shown that

EGF and insulin act synergistically to increase DNA synthesis (i.e. S phase entry) (Sand and Christoffersen, 1987). Culturing in growth medium preferentially enhanced the proliferation of the small BMOL-TAT1.1 cells (Figure 3.7B). It is hypothesised that this observation may be due to increased EGFR signalling, as this is often associated with reduced membranous expression of E-cadherin (and β-catenin) and driving oval cells towards a cholangiocyte lineage (Hazan and Norton, 1998). Therefore for optimum differentiation an initial period of oval cell proliferation, whilst preventing over-confluency, is important to increase cell numbers whilst maintaining a higher proportion of large cells. This is necessary before culturing for a longer period of time in differentiation medium containing the synthetic glucocorticoid dexamethasone, which in addition to promoting hepatocyte maturation also suppresses growth (Michalopoulos, et al., 2003). Dexamethasone has been shown to inhibit proliferation of primary hepatocytes and cell lines (Loeb, et al., 1973) through blunting EGF-stimulated DNA synthesis. It has been hypothesised dexamethasone inhibits EGF-stimulated tyrosine phosphorylation of the ErbB receptors (e.g. EGFR, ErbB2) via alteration of not only the expression and interaction of the ErbB proteins, but also up-regulates a number of negative growth regulatory proteins that either directly bind to the ErbB proteins or indirectly inhibit ErbB by inhibiting downstream signalling such as the ERK and PI-3K pathways (Scheving, et al., 2007). The inhibitory effect of dexamethasone on the DNA synthesis of adult rat hepatocytes has also been shown to be enhanced in areas of high cell density, suggesting cell contact may contribute to the inhibition (Vintermyr and Doskeland, 1989) . All BMOL-TAT1.1 cells treated with dexamethasone (i.e. differentiation medium) included areas of high cell density (Figure 3.7).

# Chapter 4 Multi-potentiality of the oval cell line BMOL-TAT1.1

## 4.1 Introduction

### 4.1.1 Multi-potentiality of oval cell inducible differentiation

During liver regeneration the key role of oval cells is to proliferate and differentiate towards hepatocytes. The hepatocytic differentiation of the oval cell line BMOL-TAT1.1 can be traced by measuring the expression of the *lacZ* transgene, which is driven by a promoter element from tyrosine aminotransferase (TAT), a marker of adult hepatocytes, suggesting that a portion of the BMOL-TAT1.1 cells under appropriate culture conditions differentiate towards a mature hepatocyte phenotype. However expression of a single hepatocyte marker, such as TAT, does not provide conclusive evidence for full hepatocyte phenotype (Christoffels, et al., 1998, Nitsch, et al., 1993), therefore further phenotypic analysis is required.

Oval cells are capable of differentiating into hepatic lineages (i.e. hepatocytes and cholangiocytes) and also some non-hepatic lineages, such as intestinal and pancreatic cell types (Leite, et al., 2007, Tatematsu, et al., 1985, Yang, et al., 2002). These observations highlight the problem associated with oval cell differentiation during liver regeneration, in that these metaplasias (i.e. the irreversible conversion of one cellular phenotype of an already differentiated cell to another type of normal differentiated cell) can be pre-cursors to neoplasia and thus carcinogenic (Okada, 1986, Quinlan, et al., 2007, Roskams, 2006b, Slack and Tosh, 2001). This possibility is further supported by the correlation between oval cell appearance and the early stages of hepatocellular carcinoma (HCC) (Alison and Lovell, 2005, Hacker, et al., 1992) and cholangiocarcinoma (Roskams, 2006b). For instance, it has been shown that in livers of rats exposed to the carcinogen 2-acetylaminofluorene, intestinal metaplasia is a common option for oval cells, and this is related to cholangiofibrosis and subsequent cholangiocarcinoma (Barut and Sarraf, 2009, Tatematsu, et al., 1985).

### 4.1.2 Experimental Aim

The aim of the research described in this part of the thesis was to perform a more detailed phenotypic analysis utilising the optimised differentiation protocol of BMOL-TAT1.1 cells determined in Chapter 3 (*Condition 5*, 3G14D) in order to address: (i) BMOL-TAT1.1 multi-potential capability via expression analysis by RT-PCR and immunofluorescent staining (with available antibodies) for a range of hepatic (hepatocyte and cholangiocyte) and non-hepatic (intestinal and pancreatic) cell type markers (ii) the influence of the individual differentiation medium components (dexamethasone, nicotinamide, EGF and ITS) on oval cell gene expression and thus ultimately (iii) identify the potential signalling and transcriptional mechanisms governing differentiation.

## 4.2 Results

### 4.2.1 Inducible hepatic differentiation of BMOL-TAT1.1 cells

Gene expression analysis was performed on BMOL-TAT1.1 cells subjected to the following culture conditions: (*Condition 1*) 6 days in growth medium, 6G; (*Condition 2*) 17 days in growth medium, 17G; (*Condition 5*) 3 days in growth medium followed by 14 days in differentiation medium, 3G14D. Cells grown for 17 days were allowed to grow to super-confluence in order to inhibit growth and promote differentiation. Comparative analysis of *Condition 2* (17G) and *Condition 5* (3G14D) revealed the small cells to become the dominanting cell type when in growth medium (i.e. EGF, IGF-II, insulin) thus significantly increasing the ratio of small cells to large cells (Figure 4.1A). In contrast when cultured in differentiation medium (EGF, dexamethasone, nicotinamide, ITS) BMOL-TAT1.1 cells maintained a larger proportion of large cells in the culture (Figure 4.1B).

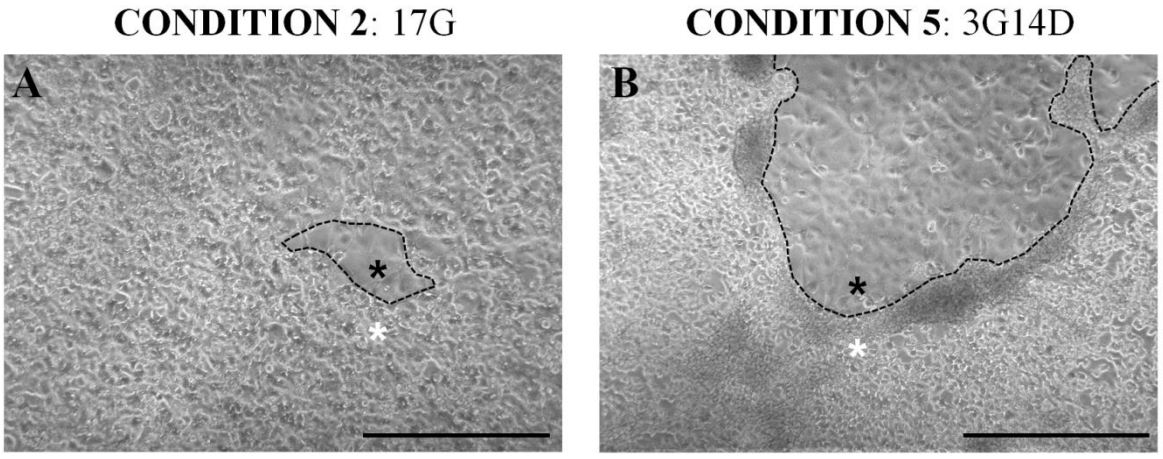



**Figure 4.1: Comparison of the proportion of small to large BMOL-TAT1.1 cells when cultured in growth or differentiation medium.** Transmitted light images showing representative areas from *Condition 2* (17 days in growth medium, **17G**) and *Condition 5* (3 days in growth medium followed by 14 days in differentiation medium, **3G14D**). Areas of large cells **(*black asterisk*)** and small cells **(*white asterisk*)** are shown **(*dashed line*)**. In contrast to differentiation medium, the growth medium resulted in the enrichment of small cells compared to large cells (scale bar = 200μm).

Given the heterogeneous nature of the cultures, RNA isolated for subsequent RT-PCR was representative of the whole culture hence comparison of *Condition 2* to *Condition 5* may allow deductions to be made about the phenotype of the small and large cells respectively. Large cells appear positive for the markers HNF4α, E-cadherin and

Cytokeratin 20 (CK20) as deduced from their down-regulation in *Condition 2* (i.e. when number of large cells is reduced) and maintenance in *Condition 5* (Figure 4.2). Small cells were revealed to be Vimentin and Sox9 positive as their expression was strongly maintained in *Condition 2* (Figure 4.2). Vimentin is often referred to as a mesenchymal cell marker, but has also been proposed as a ductal marker (Haruna, et al., 1996). Therefore to validate that the BMOL-TAT1.1 cells were not undergoing an epithelial to mesenchymal transition the gene expression of the mesenchymal cell marker α-smooth muscle actin (SMA) was determined. All cells were negative for SMA (Figure 4.2).

When cultured in differentiation medium (*Condition 5*), BMOL-TAT1.1 cells exhibited changes in gene expression (Figure 4.2). As the differentiation medium was supplemented with various factors including: EGF, dexamethasone, nicotinamide and ITS, it was necessary to culture cells in the absence of supplements and also with the individual components. This approach allowed us to determine if the gene expression changes were due to the effect of a single factor or a combination of factors. RT-PCR data was therefore generated as shown in Figure 4.3, to facilitate the elucidation of the mechanisms governing the changes in gene expression observed. Culturing BMOL-TAT1.1 to a super-confluent state whether in growth of differentiation medium resulted in the suppression of the hepatoblast marker alpha feto-protein (AFP) (Figure 4.3). However up-regulation of the mature hepatocyte markers (Albumin and TAT) and maintenance and induction of hepatocyte associated transcription factors HNF4α and C/EBPα respectively, were dependent solely on dexamethasone and enhanced by the addition of the other supplements (Figure 4.2, Figure 4.3). Cholangiocyte markers Connexin 43 (Cx43) and Osteopontin (OPN) were down-regulated under all conditions examined, with Connexin 43 most potently down-regulated in the absence of supplements or with EGF or nicotinamide alone (Figure 4.2, Figure 4.3). In addition, cholangiocyte associated transcription factors including Sox9, HNF6 and HNF1β were down-regulated in differentiation medium (Figure 4.2), with dexamethasone revealed to be crucial for Sox9 suppression (Figure 4.3). Other striking observations were that dexamethasone completely suppressed expression of the transcription factor FoxA2, but up-regulated the expression of epithelial cytoskeletal markers Cytokeratin 20 (CK20) and Cytokeratin 7(CK7) (Figure 4.3).

The RT-PCR data only provided an indication of changes in mRNA expression, therefore immunofluorescent staining was performed to look at the protein expression of several markers. From immunofluorescent staining analysis it can be seen that culturing of

BMOL-TAT1.1 cells in differentiation medium (*Condition 5*): suppressed Sox9 and FoxA2 expression in all cells (Figure 4.4). The large cells remained E-cadherin positive and up-regulated CK20 robustly and to a lesser extent CK7; while the small cells remained Vimentin positive, CK20 negative and up-regulated strongly CK7 (Figure 4.5).

Wnt/β-catenin signalling is believed to play a central role in various aspects of hepatic biology, including liver development, regeneration, growth and oncogenesis. Chapter 3 described the striking difference in β-catenin expression and localisation within small and large BMOL-TAT1.1 cells during oval cell proliferation (*Condition 1*, 6G) (Figure 3.6). When BMOL-TAT1.1 cells were cultured in differentiation medium (*Condition 5*, 3G14D), β-catenin remained localised only to the plasma membrane in the large cells (Figure 4.6A,G), however in the small cells, the nuclear expression of β-catenin was reduced with the protein predominantly found in the cytoplasm with some weakly associated with the plasma membrane (Figure 4.6D,G).

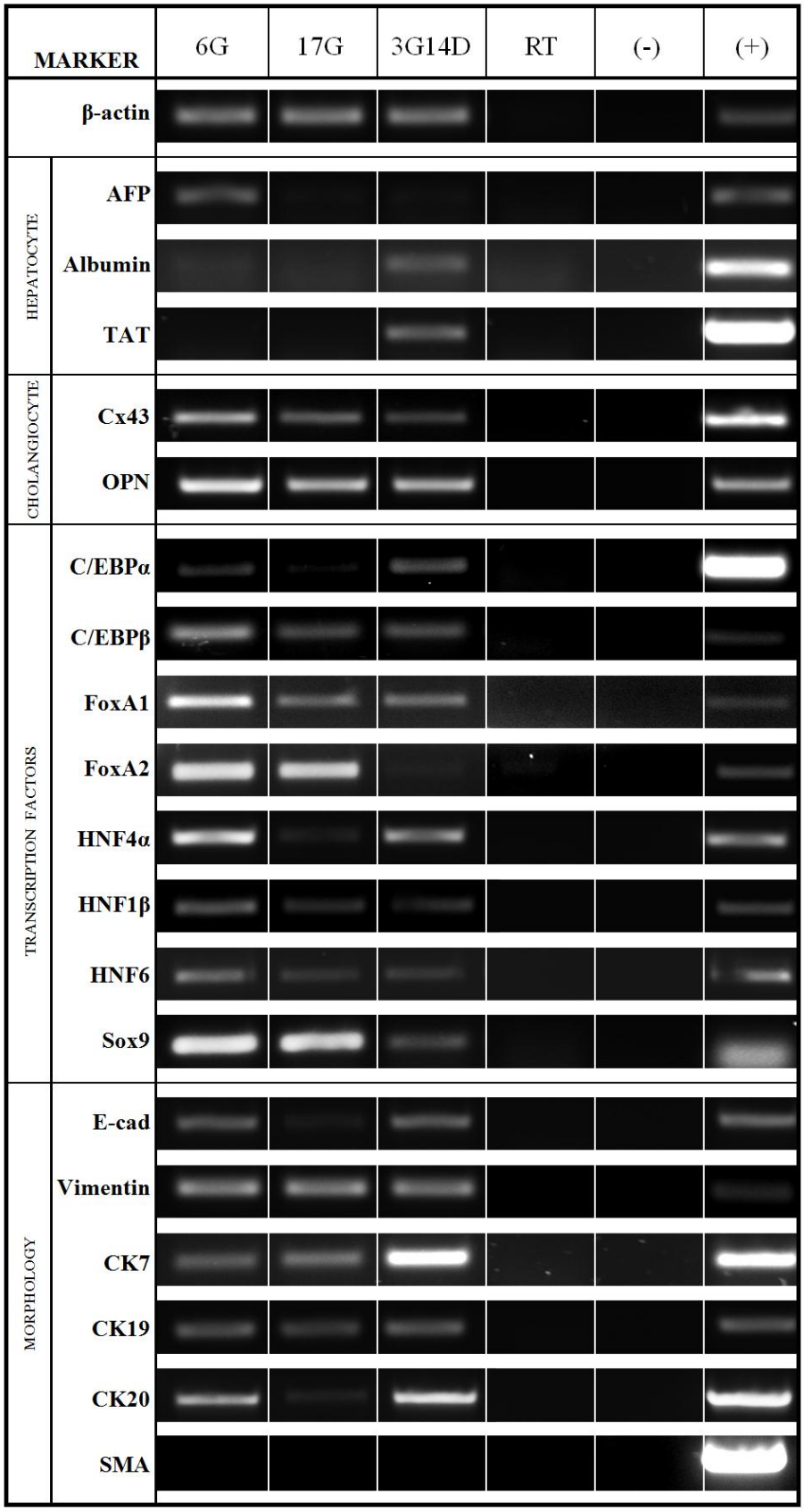



**Figure 4.2: Gene expression analysis of BMOL-TAT1.1 cells under different culture conditions.** BMOL-TAT1.1 cells were cultured in either growth medium for 6 days **(6G)** or 17 days **(17G)** and 3 days in growth medium followed by 14 days in differentiation medium **(3G14D)**. Gene expression was analysed for hepatocyte markers, cholangiocyte markers, transcription factors and morphological markers. *Abbreviations*: RT, no RT control; (-) negative control; (+), positive control.

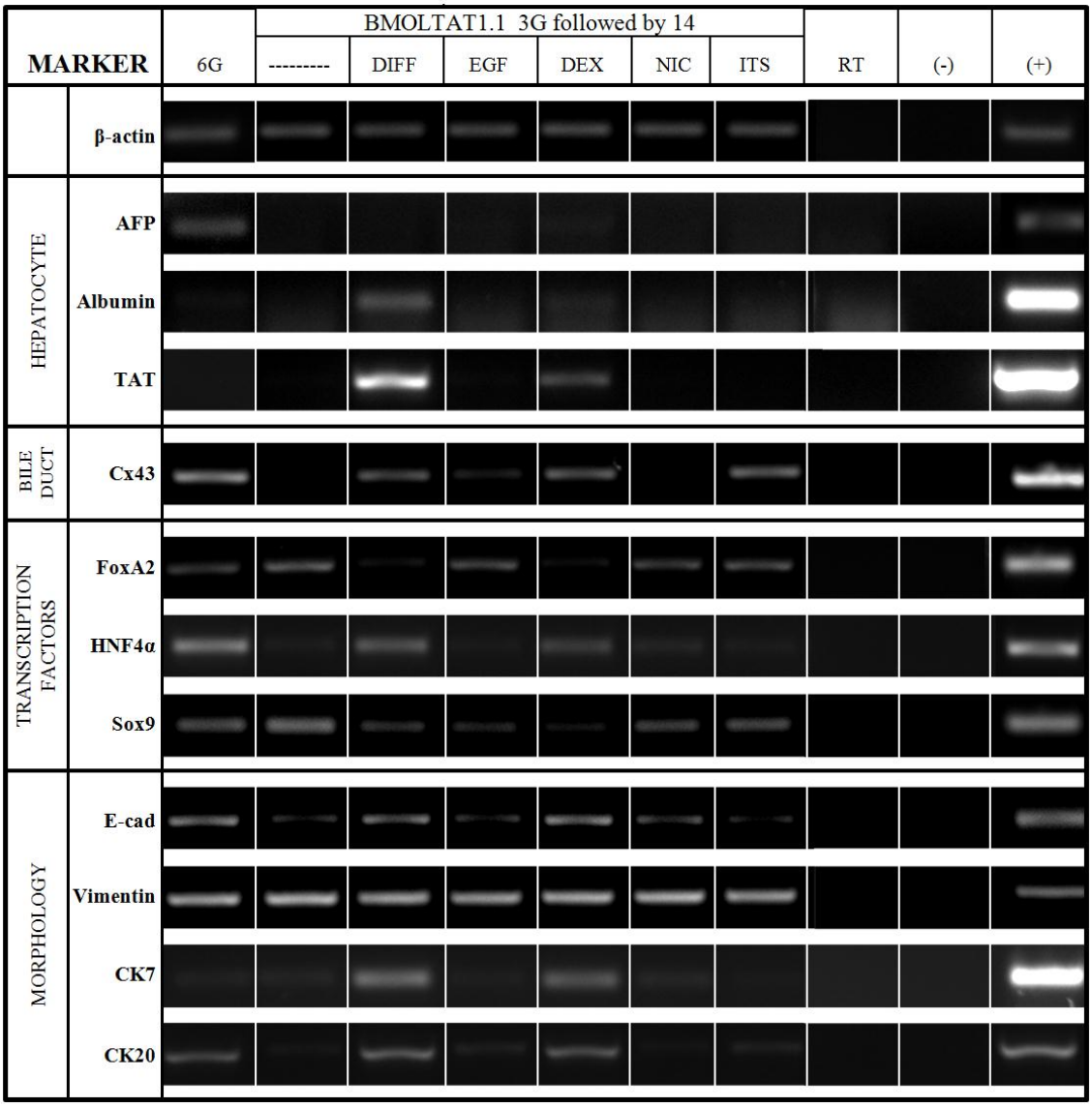



**Figure 4.3: Dissection of effect of differentiation medium on BMOL-TAT1.1 gene expression.** BMOL-TAT1.1 cells were cultured for 3 days in growth medium **(3G)** followed by 14 days with no supplementation **(----)**, full differentiation medium **(DIFF)**, EGF **(EGF)**, dexamethasone **(DEX)**, nicotinamide **(NIC)** or ITS **(ITS)**. BMOL-TAT1.1 cells cultured for 6 days in growth medium (6G) is included to represent proliferating oval cells prior to treatment. Gene expression was analysed for a range of markers for hepatocyte, cholangiocyte (bile duct), transcription factors and morphology. *Abbreviations*: RT, no RT control; (-) negative control; (+), positive control.

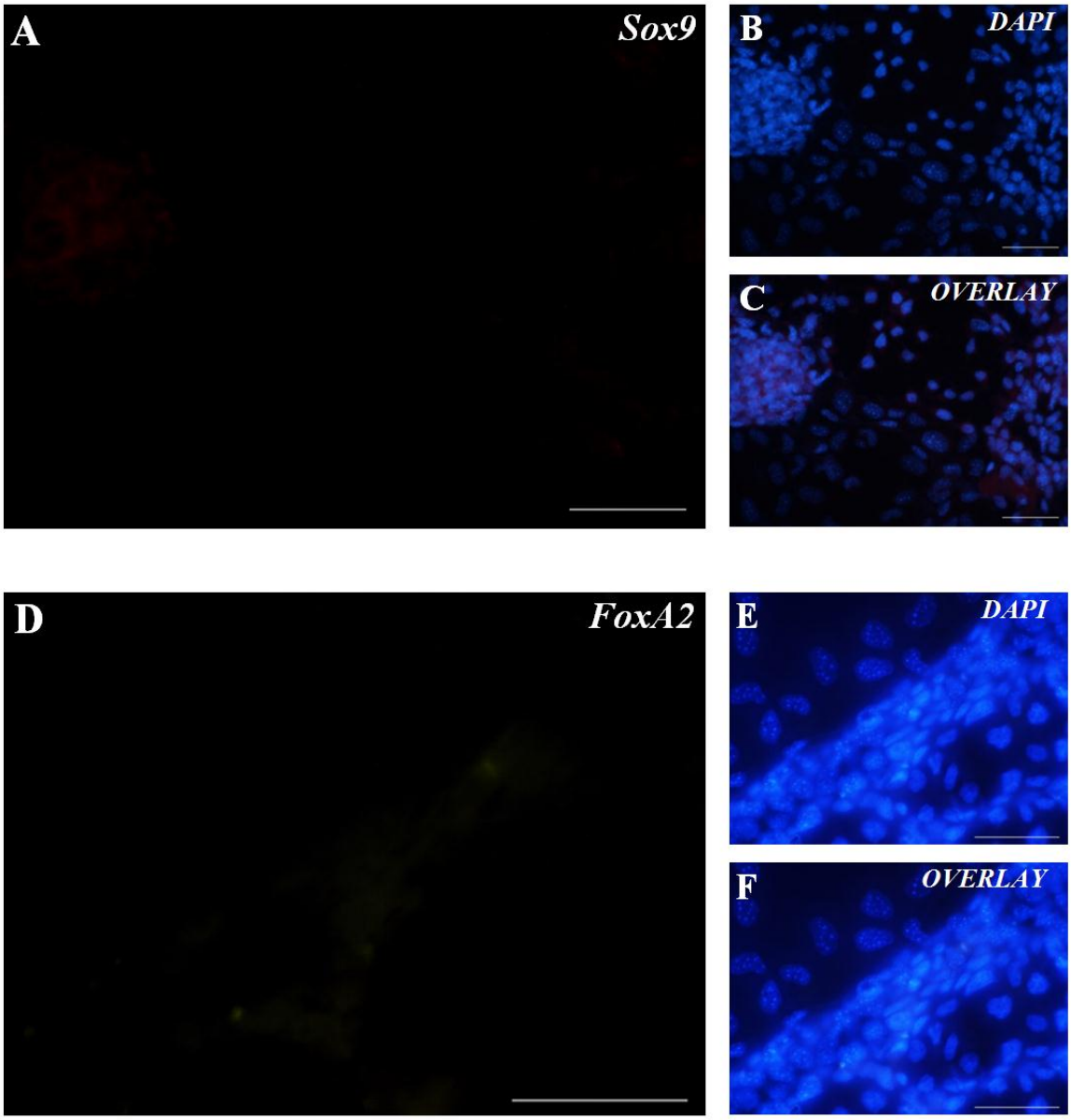



**Figure 4.4: BMOL-TAT1.1 cell line expression of transcription factors when cultured in differentiation medium.** Immunofluorescent staining to identify Sox9 **(A-C, *red*)** and FoxA2 **(D-F, *green*)** expression in BMOL-TAT1.1 cells cultured for 3 days in growth medium followed by 14 days in differentiation medium (*Condition 5*). DAPI staining **(B, E, *blue*)** is included to distinguish the two different cell types based on their nuclear morphology. All cells are now negative for Sox9 **(A)** and FoxA2 **(D).** Images **C** and **F** are overlays of **A, B** and **D, E** respectively. For all antibodies utilised, positive controls were performed in parallel and are included in *Appendix C1*. (scale bar = 50μm).

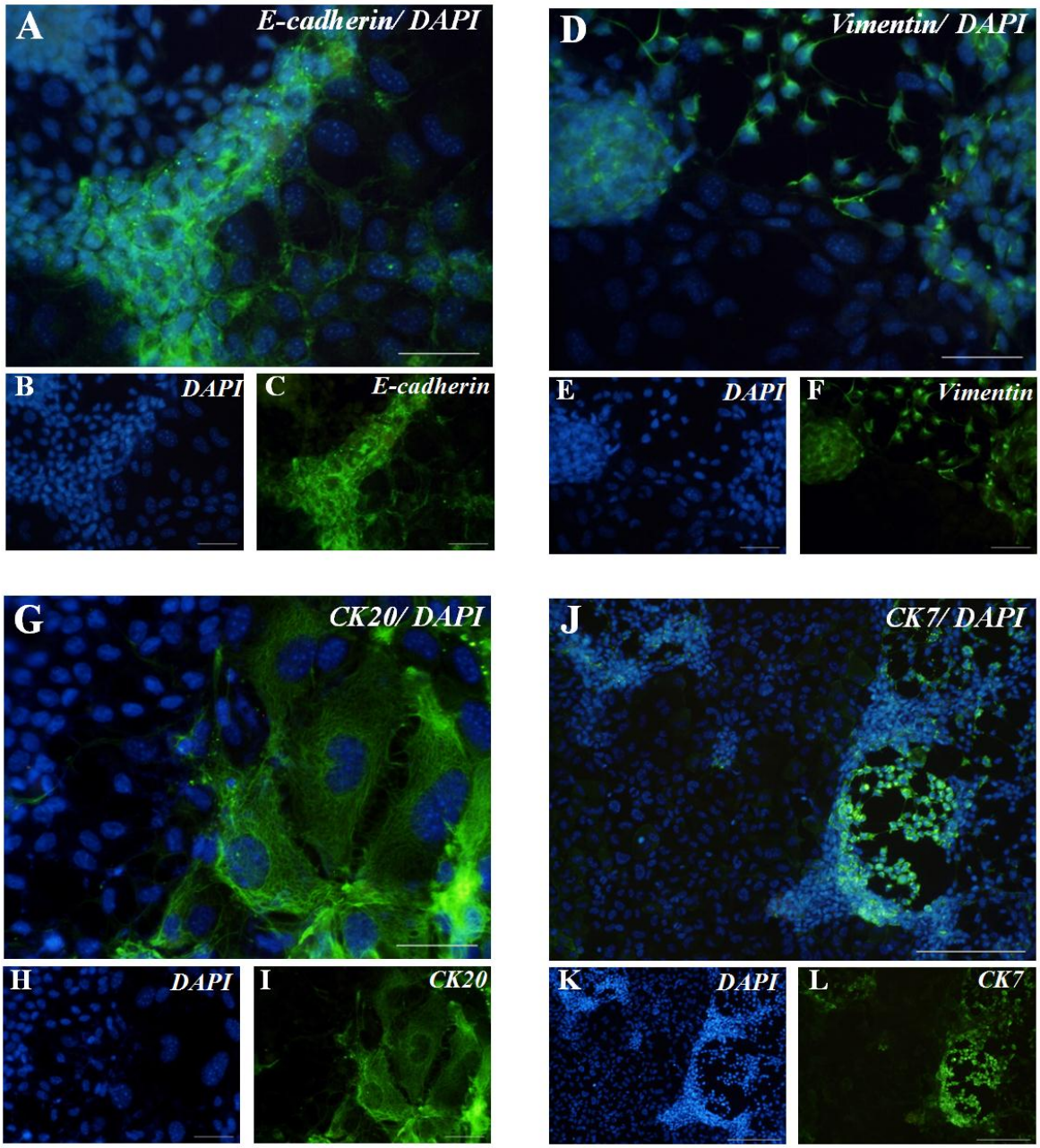



**Figure 4.5: BMOL-TAT1.1 cell line expression of morphological markers when cultured in differentiation medium.** Immunofluorescent staining to identify E-cadherin **(A-C, *green*)**, Vimentin **(D-F, *green*)**, CK20 **(G-I, *green*)** and CK 7 **(J-L, *green*)** expression in BMOL-TAT1.1 cells cultured in *Condition 5* for 3 days in growth medium followed by 14 days in differentiation medium. DAPI staining **(B, E, H, K, *blue*)** is included to distinguish the two different cell types based on their nuclear morphology. Large cells are E-cadherin and CK20 positive, Vimentin and CK7 negative, whilst the small cells are E-cadherin and CK20 negative, Vimentin and CK7 positive. Images **A**, **D, G,** and **J** are overlays of **B, C; E, F; H, I** and **K, L** respectively. *Abbreviations*: CK7, Cytokeratin 7; CK20, Cytokeratin 20. (A-I scale bar = 50µm) (J-L scale bar = 200µm).

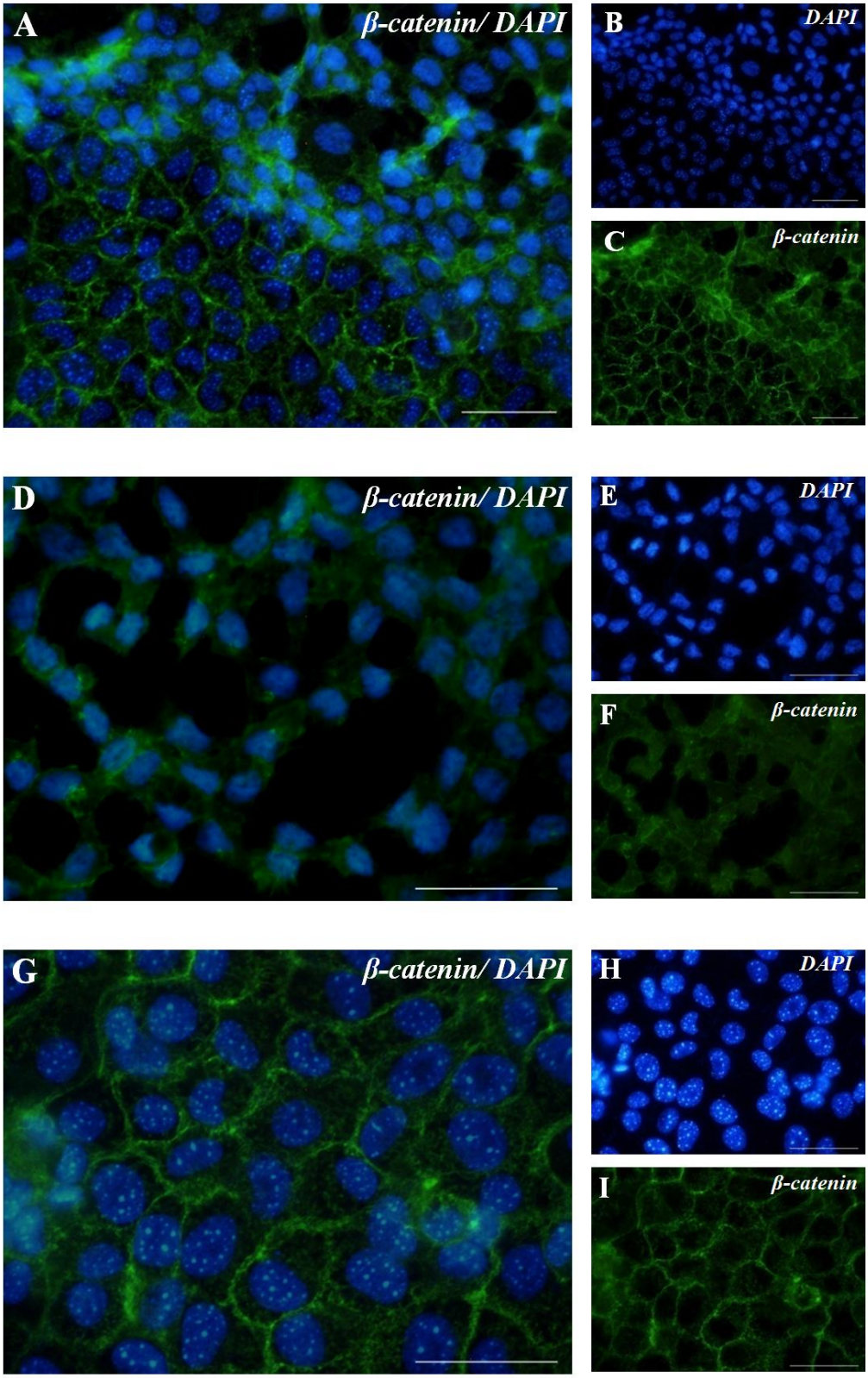



**Figure 4.6: BMOL-TAT1.1 cell line expression of β-catenin when cultured in differentiation medium.** Immunofluorescent staining to identify β-catenin **(*green*)** expression in BMOL-TAT1.1 cells cultured for 3 days in growth medium followed by 14 days in differentiation medium (*Condition 5*). DAPI staining **(B, E, H, *blue*)** is included to distinguish the two different cell types based on their nuclear morphology. Both small and large cells express β-catenin **(A).** In the small cells β-catenin is located mainly in the cytoplasm with some minor membrane localisation **(D)**. However in the large cells β–catenin is solely membrane bound completely absent from the nucleus/ cytoplasm **(G)**. Images **A**, **D** and **G** are overlays of **B, C; E, F;** and **H, I** respectively. (scale bar = 50μm).

### 4.2.2 Inducible non-hepatic differentiation of BMOL-TAT1.1 cells

Studies have shown that oval cells are capable of not only differentiating into hepatic lineages (i.e. hepatocytes and cholangiocytes) but also some non-hepatic lineages, such as intestinal and pancreatic cell types (Leite, et al., 2007, Tatematsu, et al., 1985, Yang, et al., 2002). Due to the nature of the oval cells it was therefore important to also analyse the expression of intestinal and pancreatic genes in BMOL-TAT1.1 cells subjected to the following experimental culture conditions: (*Condition 1*) 6 days in growth medium, 6G; (*Condition 2*) 17 days in growth medium, 17G; (*Condition 5*) 3 days in growth medium followed by 14 days in differentiation medium, 3G14D. Regarding pancreatic differentiation none of the culture conditions promoted expression of any key pancreatic cell markers including insulin (β-cell), glucagon (α-cell) and amylase (acinar cells) (Figure 4.7A). This suggests that under these conditions, oval cells are not amenable to differentiation to a pancreatic phenotype. To investigate the potential for intestinal differentiation of BMOL-TAT1.1 cells an array of markers were analysed for the different intestinal cell types. BMOL-TAT1.1 cells showed a weak expression for some intestinal absorptive cell markers, including Villin and Fabp2 during proliferation (6G) with the additional very weak induction of Apolipoprotein AIV (ApoAIV) and intestinal Alkaline Phosphatase (ALPi) when cultured in differentiation medium (3G14D). There was no expression of intestinal Guanylate Cyclase (GCi) detected under any conditions. The most striking change in gene expression during BMOL-TAT1.1 differentiation (3G14D), was the induction of genes Mucin 2 (Muc2), Mucin 5ac (Muc5ac) and Trefoil Factor 3 (TFF3) which are often associated with intestinal goblet cells. Culturing of the BMOL-TAT1.1 cells with the different supplements (Figure 4.7B) revealed dexamethasone as the key factor involved in the induction of all three genes, with Muc2 and TTF3 also induced to a lesser extent by EGF and nicotinamide respectively.

Lectins are sugar-binding proteins that are highly specific for their sugar moieties. Studies have suggested that specific lectins can be used as markers to determine specific cell types and that cell surface expression of glycoconjugates changes as a result of differentiation. The differential binding of two lectins, *Dolichos biflorus* agglutinin (DBA) and Peanut agglutinin (PNA) were investigated during BMOL-TAT1.1 cell differentiation (3G14D). Results show restricted binding of PNA to the small cells hence indicating the presence of galactose/N-acetylgalactosamine (Gal-β(1-3)-GalNAc) and DBA to the large cells hence indicating the presence of N-acetylgalactosamine (GalNAc) (Figure 4.8).

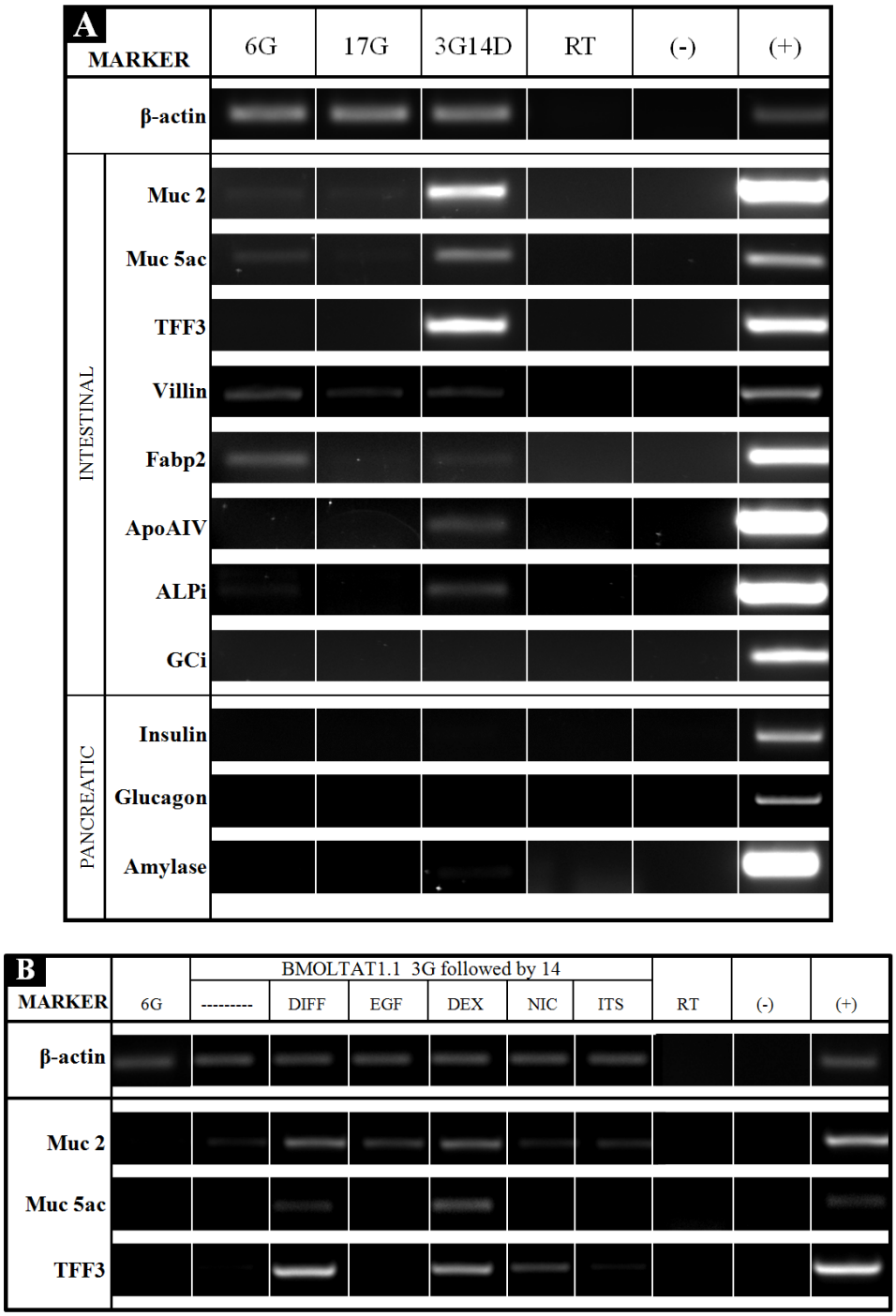



**Figure 4.7: RT-PCR analysis of non-hepatic gene expression in BMOL-TAT1.1 cells cultured in different conditions. (A)** BMOL-TAT1.1 cells were cultured in either growth medium for 6 days **(6G)** or 17 days **(17G)** as proliferating oval cell controls and 3 days in growth medium followed by 14 days in differentiation medium **(3G14D)**. **(B)** To dissect the effect of differentiation medium on BMOL-TAT1.1 cells, cells were cultured for 3 days in growth medium (3G) followed by 14 days with no supplementation (--), full differentiation medium (DIFF), EGF (EGF), dexamethasone (DEX), nicotinamide (NIC) or ITS (ITS). Gene expression was analysed for intestinal and pancreatic markers. *Abbreviations*: Muc2, mucin 2; Muc5ac, mucin 5ac, TTF3, trefoil factor 3; Fabp2, fatty acid binding protein; ApoAIV, apolipoprotein AIV, ALPi, alkaline phosphatise intestinal; GCi, guanylate cyclise intestinal; RT, no RT control; (-) negative control; (+), positive control.

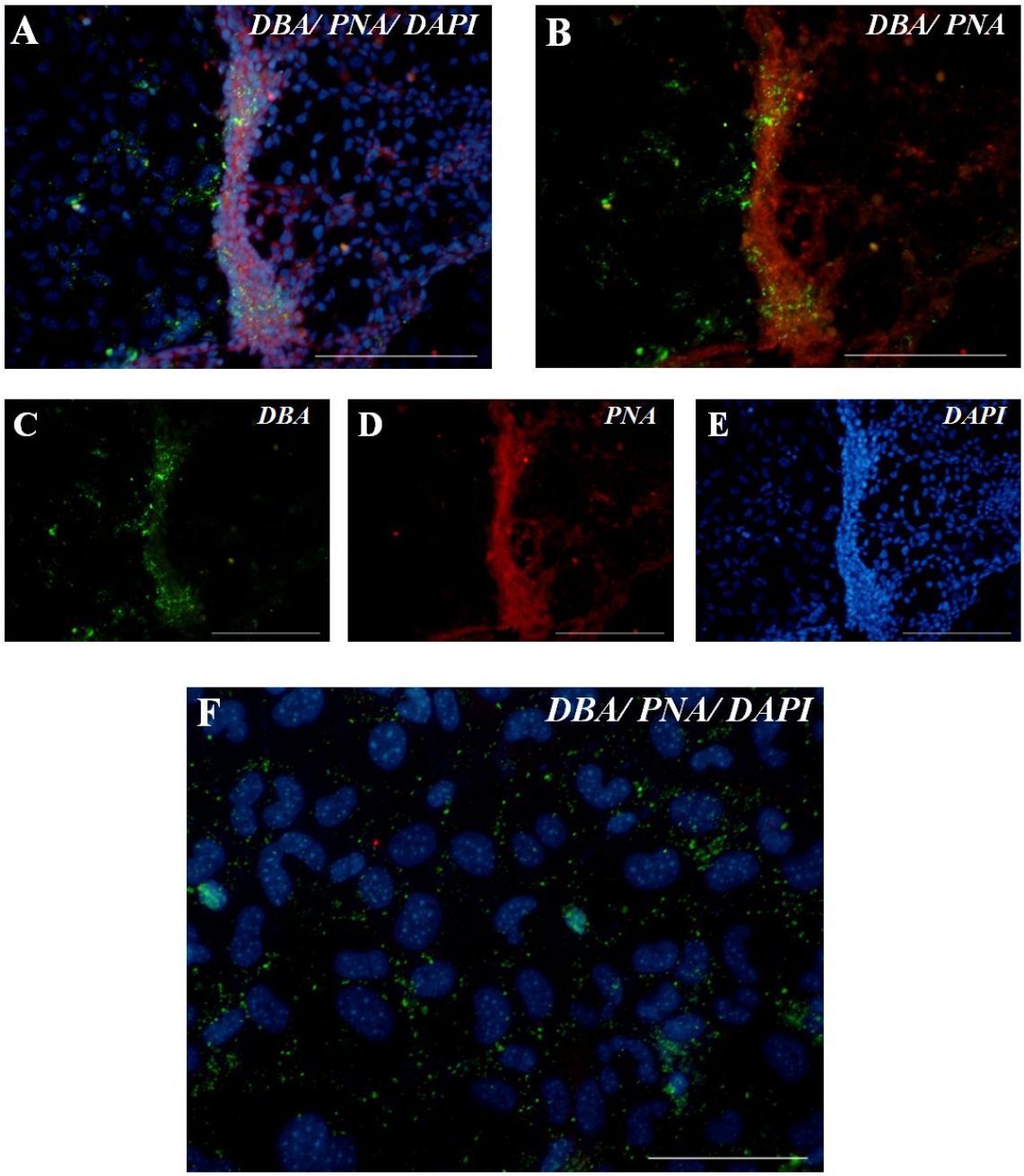



**Figure 4.8: Lectin staining of BMOL-TAT1.1 cells cultured in differentiation medium.** Immunofluorescent images identifying DBA **(C, *green*)**, PNA **(D, *red*)** lectin binding in BMOL-TAT1.1 cells cultured for 3 days in growth medium followed by 14 days in differentiation medium (*Condition 5*, 3G14D). DAPI staining **(E, *blue*)** is included to distinguish the two different cell types based on their nuclear morphology. Large cells are DBA positive PNA negative (shown in high magnification **(F)**), whilst the small cells are DBA negative PNA positive. Images **A** and **B** are overlays of **C-E** and **C, D** respectively. *Abbreviations*: DBA, *Dolichos Biflorus* Agglutinin; PNA, Peanut Agglutinin. (A-E scale bar = 200µm; F scale bar = 50µm ).

## 4.3 Discussion

### 4.3.1 Inducible hepatic differentiation of the BMOL-TAT1.1 cell line: glucocorticoid receptor and β-catenin signalling

The overall aim of the research described in this part of thesis was to perform a more detailed phenotypic analysis of inducible hepatic and non-hepatic differentiation of the BMOL-TAT1.1 cell line, in order to elucidate the potential signalling and transcriptional mechanisms governing differentiation. Analysis of BMOL-TAT1.1 inducible hepatic differentiation revealed the down-regulation of immature hepatoblast marker alpha-feto protein (AFP) and up-regulation of the mature hepatocyte markers TAT and Albumin. Up-regulation of hepatocyte markers was shown to be solely dependent on dexamethasone and enhanced by the addition of nicotinamide, ITS and EGF to the differentiation medium (Figure 4.2; Figure 4.3). The glucocorticoid dexamethasone functions via binding and activation of the glucocorticoid receptor (GR) (Munck, et al., 1984). The GR belongs to a super family of nuclear receptors, which are transcription factors that regulate diverse functions. Binding of dexamethasone to the non-DNA binding oligomer of the GR in the cytoplasm displaces chaperone Heat-Shock Protein 90 (HSP90) thus allowing GR to undergo a conformational change which leads to exposure of its nuclear localisation signal. Dexamethasone-bound GR thereby translocates to the nucleus and binds as a dimer to DNA (Figure 4.9) (Htun, et al., 1996). Nuclear receptors such as GR interact with the Wnt/β-catenin/TCF-LEF signalling axis mediating trans-repression of the signalling pathway (Olkku and Mahonen, 2009). For example the GR and its ligand, dexamethasone, can manipulate β-catenin localization, recruiting β-catenin to the plasma membrane; activating GSK3β by way of inhibition of $Akt^{Ser473}$ phosphorylation, thus promoting β-catenin degradation (Mulholland, et al., 2005). The model involving the transdifferentiation of the pancreatic exocrine cell line AR42J-B13 to functional hepatocytes utilising the established protocol of treatment with 1μM dexamethasone (Shen, et al., 2000) has been shown to result in the transient loss of constitutive Wnt3A expression, phosphorylation and depletion of β-catenin. The subsequent loss of β-catenin nuclear localisation and significant reduction in TCF/LEF transcriptional activity occurs before overt changes in phenotype to hepatocyte-like cells (Wallace, et al., 2010). This has also been confirmed by the ability of over-expression of β-catenin to block dexamethasone-dependent transdifferentiation (Wallace, et al., 2010). Analysis of β-catenin expression and localisation in the BMOL-TAT1.1 cell line treated with

differentiation medium containing dexamethasone revealed β-catenin expression remained localised to the plasma membrane in the large BMOL-TAT1.1 cells, whilst the small BMOL-TAT1.1 cells exhibited a particular loss of β-catenin nuclear localisation (Figure 4.6) when compared to the proliferation control (*Condition 1*: 6G) (Figure 3.6). This is therefore consistent with the hypothesis that the BMOL-TAT1.1 cells are differentiating towards a hepatocyte-like cell.

### 4.3.2 Inducible hepatic differentiation of the BMOL-TAT1.1 cell line: transcription factors

X-gal staining suggests culturing in the differentiation medium induces a proportion of the BMOL-TAT1.1 cells to express TAT. Associated with the induction of mature hepatocyte genes is the up-regulation of the transcription factor C/EBPα. C/EBPα may have an important role in terminal hepatocyte differentiation (Dabeva, et al., 1995), and also in the suppression of transcription factors HNF6 and Sox9. It is these two transcription factors that are thought to have a pivotal role in cholangiocyte differentiation (Figure 4.2) (Antoniou, et al., 2009). Previous published studies from our laboratories support this finding. Firstly, during the hepatic transdifferentiation of the pancreatic exocrine cell line AR42J-B13 utilising the established 1μM dexamethasone treatment, it was revealed the LETFs C/EBPβ and C/EBPα were induced first, followed by HNF4α (Shen, et al., 2000). Furthermore ectopic expression of C/EBPα in AR42J-B13 cells is sufficient to induce transdifferentiation (Burke, et al., 2006).

While C/EBPα expression is critical for hepatocyte differentiation, suppression of C/EBPα expression is important during biliary cell differentiation (Shiojiri, et al., 2004). *Yamasaki et al.,* have postulated that the absence of C/EBPα in biliary cells has an indirect (de-repressive) effect by inducing the expression of the genes HNF6 and HNF1β, both of which have important regulatory roles in biliary cell differentiation (Nagaki and Moriwaki, 2008). Liver-specific inactivation of HNF6 or HNF1β demonstrates that both are required for normal differentiation of biliary epithelial cells, for proper morphogenesis of the extra- and intra-hepatic bile ducts, and that HNF1β is controlled by HNF6 at the onset of biliary epithelial cell differentiation (Clotman, et al., 2002, Coffinier, et al., 2002). *In vitro* transdifferentiation of the AR42J-B13 cell line to hepatocytes has been shown to involve the inhibition of cholangiocyte associated transcription factors Sox9 and HNF6 (Amani Al-

Adsani Thesis). This is consistent with observations that during BMOL-TAT1.1 hepatic differentiation up-regulation of C/EBPα is accompanied by the suppression of Sox9 (Figure 4.2; Figure 4.4A) and weak down-regulation of HNF1β and HNF6 (Figure 4.2). It has been postulated there is a mutual antagonism of C/EBPα and Sox9 which is the fundamental mechanism regulating differentiation of progenitor cells into hepatocytes or cholangiocytes (Antoniou, et al., 2009), as the Sox9 promoter contains C/EBPα binding sites, hence is likely to directly repress Sox9 transcription. In relation to the cross-talk between dexamethasone stimulated GR and Wnt/β-catenin signalling, β-catenin activation via the presence of Wnt3A has been shown in studies to repress activation of C/EBPα (Kawai, et al., 2007). Therefore based on supporting studies and results contained in this chapter it is hypothesised that during BMOL-TAT1.1 induced hepatic differentiation, dexamethasone reduces β-catenin activation and associated TCF/LEF transcriptional activity which suppresses Sox9 directly or indirectly through induction of C/EBPα expression (Figure 4.9).

Treatment of the BMOL-TAT1.1 cell line with dexamethasone also revealed the striking suppression of the expression of the transcription factor FoxA2 (Figure 4.3; Figure 4.4B). FoxA2 is required for normal liver homeostasis in the adult liver, as approximately 43% of genes expressed in the liver are associated with FoxA2 binding (Wederell, et al., 2008). *Cerec et al.,* investigated the differentiation of human hepatoma-derived cell line (Cerec, et al., 2007). HepaRG cells were isolated from a well differentiated liver tumour following chronic HCV infection. HepaRG cells constitutively display morphological and immunological features of both hepatocytes and cholangiocytes resembling a hepatic progenitor cell phenotype. Immunoblot analysis of HepaRG cells cultured in medium containing the synthetic glucocorticoid hydrocortisone hemisuccinate for 2 weeks undergoing hepatic differentiation showed FoxA2 (HNF3β) expressed in HepaRG cells at the progenitor stage as in oval cells, but expression was completely suppressed by day 3 (Cerec, et al., 2007). FoxA2, has previously been shown to be induced by active Wnt/β-catenin signalling (Yu, et al., 2011). In HepaRG hepatic differentiation, FoxA2 down-regulation following glucocorticoid treatment again was correlated with β-catenin inactivation and thus a change in β-catenin localisation from nucleus to cytoplasm and plasma membrane, a characteristic often observed during hepatocyte differentiation (Micsenyi, et al., 2004, Monga, et al., 2006). Figure 4.9 shows a schematic summary of the postulated role of dexamethasone and the GR-dependent β-catenin signalling in BMOL-TAT1.1 hepatocyte differentiation.

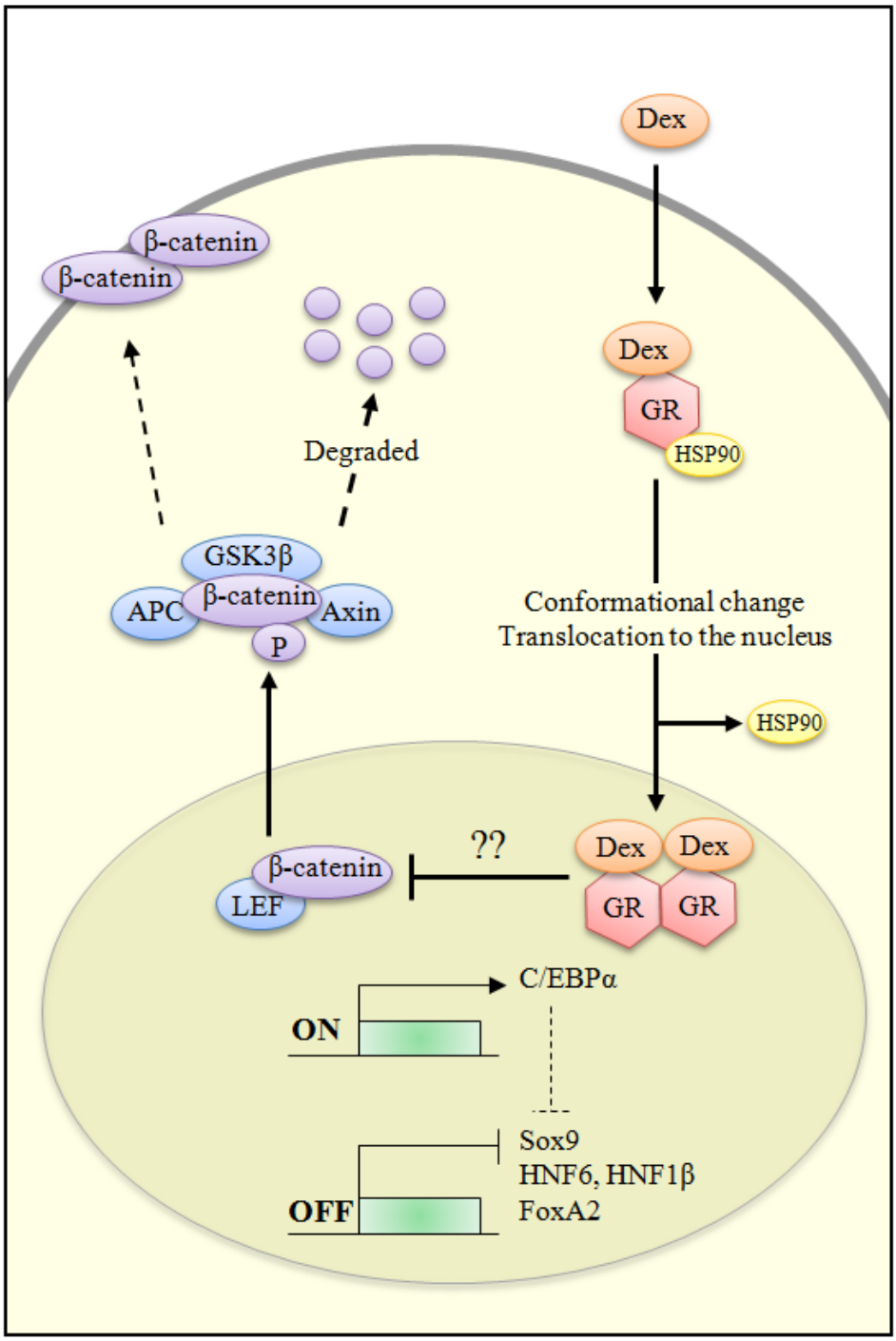



**Figure 4.9: Simplified view of the potential role dexamethasone on BMOL-TAT1.1 cells during oval cell differentiation.** The glucocorticoid dexamethasone (dex) binds to the glucocorticoid receptor (GR) in the cytosol, which displaces Heat-Shock Protein 90 (HSP90) triggering a conformational change and dimerisation of the G, and its translocation to the nucleus. Dex bound GR manipulates β-catenin signalling via GSK-3β mediated phosphorylation and degradation of β-catenin, loss of β-catenin nuclear localisation and significant reductions in LEF transcriptional activity. The exact mechanisms governing the interaction between the GR and β-catenin signalling pathways remain to be fully elucidated. However Dex treatment effects transcription factor expression. Up-regulation of hepatocyte specific C/EBPα is accompanied by the down-regulation of cholangiocyte specific transcription factors Sox9, HNF6 and HNF1β. This is consistent with other studies, for example it has been postulated there is a mutual antagonism between C/EBPα and Sox9. Suppression of the transcription factor FoxA2 is also consistent with in-activation of β-catenin signalling and hepatic differentiation. *Abbreviations*: APC, adenomatous polyposis coli ;Dex, dexamethasone; GR, glucocorticoid receptor; GSK3β, glycogen synthase kinase 3 beta; HSP90, heat-shock protein 90; LEF, lymphocyte enhancer factor; P, phosphate.

From a morphological perspective unlike hepatocyte-like cells derived from AR42J-B13 exocrine cells, the BMOL-TAT1.1 cells which expressed TAT remained negative for HNF4α and displayed no obvious change in cell morphology to resemble normal hepatocytes. This is consistent with studies showing in mice lacking HNF4α expression, hepatocytes were small, round, loosely associated, had small cytoplasmic volumes, misshapen nuclei, and disrupted cell contacts unlike normal hepatocytes which are large flat and tightly associated with canaliculi (Parviz, et al., 2003).

Recently it has also been shown that dexamethasone treatment (10 days) also has the capacity to induce in AR42J-B13 cells the expression of some markers typical of cholangiocytes including: Cytokeratin 7 (CK7) and lectin PNA. Interestingly the proportion of transdifferentiated cholangiocytes expressing these markers was enhanced by simultaneous treatment with dexamethasone and EGF (Al-Adsani, et al., 2010). This resembled treatment of the BMOL-TAT1.1 cell line with differentiation medium, containing dexamethasone and EGF, as CK7 (Figure 4.3; Figure 4.4D) and PNA (Figure 4.8) were also up-regulated in the small BMOL-TAT1.1 cells.

### 4.3.3 Inducible non-hepatic differentiation of BMOL-TAT1.1 cell line: intestinal metaplasia

Oval cells are capable of differentiation towards both hepatic lineages (e.g. hepatocytes and cholangiocytes) and non-hepatic lineages (e.g. intestinal and pancreatic cells). In order to explore the possibility in the current BMOL-TAT1.1 model, we examined the expression of a range of pancreatic and intestinal markers. From the RT-PCR data, it can be seen that under the differentiation conditions examined, the main pancreatic cell type markers were absent in all BMOL-TAT1.1 cells (Figure 4.7A). It is hypothesised this may be due to the absence of the specific extracellular factors and key pancreatic transcription factors required for transdifferentiation of hepatic oval cells into pancreatic islet cells. *Li et al.*, recently demonstrated that oval cells can be reprogrammed into β-like cells by an appropriate combination of high extracellular glucose, specific extracellular matrix proteins (laminin and fibronectin), cytokines (Activin A), and the expression of several β-cell specific transcription factors (Pdx1, Ngn3, MafA) (Li, et al., 2012).

As well as pancreatic markers we also examined the potential for the BMOL-TAT1.1 cells to undergo differentiation towards an intestinal phenotype. BMOL-TAT1.1 inducible differentiation involved the substantial induction of Muc2, Muc5ac and TFF3 expression primarily dependent on dexamethasone, and the weak induction of Villin, Fabp2, ApoAIV and ALPi (Figure 4.7). Mucins are high molecular weight, heavily O-glycosylated glycoproteins secreted by the epithelial cells in many organs. There are three types of mucins: (i) gel forming secreted types (ii) epithelial protective mucus gels and (iii) non-gel forming types. Muc2 and Muc5ac represent secreted gel forming mucins. Trefoil factor family (TFF) peptides are mucin-associated molecules co-expressed with mucins and are involved in the maintenance of the mucosal barrier and the biological behaviour of epithelial cells. TFF3, formerly known as intestinal trefoil factor ITF, is a mature peptide composed of 59 amino acids, secreted as a monomer or dimer. It promotes migration of epithelial cells *in vitro* and enhances mucosal healing *in vivo* where it co-localises with Muc2. TFF3 in combination with Muc2 are the major products synthesised in intestinal goblet cells (Poulsom, et al., 1996).

Within the normal adult liver intra-hepatic biliary system, biliary epithelial cells secrete mucins and TFF3 focally and faintly from large but not small cholangiocytes (Sasaki, et al., 2007, Sasaki, et al., 2004). Altered mucin gene expression has been reported in various non-tumourous inflammatory or cystic hepatobiliary diseases. For example increased expression of MUC5AC has recently been reported to be a highly specific tumour-associated mucin in cholangiocarcinoma (Mall, et al., 2010, Matull, et al., 2008). Most studies focus on hepatolithiasis, which involves the presence of calculi in the liver and is regarded as a model disease in cholangiocarcinogenesis arising in chronic inflammatory conditions, in which mucin is an important factor in the pathogenesis (Sasaki, et al., 2005, Sasaki, et al., 1998, Yamashita, et al., 1993). Gastric mucosal metaplasia and intestinal metaplasia are common in the intra-hepatic biliary system in hepatolithiasis; where surface epithelial cells express MUC5AC, MUC2, MUC6, and MUC5B. TFF1 and TFF3 are also augmented markedly in the biliary mucosa in hepatolithiasis co-expressed with MUC2 at the site of intestinal metaplasia (Sasaki, et al., 2004). It is believed the over-expression of TFF3 in the biliary tract increases the viscosity of the secreted mucin by interacting with the von Willebrand factor C (vWFC) cysteine-rich domain of MUC2. It has been suggested this enhances the structural integrity of the secreted mucins and that contributes to the formation of hepatoliths (Sasaki, et al., 2007, Tomasetto, et al., 2000). Both at the level of sequence homology and in the molecular

mechanisms responsible for regulating transcription and translation, the MUC2 and MUC5AC genes have much in common, thus may account for the appearance of MUC2 and MUC5AC simultaneously in mucin-secreting cells, during carcinogenesis (Van Seuningen, et al., 2001).

It has been shown that intestinal metaplasia is a common option for oval cells in livers of rats exposed to the carcinogen 2-acetylaminofluorene, and this treatment can lead to the development of cholangiocarcinoma (Barut and Sarraf, 2009, Tatematsu, et al., 1985). Oval cells reside in the Canal of Hering which is the most distal part of the biliary tree connecting the intra-hepatic biliary system with the hepatocytic canaliculi, which is consistent with the observed pathological distribution of mucin and TFF expression.

Based on the induction of mucins and TFF3 in BMOL-TAT1.1 cells it is hypothesised that the expression is in the large BMOL-TAT1.1 cells as these cells are uniquely positive for the lectin DBA (Figure 4.8). DBA binds specifically to N-acetylgalactosamine (GalNAc), one of the 5 sugars contributing to the oligosaccharide component of (goblet cell) mucin. Regarding intermediate filament expression the large BMOL-TAT1.1 cells following differentiation are strongly CK20 positive and weakly CK7 positive (Figure 4.5C,D). CK7, CK20, and MUC2 expression patterns have been reported to be useful in confirming the diagnosis of intestinal metaplasia (Schwerer and Baczako, 1996). Intestinal metaplasia is also associated with the occurrence of intestinal brush border enzymes such as ALPi, another marker up-regulated following BMOL-TAT1.1 differentiation. Additional support of the large BMOL-TAT1.1 cells displaying intestinal differentiation is two-fold. Firstly only the large BMOL-TAT1.1 cells are HNF4α positive, and HNF4α is suggested to be important in regulation of goblet cell maturation (Garrison, et al., 2006) and the expression of genes that are up-regulated during epithelial cell differentiation such as ApoAIV (Sauvaget, et al., 2002). Secondly the large BMOL-TAT1.1 cells are also uniquely positive for E-cadherin (Figure 4.5A) and studies have shown the cell surface adhesion molecule E-cadherin is essential for ApoAIV expression (Peignon, et al., 2006) and also promotes EGFR activation and mucin production (Iwashita, et al., 2011, Kim, et al., 2005).

# Chapter 5 Characterisation of a homogenous population of the BMOL-TAT1.1 cell line and over-expression of liver-enriched transcription factors

## 5.1 Introduction

### 5.1.1 Prolonged *in vitro* oval cell proliferation

As outlined in the previous chapters the BMOL-TAT1.1 cell line displays a morphologically heterogeneous phenotype consisting of two major phenotypes; small and large. It was observed that culturing of the BMOL-TAT1.1 cell line in growth medium (EGF, IGF-II and insulin) increased the ratio of small cells to large cells (Chapter 3). Therefore the initial aim of the research described in this chapter was to generate a more homogeneous population of small BMOL-TAT1.1 cells for study. This would permit more robust conclusions to be developed.

Strict control of oval cell proliferation is crucial for the oval cell response during liver regeneration. However it has been hypothesised that maintenance of a progenitor/ stem cell like population such as oval cells in a prolonged proliferative state can result in maturation arrest, in which the oval cells are blocked from terminally differentiating, hence display an immature phenotype, partially differentiated towards either hepatocytes or cholangiocytes and are unable to undergo apoptosis (Potter, 1978, Sell, 1993). The presence of arrested transitional hepatic progenitor cells has been observed in both HCC and cholangiocarcinoma (Libbrecht, et al., 2000, Shachaf, et al., 2004). Therefore it was also important to investigate the effect of prolonged proliferation on the BMOL-TAT1.1 cell line phenotype and differentiation capability.

### 5.1.2 Recombinant adenovirus

At present the most conventional differentiation protocols are aimed at inducing transcriptional changes by the application of external factors e.g. growth factors. However an alternative experimental approach has evolved over the past several years that manipulates cell fate and phenotype changes by single or combinatorial ectopic over-

expression of key transcription factors in target cell populations. Recombinant adenoviruses are a versatile tool enabling highly efficient gene delivery and expression in a broad spectrum of cell types, and have been used, both *in vitro* and *in vivo*, to achieve transient expression of specific transgenes (Howarth, et al., 2010). The most commonly used adenoviral vector is human adenovirus serotype 5, which is rendered replication deficient by the deletion of the E1 and E3 viral cassettes (AdEasy™ Adenoviral Vector System Instruction Manual). The E1 gene is essential for the assembly of infectious virus particles as it attenuates its ability to replicate, hence it is necessary to propagate the virus in a helper cell line that supplies the E1 gene *in trans*, such as the HEK-293 cell line (Graham, et al., 1977). The E3 gene encodes proteins involved in evading host immunity and hence is dispensable. Removal of the viral encoded genes allows the incorporation of approximately 7.5kb foreign DNA i.e. the specific gene of interest (Bett, et al., 1994, Verma and Weitzman, 2005). The recombinant adenovirus is produced by a double recombination event between the co-transformed adenoviral backbone plasmid vector, AdEasy™, and a shuttle vector carrying the gene of interest (Appendix D). The susceptibility of different cell types to adenoviral infection often varies and this reflected in the requirement to determine the specific conditions and multiplicity of infection (MOI) in order to achieve optimal infection, whilst preventing significant cytotoxity in infected cells, which E1-deficient adenoviruses can still produce.

### 5.1.3 Liver-enriched transcription factors in inducible hepatic differentiation of the BMOL-TAT1.1 cell line

Chapter 4 identified the liver-enriched transcription factors C/EBPα and HNF4α as potential transcription factors involved in oval cell hepatocyte differentiation (4.3.2). In proliferating small BMOL-TAT1.1 cells both C/EBPα and HNF4α are absent. Inducible hepatocyte differentiation is accompanied by the up-regulation of C/EBPα and hepatocyte specific genes (TAT, Albumin), however the cells remained negative for HNF4α and displayed no change in cell morphology to resemble normal hepatocytes. This is consistent with the known importance of C/EBPα during normal hepatocyte differentiation (Takiguchi, 1998) and importance of HNF4α in regulating morphogenesis and functional differentiation of hepatocytes (Parviz, et al., 2003). Addionally *Suetsugu et al.,* have shown that over-expression of HNF4α in fetal hepatic progenitor cells *in vitro* may enhance the therapeutic effects obtained from cell transplantation *in vivo* (Suetsugu, et al.,

2008). This work is therefore novel in one aspect as it will utilise adult ovals cells instead of fetal.

### 5.1.4 Experimental Aim

The homogenous small BMOL-TAT1.1 cell population was analysed in order to address the following questions: (1) does prolonged culturing in the growth medium effect the phenotype of the small BMOL-TAT1.1 cell type? and (2) is inducible differentiation consistent with previous low passage data? Another key aim in this chapter was to optimise adenoviral infection of BMOL-TAT1.1 cells in order to determine the effects of ectopic C/EBPα and HNF4α expression alone and in combination.

## 5.2 Results

### 5.2.1 Characterisation of the homogenous population of small BMOL-TAT1.1 cells obtained from prolonged proliferation

As stated in the results of Chapter 4 culturing of the BMOL-TAT1.1 cell line in the growth medium enriched the small BMOL-TAT1.1 cell type population. Therefore following approximately 13 serial passages in culture whilst maintained in growth medium resulted in the generation of a homogenous population of OV6 (i.e. oval cell marker) positive small cells (Figure 5.1).

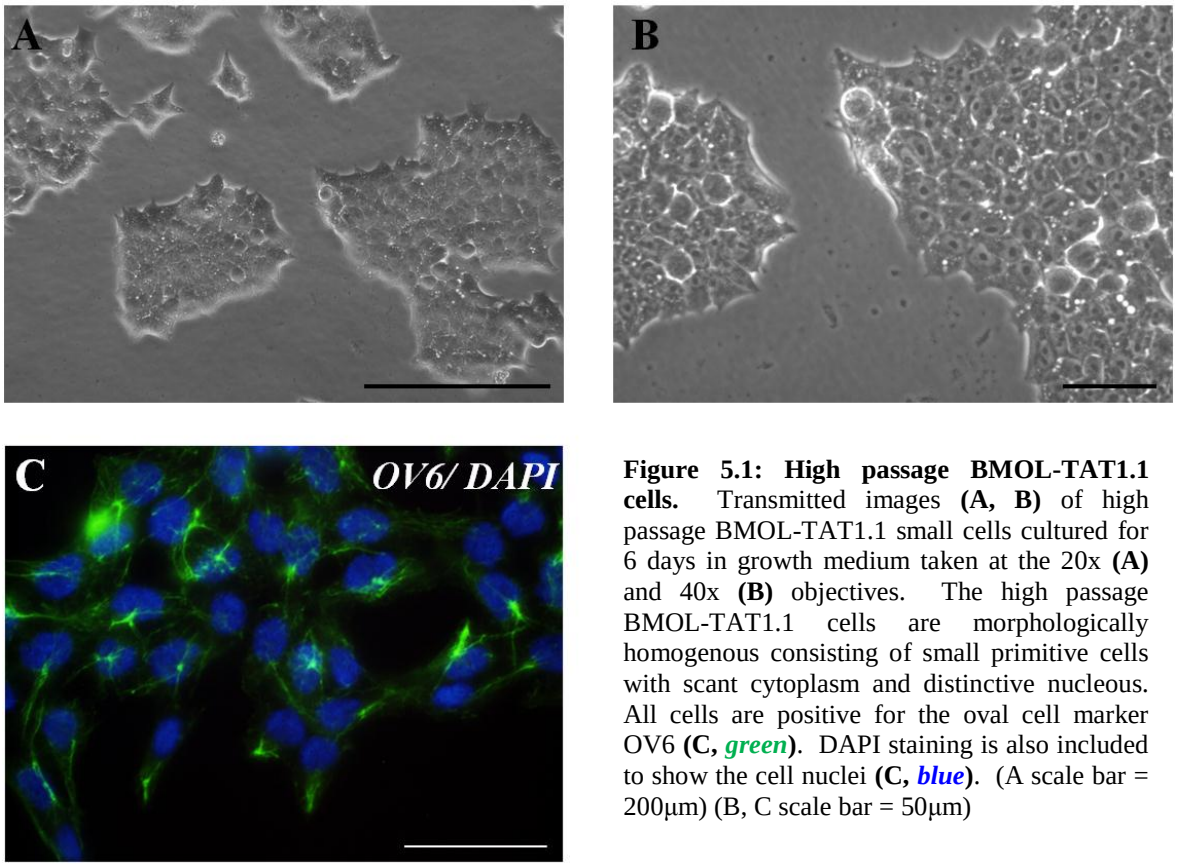



**Figure 5.1: High passage BMOL-TAT1.1 cells.** Transmitted images **(A, B)** of high passage BMOL-TAT1.1 small cells cultured for 6 days in growth medium taken at the 20x **(A)** and 40x **(B)** objectives. The high passage BMOL-TAT1.1 cells are morphologically homogenous consisting of small primitive cells with scant cytoplasm and distinctive nucleous. All cells are positive for the oval cell marker OV6 **(C, *green*)**. DAPI staining is also included to show the cell nuclei **(C, *blue*)**. (A scale bar = 200μm) (B, C scale bar = 50μm)

The homogeneous small BMOL-TAT1.1 cell population generated was characterised by RT-PCR and complementary immunofluorescent staining utilising the same culture conditions as with the heterogeneous BMOL-TAT1.1 cell population i.e. *Condition 1* (6G) and *Condition 5* (3G14D). This allowed the RT-PCR data collected to be compared to that obtained for lower passage cells with a heterogeneous population of small and large cells, as shown in Figure 5.2. These small OV6 positive cells when cultured in growth medium for 6 days (*Condition 1*) maintain the expression of an array of cholangiocyte associated transcription factors, for example HNF1β, HNF6 and Sox9 (Figure 5.3A) and weakly FoxA2, whilst markers associated with the large BMOL-TAT1.1 cells such as HNF4α and morphological markers E-cadherin and CK20 were absent (Figure 5.2). Although negative for mature cholangiocyte markers Connexin 43, Osteopontin (OPN) and CK19, they were strongly positive for Vimentin an early marker of cholangiocytes (Figure 5.3G). Vimentin is also often categorised as a mesenchymal cell marker; however these cells are negative for another mesenchymal cell marker α-smooth muscle actin (SMA) (Figure 5.2). Therefore these small BMOL-TAT1.1 cells appear to display a cholangiocyte/ ductal precursor phenotype similar to that seen in the low passage BMOL-TAT1.1 cells.

Consistent with the results obtained when the low passage cells were cultured with differentiation medium, the high passage small cells maintained Vimentin expression (Figure 5.3J), increased C/EBPα and CK7 expression (Figure 5.2), but decreased FoxA1, FoxA2 (Figure 5.2), and cholangiocyte associated markers, such as HNF1β, HNF6 and Sox9 (Figure 5.3D). The application of the differentiation medium resulted in the absence of pancreatic gene expression (Figure 5.4), and in contrast to the lower passage cells, differentiation medium failed to induce any mature hepatocyte markers, such as TAT and albumin (Figure 5.2) or intestinal markers in particular Muc2, Muc5ac and TFF3 (Figure 5.4).

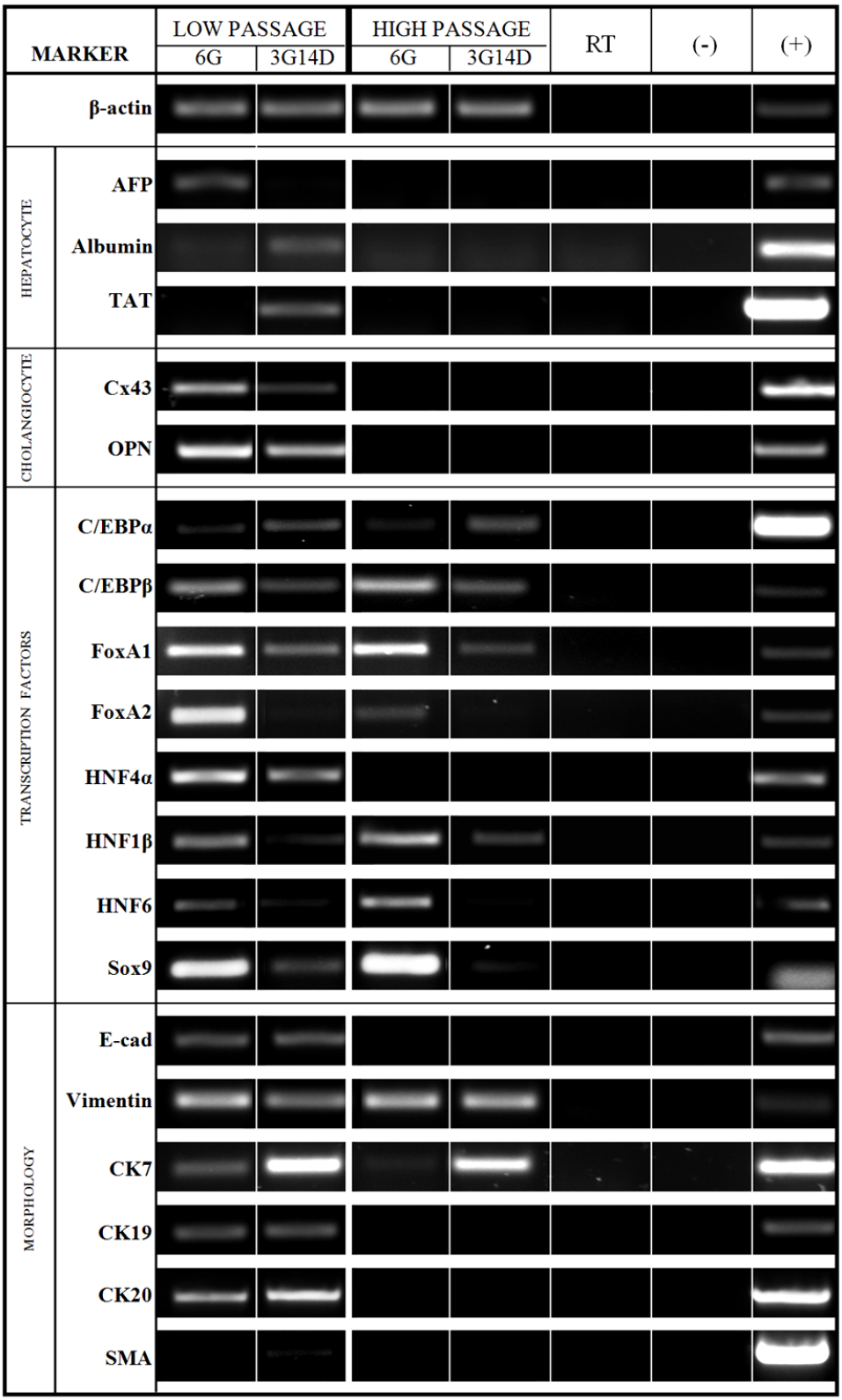



**Figure 5.2: Gene expression analysis of high passage homogenous BMOL-TAT1.1 small cells cultured in different conditions**. High passage homogenous BMOL-TAT1.1 small cells were cultured in either growth medium for 6 days **(6G)** or 3 days in growth medium followed by 14 days in differentiation medium **(3G14D)**. Gene expression was analysed for hepatocyte markers, cholangiocyte markers, transcription factors and morphological markers. Equivalent RT-PCR data from low passage heterogeneous BMOL-TAT1.1 cells is also included for comparison. *Abbreviations*: RT, no RT control; (-) negative control; (+), positive control.

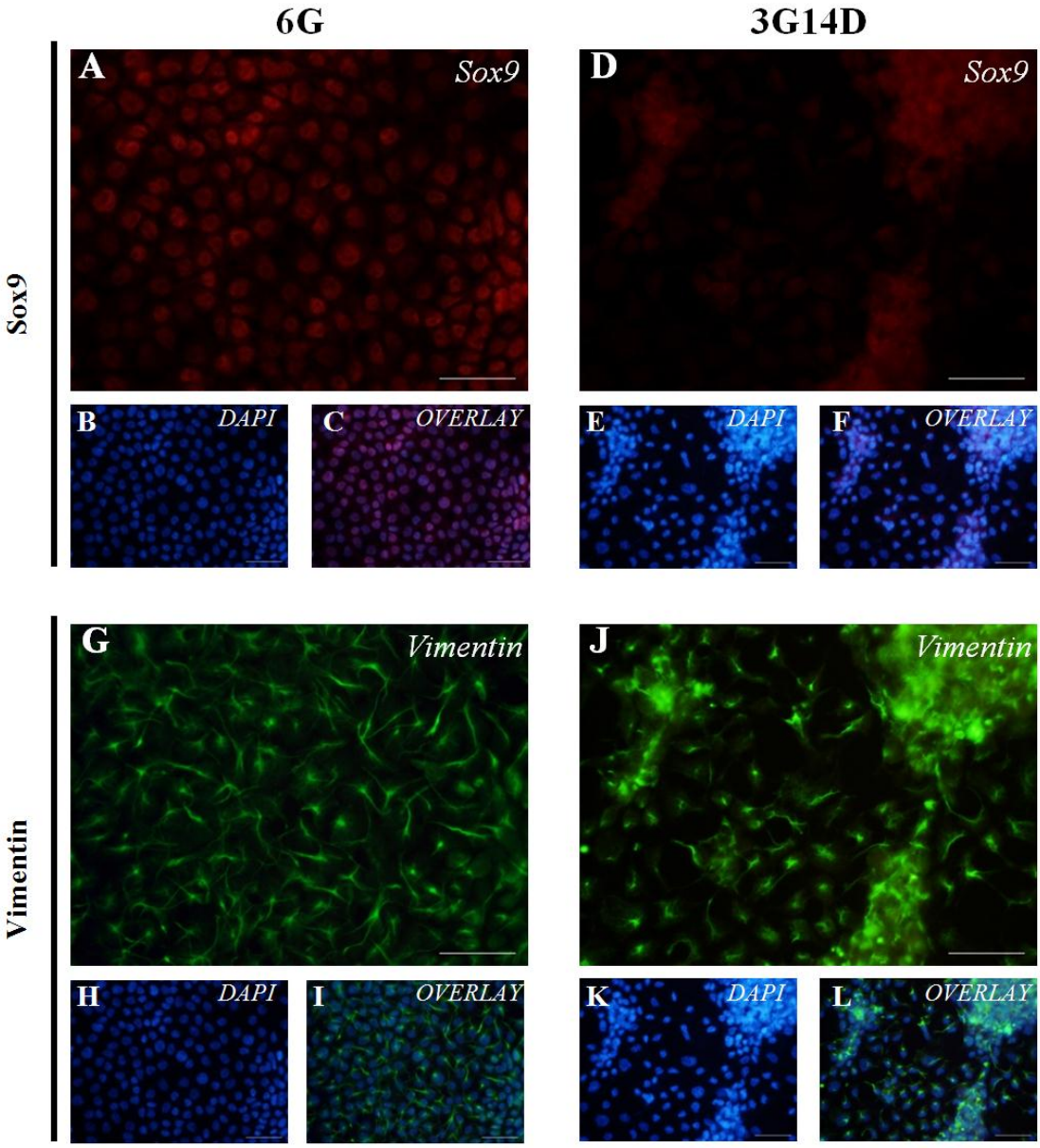



**Figure 5.3: High passage small BMOL-TAT1.1 cells expression of Sox9 and Vimentin in different culture conditions.** Immunofluorescent staining to identify Sox9 **(A, D, *red*)** and Vimentin **(G, J, *green*)** expression. BMOL-TAT1.1 cells were cultured for 6 days in growth medium (6G) or 3 days in growth medium followed by 14 days in differentiation medium (3G14D). All cells are positive for Sox9 and Vimentin when proliferating but differentiation medium induces the suppression of Sox9. DAPI staining **(B, E, H, K, *blue*)** is also included. Images **C, F, I,** and **L** are overlays of **A, B ; D, E; G, H;** and **J, K** respectively. (scale bar = 50µm).

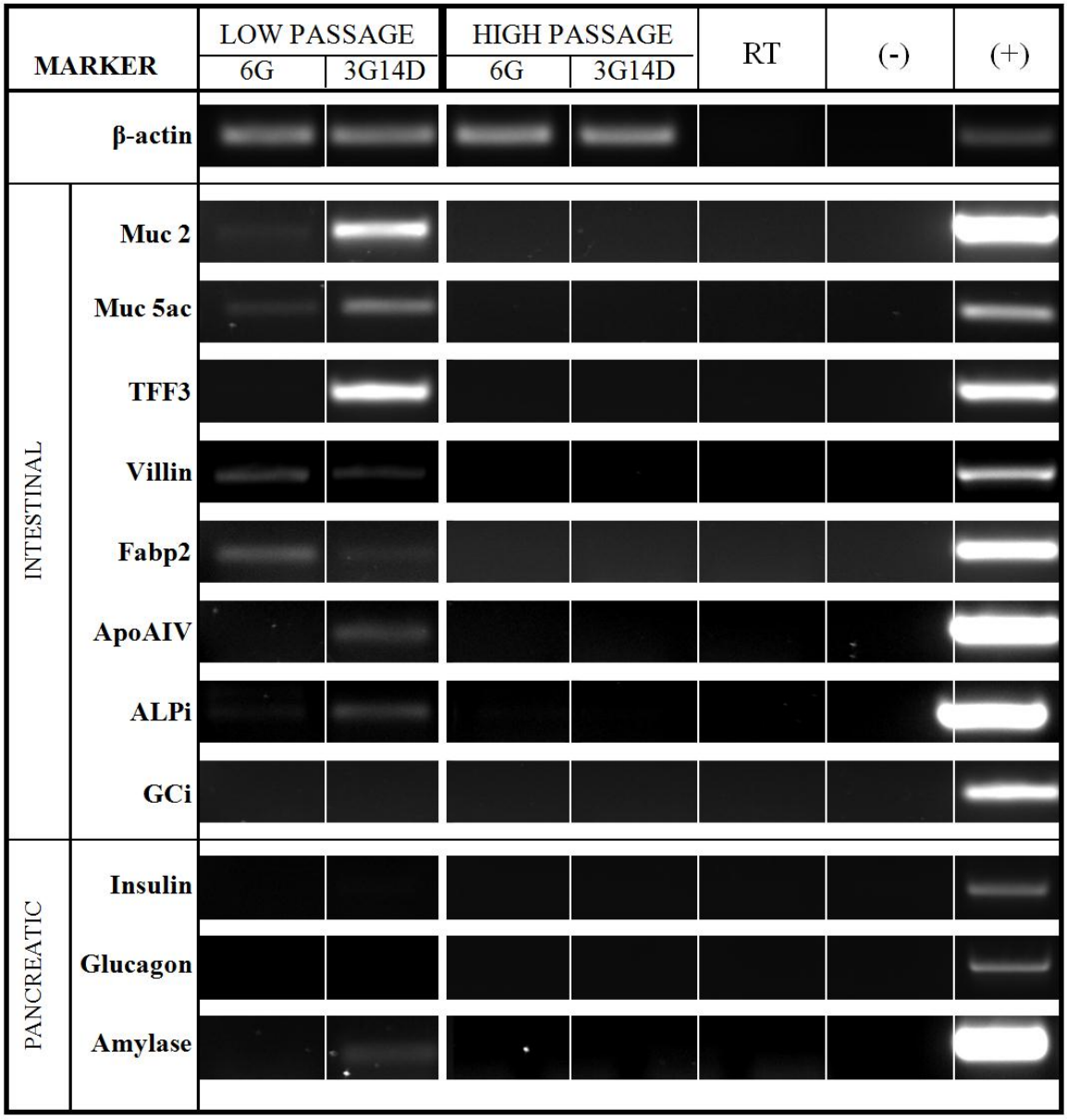



**Figure 5.4: RT-PCR analysis of non-hepatic gene expression in high passage homogenous small BMOL-TAT1.1 cells cultured in different conditions.** High passage homogenous small BMOL-TAT1.1 cells were cultured in either growth medium for 6 days **(6G)** or 3 days in growth medium followed by 14 days in differentiation medium **(3G14D)**. Gene expression was analysed for intestinal and pancreatic markers. Equivalent RT-PCR data from low passage heterogeneous BMOL-TAT1.1 cells is also included for comparison. *Abbreviations*: Muc2, mucin 2; Muc 5ac, mucin 5ac, TTF3, trefoil factor 3; Fabp2, fatty acid binding protein; ApoAIV, apolipoprotein AIV, ALPi, alkaline phosphatise intestinal; GCi, guanylate cyclise intestinal; RT, no RT control; (-) negative control; (+), positive control.

### 5.2.2 Over-expression of liver-enriched transcription factors in the homogenous population of small BMOL-TAT1.1 cells

Initially it was critical to assess if BMOL-TAT1.1 cells were first infectable by adenovirus, secondly which Multiplicity of Infection (MOI) was appropriate to produce a high infection percentage without adverse toxic effects, and finally which infection conditions (e.g. duration of exposure to virus) were optimum. BMOL-TAT1.1 cells were therefore initially infected with the adenovirus Ad-CMV-HNF4α, at a range of MOI's (data not shown) with virus incubation times of 1hour or overnight, and then cultured for 7 days in differentiation medium. BMOL-TAT1.1 cells were proven to be infectable based on the detection of HNF4α, with optimum infection efficiency obtained at an MOI of 200 (Figure 5.5). In addition, the results showed that incubation of the cells with the virus overnight (Figure 5.5G) rather than for 1hour (Figure 5.5D) dramatically increased infection efficiency, whilst still having no obvious adverse effect on cell viability.

BMOL-TAT1.1 cells were infected with adenoviral vectors containing C/EBPα and/ or HNF4α, at an MOI of 200 (each) overnight, and then cultured for 7 days in differentiation medium (Figure 5.6B,E). BMOL-TAT1.1 cells were also infected with adenovirus Ad-Null, which contained no transgene, in order to determine that any results obtained were due to the transgene expression and not the adenovirus. RT-PCR analysis showed that following 7 days culture in differentiation medium, cells ectopically expressing the C/EBPα transgene and/or the HNF4α transgene did not express hepatocyte markers, such as TAT or Albumin (Figure 5.6A). However, over-expression of HNF4α induced the mRNA expression of intestinal markers Villin, ALPi and ApoAIV, but there was no expression of other intestinal markers such as TFF3 and Muc2 (Figure 5.6A). Complementary immunofluorescent staining confirmed the induction of Villin protein expression in HNF4α-infected cells (Figure 5.7B) compared to un-infected control cells (Figure 5.7A).

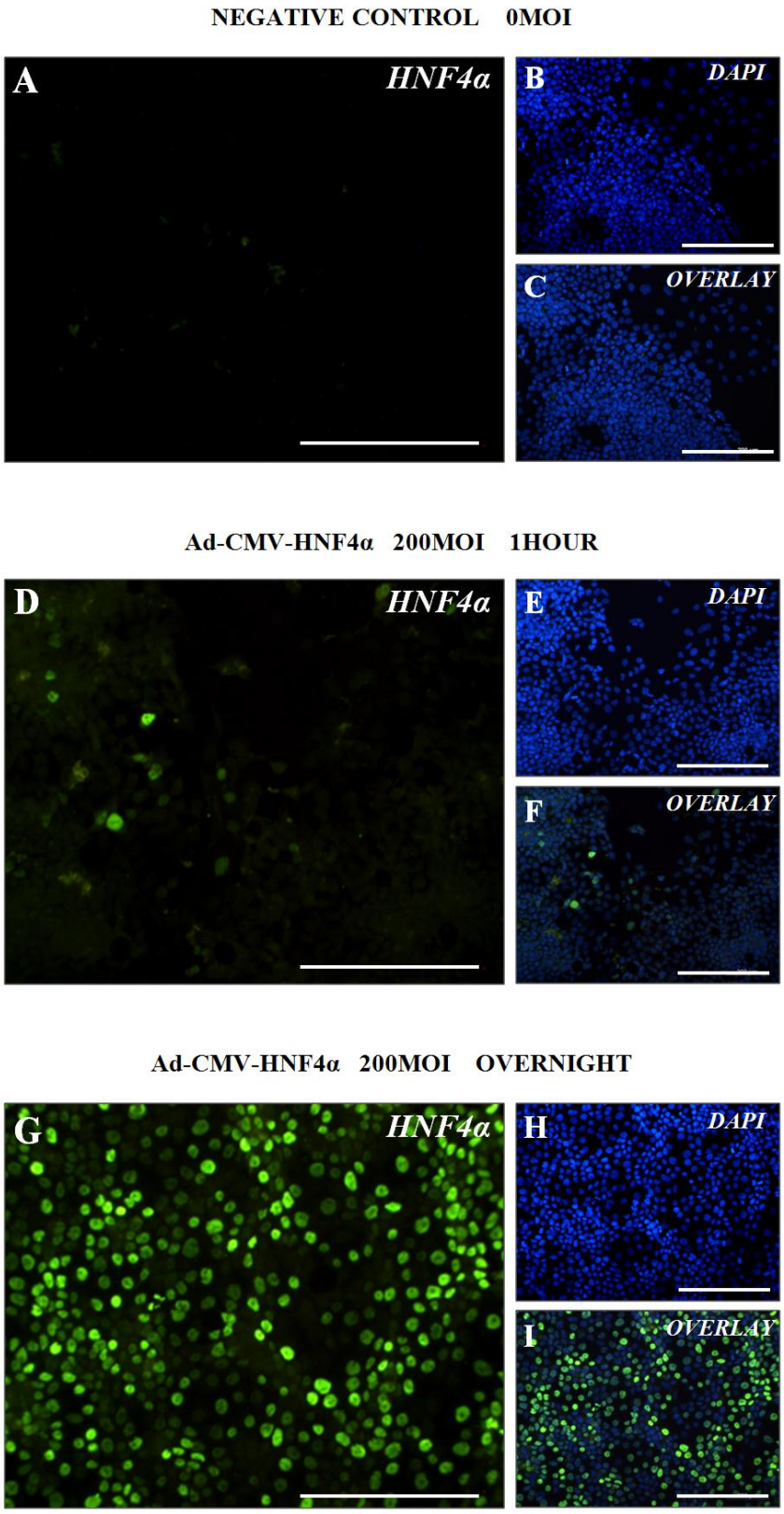



**Figure 5.5: Infection efficiency of the BMOL-TAT1.1 cell line.** Immunofluorescent staining to identify HNF4α **(A, D, G, *green*)** expression in BMOL-TAT1.1 cells infected with Ad-CMV-HNF4α and cultured for 7 days in differentiation medium. Included are a negative (un-infected) control **(A)**; BMOL-TAT1.1 cells infected with Ad-CMV-HNF4α at 200MOI for 1hour **(D)** or overnight **(G)**. DAPI staining is included to identify the cell nuclei and provide an indication of the percentage of cells infected **(B, E, H, *blue*)**. Images **C**, **F** and **I** are overlays of **A,B ; D,E** and **G, H** respectively. (scale bar = 200μm).

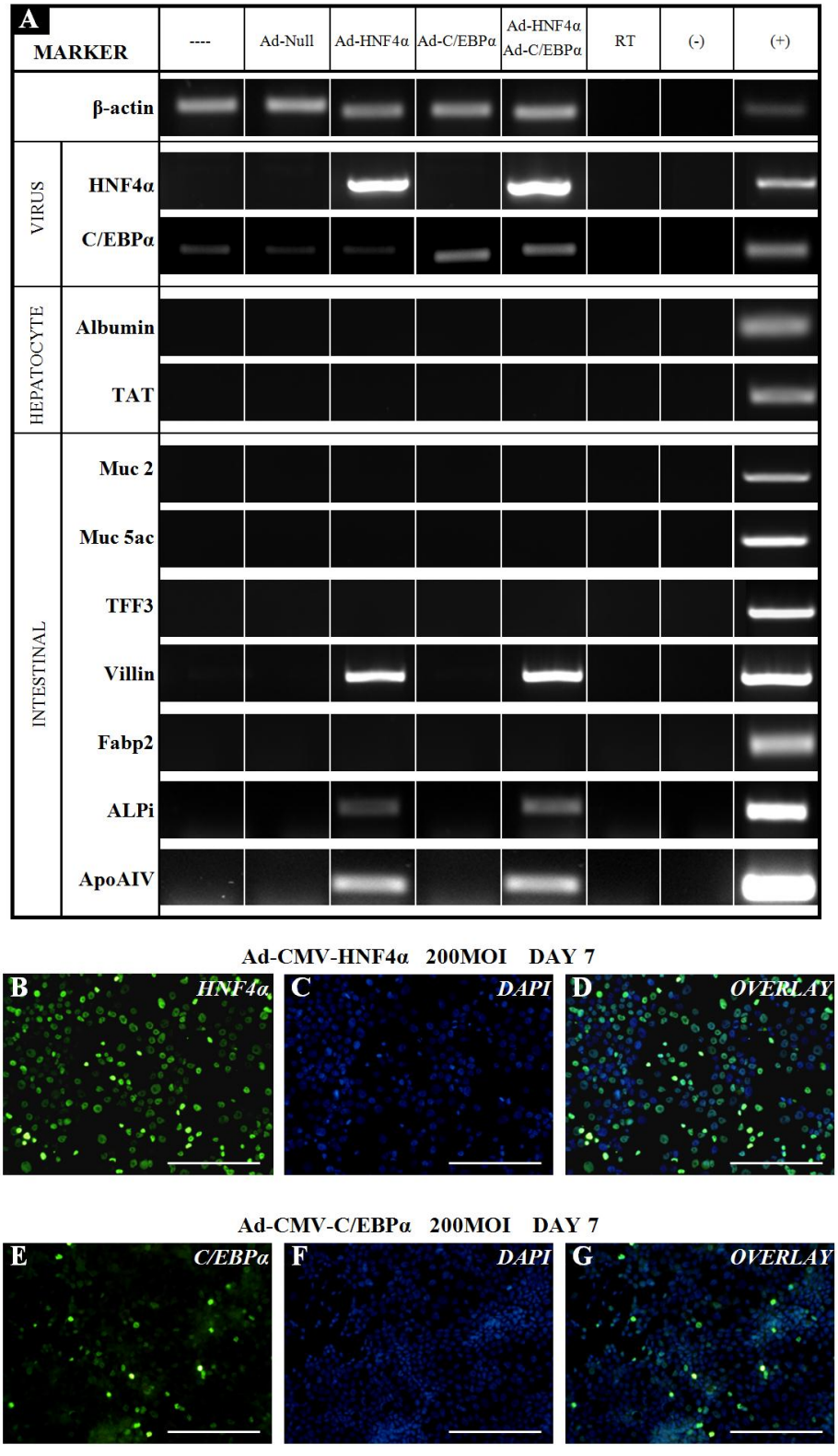



**Figure 5.6: RT-PCR analysis of over-expression of HNF4α and C/EBPα in high passage BMOL-TAT1.1 cells.** High passage BMOL-TAT1.1 small cells were infected with Ad-CMV-HNF4α and Ad-CMV-C/EBPα alone and in combination. Controls included an un-infected control (---) and cells infected with Ad-Null. All cells once infected were cultured in differentiation medium for 7 days. Gene expression was analysed for hepatocyte and intestinal markers. C/EBPα had no effect on gene expression whilst HNF4α induced the expression of Villin and intestinal Alkaline Phosphatase (ALPi) **(A)**. Immunofluorescent staining for HNF4α **(B, *green*)**, C/EBPα **(E, *green*)** and DAPI **(C, F, *blue*)** provided an indication of the percentage of cells infected. Images **D** and **G** are overlays of **B, C** and **E, F** respectively. *Abbreviations*: RT, no RT control; (-) negative control; (+), positive control. (scale bar = 50μm).

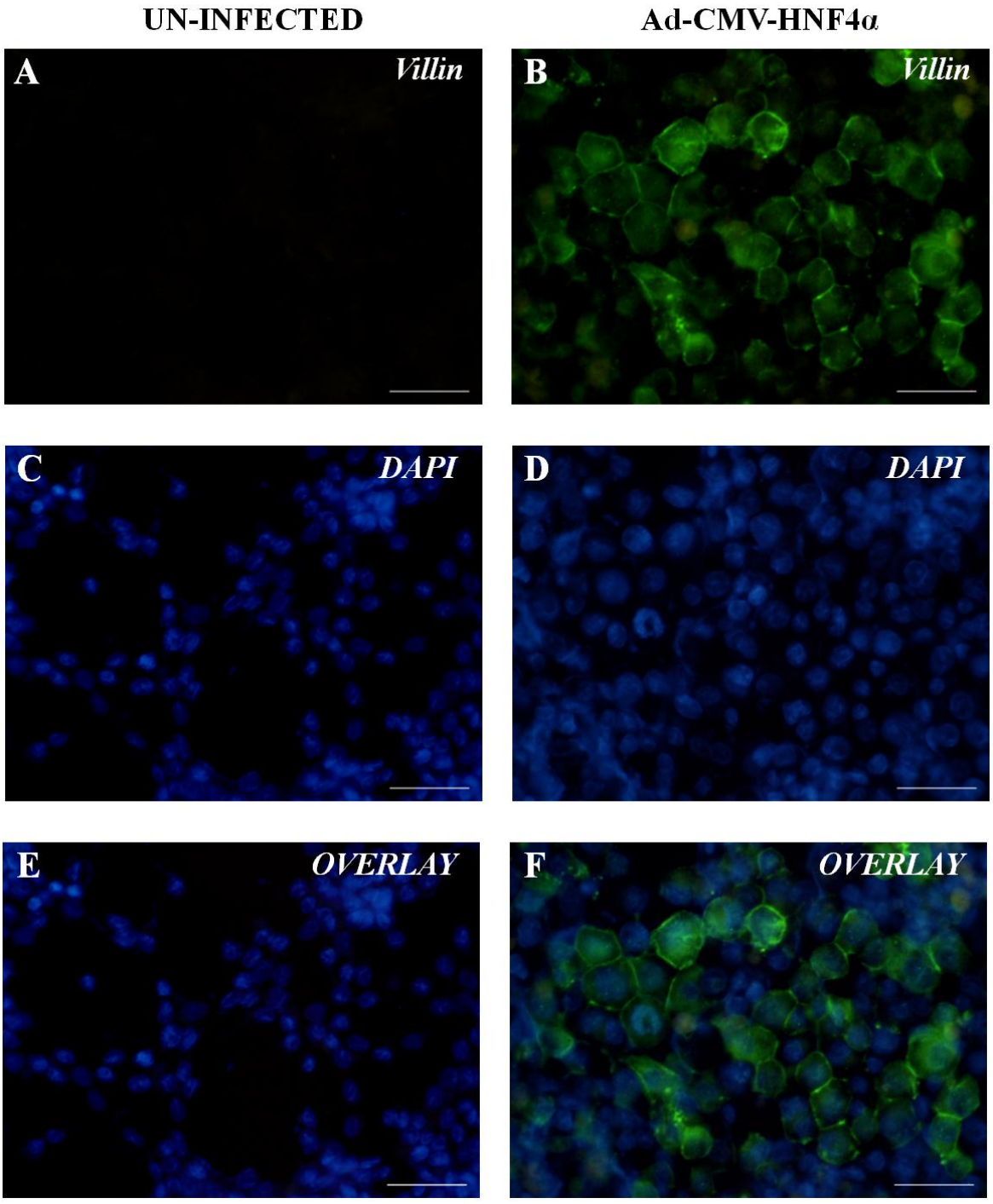



**Figure 5.7: Over-expression of HNF4α in high passage small BMOL-TAT1.1 cells induces the expression of Villin.** Immunofluorescent staining to identify Villin expression **(*green*)** in high passage small BMOL-TAT1.1 cells which were un-infected **(A)** or infected with Ad-CMV-HNF4α **(B)** prior to culture in differentiation medium for 7 days. DAPI staining is included to show the cell nuclei **(C, D, *blue*)**. Images **E** and **F** are overlays of **A, C** and **B, D** respectively. (scale bar = 50μm).

## 5.3 Discussion

### 5.3.1 Prolonged BMOL-TAT1.1 proliferation enriches for cells possessing an immature cholangiocyte phenotype.

Maintenance of the BMOL-TAT1.1 cell line under conditions that promote a proliferative state (i.e. in growth medium) for a prolonged period of time (i.e. high passage) facilitated enrichment of the small BMOL-TAT1.1 cell population, possessing an immature cholangiocyte phenotype. The phenotype was determined by RT-PCR analysis and demonstrated the potent expression of immature cholangiocyte markers including Sox9, Vimentin, HNF1β and HNF6 but the absence of mature cholangiocyte markers such as Connexin 43, Osteopontin (OPN) and CK19 (CK19) when compared to the low passage heterogeneous BMOL-TAT1.1 population (Figure 5.2). This is consistent with the components of the 'growth medium', such as EGF favouring cholangiocyte differentiation (Figure 3.10B). Similar to the heterogeneous BMOL-TAT1.1 cell population, inducible differentiation of the homogenous small BMOL-TAT1.1 cell population provoked changes in transcription factors (i.e. C/EBPα, Sox9, HNF6, FoxA2) and CK7 expression, consistent with dexamethasone treatment, as shown in Chapter 4. However, in contrast to the data from Chapter 4, inducible differentiation failed to provoke the induction of mature hepatocyte markers (e.g. TAT and Albumin) (Figure 5.2). This leads to two hypotheses that (i) hepatic differentiation requires the presence of the large BMOL-TAT1.1 cells which are present in the low passage heterogeneous BMOL-TAT1.1 cell population or (ii) prolonged proliferation of the small BMOL-TAT1.1 cells to an immature phenotype partially differentiated towards a mature cholangiocyte has resulted in maturation arrest, hence the cells are blocked from terminal differentiation (Potter, 1978, Sell, 1993).

### 5.3.2 Extended adenovirus incubation time improves infection efficiency of the BMOL-TAT1.1 cell line

Adenoviruses possess a capsid consisting of three main proteins: (i) hexon (ii) penton base and (iii) knobbed fiber proteins extending from the penton base. The infection of host cells is initiated via the high-affinity binding of the fiber protein knob domain to cell surface receptors, such as the Coxsackie Adenovirus Receptor (CAR) (Figure 5.8) (Bergelson, et al., 1997, Roelvink, et al., 1998). The efficiency of adenovirus binding and

entry to particular cell types is directly related to the distribution and availability of specific receptors on the cell membrane (Mentel, et al., 1997, Russell, 2000) and the affinity of the adenovirus to its primary receptor. Subsequent internalization of adenovirus requires a further interaction between the penton base protein of the viral capsid and α integrins on the host cells. Optimisation of the adenoviral infection protocol utilising Ad-CMV-HNF4α and BMOL-TAT1.1 cells revealed that the inefficiency of adenovirus mediated gene transfer (i.e. following 1 hour incubation) could be partially corrected when the contact time between adenovirus and cells was prolonged to overnight, as we observed a significant increase in the number of infected cells (Figure 5.5).

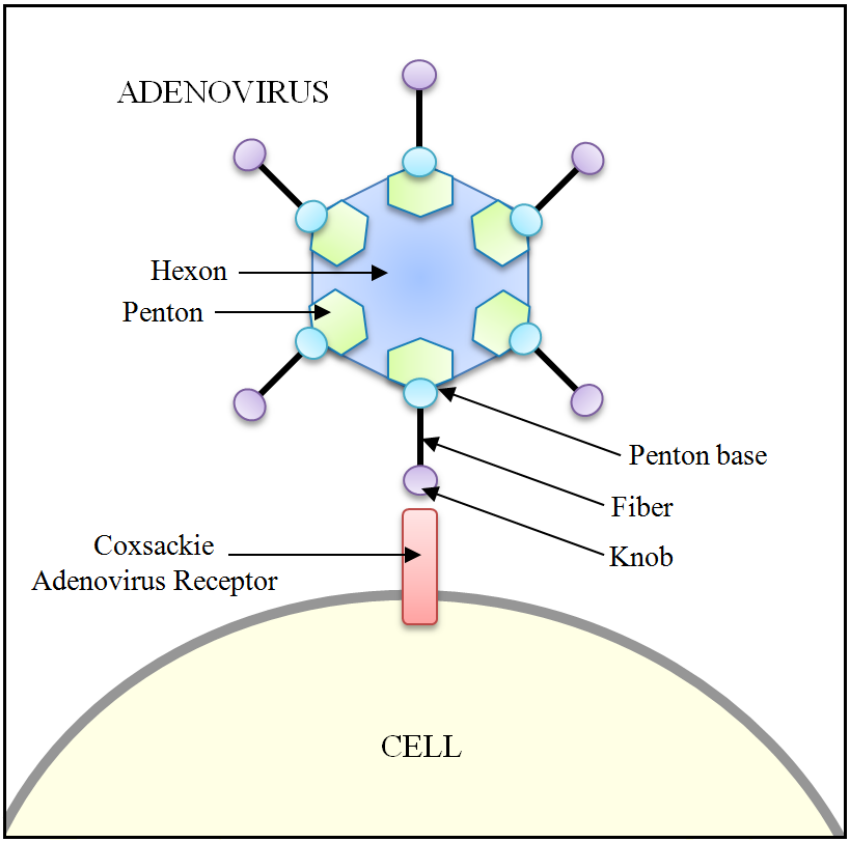



**Figure 5.8: Mechanism of adenovirus attachment to the target cell surface receptor**

### 5.3.3 Ectopic Expression of C/EBPα and/or HNF4α in the BMOL-TAT1.1 cell line is insufficient to induce hepatic differentiation

With regards to the interpretation of adenoviral infection results, it has been suggested that induction of genes may be dependent on the adenovirus itself, hence an artefact of viral infection. Utilisation of the control virus Ad-Null confirmed this not to be the case; hence the adenovirus itself was not influencing gene induction. The ectopic

expression of C/EBPα and/ or HNF4α appeared to have no effect on BMOL-TAT1.1 hepatic differentiation as identified by the lack of induction of hepatocyte associated markers (Figure 5.6). It is postulated this may be because the cells have undergone maturation arrest or that hepatic differentiation requires the concerted action of other important liver-enriched transcription factors. In a recent study *Icob and colleagues* identified that upon the sequential ectopic over-expression of three transcription factors (FoxA2, HNF4α and C/EBPα) induces an advanced mature hepatocyte phenotype in an expandable adult liver derived progenitor cell population (ALDPC) (Iacob, et al., 2011). In addition *Sekiya and Suzuki* also recently established the combined expression of HNF4α with FoxA2 is sufficient to convert other non-hepatic cell types such as embryonic and adult mouse fibroblasts into epithelial hepatocyte-like cells (Sekiya and Suzuki, 2011). Compared to these studies therefore the weak expression of FoxA2 in the high passage small BMOL-TAT1.1 cells and complete suppression following culture in dexamethasone-containing differentiation medium, may be the fundamental factor blocking hepatic differentiation. The rapid and irreversible loss of differentiated functions (i.e. de-differentiation) of primary hepatocytes in culture is related to the rapid down-regulation of LETFs such as FoxA2, during the first few days in culture. FoxA proteins have been identified to act as "competence factors" promoting chromatin modification (i.e. opening) to facilitate the binding and transcriptional activity of other transcription factors in endodermal cells (Cirillo, et al., 2002, Zaret, 2008). Also over-expression of FoxA2 in human bone marrow derived mesenchymal stem cells induced hepatic differentiation, as demonstrated by enhanced expression of albumin, AFP and TAT (Ishii, et al., 2008).

### 5.3.4 Ectopic Expression of HNF4α in the BMOL-TAT1.1 cell line induces Villin expression

The current study demonstrates for the first time that although the ectopic expression of HNF4α in BMOL-TAT1.1 cells has no impact on hepatic differentiation, HNF4α has the ability to induce intestinal genes in BMOL-TAT1.1 oval cells cultured in differentiation medium. HNF4α was sufficient to provoke the induction of Villin, which is the best characterised microfilament-associated, actin-binding protein typical of brush-border microvilli (Robine, et al., 1985), intestinal Alkaline Phosphatase (ALPi) and Apolipoprotein AIV (ApoAIV). These markers were all robustly detected by RT-PCR (Figure 5.6), with Villin confirmed by immunofluorescent staining (Figure 5.7). Most

studies have concentrated on the role of HNF4α in regulation of hepatic gene expression and hepatic lipid metabolism (Watt, et al., 2003). However there are similarities in hepatocytes and enterocytes, which are the major epithelial cell type found in the intestinal mucosa, as both are columnar epithelium concerned with metabolism of lipids and carbohydrates. The utility of HNF4α to induce an intestinal phenotype has been assessed in non-intestinal mouse embryonic fibroblast cell lines (NIH-3T3), with ApoAIV and Villin induced by stable HNF4α transfection (Babeu, et al., 2009). Also in non-intestinal HeLa cell line HNF4α increases ALPi promoter activity, which supports ALPi induction in BMOL-TAT1.1 cells ectopically expressing HNF4α (Olsen, et al., 2005). Both *in vitro* and *in vivo* ApoAIV and ALPi are associated with differentiated enterocytes and are up-regulated by HNF4α, due to possession of an over-representation of HNF4α binding sites within their promoters (Archer, et al., 2005, Babeu, et al., 2009, Stegmann, et al., 2006). Additionally positive Villin expression is frequently observed in hepatocellular carcinoma (Karabork, et al., 2010, Moll, et al., 1987), a condition often associated with the appearance of oval cells.

# Chapter 6 Characterisation of the α-TC19 cell line and inducible reprogramming to other pancreatic cell types.

## 6.1 Introduction

### 6.1.1 α-TC19 cell line

The alpha-TC1 clone 9 (α-TC19) cell line is a pancreatic α-cell line cloned from the α TC1 cell line which was derived from an adenoma created in transgenic mice expressing the SV40 large T antigen oncogene under the control of the rat pre-pro-glucagon promoter. The clone 9 line is more differentiated than the original parental α-TC1 cell line, and represents an effective α-cell model, as it maintains many characteristics of differentiated α-cells, predominantly the production of glucagon but not insulin or pre-pro-insulin mRNA (Hamaguchi and Leiter, 1990).

### 6.1.2 Over-expression of pancreatic transcription factors

Recent *in vivo* studies have demonstrated the potential for reprogramming α-cells to β-cells during pancreatic β-cell regeneration following total or near total β-cell ablation (Thorel, et al., 2010). The challenge however remains to identify and validate the 'master switch transcription factors' required for initiation of the conversion of α-cells into β-cells. Potential candidates include transcription factors involved in regulating β-cell differentiation and function, such as Paired box gene 4 (Pax4) (Collombat, et al., 2009) Pancreatic duodenal homeobox 1 (Pdx1) (Chakrabarti, et al., 2002a, Ritz-Laser, et al., 2003) and Hepatocyte nuclear factor 4 alpha (HNF4α) (Gupta, et al., 2005, Wollheim, et al., 2000). In the current chapter a recombinant adenoviral infection approach (as described in Chapter 5 (5.1.2)) was employed to transiently over-express these specific transgenes in the α-TC19 cell line.

### 6.1.3 Chromatin modification: histone acetylation

Transcriptional regulation in eukaryotes occurs within chromatin, a complex of nucleic acid and proteins condensed to form a chromosome during cell division. Chromatin is comprised of nucleosome units entailing DNA wrapped around histone octamers, of which there are four histone types H2A, H2B, H3, H4 (Kornberg, 1974). Post-translational histone modifications loosen or compact the chromatin structure and/or recruit other chromatin binding proteins in order to regulate transcription factor access and hence influence transcriptional regulation. Acetylation is one type of histone modification often observed in actively transcribed genes. It involves Histone acetyltransferases (HAT) enzymes acetylating lysine residues, hence neutralising the positive charges on histones. This consequently loosens the chromatin structure allowing easier access for transcription factors to target DNA (Figure 6.1) (Choi and Howe, 2009). HAT enzymes are balanced by Histone Deacetylase (HDAC) enzymes which deacetylate lysine residues on histones leading to chromatin compaction and transcriptional repression. Mammals possess three main HDAC classes based on phylogenetic conservation, catalytic sites and cofactor dependency. Class I HDACs (1-3, 8) are ubiquitously expressed and localised predominantly in the nucleus, whereas Class II HDACs (4-7,9,10) are only present in certain tissues (including pancreas) and shuttle between the nucleus and cytoplasm (Haberland, et al., 2009). Class II HDACs contain an N-terminal extension that links them to specific transcription factors and confers responsiveness to a variety of signal transduction pathways hence connects the environment to the genome.

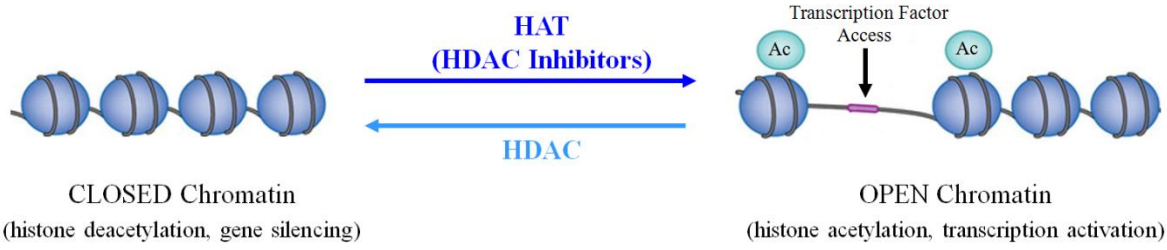



**Figure 6.1: Regulation of chromatin structure and associated transcriptional activity by histone acetylation and deacteylation.** Chromatin nucleosome buildings units comprising of histones (*blue circles*) and DNA (*grey*) are shown. Targeted enzymes, HAT and HDAC act to negotiate the acetylation status of chromatin. Hyper-acetylation is induced by HDAC inhibitors. *Abbreviations*: Ac, acetylated histone terminal domains; HAT, histone acetyltransferase; HDAC, histone deacetylase.

The acetylation state of the histones within the nucleosome is responsible for modulating chromatin structure, hence epigenetically regulating gene expression.

Pharmacological small molecule HDAC inhibitors (HDACi) are useful tools for studying the link between histone hyper-acetylation and cell lineage specification (Figure 6.1). Four types of HDACi have been identified including: (i) short chain fatty acids, (ii) hydroxamates, (iii) benzamides, and (iv) cyclic tetrapeptides. This study utilised one hydroxamate inhibitor Trichostatin A (TSA); and two short chain fatty acid inhibitors Sodium Butyrate (NaB) and Valproic Acid (VPA). VPA preferentially targets Class I HDACs (Gottlicher, et al., 2001), whereas TSA and NaB inhibit both Class I and Class II HDACs (Davie, 2003, Yoshida, et al., 1990). Confirmation that NaB, TSA and VPA are acting as HDAC inhibitors can be obtained from measuring HDAC activity utilising a HDAC enzymatic activity assay and analysis of histone acteylation by Western blotting with histone acetyl-H3 and –H4 antibodies (Haumaitre, et al., 2008).

*Scharfmann and colleagues* discovered that alteration of histone acetylation via different HDACi can modify the timing and determination of pancreatic cell fate in an *ex-vivo* rat embryonic pancreas model (Haumaitre, et al., 2008). This demonstrates that histone modifications (such as acetylation) may play a crucial role in the formation of a transcriptionally competent environment with relaxed chromatin and therefore in the control of cell differentiation. The effect of HDACi on adult pancreas and specific endocrine cell types has been extensively studied in adult β-cell lines (such as RIN cells) (Gardner, et al., 1989), however this research is novel in that it investigated the effect of HDACi on an adult pancreatic α-cell line (α-TC19).

### 6.1.4 Experimental Aim

In order to probe the molecular mechanism underlying the conversion of adult pancreatic α-cells into β-cells, required initial characterisation of the α-TC19 cell line. The major aims in this chapter were to optimise adenoviral infection of the α-TC19 cell line in order to determine the effects of ectopic expression of transcription factors commonly associated with β-cell differentiation. The final aim was to investigate if HDAC inhibition in the α-TC19 cell line impacted on regulation of the differentiation program, and through the use of different HDACi elucidate the mechanism involved.

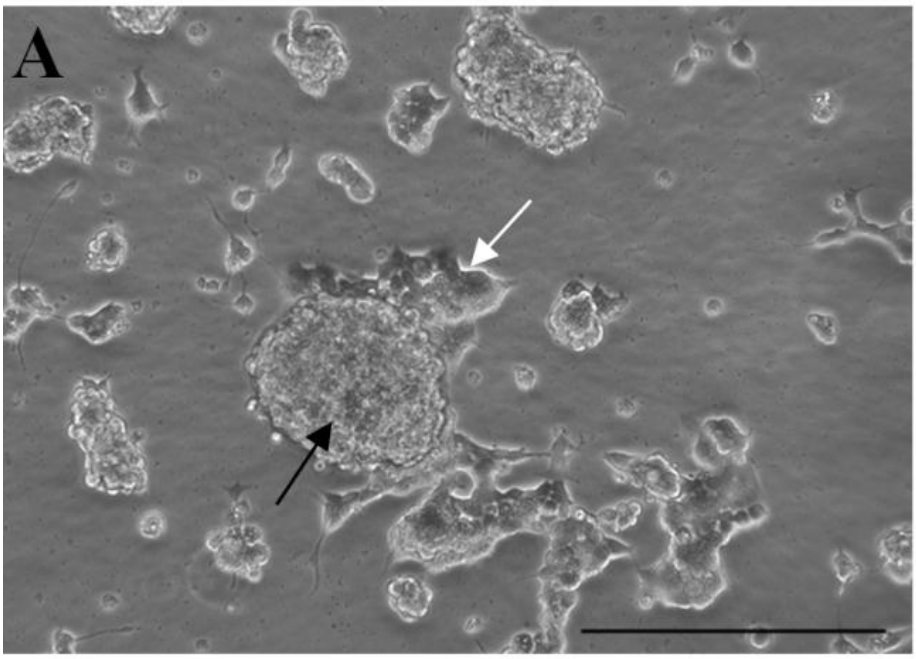
## 6.2 Results

### 6.2.1 Characterisation of the α-TC19 cell line

The α-TC19 cell line was maintained for 7 days in culture prior to morphological, gene and protein expression analyses. α-TC19 cells proliferate as adherent single cell monolayers and loosely attached cultures (Figure 6.2A). Immunofluorescent staining revealed α-TC19 cells were positive for the α-cell specific hormone glucagon but negative for the β-cell specific hormone insulin (Figure 6.2B). This observation was confirmed by RT-PCR data which showed α-TC19 cells were also negative for the hormones associated with all other pancreatic endocrine cell types (somatostatin and pancreatic polypeptide) (Figure 6.3A). We did not test for the expression of ghrelin. Finally the expression of a number of pancreatic transcription factors was assessed by RT-PCR. The α-TC19 cell line was positive for transcription factors important in α-cell specification (Nkx2.2, FoxA2, Arx, Pax6) and negative for transcription factors important in β-cell specification (HNF4α, Pdx1, Pax4, Nkx6.1) (Figure 6.3A). The transcription factors important in α- and β-cell specification are summarised in Figure 6.3B.

### 6.2.2 Expression of adenoviral vectors in the α-TC19 cell line

It was critical to assess if α-TC19 cells were first infectable, secondly which MOI was appropriate to produce a high infection percentage without adverse toxic effect, and finally which infection conditions were optimum. To address these issues, α-TC19 cells were initially infected with the Ad-RSV-GFP adenovirus which expresses Green Fluorescent Protein (GFP) under the control of the Respiratory Syncytial Virus (RSV) promoter, at a range of MOI's with virus incubation times of 1hour or overnight and with a range of DEAE-dextran concentrations (2-10μg/ml) (data not shown). α-TC19 cells were proven to be infectable, with optimum infection efficiency obtained at an MOI of 100, overnight with additional treatment with 5μg/ml DEAE-dextran. Results demonstrated adenoviral infection had no adverse effect on cell viability, with GFP expression first detectable 3 days after infection (Figure 6.4A) and maintained until day 7 (Figure 6.4D), which probably represents the minimum time point required for transdifferentiation.



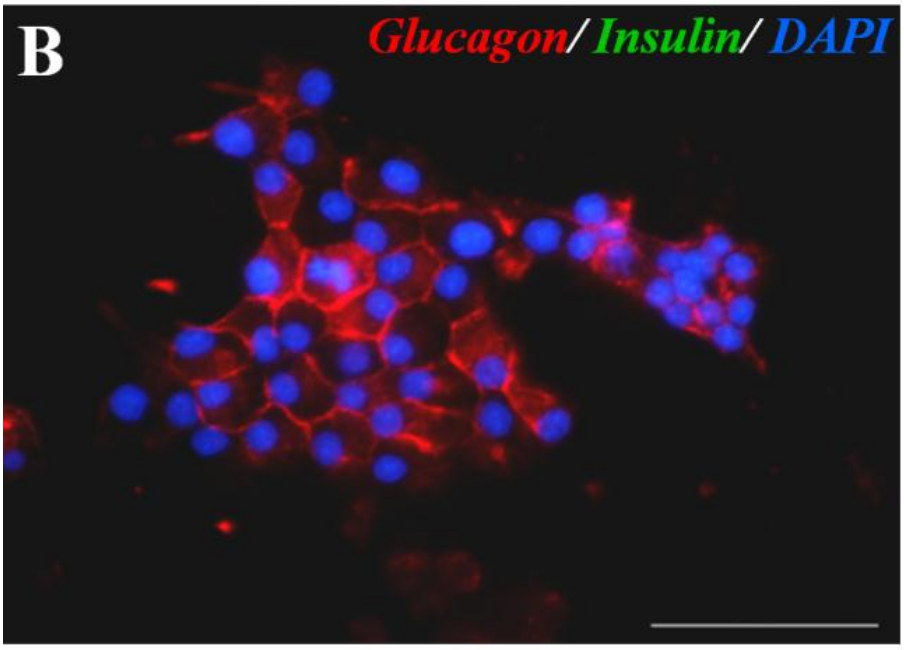
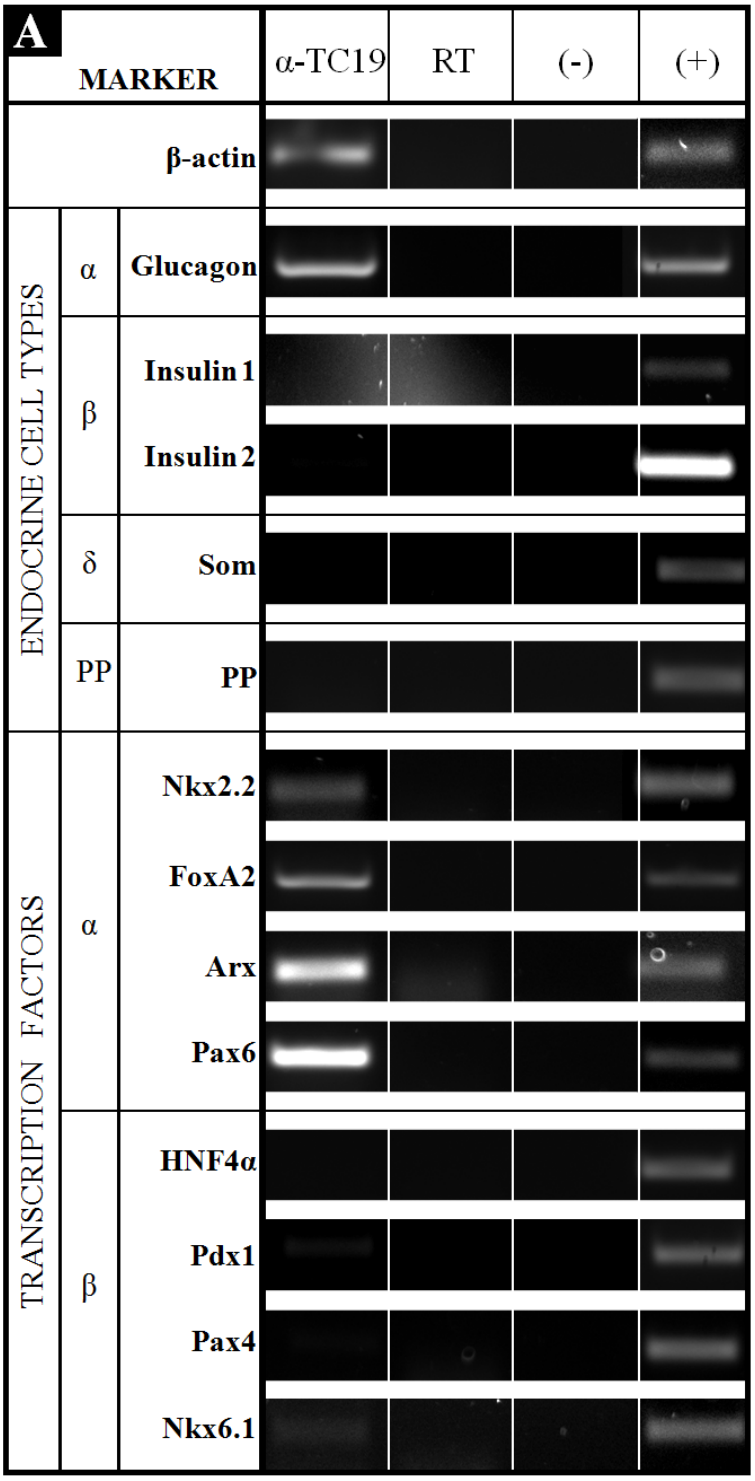




**Figure 6.2: α-TC19 cell line morphology. (A)** Transmitted light image of α-TC19 cells cultured for 7 days. α-TC19 cells grow as either monolayers **(*white arrow*)** or as loosely attached clusters **(*black arrow*)**. Immunofluorescent staining confirms α-TC19 cells to be positive for glucagon expression **(B, *red*)** and negative for insulin expression **(B, *green*)**. For all antibodies utilised, positive controls were performed in parallel and are included in *Appendix C1*. DAPI is also included to show the cell nuclei **(B, *blue*)**. (A scale bar = 200μm) (B scale bar = 50μm).



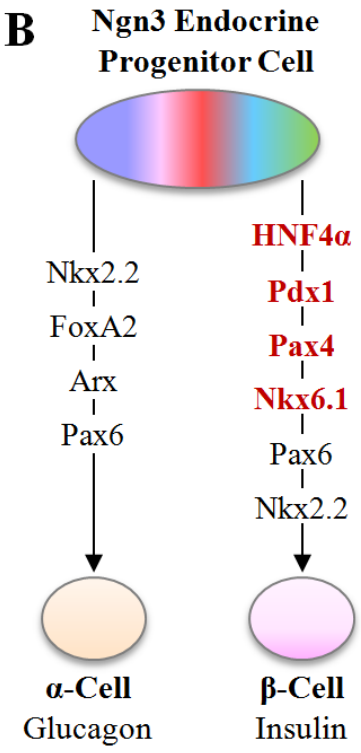




**Figure 6.3: Characterisation of the α-TC19 cell line. (A)** RT-PCR analysis of α-TC19 cells cultured for 7 days. α-TC19 cells are positive for α-cell specific transcription factors and hormone glucagon and are negative for all other endocrine hormones (i.e. insulin) and also negative for β-cell specific transcription factors. **(B)** Schematic representation of the transcription factors important in α- and β-cell type specification. Highlighted in **red** are transcription factors specific to β-cells. *Abbreviations*: RT, no RT control; (-) negative control; (+), positive control.

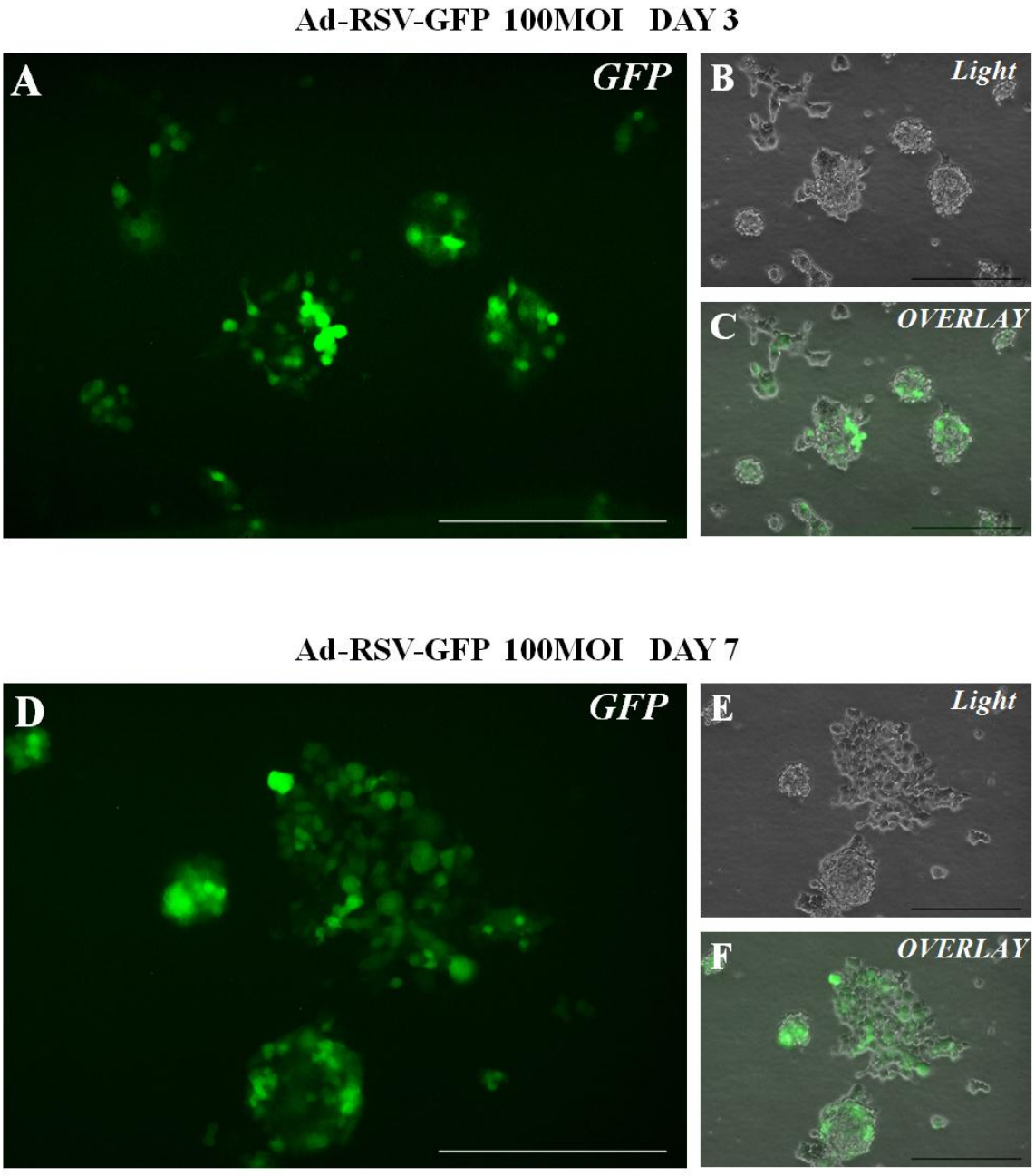



**Figure 6.4: Expression of adenoviral vectors in the α-TC19 cell line.** In order to test whether the α-TC19 cell line is amendable to adenoviral infection, we expressed Green Fluorescent Protein (GFP) in the cells under experimental conditions. Expression of GFP in α-TC19 cells infected with Ad-RSV-GFP at an Multiplicity Of Infection (MOI) of 100 overnight with 5μg/ml DEAE-dextran and cultured for 3 **(A, *green*)** and 7 **(D, *green*)** days. **(B, E)** Transmitted light images are included to provide an indication of the percentage of cells infected. Images **C** and **F** are overlays of **A, B** and **D, E** respectively. (scale bar = 200μm).

### 6.2.3 Over-expression of β-cell specific transcription factors in the α-TC19 cell line

Characterisation of the α-TC19 cell line revealed the transcription factors HNF4α, Pdx1 and Pax4, which are known to be important in β-cell differentiation and function are absent from the α-TC19 cell line (Figure 6.3A). These properties make these transcription factors possible candidates for the master switch that regulates the conversion from an α- to a β-cell phenotype. To test this, we infected α-TC19 cells with adenoviral vectors containing either HNF4α, Pdx1 or Pax4 under the experimental conditions determined utilising the Ad-RSV-GFP adenovirus (6.2.2). RT-PCR analysis showed that following 7 days in culture, cells ectopically expressing the Pax4 transgene had no change in the levels of pancreatic endocrine marker expression, ectopic expression of the Pdx1 transgene caused a weak increase in the expression of insulin1 and insulin2, whilst the most dramatic change in gene expression was observed with ectopic expression of the HNF4α transgene which weakly induced insulin1 and significantly induced expression of insulin2 and pancreatic polypeptide (PP) expression. However mRNA levels of α-cell specific hormone glucagon appeared unchanged for all conditions (Figure 6.5).

### 6.2.4 Over-expression of HNF4α in the α-TC19 cell line promotes conversion to a β-cell like phenotype

Initial investigation into the ectopic expression of HNF4α in the α-TC19 cell line was based on analysis of the transcript levels of expression, and revealed up-regulation of insulin1 and insulin2 (6.2.3). However RT-PCR data is limited as it does not take into account the expression levels of the functional protein or identify the proportion of cells expressing specific markers. Therefore our initial observation was followed up by complementary immunofluorescent staining with the available antibodies. Cells expressing the HNF4α transgene did not express the α-cell marker glucagon (Figure 6.6) suggesting that over-expression of HNF4α can suppress glucagon expression (and hence the α-cell phenotype). This result appeared inconsistent with the RT-PCR data showing no change in mRNA glucagon levels following ectopic expression of HNF4α (Figure 6.5). It is hypothesised that this is due to a large number of un-infected cells remaining which are still expressing high levels of glucagon. As the RT-PCR is only semi-quantitative and represents the whole population the levels appear unchanged. We also found that a

subpopulation of glucagon negative HNF4α infected cells were positive for insulin expression (Figure 6.7). In addition to insulin expression, over-expression of HNF4α in α-TC19 cells induced the mRNA expression of other β-cell markers Glucose transporter 2 (GLUT 2) and Glucokinase (GCK) (Figure 6.8A) which are important indirect glucose sensors involved in β-cell glucose stimulated insulin secretion. Immunofluorescent staining confirmed the induction of GLUT2 in HNF4α infected cells (Figure 6.8C).

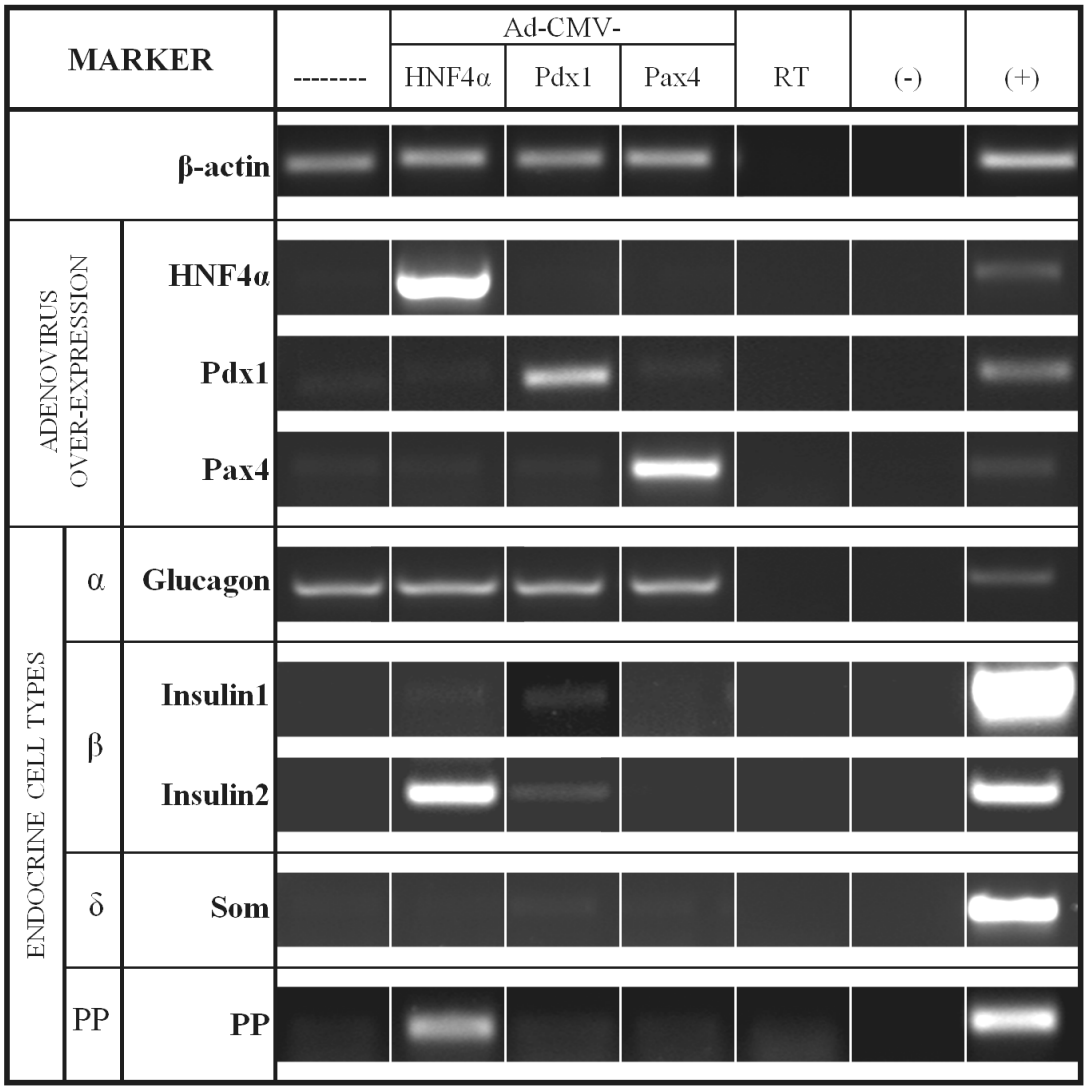



**Figure 6.5: RT-PCR analysis of over-expression of β-cell specific transcription factors in the α-TC19 cell line.** α-TC19 cells were infected overnight with either Ad-CMV-HNF4α, Ad-CMV-Pdx1 or Ad-CMV-Pax4 at an MOI of 100 with 5μg/ml DEAE-dextran and cultured for 7 days. Controls included an un-infected control (--). Gene expression was analysed for over-expressed transcription factors and endocrine hormones. *Abbreviations*: Som, somatostatin; PP, pancreatic polypeptide; RT, no RT control; (-) negative control; (+), positive control.

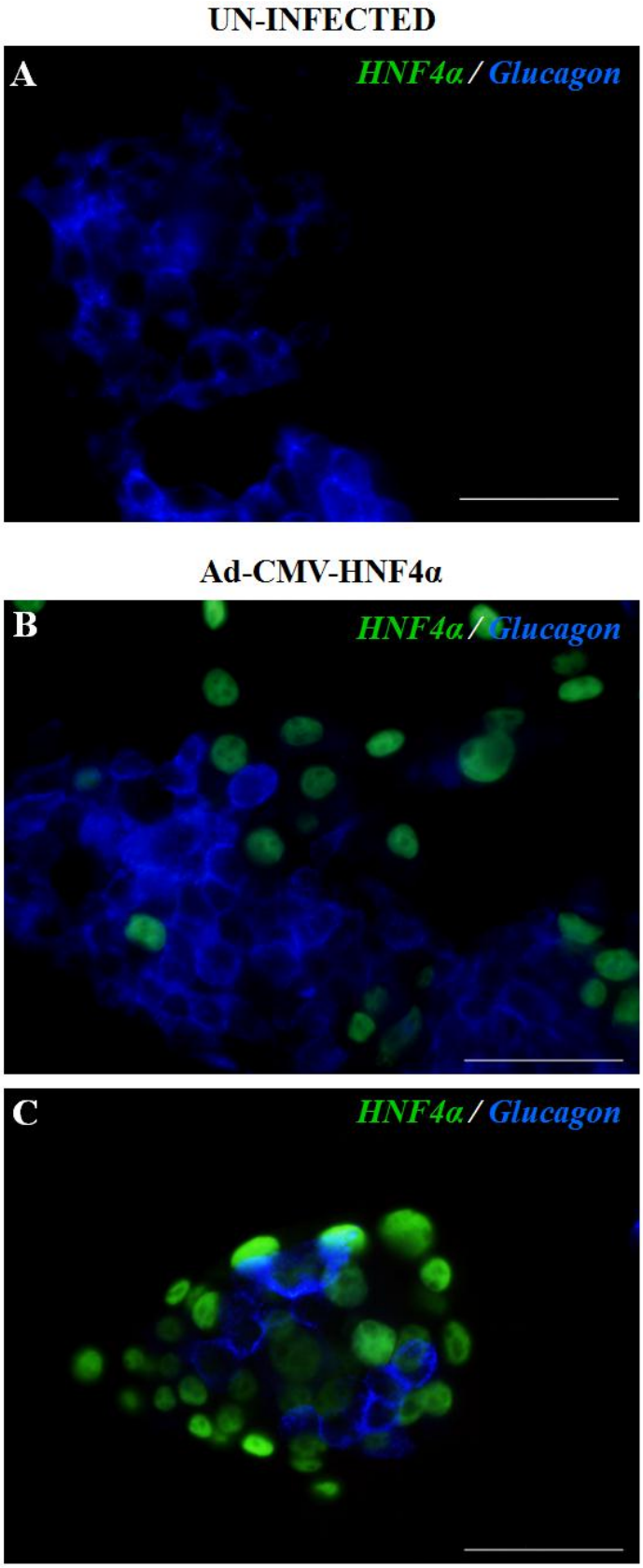



**Figure 6.6: Over-expression of HNF4α in α-TC19 cells suppresses glucagon expression.** α-TC19 cells were infected without **(A)** and with **(B, C)** Ad-CMV-HNF4α adenovirus (at an MOI of 100) with 5µg/ml DEAE-dextran and cultured for 7 days. The cells were then fixed and immunofluorescent stained for glucagon **(*blue*)** and HNF4α **(*green*)**. (scale bar =50µm ).

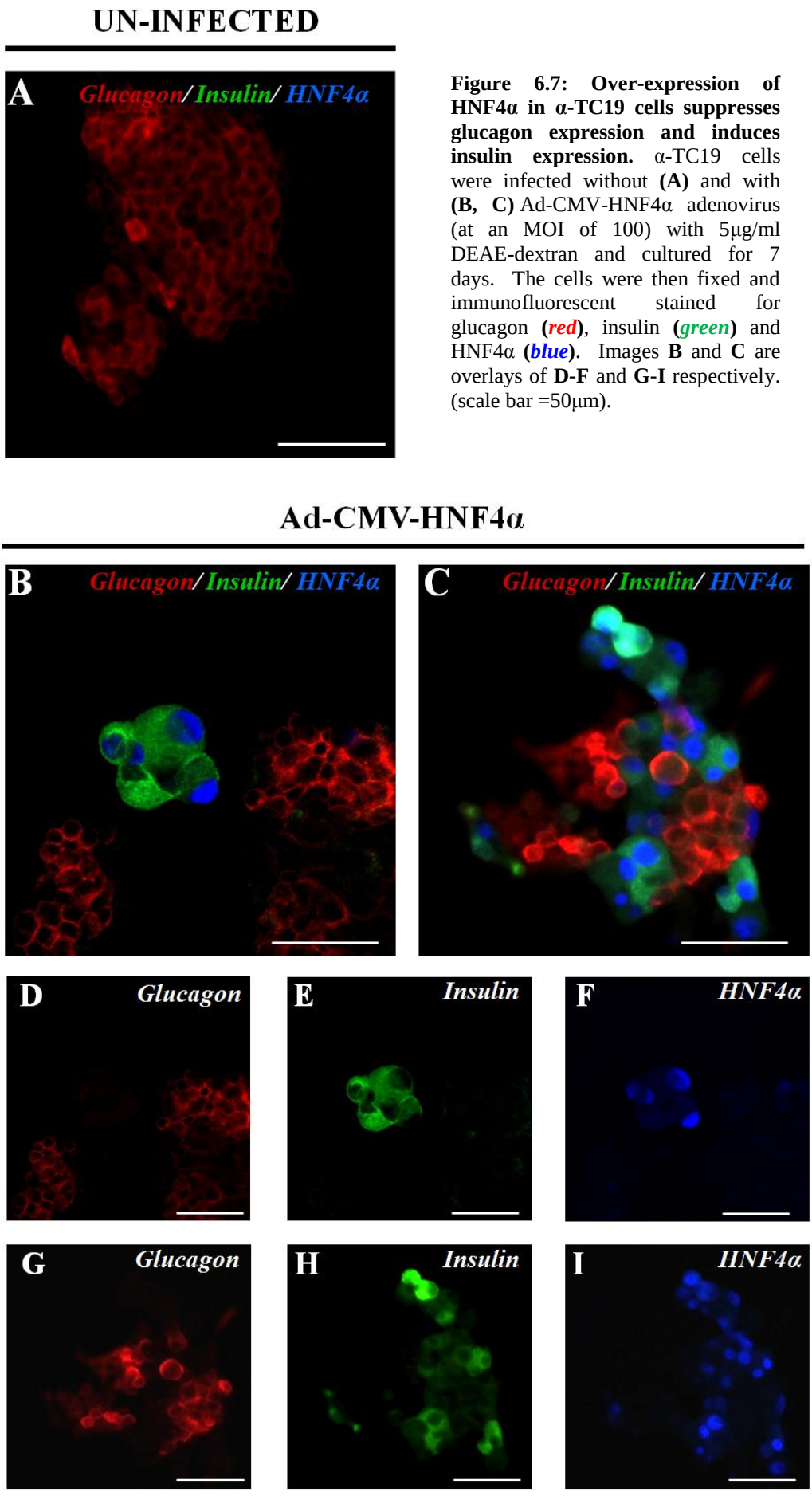
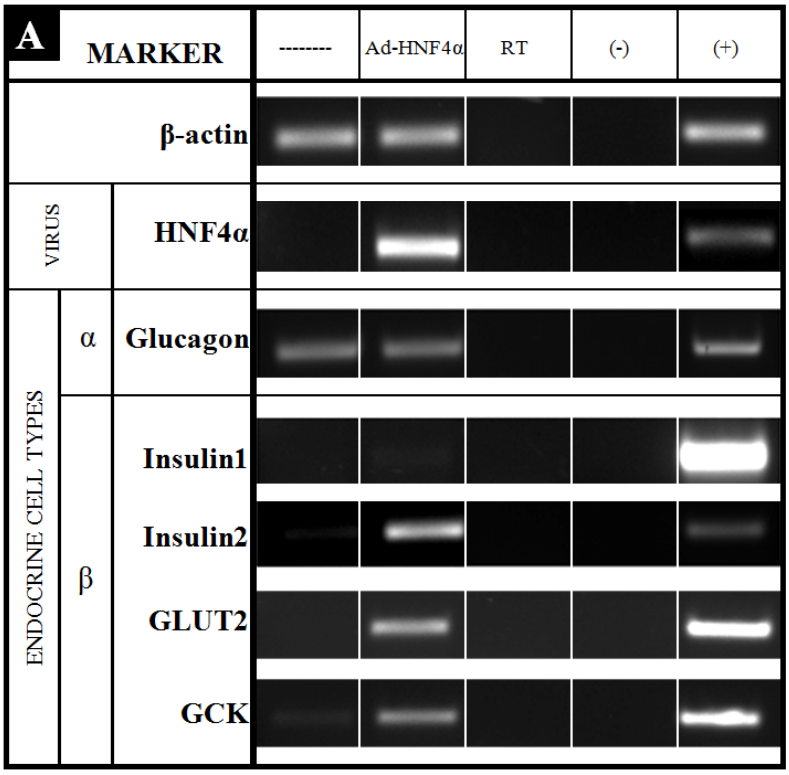



**Figure 6.7: Over-expression of HNF4α in α-TC19 cells suppresses glucagon expression and induces insulin expression.** α-TC19 cells were infected without **(A)** and with **(B, C)** Ad-CMV-HNF4α adenovirus (at an MOI of 100) with 5μg/ml DEAE-dextran and cultured for 7 days. The cells were then fixed and immunofluorescent stained for glucagon (***red***), insulin (***green***) and HNF4α (***blue***). Images **B** and **C** are overlays of **D-F** and **G-I** respectively. (scale bar =50μm).



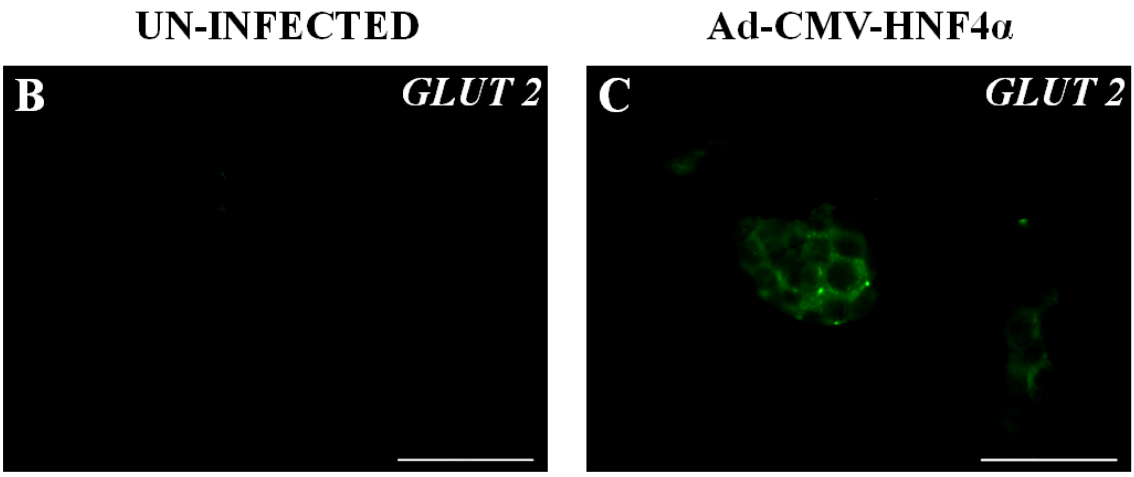
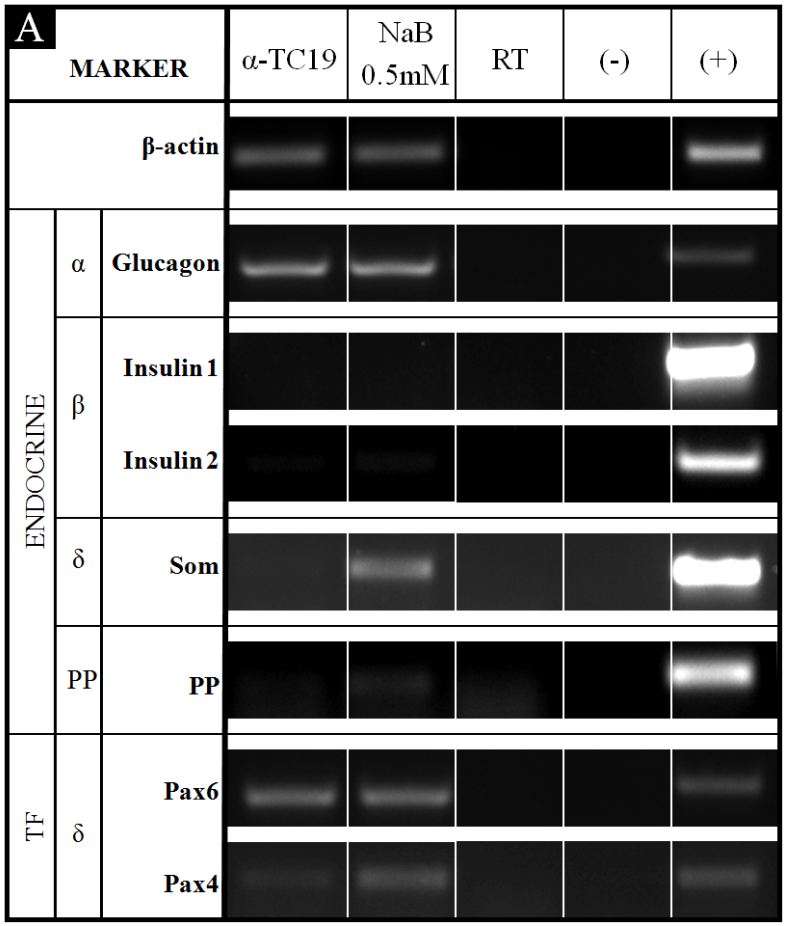




**Figure 6.8: Over-expression of HNF4α induces expression of genes important in β-cell glucose stimulated insulin secretion. (A)** RT-PCR analysis of α-TC19 cells infected with Ad-CMV-HNF4α (at an MOI of 100) with 5μg/ml DEAE-dextran and cultured for 7 days. α-TC19 cells are positive for β-cell specific hormone insulin (Insulin1/2), and markers for indirect glucose sensors GCK and GLUT2, which are involved in glucose stimulated insulin secretion. Immunofluorescent staining confirms α-TC19 infected cells **(C)** are GLUT2 positive compared to the un-infected control **(B)**. (scale bar = 50μm). *Abbreviations*: GCK, Glucokinase; GLUT2; Glucose Transporter 2; RT, no RT control; (-) negative control; (+), positive control.

### 6.2.5 Effect of HDAC inhibitors (HDACi) on the α-TC19 cell line

We were interested to see if changes in histone acetylation affect gene expression in the α-TC19 cell line. To investigate this possibility, we treated α-TC19 cells with 0.5mM NaB for 7 days in culture. RT-PCR analysis revealed that addition of this HDACi caused several changes in the gene expression mRNA levels in the α-TC19 cell line. NaB treatment appeared to cause no difference in the expression levels of glucagon mRNA, however treatment induced expression of the δ-cell specific hormone somatostatin and the transcription factor Pax4 (Figure 6.9A).

NaB inhibits both Class I and Class II HDACs, and treatment with a similar HDACi TSA had the same effect on mRNA levels as treatment with NaB. However the HDACi VPA (which preferentially only inhibits Class I HDACs) did not induce any changes in gene expression. All HDACi were tested at a range of concentrations showing the effects observed were dose dependent (Figure 6.8B). Concentrations above 1mM for NaB and VPA and 50nM for TSA, resulted in substantial cell death hence these concentrations were excluded from the results. From the original stocks NaB and VPA were diluted in water and TSA in DMSO, therefore water and DMSO controls were included in order to demonstrate that all effects observed were solely dependent on the action of the HDACi added.



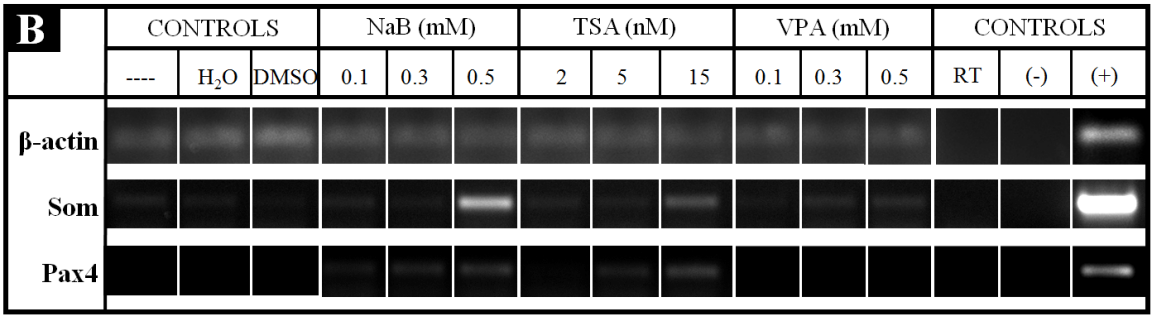




**Figure 6.9: Effect of HDAC inhibitors on gene expression in the α-TC19 cell line. (A)** RT-PCR analysis of α-TC19 cells treated with 5mM NaB for 7 days in culture. **(B)** RT-PCR analysis of α-TC19 cells treated with NaB, TSA or VPA for 7 days in culture at a range of concentrations. NaB and TSA treatment induced the expression of δ cell hormone somatostatin (Som) and transcription factor Pax4. α-TC19 cells treated with $H_2O$ and DMSO served as additional controls. *Abbreviations*: ---, untreated α-TC19 cells; $H_2O$, water; DMSO, Dimethyl Sulfoxide; NaB, Sodium Butyrate; TSA, Trichostatin A; VPA, Valporic Acid; Som, Somatostatin; PP, Pancreatic Polypeptide; TF, Transcription Factor; RT, no RT control; (-) negative control; (+) positive control.

## 6.3 Discussion

### 6.3.1 Characterisation of the α-TC19 cell line

We initially set out to characterise the phenotype of the α-TC19 cell line. From the characterisation of the murine α-TC19 cell line we identified only expression of the endocrine hormone glucagon and transcription factors important in α-cell specification (Nkx2.2, FoxA2, Arx, Pax6), thus revealing a phenotype consistent with that of a differentiated adult pancreatic α-cell (Figure 6.2B; Figure 6.3). The α-TC19 cell line was thus utilised in subsequent adenoviral infection experiments to investigate whether the over-expression of β-cell associated transcription factors could induce the conversion of α-cells into β-cells.

### 6.3.2 Enhancement of adenoviral infection of the α-TC19 cell line

Poor efficiency of adenoviral gene transfer to target cells is a major limitation to adenoviral gene therapy. Inefficient infection can occur for many reasons, for example the absence of the Coxsackie Adenovirus Receptor (CAR) on the cell surface, hence higher MOIs are required which can lead to unwanted cellular toxicity. Therefore an alternative approach to improve adenovirus transgene expression whilst minimising adverse reactions is to enhance adenoviral entry with polycationic compounds (Bonsted, et al., 2004). *In vitro* incubation of epithelial cells with a polycation such as Diethylaminoethyl (DEAE)-dextran, has been shown to enhance adenovirus infection efficiency (Clark, et al., 1999). This is consistent with results showing the presence of 5μg/ml DEAE-dextran during adenoviral adsorption increases infection efficiency of the α-TC19 cell line (Figure 6.4). It has been hypothesised that the epithelial cell membrane glycoconjugates contain sialic acid residues, thereby conferring a negative charge on the cell surface that contributes to impairment of adenovirus binding. Further investigation is required to elucidate the mechanism involved in DEAE-dextran enhancement of adenoviral infection, however several possible mechanisms have been suggested: (i) neutralisation of the cell surface negative charge (ii) facilitation of binding of the viral capsid via the fibre protein knob or an alternative protein, and (iii) increase in the target cell membrane permeability (Figure 6.10) (Arcasoy, et al., 1997).

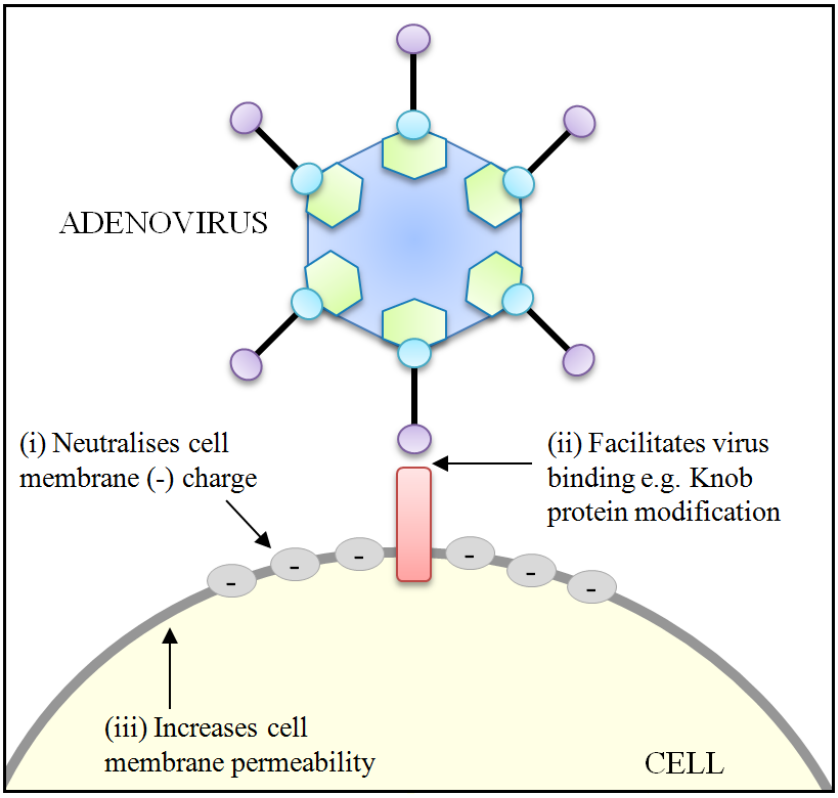



**Figure 6.10: Potential mechanisms involved in DEAE-dextran enhancement of adenoviral infection**

### 6.3.3 Ectopic expression of Pax4 in the α-TC19 cell line does not alter the α-cell phenotype

Pax4 is an important transcription factor in β-cell fate determination during pancreatic development. Pax4 knockout mice display a reduction in β- and δ-cells and an increase in α-cells (SosaPineda, et al., 1997). Ectopic expression of Pax4 in the α-TC19 cell line had no effect on gene expression. This is in contrast to previous studies by *Collombat et al.,* that showed the conditional ectopic expression of Pax4 in embryonic endocrine progenitor cells, as well as mature α-cells could induce their conversion into β-cells *in vivo* (Collombat, et al., 2009). This discrepancy between our results and the published results could be due to these experiments being performed *in vitro* instead of *in vivo*. This suggests that additional factors (other than Pax4) may contribute to the conversion of α-cells to β-cells. Also a combination of transcription factors may be required, for example Pdx1-VP16 expression alone could only induce hepatic cell transdifferentiation into pancreatic precursor cells. These cells failed to become glucose-sensitive mature insulin-producing cells with a gene expression profile similar to the rat insulinoma cell line (INS-1), without the additional activation of the pancreatic

transcription factor Pax4, which promotes late-stage β-cell differentiation and maturation (Tang, et al., 2006).

### 6.3.4 Ectopic expression of Pdx1 in the α-TC19 cell line induces weak insulin expression

Ectopic expression of Pdx1 in the α-TC19 cell line induced weak expression of both insulin1 and insulin2 mRNA (Figure 6.5). Transformation of α-cells using adenoviral transduced Pdx1 is not a new area of research. Pdx1 has been demonstrated as a potent transcriptional regulator of endogenous insulin gene expression in α-TC1 cells (Watada, et al., 1996). Control of insulin gene expression is largely exerted at the transcriptional level through well defined elements located within the promoter region that bind to β-cell restricted as well as ubiquitous transcription factors (Chakrabarti and Mirmira, 2003). Pdx1 can directly activate β-cell specific insulin gene expression, as Pdx1 binds to the A box enhancer element of the insulin promoter (Cerf, 2006), and chromatin immuno-precipitation assays in Ad-Pdx1 α-TC1 cells have demonstrated Pdx1 occupancy and the hyper-acetylation of histone H4 in the insulin promoter region (Wang, et al., 2007).

### 6.3.5 Ectopic Expression of HNF4α in the α-TC19 cell line induces a β-cell like phenotype

HNF4α has not been used previously to induce the reprogramming of adult differentiated α-cells into pancreatic β-like-cells. Glucagon mRNA levels appeared unchanged whilst glucagon protein expression was suppressed in HNF4α over-expressing α-TC19 cells (Figure 6.7). The mechanism involved in α-cell HNF4α related glucagon suppression remains to be elucidated, however it is possible HNF4α may directly/ indirectly interfere with the translation of glucagon into a functional protein. Alternatively HNF4α may directly/ indirectly interfere with the transcription of the glucagon gene, highlighting a limitation of semi-quantitative RT-PCR method, as following infection a number of un-infected cells still remain abundantly expressing glucagon, hence any minor changes in glucagon mRNA levels were un-detectable. Ectopic expression of HNF4α was capable of inducing high levels of insulin2 mRNA and insulin protein (Figure 6.5; Figure 6.8). Rat and mouse insulin1 and insulin 2 promoters contain a consensus binding site for

HNF4α (5′ACGGCAAAGTCC) located between nucleotides −69 and −57 (Hay and Docherty, 2006). *Bartoov-Shifman et al.*, have shown the direct activation of the rat insulin gene promoter by HNF4α, as computational analysis revealed potential HNF4α binding sites within the insulin promoter, which when mutated led to a 50-75% reduction in insulin expression in the transfected INS-1 β-cells (Bartoov-Shifman, et al., 2002). One of the most important paracrine mechanisms responsible for inhibiting glucagon release is conducted by insulin negatively regulating glucagon at the transcriptional level. Studies indicate that insulin, in a dose dependent fashion decreases steady-state glucagon mRNA levels in a clonal hamster glucagon producing cell line, InR1G9 (Philippe, 1989). G3, a DNA control element located in the 5'-flanking sequence of the rat glucagon gene mediates the inhibition of transcription, which occurs in response to insulin (Philippe, et al., 1995). This is consistent with studies showing that in α-cell specific insulin receptor knockout mice and siRNA-mediated knockdown of the insulin receptor in glucagon-secreting InR1G cells promotes enhanced glucagon secretion in response to L-arginine (Kawamori, et al., 2009). Specifically regarding the α-TC1 cell line, recent studies utilising α-TC1 clone 6 (i.e. α-TC16) have shown 1 nmol/L insulin concentrations slightly inhibit glucagon secretion (Shen, et al., 2012). Therefore it can be postulated that HNF4α induced insulin expression may be involved in the observed suppression of glucagon protein.

In addition to insulin, ectopic expression of HNF4α also induced high levels of GLUT2 and GCK gene expression (Figure 6.8), as well as yielding high levels of GLUT2 protein (Figure 6.7; Figure 6.8B). α-cells normally possess the high affinity, low capacity GLUT1, instead of the high capacity GLUT2 characteristic of β-cells (Gorus, et al., 1984, Heimberg, et al., 1995). Two steps are important in the control of β-cell insulin secretion: (i) the rate of glucose transport into the β-cell and (ii) the rate of glucose metabolism of which glycolysis represents a major pathway. HNF4α has been shown to regulate expression of pancreatic β-cell genes, including insulin and GLUT2, implicated in glucose metabolism and nutrient-induced insulin secretion (Wang, et al., 2000). This correlates with the ability of a mutation in the HNF4α gene on chromosome 20, to form early-onset Type 2 diabetes (MODY1), involving impaired expression of genes involved in glucose transport and glucose metabolism, such as GLUT2 and GCK respectively (Stoffel and Duncan, 1997). HNF4α infected α-TC19 cells remain negative for Pdx1 expression. Pdx1 controls not only the transcription of insulin but also expression of enzymes involved in its processing. Suppression of Pdx1 function in INS-1 cells does not alter glucose metabolism but rather inhibits insulin release by impairing steps distal to the generation of

mitochondrial coupling factors (Wang, et al., 2002a). Therefore although HNF4α induces the strong induction of insulin expression and other β-cell associated genes, gene expression does not represent function (i.e. glucose stimulated insulin secretion), hence the expression of other transcription factors such as Pdx1 or Pax4 may also be required in order to obtain fully functional β-cells.

It is worth noting that ectopic expression of HNF4α in the α-TC19 cell line also induced the expression of Pancreatic Polypeptide (PP) mRNA (Figure 6.5). In the mature pancreas HNF4α has been shown to be expressed in insulin positive β-cells and PP-cells (Nammo, et al., 2008). A mutation in the HNF4α/MODY1 gene also correlates with reduced PP secretion in response to hypoglycaemia (Ilag, et al., 2000). It is widely accepted that the production of insulin, glucagon, pancreatic polypeptide and somatostatin in islet cells is specific to β-, α-, PP- and δ-cells respectively. However a recent study has been conducted to determine if single mouse β-cells co-express multiple islet hormone genes. *Katsuta et al.,* utilised transgenic mice with GFP driven by mouse β-cell insulin1 promoter, to demonstrate that 60-80% embryonic and neonatal β-cells and 29% adult β-cells co-express PP and insulin genes (Katsuta, et al., 2010). It was hypothesised this co-expression may be due to residual PP expression from β-cell precursors, consistent with other previous studies in a variety of species showing early progenitor endocrine cells are multi-potent hence co-activate more than one islet hormone gene (Chiang and Melton, 2003, Teitelman, et al., 1993). An alternative explanation is that co-expression of insulin and PP (particularly in the adult β-cells) may be unrelated to development, but related to the idea of adult β-cell functional heterogeneity, due to some environmental factor eliminating β-cell gene expression restraints. *Herrera et al.,* showed the selective ablation of cells expressing the PP gene in transgenic mouse embryos utilising PP promoter-targeted expression of the toxigene diphtheria toxin A, resulted in a highly signigicant decrease in the volume density (i.e. development) of insulin and somatostatin producing cells. This was in contrast to the embryos lacking glucagon- or insulin-containing cells which did not exhibit any alterations in the development of the non targeted islet cell types. These results suggest that PP-expressing cells are indispensable for the differentiation to insulin producing β-cells (Herrera, et al., 1994). *Herrera and colleagues* later also showed insulin β-cell progenitors but not glucagon α-cell progenitors, transcribe the PP gene, suggesting a cell-lineage relationship (Herrera, 2000). *In toto,* our data therefore supports the hypothesis that expression of PP mRNA may be a marker of nascent β-cells during HNF4α induced reprogramming of α-cells to β-cells.

### 6.3.6 Induction of somatostatin in the α-TC19 cell line following Class II HDAC inhibition

Interestingly treatment of α-TC19 cells with Trichostatin A (TSA) and Sodium Butyrate (NaB), two inhibitors of Class I and Class II HDACs, induced mRNA expression of δ-cell specific hormone somatostatin in a dose dependent manner. A result not replicated with Valporic Acid (VPA), which is a Class I HDAC specific inhibitor (Figure 6.9B). This result is consistent with previous studies showing in an *ex vivo* rat embryonic pancreas model VPA dramatically decreased β-/ δ-cell differentiation, whilst TSA and NaB enhanced the pool of β-/ δ-cells (Haumaitre, et al., 2008). In addition NaB has been shown to stimulate somatostatin production in two different cultured cell lines, rat insulinoma cell line RIN, as well as the HeLa cell line (Ciardiello, et al., 2000, Green and Shields, 1984). Pax4 has been shown to play an important part in β-/ δ-cell lineage specification during pancreatic development, with *Pax4*-deficient mice displaying a selective loss of β- and δ-cells with a proportional increase in α-cells (Collombat, et al., 2003). Induction of β-cell marker insulin was not observed in HDACi treated α-TC19 cells (Figure 6.9A), suggesting that an *in vitro* adult α-cell model may lack other important factors such as transcription factors (Pdx1 (6.3.4), HNF4α (6.3.5)) or cues from other cell types important for β-cell differentiation. RT-PCR analysis revealed that the induction of somatostatin expression in the α-TC19 cells following 7 days of TSA and NaB treatment was accompanied by strong Pax4 expression activation, a transcription factor absent in adult differentiated α-cells (Figure 6.9B). However the mechanism of induction of somatostatin expression via HDAC inhibition requires further investigation, as it may not be solely dependent on up-regulated Pax4 expression, as ectopic expression of Pax4 in the α-TC19 cell line was insufficient to induce somatostatin expression (Figure 6.5). *Lenoir et al.,* recently identified Class II HDAC4 and HDAC5 as important in the specific control of β-cell and δ-cell mass, as HDAC4 and HDAC5 were restrictively expressed in δ-cells and loss of function experiments showed an increase in δ-cell mass (Lenoir, et al., 2011). This is supported by the present data, given the HDAC Class inhibition specificities of VPA and of TSA and NaB. Therefore i*n toto,* we propose that in α-cells Class II HDAC may possess a role in Pax4 inhibition, which prevents the reprogramming of α-cells to alternative pancreatic cell fates, such as somatostatin producing δ-cells.

# Chapter 7 Conclusions

## 7.1 Oval cell differentiation research (BMOL-TAT1.1 cell line)

One of the cell types most in demand for therapeutic purposes is the hepatocyte. This is because liver diseases are associated with a marked reduction in the viable mass of hepatocytes. The most severe cases of liver disease (liver failure) are treated by orthotopic liver transplantation or alternatively by hepatocyte transplantation, but the major problem to overcome is the shortage of organ donors. Oval cells (i.e. hepatic progenitor cells) have been proposed as an alternative source of cells, with their therapeutic potential lying in their ability to proliferate and differentiate into hepatocytes and other hepatic and non-hepatic cell types. However, using oval cells as a cell therapy cannot be exploited fully until the mechanisms governing hepatocyte differentiation are elucidated. Figure 7.1 summarises the main findings obtained from the BMOL-TAT1.1 research carried out during the progress of my PhD.

Chapter 3 detailed the in depth characterisation of the BMOL-TAT1.1 oval cell line, revealing a heterogeneous cell population consisting of two morphologically distinct cell types expressing a repertoire of hepatocyte and cholangiocyte markers. Large BMOL-TAT1.1 were identified as positive for A6, HNF4α and E-cadherin, whilst small BMOL-TAT1.1 cells were identified as positive for OV6, Sox9 and Vimentin (3.2.1). Due to the multi-potent capability of oval cells, heterogeneity of an oval cell line is a common phenomenon. *Radaeva and Steinberg* observed a similar heterogeneity in two different oval cell lines OC/CDE 6 and OC/CDE 22 (Radaeva and Steinberg, 1995). In the present study constitutive treatment with IGFII, insulin and EGF to promote oval cell proliferation resulted in enrichment of the small BMOL-TAT1.1 cell type, which was identified to express an array of cholangiocyte associated markers (3.2.1 and 4.2.1). Investigation into the heterogeneity of the BMOL-TAT1.1 cell line could be extended beyond gene and protein expression by Transmission Electron Microscopy in order to analyse the ultra-structure detail of the two distinctive morphologies. The striking difference in β-catenin cellular localisation observed allowed hypothesis of the potential mechanism involved in distinguishing the two BMOL-TAT1.1 cell types, as it is postulated that the proliferation conditions promote EGFR signalling, which subsequently activates Wnt/β-catenin signalling pathway, resulting in β-catenin nuclear accumulation (i.e. as observed in the

small BMOL-TAT1.1 cells) and altered gene expression. β-catenin localisation was identified by immunofluorescent staining but further more quantitative approaches were considered such as western blotting of protein extracted from the nuclear and cytosolic cell lysate/ fractions of treated and un-treated BMOL-TAT1.1 cells. Although this technique is limited as is prevents revelation of β-catenin localisation to a specific cell type. In order to confirm that the increase in small BMOL-TAT1.1 cells and associated β-catenin activation was due to the specific effect of EGF treatment, we could inhibit the EGF signalling pathway or Wnt/ β-catenin signalling pathway from day 1 of EGF treatment. There are two major classes of EGFR targeted inhibitors available. Tyrosine kinase inhibitors are available such as gefitinib (ZD1839), erlotinib (OSI-774), or AG1478, which competitively bind to the ATP pocket of EGFR to inhibit its activity (Ciardiello, et al., 2000,Grunwald and Hidalgo, 2003, Han, et al., 1996). Monoclonal antibodies (mAb) against EGFR such as mAb 528 and C225 (cetuximab) competitively inhibit ligand binding and thereby prevent receptor activation (Gill, et al., 1984, Masui, et al., 1984). There is also an extensive list of compounds that can inhibit the Wnt/ β-catenin pathway at different levels. Endogenous secreted Wnt antagonist Dickkopfs (Dkks), such as Dkk-1 and Dkk-4 bind to the Wnt receptor to inhibit its activation, whilst secreted Frizzled-related proteins (sFRPs), such as sFRP2, sFRP3 and Wnt inhibitory factor-1 (WIF-1) bind to Wnt proteins (Kawano and Kypta, 2003).

It remains to be elucidated if the differences in the BMOL-TAT1.1 cell phenotype are due to (i) large and small BMOL-TAT1.1 cells arising from a common precursor with EGFR and Wnt/β-catenin signalling enhanced proliferation of predominantly the small BMOL-TAT1.1 cells or (ii) EGFR and Wnt/β-catenin signalling promotes transformation of the large BMOL-TAT1.1 cells into the small BMOL-TAT1.1 cells. Analysis of the BMOL-TAT1.1 cell line does not support the second hypothesis, as the cell sub-types grow as distinct colonies in culture instead of mixed colonies and there are no cells displaying a transitional state present in the cultures. In order to confirm whether small BMOL-TAT1.1 cells arise from the transformation of the large BMOL-TAT1.1 cells, a cloning approach could be used in order to remove heterogeneity from the proliferating BMOL-TAT1.1 cell population via isolation of single cells. Although review of the literature proves this method may not be applicable to oval cells, as following cloning, the progeny of oval cells have a mixed morphology, as when daughter clones were cultured in differentiating medium, their characteristic bi-potential state was shown to be inheritable (Strick-Marchand and Weiss, 2002).

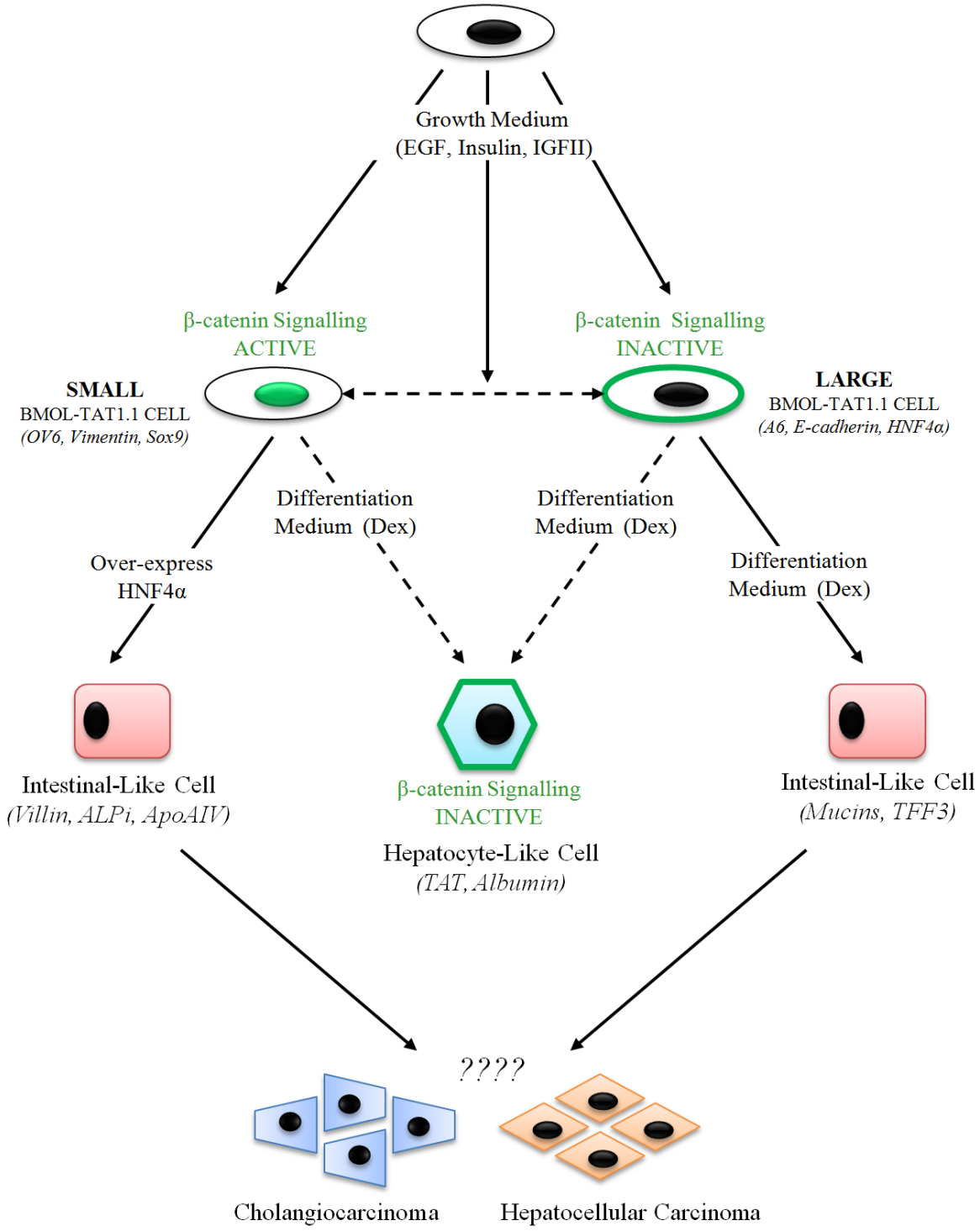



**Figure 7.1: Schematic representation of the main findings from the BMOL-TAT1.1 research.**

The overall aim of Chapter 4 and Chapter 5 was to investigate and therefore better understand the precise conditions and potential transcriptional mechanisms governing inducible oval cell differentiation in the BMOL-TAT1.1 cell line. This utilised our current optimised *in vitro* hepatic differentiation protocol involving treatment for 3 days with

growth medium (EGF, Insulin, IGFII) followed by 14 days in differentiation medium (dexamethasone, nicotinamide, EGF, ITS). Knowledge obtained could have significant implications in liver regeneration and associated liver pathologies, thus application in technologies for treatment of liver diseases, such as liver bioengineering.

The liver has a multitude of functions including the synthesis and secretion of serum proteins (Morgan and Peters, 1971), regulation of carbohydrate metabolism (Mithieux, 1997) and control of cholesterol homeostasis. Hepatocytes also mediate detoxification through activation of Phase I and Phase II enzymatic pathways. Following inducible hepatic differentiation BMOL-TAT1.1 cells expressed mature hepatocyte markers, such as TAT and Albumin, and appeared PAS positive suggesting the ability to store glycogen. However the data does not provide conclusive evidence for full hepatocyte phenotype and function (Christoffels, et al., 1998, Nitsch, et al., 1993). Therefore further work should determine if the hepatocyte-like cells derived from BMOL-TAT1.1 cells express other mature hepatocyte markers and are able to exhibit more active indicators of hepatocyte function including: (i) Albumin synthesis and secretion measured with a mouse albumin ELISA based assay (ii) Ureagenesis, detection of urea secretion and arginase activity (Corraliza, et al., 1994, Meng, et al., 2004) and (iii) Appropriate response to xenobiotics (e.g. Phenobarbital induction of Phase I (cytochrome P450) and Phase II( testosterone/ 4-nitrophenol UGT) enzymes and ciprofibrate induction of catalase enzyme) (Burke, et al., 2006, Ritter, et al., 1999, Tosh, et al., 2002). Furthermore it is believed that the current hepatic differentiation protocol could be improved further. One approach might be to use our knowledge of the fact that functional hepatocytes have the ability to synthesise glucose from non-glucose precursors (gluconeogenesis). We propose that by placing differentiating hepatocyte-like cells into a new medium containing reduced levels of glucose (with pyruvate or oxaloacetate as substrates), only functional hepatocytes should survive based on their ability to synthesise their own glucose.

Elucidation of the mechanism of action of the extracellular growth factors included in the specialised ‘differentiation medium’ in BMOL-TAT1.1 cells is extrapolated from data collected utilising the *in vitro* model with addition of individual growth factors. However liver regeneration *in vivo* may entail complex interactions of growth factors in orchestrated processes which result in novel cellular responses not previously identified. Induction of hepatic differentiation and suppression of cholangiocyte differentiation of the BMOL-TAT1.1 cell line was solely dependent on dexamethasone treatment.

Glucocorticoid Receptor (GR) signalling activation blocked β-catenin signalling activation and was accompanied by loss of β-catenin nuclear localisation. This is consistent with over-expression of β-catenin blocking dexamethasone dependent transdifferentiation of pancreatic acinar cells to hepatocytes (Wallace, et al., 2010). More detailed experiments should be performed to dissect out the affect of dexamethasone on the β-catenin signalling pathway in the BMOL-TAT1.1 cell line.

Oval cell differentiation towards hepatocytes can be divided into two distinct phases, with *phase 1* involving extracellular factors (e.g. EGF, dexamethasone) stimulating signalling pathways (e.g. GR and β-catenin signalling) which subsequently induce expression of *phase 2* specific intracellular liver-enriched transcription factors responsible for regulating the expression of key mature liver proteins. Therefore an important aim of this research was to investigate the potential transcription factors required during inducible BMOL-TAT1.1 hepatic differentiation. C/EBPα and HNF4α have both been suggested in previous studies to be important in the onset of the hepatocyte differentiation program in oval cells (Nagy, et al., 1994, Suetsugu, et al., 2008). Induction of hepatic differentiation in the BMOL-TAT1.1 cell line by differentiation medium and specifically dexamethasone coincided with induced expression of C/EBPα. The small BMOL-TAT1.1 cells displaying a hepatocyte-like phenotype (i.e. TAT positive) showed no characteristic hepatocyte morphology and were negative for HNF4α expression. This correlates with HNF4α's known critical role in the development of the normal liver architecture, as the regulation of epithelial morphogenesis is crucial for correct liver function (Parviz, et al., 2003). Therefore C/EBPα and HNF4α were identified from initial BMOL-TAT1.1 differentiation studies as potential master transcription factor candidates. In order to test this hypothesis BMOL-TAT1.1 cells were cultured to a high passage in order to enrich the small BMOL-TAT1.1 cell type population and adenoviral mediated over-expression experiments performed. Ectopic expression of C/EBPα and HNF4α alone or in combination failed to induce any hepatocyte marker gene expression in the high passage small BMOL-TAT1.1 cells but also surprisingly culturing for these cells under the same inducible differentiation conditions (i.e. 3 days growth medium, 14 days differentiation medium) did not result in any signs of hepatic differentiation. There are several possible hypotheses that can be suggested to explain this result. Firstly hepatic differentiation may require the low passage heterogeneous cell environment hence displaying 'Community Effect', a term introduced by John Gurdon which denotes intra-territorial signalling amongst cells which constitute a particular tissue or progenitor field (Gurdon, 1988). For instance cell-interactions or

factors secreted by the large BMOL-TAT1.1 cells may be important in hepatic differentiation of the small BMOL-TAT1.1 cells. To test this hypothesis, high passage small BMOL-TAT1.1 cells should be cultured with an isolated homogenous cell population of low passage large BMOL-TAT1.1 cells, and hepatic differentiation monitored. A review by Erker and Grompe emphasises the importance of factors secreted by surrounding cells types, such as inflammatory, Kuppfer and hepatic stellate cells in stimulating oval cell proliferation and differentiation (Erker and Grompe, 2008). Indeed, differentiation of oval cells into mature hepatocytes has been shown to be induced by hepatic stellate cells (Chen, et al., 2009). Alternatively maintenance of the small BMOL-TAT1.1 cells in a prolonged proliferative state may have altered the competence of the cells for hepatic differentiation (maturation arrest) via specific changes in gene expression in particular transcription factor expression. For instance, analysis of the expression of the transcription factor FoxA2 in the high passage small BMOL-TAT1.1 cells revealed very weak expression of the transcription factor. FoxA2 is required for normal liver homeostasis in the adult liver, as approximately 43% of genes expressed in the liver are associated with FoxA2 binding (Wederell, et al., 2008). Therefore ectopic expression of additional transcription factors such as FoxA2 may be required for hepatic differentiation. *Sekiya and Suzuki* recently established the combined expression of HNF4α with FoxA2 is sufficient to convert other non-hepatic cell types such as embryonic and adult mouse fibroblasts into epithelial hepatocyte-like cells (Sekiya and Suzuki, 2011). Whilst it has also been identified that upon the sequential ectopic over-expression of the three transcription factors FoxA2, HNF4α and C/EBPα an expandable adult liver derived progenitor cell population (ALDPC) can be converted into an advanced mature hepatocyte phenotype (Iacob, et al., 2011).

*Iacob and colleagues* cloned the murine liver enriched transcription factors FoxA2, HNF4α and C/EBPα into lentiviral vectors (Iacob, et al., 2011). Ectopic over-expression studies on the BMOL-TAT1.1 could be improved by utilisation of lentivirus instead of adenovirus. Adenoviral vectors induce large quantities of the protein of interest but the duration of protein production is short (2–3 weeks). This is because adenoviral DNA does not integrate into the genome and hence is not replicated during cell division. Therefore the concern is that adenoviral transient transcription factor expression may be insufficient to induce hepatic differentiation in the BMOL-TAT1.1 cell line in a biologically stringent environment. Accordingly, there is interest in developing gene therapy strategies with vectors that can produce more prolonged transgene expression. Lentiviruses represent a

subgroup of retroviruses, and comprise a lipid enveloped RNA virus (virion) which surrounds an inner core (nucleocapsid) of two identical copies of the viral RNA genome, reverse transcriptase, integrase and protease (Howarth, et al., 2010). Their genomes are slightly more complicated, containing accessory genes that regulate viral gene expression, control the assembly of infectious particles, modulate viral replication in infected cells and contribute to the persistence of infection (Kay, et al., 2001). Lentiviruses have evolved several remarkable features and advantages over adenoviral vectors in that they can stably transduce non-dividing cells (Naldini, et al., 1996); *integrase* integrates the DNA into the target host cell genome to enable long lasting stable gene expression (weeks to months); and also transfection shows little or no cell toxicity, hence do not elicit any detectable immune or inflammatory response. For safety reasons lentiviral vectors never carry the genes required for their replication hence production involves the removal of all viral genes, except those required in order to complete a single round of replication. All other components are supplied *in trans* from transient co-transfection of a stable packaging cell line such as HEK 293 with several plasmids. The lentiviral vector particles are produced and released into the media by the budding process, and are then purified and concentrated by ultracentrifugation for use in experiments.

Although it is now accepted that the function of oval cell activation during chronic liver injury is to facilitate liver regeneration, years of experimental evidence has exposed a second, seemingly paradoxical role for these cells during liver injury, carcinogenesis (Knight, et al., 2005). There is a strong correlation between oval cell appearance and the early stages of hepatocellular carcinoma (HCC) and cholangiocarcinoma (Alison and Lovell, 2005, Hacker, et al., 1992). Expression of Villin, the best characterised microfilament-associated, actin-binding protein typical of brush-border microvilli in intestinal enterocytes (Robine, et al., 1985), is frequently observed in HCC (Karabork, et al., 2010, Moll, et al., 1987). Ectopic expression of HNF4α in the high passage small BMOL-TAT1.1 cells induced the potent expression of Villin (5.2.2). Transmission Electron Microscopy would be beneficial to analyse the ultra-structure detail in the HNF4α infected cells, and identify if these cells exhibit microvilli, which may have implications regarding cell function. Although the BMOL-TAT1.1 cell line has been shown to be non-tumorogeneic in a nude mouse assay, further work is required to validate whether transplantation of the modified HNF4α and Villin positive BMOL-TAT1.1 cells contributes to development of HCC in an *in vivo* mouse model. Induction of several liver markers in the low passage heterogeneous BMOL-TAT1.1 cell line following culture with

differentiation medium and in particular dexamethasone was accompanied by potent induction of Mucin2, Mucin5ac and TFF3 expression. Increased expression of MUC5AC has recently been reported to be a highly specific tumour-associated mucin in cholangiocarcinoma (Mall, et al., 2010, Matull, et al., 2008) and hepatolithiasis, which is regarded as a model disease in cholangiocarcinogenesis arising in chronic inflammatory conditions, in which mucin is an important factor in the pathogenesis (Sasaki, et al., 2005, Sasaki, et al., 1998, Yamashita, et al., 1993). Further work could extend analysis of the differentiated BMOL-TAT1.1 cells to look at the expression levels of a wider repertoire of mucins (Muc6, and Muc5b) and TFFs (TFF1) and also measure synthesis and secretion with a mouse mucin ELISA based assay. To validate if it is the large BMOL-TAT1.1 cells which are expressing the mucins and TFF3, Fluorescent activated cell sorting (FACS) or Magnetic activated cell sorting (MACS) could be utilised due to the reciprocal expression of cell surface markers DBA and PNA in order to isolate homogenous small and large BMOL-TAT1.1 cell type populations without compromising cell integrity. For example cells labelled with the FITC–conjugated PNA or FITC-conjugated DBA antibodies could be sorted using the MiniMACS system. Again long term future work could involve transplantation of isolated mucin and TFF positive BMOL-TAT1.1 cells into an *in vivo* mouse model, to determine if they contribute to cholangiocarcinoma. *In toto* this research highlights the major advantage of *in vitro* transformation of cells providing greater quality control, as cells can be specifically selected for transplantation which are not potentially carcinogenic.

An overall limitation of studies involving a specific oval cell line (i.e. BMOL-TAT1.1) is that it is not phenotypically identical to other oval cell lines established in different laboratories, hence results cannot be generalized. Therefore in order to ultimately validate results, experiments should be replicated in other available oval cell lines, primary oval cells and oval cells from other species preferably humans.

## 7.2 α-cell reprogramming research (α-TC19 cell line)

One of the cell types most in demand for therapeutic purposes is the pancreatic β-cell. This is because Type 1 diabetes is a major healthcare problem in the world. Type 1 diabetes can be treated by islet transplantation, but the major limitation is the shortage of organ donors. To overcome the shortfall in donors, alternative sources of pancreatic β-

cells must be found. There is now a startling new addition to this list of sources: the pancreatic α-cell. *Thorel and colleagues* recently showed that under circumstances of extreme pancreatic β-cell loss, during pancreas regeneration α-cells may serve to transdifferentiate and replenish the insulin-producing compartment. Chapter 6 focused on understanding the molecular basis behind α- to β-cell transdifferentiation utilising a murine adult pancreatic α-cell line (α-TC19). This entailed initial characterisation of the α-TC19 cell line and subsequent experiments to investigate the effect of chromatin modification (i.e. histone hyper-acetylation) and ectopic expression of β-cell specific transcription factors on α-cell reprogramming. The knowledge obtained from this research may help enhance the generation of β-cells for the treatment of Type 1 diabetes.

Chromatin modification via histone acetylation has been shown to be important in determination of pancreatic cell fate. Inhibition of Class II HDACs (i.e. chromatin histone hyper-acetylation) has direct effects on endocrine cell fate regulation, promoting δ-cell somatostatin expression but not β-cell insulin expression in the adult α-TC19 cell line, possibly via a Pax4-dependent mechanism. Unfortunately, no Class II specific HDACi were easily available during the commencement of this research in order to test this hypothesis. However a selective Class IIa HDAC inhibitor MC1568 has since been identified in the literature, which could be employed in future work (Lenoir, et al., 2011). Also to support the hypothesis it will also be important to determine if the effect of Class II HDAC inhibition is transient and thus would be reversed on withdrawal of the treatment (Haumaitre, et al., 2008). The precise role of somatostatin in islet function is unclear and currently studies are in progress with a somatostatin gene knock-out mouse model in order to investigate the role of somatostatin in islet development and the regulation of insulin secretion, as it has been shown somatostatin possesses potent anti-secretory activity, directly inhibiting insulin synthesis and release (Philippe, 1993). *In toto* the knowledge regarding the impact of chromatin modification on endocrine fate regulation should be considered for enhancement of α- to β-cell reprogramming for therapeutic purposes.

Chapter 6 identified that HNF4α may have the potential to promote the reprogramming of α-cells to β-cells. The mechanism of HNF4α reprogramming may involve suppression of glucagon expression and the induction of a phenotype resembling a β-cell. Given that we also observe expression of pancreatic polypeptide (PP) the reprogramming may also occur through a PP β-cell progenitor state. HNF4α induced suppression of glucagon was detected at the protein level but not at the mRNA level, this

highlights that experiments could be repeated by Quantitative (QT)-PCR, as RT-PCR is limited as only semi-quantitative and hence as a proportion of cells are un-infected (HNF4α negative) minor changes in gene expression levels are undetectable. Additionally we also believe an element of further refinement to the *in vitro* reprogramming protocol utilising several methods will ensure the number of β-like cells is enriched and maximised. Firstly adenoviral vectors expressing HNF4α could be replaced with lentiviral vectors expressing HNF4α because of the advantages detailed above and this would also help address if HNF4α is required transiently or permanently during reprogramming of α-cells to a stable β-cell phenotype. Current demonstrations showing the reprogramming of pancreatic α-cells into pancreatic β-cells are all *in vivo*, therefore a long term goal based on this research would be to investigate the ability of HNF4α to drive reprogramming of mature adult α-cells *in vivo*. Transgenic mice conditionally expressing HNF4α in glucagon producing α-cells utilising the Cre-ER/loxP system (i.e. glucagon-Cre-ER and loxP-stop-loxP-HNF4α mice) could be generated for studies. Extracellular growth factors have been reported to exert a stimulatory effect on β-cell replication *in vivo* (Bouwens and Rooman, 2005, Garcia-Ocana, et al., 2000, Soria, 2001). For example HGF and betacellulin can convert pancreatic acinar cells (AR42J cell line) to β-cells (Mashima, et al., 1996a, Mashima, et al., 1996b) and in α-TC16 cells Pdx1 expression in combination with betacellulin treatment, induces expression of several key β-cell markers (Watada, et al., 1996). Therefore the effect of addition of extracellular growth factors including betacellulin, IGF-II, HGF, EGF and nicotinamide on HNF4α induced α- to β-cell reprogramming and β-cell maturation should also be investigated (Cho, et al., 2008, Tsaniras and Jones, 2010).

Functional characterisation will also need to be carried out on the nascent β-cells in order to assess quantification of hormone content (e.g. ELISA hormone secretion assays) and monitor glucose homeostasis, including demonstration and measurement of dynamic biphasic insulin release upon glucose challenge. Functional characterisation and certainly from the point of view of application for treatment, a more homogeneous population of generated β-cells using a functional selection strategy would be desirable. This may involve strategies to: (i) isolate the β-cell population e.g. Fluorescent Activated Cell Sorting (FACS) using the β-cell specific surface marker GLUT2 or (ii) remove the remaining α-cell population e.g. cell trapping by stably tranfecting the α-TC19 cells with a construct of a drug- resistance gene downstream of a promoter that is only active in β-cells, for example the promoter of the insulin gene. Therefore following the reprogramming

protocol generated β-cells could be selected by incubation with the chosen drug which only the β-cells would be resistant to.

Importantly, we will need to determine whether the nascent β-cells produced from the conditional expression of HNF4α in glucagon producing α-cells are capable of restoring functional β-cell mass and normoglycaemia in mice where diabetes has been induced by administration of the β-cell toxin streptozotocin. Streptozotocin is an antibiotic that can cause pancreatic β-cell destruction, so it is widely used experimentally as an agent capable of inducing Type 1 diabetes (Wu and Huan, 2008).

Unfortunately limitations of this research are that α-TC19 cells are from a clonal cell line derived from an adenoma, hence may not completely recapitulate an adult α-cell in phenotype and function e.g. response to glucose, insulin, L-arginine and Kainate. Future work will therefore be crucial to validate our results obtained; it is warranted to perform comparative experiments in primary murine α-cells. Also mice/ rodents are not always reliable as models for human disease and the scientific literature is littered with examples of differences between rodents and human. For instance the rat insulin 1 promoter has been shown to be activated directly by HNF4α, but in contrast, the HNF4α binding site does not exist in the human insulin promoter, and HNF4α fails to activate the gene in reporter assays (Matys, et al., 2003). There is also no evidence of any HNF4α binding sites in other insulin promoters, thus HNF4α transactivation may be unique to rodents and may not have a function in insulin regulation (Hay and Docherty, 2006), therefore comparative experiments to support this research would ideally need to be performed with human α-cells.

# References

Aberle H, Butz S, Stappert J, Weissig H, Kemler R, Hoschuetzky H (1994) Assembly of the cadherin catenin complex in-vitro with recombinant proteins. Journal of Cell Science 107:3655-3663

Ackermann AM, Gannon M (2007) Molecular regulation of pancreatic beta-cell mass development, maintenance, and expansion. Journal of Molecular Endocrinology 38:193-206

Akhurst B, Croager EJ, Farley-Roche CA, Ong JK, Dumble ML, Knight B, Yeoh GC (2001) A modified choline-deficient, ethionine-supplemented diet protocol effectively induces oval cells in mouse liver. Hepatology 34:519-522

Al-Adsani A, Burke ZD, Eberhard D, Lawrence KL, Shen C-N, Rustgi AK, Sakaue H, Farrant JM, Tosh D (2010) Dexamethasone treatment induces the reprogramming of pancreatic acinar cells to hepatocytes and ductal cells. PloS one 5:e13650

Alison MR, Lovell MJ (2005) Liver cancer: the role of stem cells. Cell Proliferation 38:407-421

Allen JW, Hassanein T, Bhatia SN (2001) Advances in bioartificial liver devices. Hepatology 34:447-455

Alpini G, Glaser S, Robertson W, Rodgers RED, Phinizy JL, Lasater J, Lesage GD (1997) Large but not small intrahepatic bile ducts are involved in secretin-regulated ductal bile secretion. American Journal of Physiology 272:G1064-G1074

Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, Jacquemin P, Pierreux CE, Clotman F, Lemaigre FP (2009) Intrahepatic Bile Ducts Develop According to a New Mode of Tubulogenesis Regulated by the Transcription Factor SOX9. Gastroenterology 136:2325-2333

Arcasoy SM, Latoche JD, Gondor M, Pitt BR, Pilewski JM (1997) Polycations increase the efficiency of adenovirus-mediated gene transfer to epithelial and endothelial cells in vitro. Gene Therapy 4:32-38

Archer A, Sauvaget D, Chauffeton V, Bouchet PE, Chambaz J, Pincon-Raymond M, Cardot P, Ribeiro A, Lacasa M (2005) Intestinal apolipoprotein A-IV gene transcription is controlled by two hormone-responsive elements: A role for hepatic nuclear factor-4 isoforms. Molecular Endocrinology 19:2320-2334

Arden C, Harbottle A, Baltrusch S, Tiedge M, Agius L (2004) Glucokinase is an integral component of the insulin granules in glucose-responsive insulin secretory cells and does not translocate during glucose stimulation. Diabetes 53:2346-2352

Arii S, Imamura M (2000) Physiological role of sinusoidal endothelial cells and Kupffer cells and their implication in the pathogenesis of liver injury. Journal of hepato-biliary-pancreatic surgery 7:40-48

Auth M-KH, Boost KA, Leckel K, Beecken W-D, Engl T, Jonas D, Oppermann E, Hilgard P, Markus BH, Bechstein W-O, Blaheta RA (2005) Controlled and reversible induction of differentiation and activation of adult human hepatocytes by a biphasic culture technique. World J Gastroenterol 11:2080-2087

Babeu JP, Darsigny M, Lussier CR, Boudreau F (2009) Hepatocyte nuclear factor 4 alpha contributes to an intestinal epithelial phenotype in vitro and plays a partial role in mouse intestinal epithelium differentiation. American Journal of Physiology-Gastrointestinal and Liver Physiology 297:G124-G134

Barbu A, Welsh N (2007) Lipofection of insulin-producing RINm5F cells: Methodological improvements. Journal of Liposome Research 17:49-62

Bardeesy N, DePinho RA (2002) Pancreatic cancer biology and genetics. Nature Reviews Cancer 2:897-909

Bartoov-Shifman R, Hertz R, Wang HY, Wollheim CB, Bar-Tana J, Walker MD (2002) Activation of the insulin gene promoter through a direct effect of hepatocyte nuclear factor 4 alpha. Journal of Biological Chemistry 277:25914-25919

Barut V, Sarraf CE (2009) Intestinal metaplasia in liver of rats after partial hepatectomy and treatment with acetylaminofluorene. Cell Proliferation 42:657-660

Batterham RL, Le Roux CW, Cohen MA, Park AJ, Ellis SM, Patterson M, Frost GS, Ghatei MA, Bloom SR (2003) Pancreatic polypeptide reduces appetite and food intake in humans. Journal of Clinical Endocrinology & Metabolism 88:3989-3992

Bergelson JM, Cunningham JA, Droguett G, KurtJones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW (1997) Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science 275:1320-1323

Besnard V, Wert SE, Hull WM, Whitsett JA (2004) Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. Gene Expression Patterns 5:193-208

Bett AJ, Haddara W, Prevec L, Graham FL (1994) An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early region-1 and region-3. Proceedings of the National Academy of Sciences of the United States of America 91:8802-8806

Blouin A, Bolender RP, Weibel ER (1977) Distribution of organelles and membranes between hepatocytes and non-hepatocytes in rat-liver parenchyma - stereological study. Journal of Cell Biology 72:441-455

Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, Wobus AM (2003) Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. Proceedings of the National Academy of Sciences of the United States of America 100:998-1003

Bonsted A, Engesaeter BO, Hogset A, Maelandsmo GM, Prasmickaite L, Kaalhus O, Berg K (2004) Transgene expression is increased by photochemically mediated transduction of polycation-complexed adenoviruses. Gene Therapy 11:152-160

Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, Ridgway RA, Seo SS, Spee B, Van Rooijen N, Sansom OJ, Iredale JP, Lowell S, Roskams T, Forbes SJ (2012) Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nature Medicine 18:572-579

Bouwens L, Debleser P, Vanderkerken K, Geerts B, Wisse E (1992) Liver-cell heterogeneity - functions of nonparenchymal cells. Enzyme 46:155-168

Bouwens L, Rooman I (2005) Regulation of pancreatic beta-cell mass. Physiological Reviews 85:1255-1270

Bouwens L, Wisse E (1992) Pit cells in the liver. Liver 12:3-9

Brissova M, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM, Powers AC (2005) Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. Journal of Histochemistry & Cytochemistry 53:1087-1097

Burke ZD, Shen CN, Ralphs KL, Tosh D (2006) Characterization of liver function in transdifferentiated hepatocytes. Journal of Cellular Physiology 206:147-159

Burke ZD, Tosh D (2006) The Wnt/beta-catenin pathway: master regulator of liver zonation? Bioessays 28:1072-1077

Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggrern PO, Caicedo A (2006) The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proceedings of the National Academy of Sciences of the United States of America 103:2334-2339

Cano DA, Rulifson IC, Heiser PW, Swigart LB, Pelengaris S, German M, Evan GI, Bluestone JA, Hebrok M (2008) Regulated beta-cell regeneration in the adult mouse pancreas. Diabetes 57:958-966

Cerec V, Glaise D, Garnier D, Morosan S, Turlin B, Drenou B, Gripon P, Kremsdorf D, Guguen-Guillouzo C, Corlu A (2007) Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. Hepatology 45:957-967

Cerf ME (2006) Transcription factors regulating beta-cell function. European Journal of Endocrinology 155:671-679

Chakrabarti SK, James JC, Mirmira RG (2002a) Genetic targeting in vivo and in vitro by the pancreas development factor, Pdx1. Faseb Journal 16:A1-A1

Chakrabarti SK, James JC, Mirmira RG (2002b) Quantitative assessment of gene targeting in vitro and in vivo by the pancreatic transcription factor, Pdx1. Importance of chromatin structure in directing promoter binding. Journal of Biological Chemistry 277:13286-13293

Chakrabarti SK, Mirmira RG (2003) Transcription factors direct the development and function of pancreatic beta cells. Trends in Endocrinology and Metabolism 14:78-84

Chen L, Chen X-P, Zhang W, Liang H-F, Lin Y-Z, Dong H-H, Zhou Q-D (2009) Differentiation of hepatic oval cell into mature hepatocyte induced by hepatic stellate cells. Zhonghua Gan Zang Bing Za Zhi 17:765-770

Chiang MK, Melton DA (2003) Single-cell transcript analysis of pancreas development. Developmental Cell 4:383-393

Chidgey AP, Layton D, Trounson A, Boyd RL (2008) Tolerance strategies for stem-cell-based therapies. Nature 453:330-337

Cho YM, Lim JM, Yoo DH, Kim JH, Chung SS, Park SG, Kim TH, Oh SK, Choi YM, Moon SY, Park KS, Lee HK (2008) Betacellulin and nicotinamide sustain PDX1 expression and induce pancreatic beta-cell differentiation in human embryonic stem cells. Biochemical and Biophysical Research Communications 366:129-134

Choi JK, Howe LJ (2009) Histone acetylation: truth of consequences? Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire 87:139-150

Christoffels VM, Grange T, Kaestner KH, Cole TJ, Darlington GJ, Croniger CM, Lamers WH (1998) Glucocorticoid receptor, C/EBP, HNF3, and protein kinase A coordinately activate the glucocorticoid response unit of the carbamoylphosphate synthetase I gene. Molecular and Cellular Biology 18:6305-6315

Ciardiello F, Caputo R, Pomatico G, Damiano V, Bianco R, Fontanini G, Cuccato S, De Placido S, Bianco AR, Tortora G (2000) Inhibition of growth factor production and angiogenesis in human cancer cell lines by ZD1839 (Iressa (TM)), an EGFR-selective tyrosine kinase inhibitor. Clinical Cancer Research 6:4542S-4542S

Cirillo LA, Lin FR, Cuesta I, Friedman D, Jarnik M, Zaret KS (2002) Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. Molecular Cell 9:279-289

Clark PR, Stopeck AT, Brailey JL, Wang Q, McArthur J, Finer MH, Hersh EM (1999) Polycations and cationic lipids enhance adenovirus transduction and transgene expression in tumor cells. Cancer Gene Therapy 6:437-446

Clotman F, Lannoy VJ, Reber M, Cereghini S, Cassiman D, Jacquemin P, Roskams T, Rousseau GG, Lemaigre FR (2002) The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 129:1819-1828

Coffinier C, Gresh L, Fiette L, Tronche F, Schutz W, Babinet C, Pontoglio M, Yaniv M, Barra J (2002) Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1 beta. Development 129:1829-1838

Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, Gradwohl G, Gruss P (2003) Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes & Development 17:2591-2603

Collombat P, Xu X, Ravassard P, Sosa-Pineda B, Dussaud S, Billestrup N, Madsen OD, Serup P, Heimberg H, Mansouri A (2009a) The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. Cell 138:449-462

Collombat P, Xu X, Ravassard P, Sosa-Pineda B, Dussaud S, Billestrup N, Madsen OD, Serup P, Heimberg H, Mansouri A (2009b) The Ectopic Expression of Pax4 in the Mouse Pancreas Converts Progenitor Cells into alpha and Subsequently beta Cells. Cell 138:449-462

Corraliza IM, Campo ML, Soler G, Modolell M (1994) Determination of arginase activity in macrophages - a micromethod. Journal of Immunological Methods 174:231-235

Costa RH, Kalinichenko VV, Holterman AXL, Wang XH (2003) Transcription factors in liver development, differentiation, and regeneration. Hepatology 38:1331-1347

Crosby HA, Hubscher SG, Joplin RE, Kelly DA, Strain AJ (1998) Immunolocalization of OV-6, a putative progenitor cell marker in human fetal and diseased pediatric liver. Hepatology 28:980-985

Dabeva MD, Hurston E, Shafritz DA (1995) Transcription factor and liver specific messenger RNA expression in facultative epithelial progenitor cells of liver and pancreas. American Journal of Pathology 147:1633-1648

Darlington GJ (1999) Molecular mechanisms of liver development and differentiation. Current Opinion in Cell Biology 11:678-682

Davie JR (2003) Inhibition of histone deacetylase activity by butyrate. Journal of Nutrition 133:2485S-2493S

Decaens T, Godard C, de Reynies A, Rickman DS, Tronche F, Couty J-P, Perret C, Colnot S (2008) Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. Hepatology 47:247-258

Dezaki K, Damdindorj B, Kakei M, Yada T (2010) Ghrelin is a physiological regulator of insulin release in islet beta-cells. Journal of Physiological Sciences 60:S62-S62

Diao JY, Asghar Z, Chan CB, Wheeler MB (2005) Glucose-regulated glucagon secretion requires insulin receptor expression in pancreatic alpha-cells. Journal of Biological Chemistry 280:33487-33496

Diehl AM (1998) Roles of CCAAT/Enhancer-binding proteins in regulation of liver regenerative growth. Journal of Biological Chemistry 273:30843-30846

Dolle L, Best J, Mei J, Al Battah F, Reynaert H, van Grunsven LA, Geerts A (2010) The quest for liver progenitor cells: A practical point of view. Journal of Hepatology 52:117-129

Dorrell C, Erker L, Lanxon-Cookson KM, Abraham SL, Victoroff T, Ro S, Canaday PS, Streeter PR, Grompe M (2008) Surface markers for the murine oval cell response. Hepatology 48:1282-1291

Dunne MJ (2000) Ions, genes and insulin release: from basic science to clinical disease. Diabetic Medicine 17:91-104

Eberhard D, Tosh D (2008) Transdifferentiation and metaplasia as a paradigm for understanding development and disease. Cellular and Molecular Life Sciences 65:33-40

Edlund H (2002) Pancreatic organogenesis - Developmental mechanisms and implications for therapy. Nature Reviews Genetics 3:524-532

Eguchi G, Kodama R (1993) Transdifferentiation. Current Opinion in Cell Biology 5:1023-1028

Erker L, Grompe M (2008) Signaling networks in hepatic oval cell activation. Stem Cell Research 1:90-102

Espanol-Suner R, Carpentier R, Van Hul N, Legry V, Achouri Y, Cordi S, Jacquemin P, Lemaigre F, Leclercq IA (2012) Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 12: 1244-1249.

Factor VM, Radaeva SA, Thorgeirsson SS (1994) Origin and fate of oval cells in dipin-induced hepatocarcinogenesis in the mouse. American Journal of Pathology 145:409-422

Fajans SS, Bell GI, Polonsky KS (2001) Mechanisms of disease: Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. New England Journal of Medicine 345:971-980

Farber E (1956) Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylamino-fluorene, and 3'-methyl-4-dimethylaminoazobenzene. Cancer Research 16:142-&

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. Nature Medicine 6:568-572

Finegood DT, Scaglia L, Bonnerweir S (1995) Dynamics of beta-cell mass in the growing rat pancreas - estimation with a simple mathematical-model. Diabetes 44:249-256

Flodby P, Antonson P, Barlow C, Blanck A, Porschhallstrom I, Xanthopoulos KG (1993) Differential patterns of expression of 3 c/ebp isoforms, hnf-1, and hnf-4 after partial-hepatectomy in rats. Experimental Cell Research 208:248-256

Friedman SL (2000) Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. Journal of Biological Chemistry 275:2247-2250

Friedman SL, Arthur MJP (1989) Activation of cultured rat hepatic lipocytes by kupffer cell conditioned medium - direct enhancement of matrix synthesis and stimulation of cell-proliferation via induction of platelet-derived growth-factor receptors. Journal of Clinical Investigation 84:1780-1785

Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M, Masui T, Kawaguchi M, Takaori K, Doi R, Nishi E, Kakinoki R, Deng JM, Behringer RR, Nakamura T, Uemoto S (2011) Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nature Genetics 43:34-U52

Galvao FH, de Andrade Junior DR, de Andrade DR, Martins BC, Marson AG, Bernard CV, Dos Santos SA, Bacchella T, Machado MC (2006) Hepatocyte transplantation: State of the art. Hepatol Res 36:237-247

Garcia-Ocana A, Takane KK, Syed MA, Philbrick WM, Vasavada RC, Stewart AF (2000) Hepatocyte growth factor overexpression in the islet of transgenic mice increases beta cell proliferation, enhances islet mass, and induces mild hypoglycemia. Journal of Biological Chemistry 275:1226-1232

Gardner SL, Dotta F, Nayak RC, George KL, Eisenbarth GS, Halban PA (1989) Modulation by sodium-butyrate of the differentiated status of a clonal pancreatic b-cell line (rin). Diabetes Research Clinical and Experimental 12:93-99

Garrison WD, Battle MA, Yang CH, Kaestner KH, Sladek FM, Duncan SA (2006) Hepatocyte nuclear factor 4 alpha is essential for embryonic development of the mouse colon. Gastroenterology 130:1207-1220

Gebhardt R (1992) Metabolic zonation of the liver - regulation and implications for liver-function. Pharmacology & Therapeutics 53:275-354

Gerich JE, Lorenzi M, Bier DM, Schneider V, Tsalikian E, Karam JH, Forsham PH (1975) Prevention of human diabetic ketoacidosis by somatostatin - evidence for an essential role of glucagon. New England Journal of Medicine 292:985-989

Gill GN, Kawamoto T, Cochet C, Le A, Sato JD, Masui H, McLeod C, Mendelsohn J (1984) Monoclonal anti-epidermal growth-factor receptor antibodies which are inhibitors of epidermal growth-factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein-kinase activity. Journal of Biological Chemistry 259:7755-7760

Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, Foidart JM (2003) Transactivation of vimentin by beta-catenin in human breast cancer cells. Cancer Research 63:2658-2664

Goke B (2008) Islet cell function: alpha and beta cells--partners towards normoglycaemia. International journal of clinical practice Supplement 2-7

Gorus FK, Malaisse WJ, Pipeleers DG (1984) Differences in glucose handling by pancreatic a-cells and b-cells. Journal of Biological Chemistry 259:1196-1200

Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzel T (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. Embo Journal 20:6969-6978

Graham FL, Smiley J, Russell WC, Nairn R (1977) Characteristics of a human cell line transformed by dna from human adenovirus type-5. Journal of General Virology 36:59-72

Green R, Shields D (1984) Sodium-butyrate stimulates somatostatin production by cultured-cells. Endocrinology 114:1990-1994

Greenbaum CJ, Havel PJ, Taborsky GJ, Klaff LJ (1991) Intra-islet insulin permits glucose to directly suppress pancreatic a-cell function. Journal of Clinical Investigation 88:767-773

Gressner AM (1995) Cytokines and cellular crosstalk involved in the activation of fat-storing cells. Journal of Hepatology 22:28-36

Grisham JW, Nopanitaya W, Compagno J, Nagel AEH (1975) Scanning electron-microscopy of normal rat-liver - surface-structure of its cells and tissue components. American Journal of Anatomy 144:295-321

Gromada J, Franklin I, Wollheim CB (2007) alpha-Cells of the endocrine pancreas: 35 years of research but the enigma remains. Endocrine Reviews 28:84-116

Grunwald V, Hidalgo M (2003) Development of the epidermal growth factor receptor inhibitor OSI-774. Seminars in Oncology 30:23-31

Gu MJ, Choi JH (2012) Clinicopathological Significance of E-Cadherin, beta-Catenin and Epidermal Growth Factor Receptor Expression in Intrahepatic Cholangiocarcinoma. Hepato-gastroenterology 59:1241-1244

Gupta RK, Gao N, Gorski RK, White P, Hardy OT, Rafiq K, Brestelli JE, Chen G, Stoeckert CJ, Jr., Kaestner KH (2007) Expansion of adult beta-cell mass in response to increased metabolic demand is dependent on HNF-4alpha. Genes Dev 21:756-769

Gupta RK, Vatamaniuk MZ, Lee CS, Flaschen RC, Fulmer JT, Matschinsky FM, Duncan SA, Kaestner KH (2005) The MODY1 gene HNF-4alpha regulates selected genes involved in insulin secretion. J Clin Invest 115:1006-1015

Gurdon JB (1988) A community effect in animal development. Nature 336:772-774

Haberland M, Montgomery RL, Olson EN (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nature Reviews Genetics 10:32-42

Hacker HJ, Steinberg P, Toshkov I, Oesch F, Bannasch P (1992) Persistence of the cholangiocellular and hepatocellular lesions observed in rats fed a choline-deficient/ DL-ethionine-supplemented diet. Carcinogenesis 13:271-276

Halban PA (1994) Proinsulin processing in the regulated and the constitutive secretory pathway. Diabetologia 37:S65-S72

Hamaguchi K, Leiter EH (1990) Comparison of cytokine effects on mouse pancreatic alpha-cell and beta-cell lines - viability, secretory function, and mhc antigen expression. Diabetes 39:415-425

Hameeteman W, Tytgat GNJ, Houthoff HJ, Vandentweel JG (1989) Barretts esophagus - development of dysplasia and adenocarcinoma. Gastroenterology 96:1249-1256

Han YC, Caday CG, Nanda A, Cavenee WK, Huang HJS (1996) Tyrphostin AG 1478 preferentially inhibits human glioma cells expressing truncated rather than wild-type epidermal growth factor receptors. Cancer Research 56:3859-3861

Harper ME, Ullrich A, Saunders GF (1981) Localization of the human insulin gene to the distal end of the short arm of chromosome-11. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 78:4458-4460

Haruna Y, Saito K, Spaulding S, Nalesnik MA, Gerber MA (1996) Identification of bipotential progenitor cells in human liver development. Hepatology 23:476-481

Hauge-Evans AC, King AJ, Carmignac D, Richardson CC, Robinson ICAF, Low MJ, Christie MR, Persaud SJ, Jones PM (2009) Somatostatin Secreted by Islet delta-Cells Fulfills Multiple Roles as a Paracrine Regulator of Islet Function. Diabetes 58:403-411

Haumaitre C, Lenoir O, Scharfmann R (2008) Histone deacetylase inhibitors modify pancreatic cell fate determination and amplify endocrine progenitors. Molecular and Cellular Biology 28:6373-6383

Hay CW, Docherty K (2006) Comparative analysis of insulin gene promoters - Implications for diabetes research. Diabetes 55:3201-3213

Hazan RB, Norton L (1998) The epidermal growth factor receptor modulates the interaction of E-cadherin with the actin cytoskeleton. Journal of Biological Chemistry 273:9078-9084

Heimberg H, Devos A, Pipeleers D, Thorens B, Schuit F (1995) Differences in glucose-transporter gene-expression between rat pancreatic alpha-cells and beta-cells are correlated to differences in glucose-transport but not in glucose-utilization. Journal of Biological Chemistry 270:8971-8975

Herold KC, Taylor L (2003) Treatment of type 1 diabetes with anti-CD3 monoclonal antibody - Induction of immune regulation? Immunologic Research 28:141-150

Herrera PL (2000) Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 127:2317-2322

Herrera PL, Huarte J, Zufferey R, Nichols A, Mermillod B, Philippe J, Muniesa P, Sanvito F, Orci L, Vassalli JD (1994) Ablation of islet endocrine-cells by targeted expression of hormone-promoter-driven toxigenes. Proceedings of the National Academy of Sciences of the United States of America 91:12999-13003

Howarth JL, Lee YB, Uney JB (2010) Using viral vectors as gene transfer tools (Cell Biology and Toxicology Special Issue: ETCS-UK 1 day meeting on genetic manipulation of cells). Cell Biology and Toxicology 26:1-20

Htun H, Barsony J, Renyi I, Gould DL, Hager GL (1996) Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. Proceedings of the National Academy of Sciences of the United States of America 93:4845-4850

Hu T, Li C (2010) Convergence between Wnt-beta-catenin and EGFR signaling in cancer. Molecular Cancer 9:

Hui HX, 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 50:785-796

Hussain SZ, Sneddon T, Tan XP, Micsenyi A, Michalopoulos GK, Monga SPS (2004) Wnt impacts growth and differentiation in ex vivo liver development. Experimental Cell Research 292:157-169

Iacob R, Ruedrich U, Rothe M, Kirsch S, Maasoumy B, Narain N, Verfaillie CM, Sancho-Bru P, Iken M, Popescu I, Schambach A, Manns MP, Bock M (2011) Induction of a mature hepatocyte phenotype in adult liver derived progenitor cells by ectopic expression of transcription factors. Stem Cell Research 6:251-261

Ilag LL, Tabaei BP, Herman WH, Zawacki CM, D'Souza E, Bell GI, Fajans SS (2000) Reduced pancreatic polypeptide response to hypoglycemia and amylin response to arginine in subjects with a mutation in the HNF-4 alpha/MODY1 gene. Diabetes 49:961-968

Inoue C, Yamamoto H, Nakamura T, Ichihara A, Okamoto H (1989) Nicotinamide prolongs survival of primary cultured-hepatocytes without involving loss of hepatocyte-specific functions. Journal of Biological Chemistry 264:4747-4750

Isfort RJ, Cody DB, Stuard SB, Randall CJ, Miller C, Ridder GM, Doersen CJ, Richards WG, Yoder BK, Wilkinson JE, Woychik RP (1997) The combination of epidermal growth factor and transforming growth factor-beta induces novel phenotypic changes in mouse liver stem cell lines. Journal of Cell Science 110:3117-3129

Ishii K, Yoshida Y, Akechi Y, Sakabe T, Nishio R, Ikeda R, Terabayashi K, Matsumi Y, Gonda K, Okamoto H, Takubo K, Tajima F, Tsuchiya H, Hoshikawa Y, Kurimasa A, Umezawa A, Shiota G (2008) Hepatic differentiation of human bone marrow-derived mesenchymal stem cells by tetracycline-regulated hepatocyte nuclear factor 3 beta. Hepatology 48:597-606

Ishii T, Fukano K, Shimada K, Kamikawa A, Okamatsu-Ogura Y, Terao A, Yoshida T, Saito M, Kimura K (2012) Proinsulin C-peptide activates alpha-enolase: implications for C-peptide-cell membrane interaction. Journal of Biochemistry 152:

Ishikawa T, Factor VM, Marquardt JU, Raggi C, Seo D, Kitade M, Conner EA, Thorgeirsson SS (2012) Hepatocyte growth factor/c-met signaling is required for stem-cell-mediated liver regeneration in mice. Hepatology 55:1215-1226

Iwashita J, Ose K, Ito H, Murata J, Abe T (2011) Inhibition of E-Cadherin Dependent Cell-Cell Contact Promotes MUC5AC Mucin Production through the Activation of Epidermal Growth Factor Receptors. Bioscience Biotechnology and Biochemistry 75:688-693

Jensen J, Serup P, Karlsen C, Nielsen TF, Madsen OD (1996) mRNA profiling of rat islet tumors reveals Nkx 6.1 as a beta-cell-specific homeodomain transcription factor. Journal of Biological Chemistry 271:18749-18758

Johnson PF (2005) Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. Journal of Cell Science 118:2545-2555

Jonsson J, Carlsson L, Edlund T, Edlund H (1994) Insulin-promoter-factor-1 is required for pancreas development in mice. Nature 371:606-609

Jungermann K, Kietzmann T (1996) Zonation of parenchymal and nonparenchymal metabolism in liver. Annual Review of Nutrition 16:179-203

Kaestner KH, Hiemisch H, Luckow B, Schutz G (1994) The HNF-3 gene family of transcription factors in mice - gene structure, cdna sequence, and messenger-rna distribution. Genomics 20:377-385

Kaneda K, Dan C, Wake K (1983) Pit cells as natural-killer cells. Biomedical Research-Tokyo 4:567-576

Kanno N, LeSage G, Glaser S, Alpini G (2001) Regulation of cholangiocyte bicarbonate secretion. American Journal of Physiology-Gastrointestinal and Liver Physiology 281:G612-G625

Kanno N, LeSage G, Glaser S, Alvaro D, Alpini G (2000) Functional heterogeneity of the intrahepatic biliary epithelium. Hepatology 31:555-561

Karabork A, Kaygusuz G, Ekinci C (2010) The best immunohistochemical panel for differentiating hepatocellular carcinoma from metastatic adenocarcinoma. Pathology Research and Practice 206:572-577

Katsuta H, Akashi T, Katsuta R, Nagaya M, Kim D, Arinobu Y, Hara M, Bonner-Weir S, Sharma AJ, Akashi K, Weir GC (2010) Single pancreatic beta cells co-express multiple islet hormone genes in mice. Diabetologia 53:128-138

Kawai M, Mushiake S, Bessho K, Murakami M, Namba N, Kokubu C, Michigami T, Ozono K (2007) Wnt/Lrp/beta-catenin signaling suppresses adipogenesis by inhibiting mutual activation of PPAR gamma and C/EBP alpha. Biochemical and Biophysical Research Communications 363:276-282

Kawamori D, Kurpad AJ, Hu J, Liew CW, Shih JL, Ford EL, Herrera PL, Polonsky KS, McGuinness OP, Kulkarni RN (2009) Insulin Signaling in alpha Cells Modulates Glucagon Secretion In Vivo. Cell Metabolism 9:350-361

Kawano Y, Kypta R (2003) Secreted antagonists of the Wnt signalling pathway. Journal of Cell Science 116:2627-2634

Kay MA, Glorioso JC, Naldini L (2001) Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nature Medicine 7:33-40

Kemler R (1993) From cadherins to catenins - cytoplasmic protein interactions and regulation of cell-adhesion. Trends in Genetics 9:317-321

Kim S, Schein AJ, Nadel JA (2005) E-cadherin promotes EGFR-mediated cell differentiation and MUC5AC mucin expression in cultured human airway epithelial cells. American Journal of Physiology-Lung Cellular and Molecular Physiology 289:L1049-L1060

Kmiec Z (2001) Cooperation of liver cells in health and disease. Advances in anatomy, embryology, and cell biology 161:III

Knight B, Matthews VB, Olynyk JK, Yeoh GC (2005) Jekyll and Hyde: evolving perspectives on the function and potential of the adult liver progenitor (oval) cell. Bioessays 27:1192-1202

Ko SH, Suh SH, Kim BJ, Ahn YB, Song KH, Yoo SJ, Son HS, Cha BY, Lee KW, Son HY, Kang SK, Bonner-Weir S, Weir GC, Yoon KH, Park CG (2004) Expression of the intermediate filament vimentin in proliferating duct cells as a marker of pancreatic precursor cells. Pancreas 28:121-128

Komuta M, Spee B, Borght SV, De Vos R, Verslype C, Aerts R, Yano H, Suzuki T, Matsuda M, Fujii H, Desmet VJ, Kojiro M, Roskams T (2008) Clinicopathological study on cholangiolocellular carcinoma suggesting hepatic progenitor cell origin. Hepatology 47:

Kornberg RD (1974) Chromatin structure - repeating unit of histones and dna. Science 184:868-871

Kuhlmann WD, Peschke P (2006) Hepatic progenitor cells, stem cells, and AFP expression in models of liver injury. International Journal of Experimental Pathology 87:

Kurash JK, Shen CN, Tosh D (2004) Induction and regulation of acute phase proteins in transdifferentiated hepatocytes. Experimental Cell Research 292:342-358

Lazaridis KN, Pham L, Tietz P, Marinelli PA, deGroen PC, Levine S, Dawson PA, LaRusso NF (1997) Rat cholangiocytes absorb bile acids at their apical domain via the ileal sodium-dependent bile acid transporter. Journal of Clinical Investigation 100:2714-2721

Lazaro CA, Rhim JA, Yamada Y, Fausto N (1998) Generation of hepatocytes from oval cell precursors in culture. Cancer Research 58:5514-5522

Leite AR, Correa-Giannella ML, Dagli ML, Fortes MA, Vegas VM, Giannella-Neto D (2007) Fibronectin and laminin induce expression of islet cell markers in hepatic oval cells in culture. Cell and Tissue Research 327:529-537

Lekstrom-Himes J, Xanthopoulos KG (1998) Biological role of the CCAAT enhancer-binding protein family of transcription factors. Journal of Biological Chemistry 273:28545-28548

Lemaigre F, Zaret KS (2004) Liver development update: new embryo models, cell lineage control, and morphogenesis. Current Opinion in Genetics & Development 14:582-590

Lemire JM, Shiojiri N, Fausto N (1991) Oval cell-proliferation and the origin of small hepatocytes in liver-injury induced by D-galactosamine. American Journal of Pathology 139:535-552

Lenoir O, Flosseau K, Ma FX, Blondeau B, Mai A, Bassel-Duby R, Ravassard P, Olson EN, Haumaitre C, Scharfmann R (2011) Specific Control of Pancreatic Endocrine beta- and delta-Cell Mass by Class IIa Histone Deacetylases HDAC4, HDAC5, and HDAC9. Diabetes 60:2861-2871

Li JX, Ning G, Duncan SA (2000) Mammalian hepatocyte differentiation requires the transcription factor HNF-4 alpha. Genes & Development 14:464-474

Li WC, Horb ME, Tosh D, Slack JMW (2005a) In vitro transdifferentiation of hepatoma cells into functional pancreatic cells. Mechanisms of Development 122:835-847

Li WC, Ralphs KL, Slack JMW, 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. International Journal of Biochemistry & Cell Biology 39:541-553

Li WC, Yu WY, Quinlan JM, Burke ZD, Tosh D (2005b) The molecular basis of transdifferentiation. Journal of Cellular and Molecular Medicine 9:569-582

Li Y, Zhao L-J, Xia F-Z, Li Y-X, Lu Y-l (2012) Transdifferentiation of hepatic oval cells into pancreatic islet beta-cells. Frontiers in bioscience : a journal and virtual library 17:

Libbrecht L, Desmet V, Van Damme B, Roskams T (2000) The immunohistochemical phenotype of dysplastic foci in human liver: correlation with putative progenitor cells. Journal of Hepatology 33:76-84

Libbrecht L, Roskams T (2002) Hepatic progenitor cells in human liver diseases. Seminars in Cell & Developmental Biology 13:

Lim SC, Lee MS (2002) Significance of E-cadherin/B-catenin complex and cyclin D1 in breast cancer. Oncology Reports 9:915-928

Liu YY, Rajkumar K, Murphy LJ (1999) Hepatic regeneration in insulin-like growth factor binding protein-1 transgenic mice. Journal of Hepatology 30:674-680

Loeb JN, Borek C, Yeung LL (1973) Suppression of dna-synthesis in hepatoma-cells exposed to glucocorticoid hormone in-vitro. Proceedings of the National Academy of Sciences of the United States of America 70:3852-3856

Lorenzini S, Bird TG, Boulter L, Bellamy C, Samuel K, Aucott R, Clayton E, Andreone P, Bernardi M, Golding M, Alison MR, Iredale JP, Forbes SJ (2010) Characterisation of a stereotypical cellular and extracellular adult liver progenitor cell niche in rodents and diseased human liver. Gut 59:645-654

Lowes KN, Brennan BA, Yeoh GC, Olynyk JK (1999) Oval cell numbers in human chronic liver diseases are directly related to disease severity. American Journal of Pathology 154:537-541

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 292:1389-1394

Mall AS, Tyler MG, Ho SB, Krige JEJ, Kahn D, Spearman W, Myer L, Govender D (2010) The expression of MUC mucin in cholangiocarcinoma. Pathology Research and Practice 206:805-809

Martinez-Palacian A, del Castillo G, Herrera B, Fernandez M, Roncero C, Fabregat I, Sanchez A (2012) EGFR is dispensable for c-Met-mediated proliferation and survival activities in mouse adult liver oval cells. Cellular Signalling 24:505-513

Maruyama H, Hisatomi A, Orci L, Grodsky GM, Unger RH (1984) Insulin within islets is a physiologic glucagon-release inhibitor. Journal of Clinical Investigation 74:2296-2299

Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I (1996a) Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. Journal of Clinical Investigation 97:1647-1654

Mashima H, Shibata H, Mine T, Kojima I (1996b) Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. Endocrinology 137:3969-3976

Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J (1984) Growth-inhibition of human-tumor cells in athymic mice by anti-epidermal growth-factor receptor monoclonal-antibodies. Cancer Research 44:1002-1007

Matull WR, Andreola F, Loh A, Adiguzel Z, Deheragoda M, Qureshi U, Batra SK, Swallow DM, Pereira SP (2008) MUC4 and MUC5AC are highly specific tumour-associated mucins in biliary tract cancer. British Journal of Cancer 98:1675-1681

Matys V, Fricke E, Geffers R, Gossling E, Haubrock M, Hehl R, Hornischer K, Karas D, Kel AE, Kel-Margoulis OV, Kloos DU, Land S, Lewicki-Potapov B, Michael H,

Munch R, Reuter I, Rotert S, Saxel H, Scheer M, Thiele S, Wingender E (2003) TRANSFAC (R): transcriptional regulation, from patterns to profiles. Nucleic Acids Research 31:374-378

Meng Q, Zhang GL, Wu DQ (2004) Hepatocyte culture in bioartificial livers with different membrane characteristics. Biotechnology Letters 26:1407-1412

Mentel R, Dopping G, Wegner U, Seidel W, Liebermann H, Dohner L (1997) Adenovirus-receptor interaction with human lymphocytes. Journal of Medical Virology 51:252-257

Michalopoulos GK, Bowen MC, Mule K, Luo JH (2003) HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures. Gene Expression 11:55-75

Michalopoulos GK, Khan Z (2005) Liver regeneration, growth factors, and amphiregulin. Gastroenterology 128:503-506

Micsenyi A, Tan XP, Sneddon T, Luo JH, Michalopoulos GK, Monga SPS (2004) beta-catenin is temporally regulated during normal liver development. Gastroenterology 126:1134-1146

Milani S, Herbst H, Schuppan D, Niedobitek G, Kim KY, Stein H (1989) Vimentin expression of newly formed rat bile-duct epithelial-cells in secondary biliary fibrosis. Virchows Archiv a-Pathological Anatomy and Histopathology 415:237-242

Miller JR, Hocking AM, Brown JD, Moon RT (1999) Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. Oncogene 18:7860-7872

Mithieux G (1997) New knowledge regarding glucose-6 phosphatase gene and protein and their roles in the regulation of glucose metabolism. European Journal of Endocrinology 136:137-145

Miyazaki S, Yamato E, Miyazaki J (2004) Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells. Diabetes 53:1030-1037

Moll R, Robine S, Dudouet B, Louvard D (1987) Villin - a cytoskeletal protein and a differentiation marker expressed in some human adenocarcinomas. Virchows Archiv B-Cell Pathology Including Molecular Pathology 54:155-169

Monga SPS, Micsenyi A, Germinaro M, Apte U, Bell A (2006) beta-catenin regulation during matrigel-induced rat hepatocyte differentiation. Cell and Tissue Research 323:71-79

Montoliu L, Blendy JA, Cole TJ, Schutz G (1995) Analysis of perinatal gene-expression - hormone response elements mediate activation of a lacz reporter gene in liver of transgenic mice. Proceedings of the National Academy of Sciences of the United States of America 92:4244-4248

Moreira RK (2007) Hepatic stellate cells and liver fibrosis. Archives of Pathology & Laboratory Medicine 131:1728-1734

Morgan EH, Peters T (1971) Biosynthesis of rat serum albumin .5. effect of protein depletion and refeeding on albumin and transferrin synthesis. Journal of Biological Chemistry 246:3500-&

Mulholland DJ, Dedhar S, Coetzee GA, Nelson CC (2005) Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? Endocrine Reviews 26:898-915

Munck A, Guyre PM, Holbrook NJ (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Reviews 5:25-44

Murtaugh LC (2007) Pancreas and beta-cell development: from the actual to the possible. Development 134:427-438

Nagaki M, Moriwaki H (2008) Transcription factor HNF and hepatocyte differentiation. Hepatology Research 38:961-969

Nagy P, Bisgaard HC, SantoniRugiu E, Thorgeirsson SS (1996) In vivo infusion of growth factors enhances the mitogenic response of rat hepatic ductal (oval) cells after administration of 2-acetylaminofluorene. Hepatology 23:71-79

Nagy P, Bisgaard HC, Thorgeirsson SS (1994) Expression of hepatic transcription factors during liver development and oval cell-differentiation. Journal of Cell Biology 126:223-233

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263-267

Nammo T, Yamagata K, Tanaka T, Kodama T, Sladek FM, Fukui K, Katsube F, Sato Y, Miyagawa J-i, Shimomura I (2008) Expression of HNF-4 alpha (MODY1), HNF-1 beta (MODY5), and HNF-1 alpha (MODY3) proteins in the developing mouse pancreas. Gene Expression Patterns 8:96-106

Neuberger J (2000) Liver transplantation. Journal of Hepatology 32:198-207

Nir T, Melton DA, Dor Y (2007) Recovery from diabetes in mice by beta cell regeneration. Journal of Clinical Investigation 117:2553-2561

Nishiyori A, Tashiro H, Kimura A, Akagi K, Yamamura K, Mori M, Takiguchi M (1994) Determination of tissue-specificity of the enhancer by combinatorial operation of tissue-enriched transcription factors - both hnf-4 and c/ebp-beta are required for liver-specific activity of the ornithine transcarbamylase enhancer. Journal of Biological Chemistry 269:1323-1331

Nitsch D, Boshart M, Schutz G (1993) Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. Proceedings of the National Academy of Sciences of the United States of America 90:5479-5483

Nomoto K, Tsuneyama K, Cheng CM, Takahashi H, Hori R, Murai Y, Takano Y (2006) Intrahepatic cholangiocarcinoma arising in cirrhotic liver frequently expressed p63-positive basal/stem-cell phenotype. Pathology Research and Practice 202:71-76

O'Neill KE, Eberhard D, Tosh D (2008) Origin of beta-cells in regenerating pancreas. Bioessays 30:617-620

Okada TS (1986) Transdifferentiation in animal cells: fact or artifact? Development Growth & Differentiation 28:213-221

Olkku A, Mahonen A (2009) Calreticulin mediated glucocorticoid receptor export is involved in beta-catenin translocation and Wnt signalling inhibition in human osteoblastic cells. Bone 44:555-565

Olsen L, Bressendorff S, Troelsen JT, Olsen J (2005) Differentiation-dependent activation of the human intestinal alkaline phosphatase promoter by HNF-4 in intestinal cells. American Journal of Physiology-Gastrointestinal and Liver Physiology 289:G220-G226

Orsulic S, Huber O, Aberle H, Arnold S, Kemler R (1999) E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. Journal of Cell Science 112:1237-1245

Pack R, Heck R, Dienes HP, Oesch F, Steinberg P (1993) Isolation, biochemical-characterization, long-term culture, and phenotype modulation of oval cells from carcinogen-fed rats. Experimental Cell Research 204:198-209

Padgham CRW, Boyle CC, Wang XJ, Raleigh SM, Wright MC, Paine AJ (1993) Alteration of transcription factor messenger-rnas during the isolation and culture of rat hepatocytes suggests the activation of a proliferative mode underlies their dedifferentiation. Biochemical and Biophysical Research Communications 197:599-605

Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA (2003) Hepatocyte nuclear factor 4 alpha controls the development of a hepatic epithelium and liver morphogenesis. Nature Genetics 34:292-296

Peignon G, Thenet S, Schreider C, Fouquet S, Ribeiro A, Dussaulx E, Chambaz J, Cardot P, Pincon-Raymond M, Le Beyec J (2006) E-cadherin-dependent transcriptional control of apolipoprotein A-IV gene expression in intestinal epithelial cells - A role for the hepatic nuclear factor 4. Journal of Biological Chemistry 281:3560-3568

Pertusa JAG, Sanchez-Andres JV, Martin F, Soria B (1999) Effects of calcium buffering on glucose-induced insulin release in mouse pancreatic islets: an approximation to the calcium sensor. Journal of Physiology-London 520:473-483

Philippe J (1989) Glucagon gene-transcription is negatively regulated by insulin in a hamster islet cell-line. Journal of Clinical Investigation 84:672-677

Philippe J (1993) Somatostatin inhibits insulin-gene expression through a posttranscriptional mechanism in a hamster islet cell-line. Diabetes 42:244-249

Philippe J, Morel C, Cordierbussat M (1995) Islet-specific proteins interact with the insulin-response element of the glucagon gene. Journal of Biological Chemistry 270:3039-3045

Pinzani M, Marra F, Carloni V (1998) Signal transduction in hepatic stellate cells. Liver 18:2-13

Potten CS, Booth C, Pritchard DM (1997) The intestinal epithelial stem cell: the mucosal governor. Int J Exp Pathol 78:219-243

Potter VR (1978) Phenotypic diversity in experimental hepatomas the concept of partially blocked ontogeny. British Journal of Cancer 38:1-23

Poulsom R, Begos DE, Modlin IM (1996) Molecular aspects of restitution: Functions of trefoil peptides. Yale Journal of Biology and Medicine 69:137-146

Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L (2004) Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proceedings of the National Academy of Sciences of the United States of America 101:2924-2929

Quinlan JM, Colleypriest BJ, Farrant M, Tosh D (2007) Epithelial metaplasia and the development of cancer. Biochimica Et Biophysica Acta-Reviews on Cancer 1776:10-21

Radaeva S, Steinberg P (1995) Phenotype and differentiation patterns of the oval cell-lines oc/cde-6 and oc/cde-22 derived from the livers of carcinogen-treated rats. Cancer Research 55:1028-1038

Ramji DP, Foka P (2002) CCAAT/enhancer-binding proteins: structure, function and regulation. Biochemical Journal 365:561-575

Ravier MA, Rutter GA (2005) Glucose or insulin, but not zinc ions, inhibit glucagon secretion from mouse pancreatic alpha-cells. Diabetes 54:1789-1797

Richards WG, Yoder BK, Isfort RJ, Detilleux PG, Foster C, Neilsen N, Woychik RP, Wilkinson JE (1997) Isolation and characterization of liver epithelial cell lines from wild-type and mutant TgN737Rpw mice. American Journal of Pathology 150:1189-1197

Rigler R, Pramanik A, Jonasson P, Kratz G, Jansson OT, Nygren PA, Stahl S, Ekberg K, Johansson BL, Uhlen S, Uhlen M, Jornvall H, Wahren J (1999) Specific binding of proinsulin C-peptide to human cell membranes. Proceedings of the National Academy of Sciences of the United States of America 96:

Ritter JK, Kessler FK, Thompson ET, Grove AD, Auyeung DJ, Fisher RA (1999) Expression and inducibility of the human bilirubin UDP-glucuronosyltransferase UGTLA1 in liver and cultured primary hepatocytes: Evidence for both genetic and environmental influences. Hepatology 30:476-484

Ritz-Laser B, Gauthier BR, Estreicher A, Mamin A, Brun T, Ris F, Salmon P, Halban PA, Trono D, Philippe J (2003) Ectopic expression of the beta-cell specific transcription factor Pdx1 inhibits glucagon gene transcription. Diabetologia 46:810-821

Robine S, Huet C, Moll R, Sahuquillomerino C, Coudrier E, Zweibaum A, Louvard D (1985) Can villin be used to identify malignant and undifferentiated normal digestive epithelial-cells. Proceedings of the National Academy of Sciences of the United States of America 82:8488-8492

Roelvink PW, Lizonova A, Lee JGM, Li Y, Bergelson JM, Finberg RW, Brough DE, Kovesdi I, Wickham TJ (1998) The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. Journal of Virology 72:7909-7915

Roskams T (2006a) Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. Oncogene 25:3818-3822

Roskams T (2006b) Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. Oncogene 25:3818-3822

Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, Brunt EM, Crawford JM, Crosby HA, Desmet V, Finegold MJ, Geller SA, Gouw ASH, Hytiroglou P, Knisely AS, Kojiro M, Letkowitch JH, Nakanuma Y, Olynyk JK, Park YN, Portmann B, Saxena R, Scheuer PJ, Strain AJ, Thung SN, Wanless IR, West AB (2004) Nomenclature of the finer branches of the biliary tree: Canals, ductules, and ductular reactions in human livers. Hepatology 39:1739-1745

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods in molecular biology (Clifton, NJ) 132:365-386

Russell WC (2000) Update an adenovirus and its vectors. Journal of General Virology 81:2573-2604

Samson SL, Chan L (2006) Gene therapy for diabetes: reinventing the islet. Trends in Endocrinology and Metabolism 17:92-100

Sand TE, Christoffersen T (1987) Temporal requirement for epidermal growth-factor and insulin in the stimulation of hepatocyte dna-synthesis. Journal of Cellular Physiology 131:141-148

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 127:5533-5540

Sangan CB, Tosh D (2010a) A new paradigm in cell therapy for diabetes: Turning pancreatic alpha-cells into beta-cells. Bioessays 32:881-884

Sangan CB, Tosh D (2010b) Hepatic progenitor cells. Cell and Tissue Research 342:131-137

Sasaki M, Ikeda H, Nakanuma Y (2005) Expression profiles of MUC mucin core protein in the intrahepatic biliary system: Physiological distribution and pathological significance. Acta Histochemica Et Cytochemica 38:295-303

Sasaki M, Ikeda H, Nakanuma Y (2007) Expression profiles of MUC mucins and trefoil factor family (TFF) peptides in the intrahepatic biliary system: Physiological distribution and pathological significance. Progress in Histochemistry and Cytochemistry 42:61-110

Sasaki M, Nakanuma Y, Kim YS (1998) Expression of apomucins in the intrahepatic biliary tree in hepatolithiasis differs from that in normal liver and extrahepatic biliary obstruction. Hepatology 27:54-61

Sasaki M, Tsuneyama K, Saito T, Kataoka H, Mollenhauer J, Poustka A, Nakanuma Y (2004) Site-characteristic expression and induction of trefoil factor family 1, 2 and 3 and malignant brain tumor-1 in normal and diseased intrahepatic bile ducts relates to biliary pathophysiology. Liver International 24:29-37

Sato M, Suzuki S, Senoo H (2003) Hepatic stellate cells: Unique characteristics in cell biology and phenotype. Cell Structure and Function 28:105-112

Sauvaget D, Chauffeton V, Citadelle D, Chatelet FP, Cywiner-Golenzer C, Chambaz J, Pincon-Raymond M, Cardot P, Le Beyec J, Ribeiro A (2002) Restriction of apolipoprotein A-IV gene expression to the intestine villus depends on a-hormone-responsive element and parallels differential expression of the hepatic nuclear factor 4 alpha and gamma isoforms. Journal of Biological Chemistry 277:34540-34548

Scheving LA, Buchanan R, Krause MA, Zhang X, Stevenson MC, Russell WE (2007) Dexamethasone modulates ErbB tyrosine kinase expression and signaling through multiple and redundant mechanisms in cultured rat hepatocytes. American Journal of Physiology-Gastrointestinal and Liver Physiology 293:G552-G559

Schisler JC, Jensen PB, Taylor DG, Becker TC, Knop FK, Takekawa S, German M, Weir GC, Lu D, Mirmira RG, Newgard CB (2005) The Nkx6.1 homeodomain transcription factor suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. Proc Natl Acad Sci U S A 102:7297-7302

Schmittgraff A, Kruger S, Bochard F, Gabbiani G, Denk H (1991) Modulation of alpha smooth-muscle actin and desmin expression in perisinusoidal cells of normal and diseased human livers. American Journal of Pathology 138:1233-1242

Schwerer MJ, Baczako K (1996) Immunohistochemical evaluation of keratin 20 expression in intestinal metaplasia types I to III. Journal of Clinical Pathology 49:791-794

Sekiya S, Suzuki A (2011) Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 475:390-U148

Sell S (1993) Cellular-origin of cancer - dedifferentiation or stem-cell maturation arrest. Environmental Health Perspectives 101:15-26

Shachaf CM, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, Bachmann MH, Borowsky AD, Ruebner B, Cardiff RD, Yang QW, Bishop JM, Contag CH, Felsher DW (2004) MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. Nature 431:1112-1117

Shafritz DA, Oertel M (2010) Model systems and experimental conditions that lead to effective repopulation of the liver by transplanted cells. Int J Biochem Cell Biol

Shapiro AMJ, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson P, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems J-A, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DER, Eisenbarth G, Segal M, Preiksaitis J, Korbutt GS, Barton FB, Viviano L, Seyfert-Margolis V, Bluestone J, Lakey JRT (2006) International trial of the edmonton protocol for islet transplantation. New England Journal of Medicine 355:1318-1330

Shapiro AMJ, Ryan EA, Lakey JRT (2001) Pancreatic islet transplantation in the treatment of diabetes mellitus. Best Practice & Research Clinical Endocrinology & Metabolism 15:241-264

Shen CN, Horb ME, Slack JMW, Tosh D (2003) Transdifferentiation of pancreas to liver. Mechanisms of Development 120:107-116

Shen CN, Slack JMW, Tosh D (2000) Molecular basis of transdifferentiation of pancreas to liver. Nature Cell Biology 2:879-887

Shen X-x, Li H-l, Pan L, Hong J, Xiao J, Hermansen K, Jeppesen PB, Li G-W (2012) Glucotoxicity and alpha Cell Dysfunction: Involvement of the PI3K/Akt Pathway in Glucose-Induced Insulin Resistance in Rat Islets and Clonal alpha TC1-6 Cells. Endocrine Research 37:12-24

Shiao M-S, Liao B-Y, Long M, Yu H-T (2008) Adaptive evolution of the insulin two-gene system in mouse. Genetics 178:1683-1691

Shiojiri N, Takeshita K, Yamasaki H, Iwata T (2004) Suppression of C/EBP alpha expression in biliary cell differentiation from hepatoblasts during mouse liver development. Journal of Hepatology 41:790-798

Shiroi A, Ueda S, Ouji Y, Saito K, Moriya K, Sugie Y, Fukui H, Ishizaka S, Yoshikawa M (2005) Differentiation of embryonic stem cells into insulin-producing cells promoted by Nkx2.2 gene transfer. World journal of gastroenterology : WJG 11:4161-4166

Slack JMW (1995) Developmental biology of the pancreas. Development 121:1569-1580

Slack JMW (2003) Regeneration research today. Developmental Dynamics 226:162-166

Slack JMW, Tosh D (2001) Transdifferentiation and metaplasia - switching cell types. Current Opinion in Genetics & Development 11:581-586

Sladek FM, Zhong WM, Lai E, Darnell JE (1990) Liver-enriched transcription factor hnf-4 is a novel member of the steroid-hormone receptor superfamily. Genes & Development 4:2353-2365

Smedsrod B, Debleser PJ, Braet F, Lovisetti P, Vanderkerken K, Wisse E, Geerts A (1994) Cell biology of liver endothelial and kupffer cells. Gut 35:1509-1516

Smith A (2006) A glossary for stem-cell biology. Nature 441:1060-1060

Soria B (2001) In-vitro differentiation of pancreatic beta-cells. Differentiation 68:205-219

SosaPineda 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

Stagner JI, Samols E (1986) Retrograde perfusion as a model for testing the relative effects of glucose versus insulin on the a-cell. Journal of Clinical Investigation 77:1034-1037

Stegmann A, Hansen M, Wang YL, Larsen JB, Lund LR, Ritie L, Nicholson JK, Quistorff B, Simon-Assmann P, Troelsen JT, Olsen J (2006) Metabolome, transcriptome, and bioinformatic cis-element analyses point to HNF-4 as a central regulator of gene expression during enterocyte differentiation. Physiological Genomics 27:141-155

Steinberg P, Steinbrecher R, Radaeva S, Schirmacher P, Dienes HP, Oesch F, Bannasch P (1994) Oval cell-lines oc/cde-6 and oc/cde-22 give rise to cholangio-cellular and undifferentiated carcinomas after transformation. Laboratory Investigation 71:700-709

Steiner DF, Cunningh.D, Spigelma.L, Aten B (1967) Insulin biosynthesis - evidence for a precursor. Science 157:697-&

Stoffel M, Duncan SA (1997) The maturity-onset diabetes of the young (MODY1) transcription factor HNF4 alpha regulates expression of genes required for glucose transport and metabolism. Proceedings of the National Academy of Sciences of the United States of America 94:13209-13214

Strick-Marchand H, Weiss MC (2002) Inducible differentiation and morphogenesis of bipotential liver cell lines from wild-type mouse embryos. Hepatology 36:794-804

Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriwaki H (2008) Differentiation of Mouse Hepatic Progenitor Cells Induced by Hepatocyte Nuclear Factor-4 and Cell Transplantation in Mice With Liver Fibrosis. Transplantation 86:1178-1186

Takiguchi M (1998) The C/EBP family of transcription factors in the liver and other organs. International Journal of Experimental Pathology 79:369-391

Tang DQ, Cao LZ, Chou W, Shun L, Farag C, Atkinson MA, Li SW, Chang LJ, Yang LJ (2006) Role of Pax4 in Pdx1-VP16-mediated liver-to-endocrine pancreas transdifferentiation. Laboratory Investigation 86:829-841

Tatematsu M, Kaku T, Medline A, Farber E (1985) Intestinal metaplasia as a common option of oval cells in relation to cholangiofibrosis in liver of rats exposed to 2-acetylaminofluorene. Laboratory Investigation 52:354-362

Tavoloni N (1987) The intrahepatic biliary epithelium - an area of growing interest in hepatology. Seminars in Liver Disease 7:280-292

Teitelman G, Alpert S, Polak JM, Martinez A, Hanahan D (1993) Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine-hydroxylase and neuropeptide-y, but not pancreatic-polypeptide. Development 118:1031-1039

Thorel F, Nepote V, Avril I, Kohno K, Desgraz R, Chera S, Herrera PL (2010) Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 464:1149-1154

Thowfeequ S, Myatt EJ, Tosh D (2007) Transdifferentiation in developmental biology, disease, and in therapy. Developmental Dynamics 236:3208-3217

Tietz PS, Alpini G, Pham LD, Larusso NF (1995) Somatostatin inhibits secretin-induced ductal hypercholeresis and exocytosis by cholangiocytes. American Journal of Physiology-Gastrointestinal and Liver Physiology 269:G110-G118

Tirnitz-Parker JEE, Tonkin JN, Knight B, Olynyk JK, Yeoh GCT (2007) Isolation, culture and immortalisation of hepatic oval cells from adult mice fed a choline-deficient, ethionine-supplemented diet. International Journal of Biochemistry & Cell Biology 39:2226-2239

Tomasetto C, Masson R, Linares JL, Wendling C, Lefebvre O, Chenard MP, Rio MC (2000) pS2/TFF1 interacts directly with the VWFC cysteine-rich domains of mucins. Gastroenterology 118:70-80

Tosh D, Shen CN, Slack JMW (2002) Differentiated properties of hepatocytes induced from pancreatic cells. Hepatology 36:534-543

Tsaniras SC, Jones PM (2010) Generating pancreatic beta-cells from embryonic stem cells by manipulating signaling pathways. Journal of Endocrinology 206:13-26

Tsao MS, Grisham JW (1987) Hepatocarcinomas, cholangiocarcinomas, and hepatoblastomas produced by chemically transformed cultured rat-liver epithelial-cells - a light-microscopic and electron-microscopic analysis. American Journal of Pathology 127:168-181

van de Kerkhove MP, Hoekstra R, Chamuleau RA, van Gulik TM (2004) Clinical application of bioartificial liver support systems. Ann Surg 240:216-230

Van Seuningen I, Pigny P, Perrais M, Porchet N, Aubert JP (2001) Transcriptional regulation of the 11p15 mucin genes. Towards new biological tools in human therapy, in inflammatory diseases and cancer? Frontiers in Bioscience 6:D1216-D1234

Verma IM, Weitzman MD (2005) Gene therapy: Twenty-first century medicine. Annual Review of Biochemistry 74:711-738

Vintermyr OK, Doskeland SO (1989) Characterization of the inhibitory effect of glucocorticoids on the dna-replication of adult-rat hepatocytes growing at various cell densities. Journal of Cellular Physiology 138:29-37

Wahren J, Ekberg K, Johansson J, Henriksson M, Pramanik A, Johansson BL, Rigler R, Jornvall H (2000) Role of C-peptide in human physiology. American Journal of Physiology-Endocrinology and Metabolism 278:E759-E768

Wahren J, Jornvall H (2003) C-peptide makes a comeback. Diabetes-Metabolism Research and Reviews 19:

Wake K (1971) Sternzellen in liver - perisinusoidal cells with special reference to storage of vitamin-a. American Journal of Anatomy 132:429-&

Wallace K, Marek CJ, Hoppler S, Wright MC (2010) Glucocorticoid-dependent transdifferentiation of pancreatic progenitor cells into hepatocytes is dependent on transient suppression of WNT signalling. Journal of Cell Science 123:2102-2109

Wang HM, Iakova P, Wilde M, Welm A, Goode T, Roesler WJ, Timchenko NA (2001) C/EBP alpha arrests cell proliferation through direct inhibition of cdk2 and cdk4. Molecular Cell 8:817-828

Wang HW, Breslin MB, Lan MS (2007) Pdx-1 modulates histone H4 acetylation and insulin gene expression in terminally differentiated alpha-TC-1 cells. Pancreas 34:248-253

Wang HY, Hagenfeldt-Johansson K, Otten LA, Gauthier BR, Herrera PL, Wollheim CB (2002a) Experimental models of transcription factor-associated maturity-onset diabetes of the young. Diabetes 51:S333-S342

Wang HY, Maechler P, Antinozzi PA, Hagenfeldt KA, Wollheim CB (2000) Hepatocyte nuclear factor 4 alpha regulates the expression of pancreatic beta-cell genes implicated in glucose metabolism and nutrient-induced insulin secretion. Journal of Biological Chemistry 275:35953-35959

Wang Q, Sun ZX, Allgayer H, Yang HS (2010) Downregulation of E-cadherin is an essential event in activating beta-catenin/Tcf-dependent transcription and expression of its target genes in Pdcd4 knockdown cells. Oncogene 29:128-138

Wang RN, Bouwens L, Kloppel G (1996) Beta-cell growth in adolescent and adult rats treated with streptozotocin during the neonatal period. Diabetologia 39:548-557

Wang X, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M (2002b) The origin and liver repopulating capacity of murine oval cells. Arthur M Sackler Colloquium of the National-Academy-of-Sciences on Regenerative Medicine, Irvine, California, pp 11881-11888

Watada H, Kajimoto Y, Miyagawa J, Hanafusa T, Hamaguchi K, Matsuoka TA, Yamamoto K, Matsuzawa Y, Kawamori R, Yamasaki Y (1996) PDX-1 induces insulin and glucokinase gene expressions in alpha TC1 clone 6 cells in the presence of betacellulin. Diabetes 45:1826-1831

Watt AJ, Garrison WD, Duncan SA (2003) HNF4: A central regulator of hepatocyte differentiation and function. Hepatology 37:1249-1253

Wederell ED, Bilenky M, Cullum R, Thiessen N, Dagpinar M, Delaney A, Varhol R, Zhao Y, Zeng T, Bernier B, Ingham M, Hirst M, Robertson G, Marra MA, Jones S, Hoodless PA (2008) Global analysis of in vivo Foxa2-binding sites in mouse adult liver using massively parallel sequencing. Nucleic Acids Research 36:4549-4564

Wells JM, Melton DA (1999) Vertebrate endoderm development. Annual Review of Cell and Developmental Biology 15:393-410

Westmacott A, Burke ZD, Oliver G, Slack JMW, Tosh D (2006) C/EBP alpha and C/EBP beta are markers of early liver development. International Journal of Developmental Biology 50:653-657

Wierup N, Svensson H, Mulder H, Sundler F (2002) The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. Regulatory Peptides 107:63-69

Wisse E, Braet F, Luo D, Jans D, Vermijlen D, Kanellopoulou C, Spapen H, De Zanger R (1997) On the tumoricide function of pit cells. The NK cells of the liver. In: Vidal-Vanclocha F (ed) Medical Intelligence Unit; Functional heterogeneity of liver tissue: From cell lineage diversity to sublobular compartment-specific pathogenesis. Chapman and Hall, Inc., 29 West 35th Street, New York, New York, USA; Chapman and Hall Ltd., 2-6 Boundary Row, London SE1 8HN, England, pp 207-235

Wollheim CB, Wang HY, Maechler P, Antinozzi PA, Hagenfeldt KA (2000) Hepatocyte nuclear factor 4 alpha regulates the expression of pancreatic beta-cell genes implicated in glucose metabolism and nutrient-induced insulin secretion. Journal of Biological Chemistry 275:35953-35959

Wu KJ, Wilson DR, Shih CH, Darlington GJ (1994) The transcription factor hnf1 acts with c/ebp-alpha to synergistically activate the human albumin promoter through a novel domain. Journal of Biological Chemistry 269:1177-1182

Wu KK, Huan Y (2008) Streptozotocin-induced diabetic models in mice and rats. Current protocols in pharmacology / editorial board, SJ Enna (editor-in-chief) [et al] Chapter 5:

Xu G, Stoffers DA, Habener JF, Bonner-Weir S (1999) Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270-2276

Xu X, D'Hoker J, Stange G, Bonne S, De Leu N, Xiao X, De Casteele MV, 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

Yamasaki H, Sada A, Iwata T, Niwa T, Tomizawa M, Xanthopoulos KG, Koike T, Shiojiri N (2006) Suppression of C/EBP alpha expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased Hnf6 and Hnf1b expression. Development 133:4233-4243

Yamashita K, Yonezawa S, Tanaka S, Shirahama H, Sakoda K, Imai K, Xing PX, McKenzie IFC, Hilkens J, Kim YS, Sato E (1993) Immunohistochemical study of

mucin carbohydrates and core proteins in hepatolithiasis and cholangiocarcinoma. International Journal of Cancer 55:82-91

Yang LJ, Li SW, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB (2002) In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proceedings of the National Academy of Sciences of the United States of America 99:8078-8083

Yang W, Yan HX, Chen L, Liu Q, He YQ, Yu LX, Zhang SH, Huang DD, Tang L, Kong XN, Chen C, Liu SQ, Wu MC, Wang HY (2008) Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. Cancer Research 68:4287-4295

Yanukakashles O, Cohen H, Trus M, Aran A, Benvenisty N, Reshef L (1994) Transcriptional regulation of the phosphoenolpyruvate carboxykinase gene by cooperation between hepatic nuclear factors. Molecular and Cellular Biology 14:7124-7133

Yeoh GCT, Bennett FA, Oliver IT (1979) Hepatocyte differentiation in culture - appearance of tyrosine aminotransferase. Biochemical Journal 180:153-160

Yin L, Sun MZ, Ilic Z, Leffert HL, Sell S (2002) Derivation, characterization, and phenotypic variation of hepatic progenitor cell lines isolated from adult rats. Hepatology 35:315-324

Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific-inhibition of mammalian histone deacetylase both invivo and invitro by trichostatin-a. Journal of Biological Chemistry 265:17174-17179

Yovchev MI, Grozdanov PN, Zhou HC, Racherla H, Guha C, Dabeva MD (2008) Identification of adult hepatic progenitor cells capable of repopulating injured rat liver. Hepatology 47:636-647

Yu X, Wang Y, DeGraff DJ, Wills ML, Matusik RJ (2011) Wnt/beta-Catenin activation promotes prostate tumor progression in a mouse model. Oncogene 30:1868-1879

Zaret KS (2002) Regulatory phases of early liver development: Paradigms of organogenesis. Nature Reviews Genetics 3:499-512

Zaret KS (2008) Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nature Reviews Genetics 9:329-340

Zhang N, Siegel K, Odenthal N, Becker R, Oesch F, Dienes HP, Schirmacher P, Steinberg P (1997) The role of insulin-like growth factor II in the malignant transformation of rat liver oval cells. Hepatology 25:900-905

Zhao R, Duncan SA (2005) Embryonic development of the liver. Hepatology 41:956-967

Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455:627-U630

# Appendix A: List of commercial suppliers

| Supplier | Distributor Location | Website Information |
|---|---|---|
| Abcam | Cambridge, UK | www.abcamplc.com |
| Acris | Herford, Germany | www.acris-antibodies.com |
| Ambion | Warrington, UK | www.ambion.com |
| Amersham Bioscience | Buckinghamshire, UK | www.gelifesciences.com |
| BD Bioscience | Oxford,UK | www.bdbiosciences.com |
| BD Transduction Laboratories | Oxford, UK | www.bdbiosciences.com |
| Beckman Coulter Inc. | Buckinghamshire, UK | www.beckmancoulter.co.uk |
| Biogenesis | Poole, UK | www.biogenesis.co.uk |
| Carl Zeiss Ltd | Hertfordshire, UK | www.zeiss.co.uk |
| Cell Signalling | Danvers, MA, USA | www.cellsignal.com |
| Clontech Laboratories | Basingstoke, UK | www.clontech.com |
| Cypex Ltd | Dundee, UK | www.cypex.co.uk |
| DAKO Cytomation | Ely, UK | www.dako.co.uk |
| Fisher Scientific | Leicestershire, UK | www.fisher.co.uk |
| Gibco, Invitrogen Life Technologies | Paisley, UK | www.invitrogen.org.uk |
| Jencons (Scientific) Ltd | West Sussex, UK | www.jencons.co.uk |
| Leica | Milton Keynes, UK | www.leica-microsystems.com |
| Millipore Corporation (Chemicon, Upstate Biotechnology, Calbiochem) | Co Durham, UK | www.millipore.co.uk |
| Eurofins MWG Operon | Ebersberg, Germany | www.eurofinsdna.com |
| New England Biolabs | Hertfordshire, UK | www.neb.com |
| National Diagnostics | Atlanta, GA, USA | www.nationaldiagnostics.com |
| Promega | Southampton, UK | www.promega.com/uk |
| R&D Systems | Abingdon, UK | www.rndsystems.com |
| Roche (Boehringer Mannheim) | West Sussex, UK | www.roche.com |
| Santa Cruz Biotechnology | Santa Cruz, CA, USA | www.scbt.com |
| Scientific Laboratory Supplies Ltd | East Yorkshire, UK | www.flowgen.net |
| Sigma (-Aldrich) | Poole, UK | www.sigmaaldrich.com |
| Thermo Scientific (Lab Vision Corp.) | Winsford, UK | www.thermoscientific.com |
| Vector Laboratories | Peterborough, UK | www.vectorlabs.com |
| Zymed Laboratories Inc. | Cambridge, UK | www.zymed.com |

**Table A.1: List of commercial suppliers, distributor address and website information.**

# Appendix B: RNA quantification and integrity analysis

| Samples | Concentration (µg/µl) | $A_{260}/A_{280}$ (<2) | $A_{260}$ (<1) |
|---|---|---|---|
| *Condition 1*: 6G | 0.66 | 1.64 | 0.164 |
| *Condition 2*: 17G | 3.46 | 1.53 | 0.864 |
| *Condition 3*: 10G7D | 2.01 | 1.60 | 0.503 |
| *Condition 4*: 7G10D | 2.56 | 1.62 | 0.640 |
| *Condition 5*: 3G14D | 2.88 | 1.53 | 0.720 |
| *Condition 6*: 17D | 1.55 | 1.64 | 0.387 |

**Table B. 1:RNA quantification and integrity data for Figure 3.8**

| Samples | Concentration (µg/µl) | $A_{260}/A_{280}$ (<2) | $A_{260}$ (<1) |
|---|---|---|---|
| *Condition 1*: 6G | 1.57 | 1.46 | 0.393 |
| *Condition 2*: 17G | 2.39 | 1.46 | 0.599 |
| *Condition 5*: 3G14D | 1.88 | 1.46 | 0.472 |

**Table B. 2: RNA quantification and integrity data for Figure 4.2 and Figure 4.7A Figure 3.8**

| Samples | Concentration (µg/µl) | $A_{260}/A_{280}$ (<2) | $A_{260}$ (<1) |
|---|---|---|---|
| *Condition 1*: 6G | 0.87 | 1.74 | 0.434 |
| *Condition 7*: 3G14---- | 0.92 | 1.86 | 0.461 |
| *Condition 5*: 3G14DIFF | 1.08 | 1.83 | 0.542 |
| *Condition 8*: 3G14EGF | 1.47 | 1.88 | 0.741 |
| *Condition 9*: 3G14DEX | 1.23 | 1.81 | 0.619 |
| *Condition 10*: 3G14NIC | 1.38 | 1.88 | 0.695 |
| *Condition 11*: 3G14ITS | 1.98 | 1.87 | 0.994 |

**Table B. 3: RNA quantification and integrity data for Figure 4.3 and Figure 4.7B**

| Samples | Concentration (µg/µl) | $A_{260}/A_{280}$ (<2) | $A_{260}$ (<1) |
|---|---|---|---|
| *Low Passage:* 6G | 1.57 | 1.46 | 0.393 |
| *Low Passage:* 3G14D | 1.88 | 1.46 | 0.472 |
| *High Passage:* 6G | 1.52 | 1.45 | 0.380 |
| *High Passage:* 3G14D | 1.97 | 1.46 | 0.492 |

**Table B. 4: RNA quantification and integrity data for Figure 5.2 and Figure 5.4**

| Samples | Concentration (µg/µl) | $A_{260}/A_{280}$ (<2) | $A_{260}$ (<1) |
|---|---|---|---|
| Un-infected (----) | 0.86 | 1.84 | 0.427 |
| Ad-Null | 0.78 | 1.85 | 0.389 |
| Ad-CMV-HNF4α | 0.72 | 1.84 | 0.359 |
| Ad-CMV-C/EBPα | 0.79 | 1.83 | 0.396 |
| Ad-CMV-HNF4α<br>Ad-CMV-C/EBPα | 0.55 | 1.80 | 0.275 |

**Table B. 5: RNA quantification and integrity data for Figure 5.6A**

| Samples | Concentration (µg/µl) | $A_{260}/A_{280}$ (<2) | $A_{260}$ (<1) |
|---|---|---|---|
| α-TC19 | 0.79 | 1.87 | 0.397 |

**Table B. 6: RNA quantification and integrity data for Figure 6.3A**

| Samples | Concentration (µg/µl) | $A_{260}/A_{280}$ (<2) | $A_{260}$ (<1) |
|---|---|---|---|
| α-TC19 (------) | 2.01 | 1.90 | 0.991 |
| Ad-CMV-HNF4α | 0.67 | 1.80 | 0.337 |
| Ad-CMV-Pdx1 | 1.71 | 1.91 | 0.859 |
| Ad-CMV-Pax4 | 1.15 | 1.85 | 0.575 |

**Table B. 7: RNA quantification and integrity data for Figure 6.5**

| Samples | Concentration (µg/µl) | $A_{260}/A_{280}$ (<2) | $A_{260}$ (<1) |
|---|---|---|---|
| α-TC19 (------) | 1.64 | 1.91 | 0.822 |
| Ad-CMV-HNF4α | 0.71 | 1.83 | 0.355 |

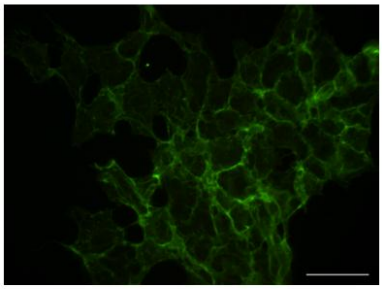
**Table B. 8: RNA quantification and integrity data for Figure 6.8A**

# Appendix C: Staining controls

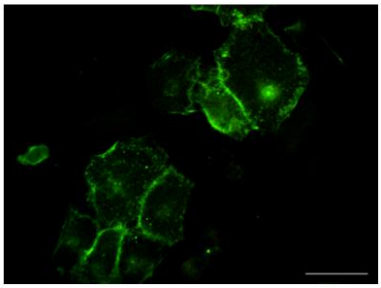
## C.1 Immunofluorescent staining

### Positive controls

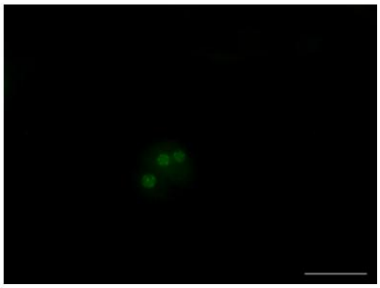
All images detail the primary antibody, manufacturer and tissue utilised (scale bar = 50μm).



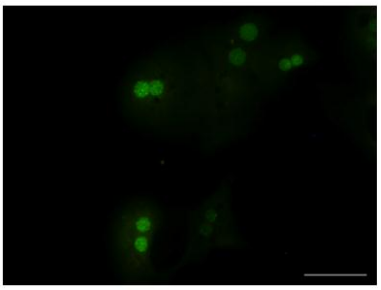
**mouse anti-A6**
Gift, Dr V.Factor
Mouse oval cell line (BMOL1.1)



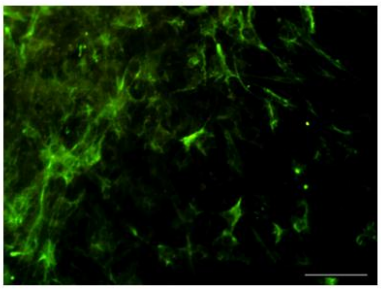
**mouse anti-β-catenin**
BD Transduction Laboratories
Mouse pancreatic ductal cells



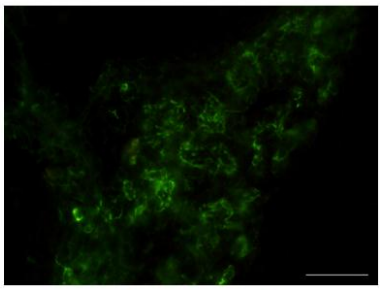
**rabbit anti-C/EBPα**
Santa Cruz Biotechnology
Mouse hepatocytes



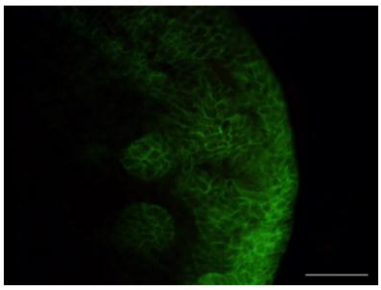
**mouse anti-C/EBPβ**
Santa Cruz Biotechnology
Mouse hepatocytes



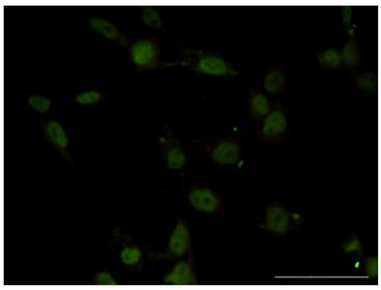
**mouse anti-Cytokeratin 7**
Abcam
Embryonic mouse pancreas



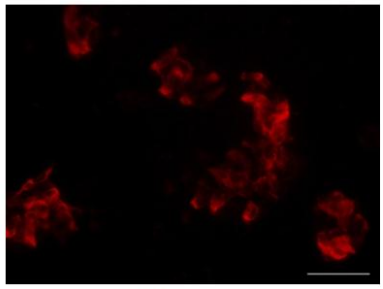
**mouse anti-Cytokeratin 20**
Dako
Embryonic mouse intestine



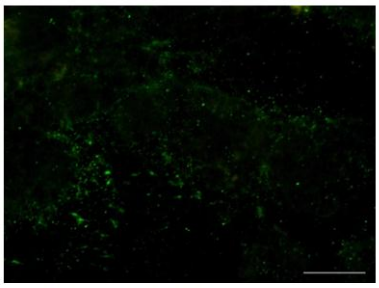
**mouse anti-E-cadherin**
BD Transduction Laboratories
Embryonic mouse intestine



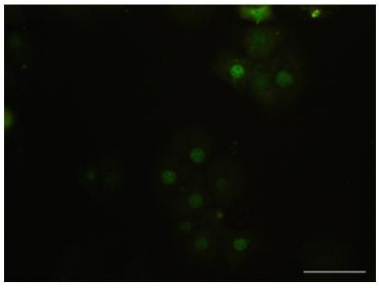
**goat anti-FoxA2**
Santa Cruz Biotechnology
Mouse oval cell line (BMOL1.1)



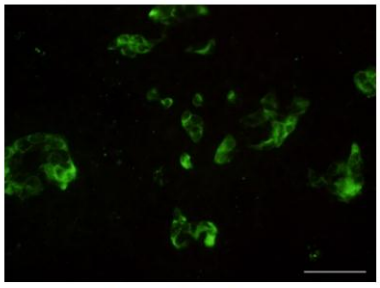
**mouse anti-Glucagon**
Sigma
Embryonic mouse pancreas



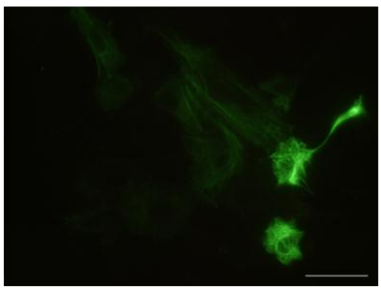
**rabbit anti-GLUT2**
Biogenesis
Embryonic mouse pancreas



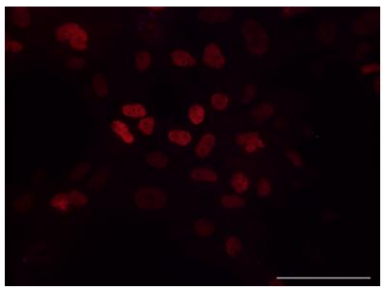
**rabbit anti-HNF4α**
Santa Cruz Biotechnology
Mouse hepatocytes



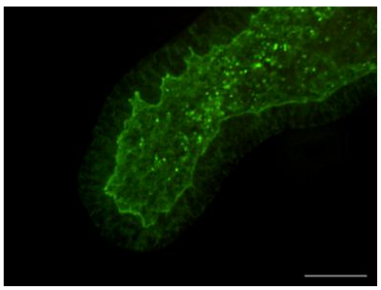
**guinea pig anti-Insulin**
Sigma
Embryonic mouse pancreas



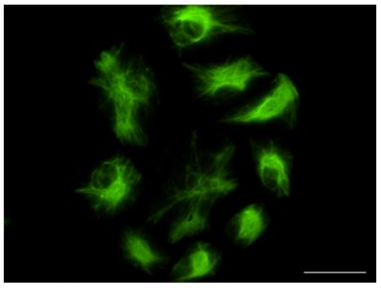
**mouse anti-OV6**
Gift, Dr Sell
Embryonic mouse liver



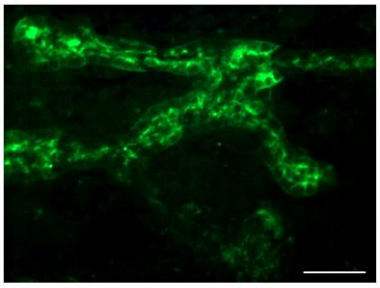
**rabbit anti-Sox9**
Chemicon
Mouse biliary epithelial cell line



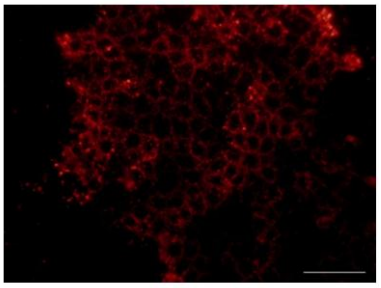
**mouse anti-Villin**
Abcam
Embryonic mouse intestine



**mouse anti-Vimentin**
Sigma
Mouse biliary epithelial cell line



**fluorescein-DBA**
Vector Laboratories
Embryonic mouse pancreas



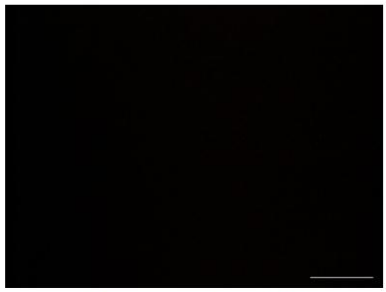
**rhodamine-PNA**
Vector Laboratories
Mouse biliary epithelial cell line

## Negative controls

All images detail the secondary antibody utilised. All antibodies were from Vector Laboratories (scale bar = 50μm).

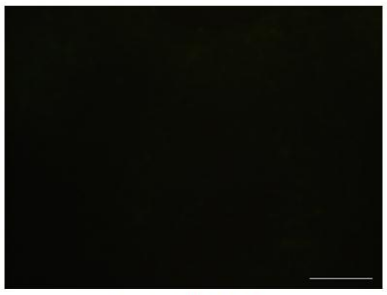
**Anti-guinea-pig FITC**

**Anti-mouse FITC**



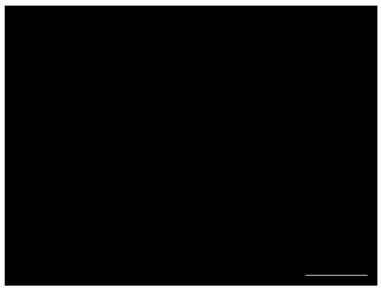
**Anti-mouse Texas Red**

**Anti-rabbit AMCA**



**Anti-rabbit FITC**

**Anti-rabbit Texas Red**



**Anti-rat FITC**

# C.2 X-gal staining for beta-galactosidase

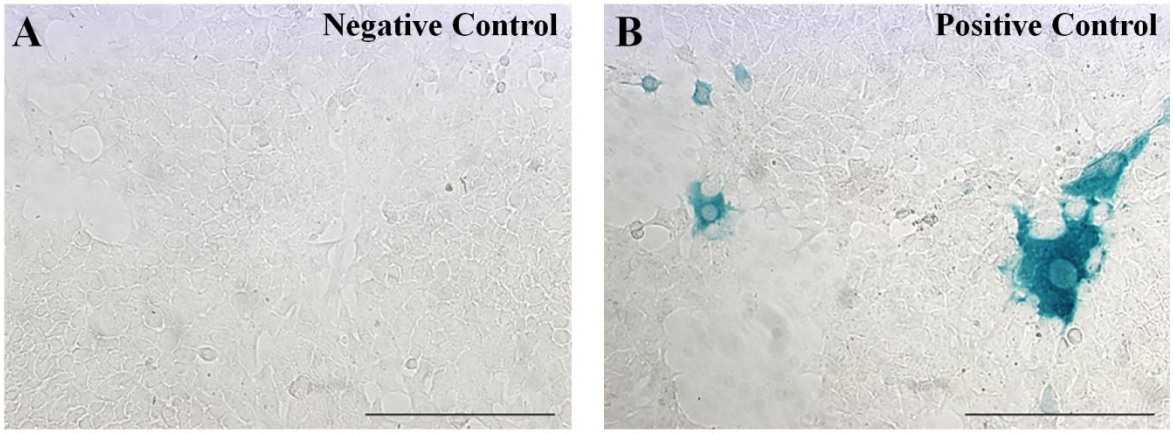



**Figure C.2: X-gal staining controls.** BMOL-TAT1.1 cells un-infected **(A)** and infected with Ad-CMV-LacZ **(B)** served as a negative and positive control respectively for the X-gal staining protocol. (scale bar = 200μm).

# C.3 Periodic acid schiff (PAS) staining

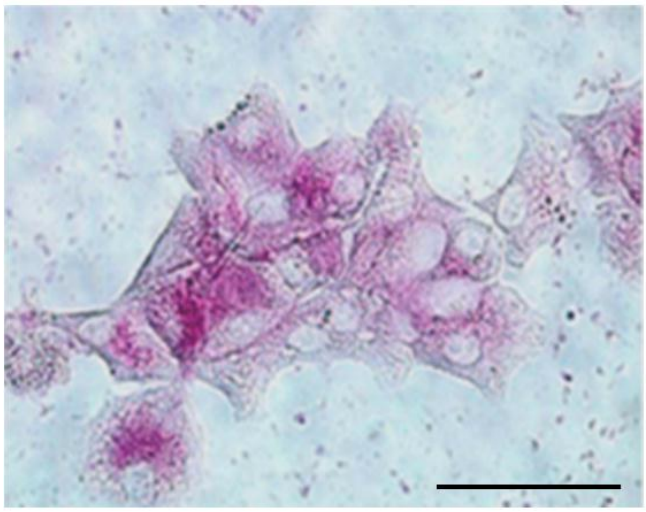


**Figure C.3: Period acid Schiff (PAS) staining positive control.** Rat hepatocytes can serve as a positive control for the PAS staining protocol (Li, et al., 2007). (scale bar = 50μm).

# Appendix D: Vector maps for the adenoviral vector utilised

Vector Maps for the adenoviral vectors utilised. All vector maps included are taken from AdEasy™ Adenoviral Vector System Instruction Manual. The genes of interest were initially cloned into pShuttle vectors.

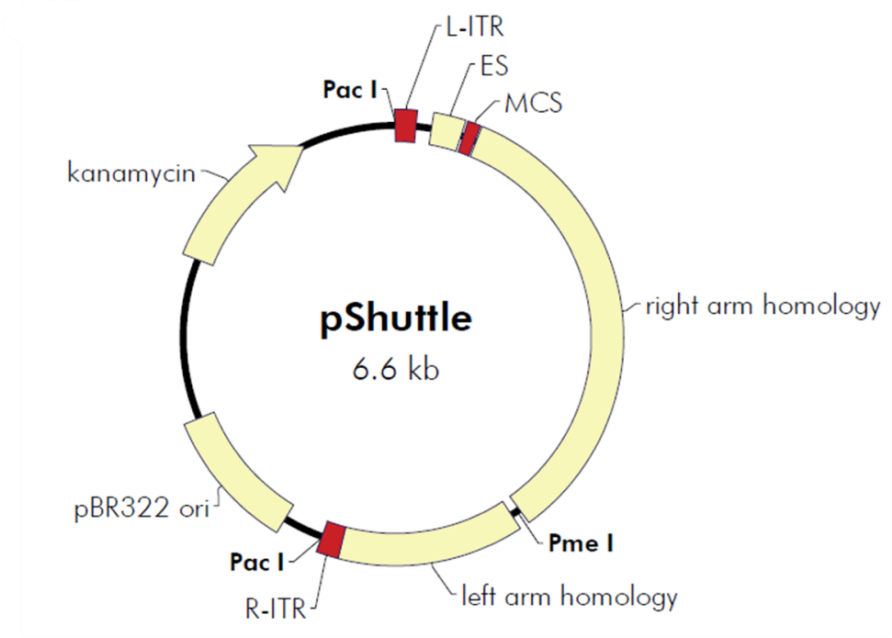



**Figure D. 1: pShuttle vector map.** For Ad-Null and Ad-RSV-GFP

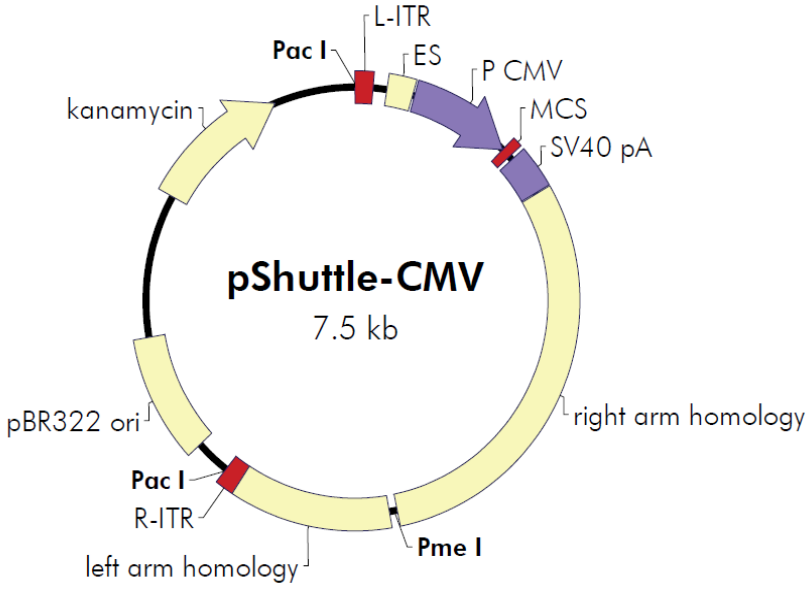



**Figure D. 2: pShuttle-CMV vector map.** For Ad-CMV-Pax4; Ad-CMV-Pdx1; Ad-CMV-C/EBPα and Ad-CMV-HNF4α.

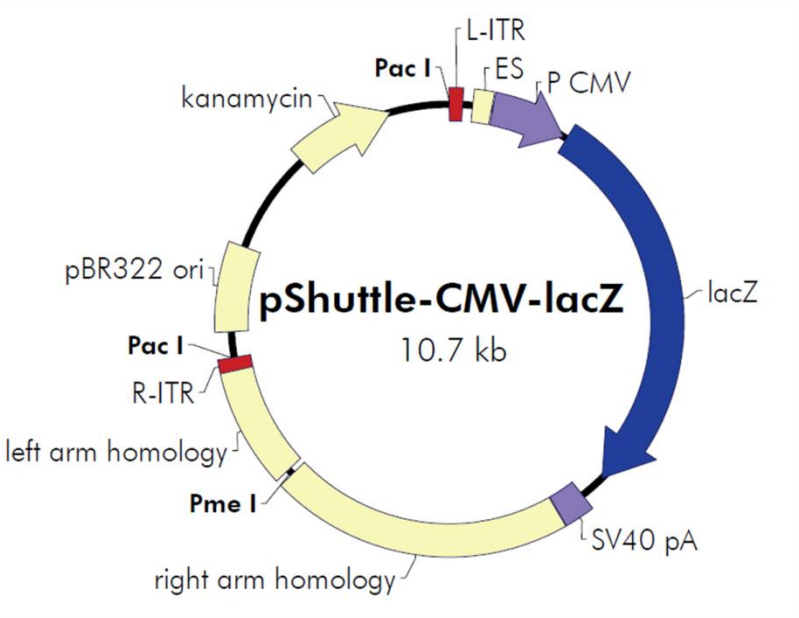



**Figure D. 3: pShuttle-CMV-*lacZ* vector map.** For Ad-CMV-LacZ

The pShuttle vectors were linearised with PmeI 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 adenovirus plasmid DNA was then subjected to PacI digestion before HEK-293 transfection for adenovirus construction.

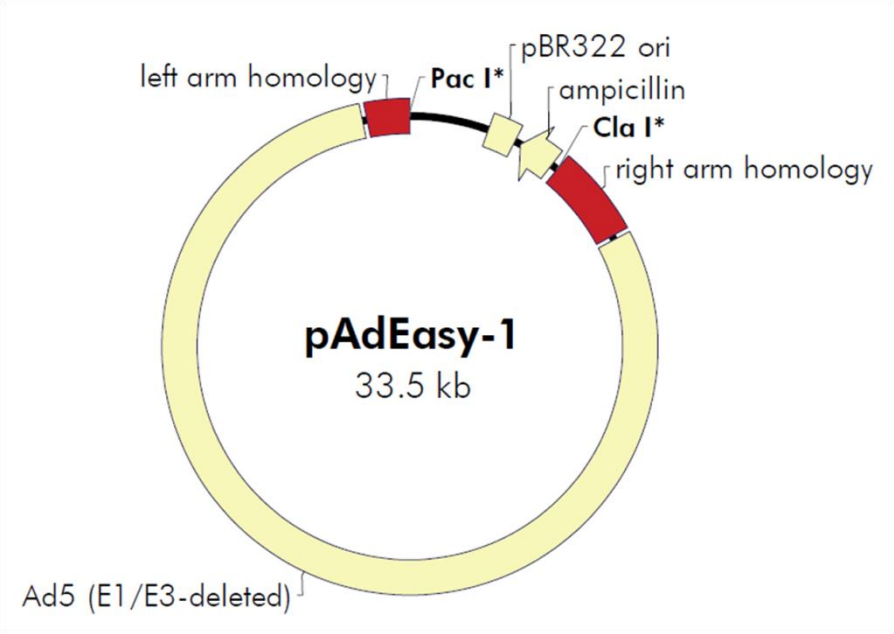



**Figure D. 4: pAdEasy-1 vector map**