Characterisation of novel cell surface-reactive antibodies for identifying and isolating liver and pancreas progenitor cells

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Supervisors:  Professor Grant Morahan (Co-ordinating supervisor)
Professor George Yeoh (Co-supervisor)
-Declaration-

The research contained in this thesis is my own unless otherwise stated. This work was carried out at the Centre for Diabetes Research at the Harry Perkins Institute of Medical Research and the School of Biomedical, Biomolecular and Chemical Sciences at the University of Western Australia. The material presented in this thesis has not been submitted for any other degree.

Benjamin Dwyer
24th June 2014
This PhD has been an unexpected adventure from start to finish. Thank you to the many people who helped make the journey possible.

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-Abstract-

The use of renewable stem cell populations to generate hepatocytes and ß-cells for therapy is a beguiling prospect to overcome the challenge of limited supply of donor organs used to treat end-stage liver disease and Type 1 Diabetes. Although progress towards this goal has been significant, generating mature cells in vitro, and overcoming the potential tumourigenicity of stem cells, are problems which must be addressed before the promise of stem-cell based therapies can be translated from the laboratory to the clinic. Understanding the mechanisms involved in expansion, differentiation and transformation are therefore vital. The liver and pancreas share both a common origin and developmental mechanisms, so studying their development in parallel should help make progress towards the goal of cell based therapies.

The ability to study the biology of liver and pancreas progenitors is currently hampered by a lack of markers to identify and isolate their progenitors. To help overcome this problem, the goals of my project were to (i) develop novel cell surface-reactive monoclonal antibodies marking liver and pancreas progenitors; (ii) to characterise the ability of these antibodies for identifying and isolating liver and pancreas progenitor cells; and (iii) to identify protein targets of useful antibodies in order to facilitate translational and functional studies. A panel of monoclonal antibodies, termed ‘Western Australian Monoclonal’ (WAM) antibodies, was generated. These antibodies were specific for antigens on the cell surface of immature liver and pancreas cells. From this panel, two antibodies, WAM18 and WAM21, recognised antigens common to immature liver and pancreas cells.

These antibodies were further characterised. First, their utility was tested in studies of the biology of liver progenitor cells (LPCs) in cell lines and in diet-induced mouse liver injury models. Double immunofluorescence with WAMs and the ‘gold standard’ LPC marker, A6, demonstrated that both antibodies marked A6-defined LPCs induced in the CDE and DDC chronic liver injury models. Critically, unlike A6, the WAM antibodies were compatible with
enzyme-dissociation and antibody-based purification of liver cells from normal and CDE-injured livers. The WAM antibodies facilitated purification of a subset of cells from the PIL-4 LPC line which co-expressed hepatocyte and ductal markers, a feature of bipotential progenitors. Interrogation of microarray data from LPC lines differentially expressing WAM18 and WAM21 antigens led to the definition of two novel candidate LPC surface markers whose expression was induced in chronic liver injury.

Having shown that WAM18 and WAM21 were useful in studies of hepatic progenitors, their capacity for isolating islet progenitor cells from the developing mouse pancreas was assessed. Initial screening studies using Ngn3-GFP/RIP-dsRed mice suggested that WAM21 would be most useful for enriching Ngn3-GFP+ islet progenitors. Characterisation of gene expression by RT-PCR confirmed that WAM21 defines immature pancreas cells with islet progenitor characteristics. Under defined culture conditions, WAM21, either alone or in combination with Cd49f, could be used for significant enrichment of pancreatic precursors capable of forming β-like cells, as defined by insulin promoter-driven fluorescent protein expression and RT-PCR analysis of gene expression.

To fulfil the final aim of this thesis, an expression cloning approach was employed to identify cDNAs encoding the WAM18 and WAM21 antigens. This led to the discovery that a single cDNA encoded a protein bearing both antigens. Inhibition of this transcript’s expression in an LPC line resulted in its reduced proliferation. Finally, a commercial antibody against the human WAM18/21 protein equivalent could enrich human foetal islet pancreas cells.

In summary, novel cell surface antibodies with demonstrated ability to identify and isolate mouse liver and pancreatic islet progenitors were developed. These new antibodies, WAM18 and WAM21, will be of great benefit for researchers studying the biology of hepatic and pancreatic progenitors for the purpose of developing methods to derive mature cells for transplantation therapy.
DECLARATION FOR THESES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION

This thesis contains published work and/or work prepared for publication, some of which has been co-authored. The bibliographical details of the work and where it appears in the thesis are outlined below.

1. Benjamin Dwyer, Emma Jamieson, Tamara Jacoby-Alner, Jasmine Low, Janina Tirnitz-Parker, Kathleen Davern, George Yeoh and Grant Morahan. *Generation and characterisation of novel cell surface antibodies to mouse liver progenitor cells.*

Manuscript prepared for submission to *Stem Cell Research.*  
Presented in Chapter 2.  
*This study describes the generation and characterisation of a panel of monoclonal antibodies and the development of two of these antibodies, WAM18 and WAM21, for identifying and isolating liver progenitor cells from progenitor cell lines and chronically injured liver.*

**Contribution statement:** BD designed and carried out experiments (animal studies, Immunostaining, flow cytometry, PCR and data mining) and drafted the manuscript. EJ, BD and TJA performed antibody screening studies. JL and JTP performed microarrays on progenitor cell lines and CDE tissue respectively. TJA and KD produced and purified antibodies used in this study. All authors read and approved the final manuscript. GY and GM conceived the study and contributed to experimental design and critical evaluation of the manuscript.

Manuscript prepared for submission to Diabetologia. Presented in Chapter 3.

This study describes the use of one of our antibodies, WAM21, for isolating islet progenitor cells which can differentiate to insulin-producing cells from the developing mouse pancreas as a model for studying beta cell development in vitro.

Contribution statement: BD designed and carried out WAM21-based experiments and drafted the manuscript. EJ performed antibody screening studies. TJA and KD produced and purified antibodies used in this study. FXJ developed the foetal pancreas differentiation protocol and contributed to experimental design and critical evaluation of the manuscript. MM developed and provided Ngn3-GFP/RIP-dsRed mice. GM conceived the study and contributed to experimental design and critical evaluation of the manuscript. All authors read and approved the final manuscript.
3. Benjamin Dwyer, Emma Jamieson, Erika Bosio, Nadine Dudek, Tamara Jacoby-Alner, Kathleen Davern and Grant Morahan. Expression cloning a single cDNA encoding the WAM18 and WAM21 antigens.

**Manuscript in preparation. Presented in Chapter 4, Results 3**

This study describes the identification of a cDNA encoding the WAM18 and WAM21 antigens using an expression cloning approach and subsequent use of a human equivalent antibody in isolating islet progenitor cells from developing human pancreas.

**Contribution statement:** BD designed and carried out experiments (expression cloning, Immunostaining, PCR, data mining, Western blotting, isolation and FACS analysis of foetal tissues, glycosylation studies, RNAi studies, cell proliferation analyses) and drafted the manuscript. EJ and EB isolated human foetal pancreas cells. ND performed 2D Western blotting, TJA and KD produced and purified antibodies used in this study. BD and GM conceived the study. GM conceived the study and contributed to experimental design and critical evaluation of the manuscript.

Benjamin Dwyer (Candidate)

Professor Grant Morahan (Co-ordinating supervisor)
Abbreviations

A absorbance
AFP alpha-fetoprotein
Alb albumin
ALT alanine transaminase
ANOVA analysis of variance
αSMA alpha-smooth muscle actin
BMOL bipotential murine oval liver
BMP bone morphogenetic protein
bp base pairs
BSA bovine serum albumin
CD cluster of differentiation
CDE choline deficient, ethionine-supplemented
cDNA complimentary deoxyribonucleic acid
C/EBP CCAAT/enhancer-binding protein
CK cytokeratin
COS CV-1 (simian) in Origin, and carrying SV40 genetic material
CX connexin

DAPI 4’6-diamidino-2-phenylindole
DDC 3,5-diethoxycarbonyl-1,4-dihydrocollidine
DM diabetes mellitus
DMSO dimethylsulphoxide
DMEM Dulbecco’s modified eagle medium
DNA deoxyribonucleic acid
DNase deoxyribonuclease

E embryonic day
EDTA ethylene diamine tetraacetic acid
EGTA ethylene glycol tetraacetic acid
<table>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
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<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
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<td>foetal bovine serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>forkhead box A</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma-glutamyl-transpeptidase</td>
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<tr>
<td>GLUT</td>
<td>glucose transporter</td>
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<td>glucocorticoid response element</td>
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<tr>
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<td>hepatocyte growth factor</td>
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<td>hepatocyte nuclear factor</td>
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<td>hepatic stellate cell</td>
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<td>IGF</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>i/p</td>
<td>intraperitoneal</td>
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<tr>
<td>iPSC</td>
<td>induced pluripotent cell</td>
</tr>
<tr>
<td>ITS</td>
<td>insulin, transferrin, selenium</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KLF</td>
<td>Kruppel-like factor</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>LPC</td>
<td>liver progenitor cell</td>
</tr>
<tr>
<td>LT</td>
<td>lymphotoxin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-------------</td>
<td>-----------------------------------------------------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>M(_2)PK</td>
<td>muscle pyruvate kinase 2</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger-ribonucleic acid</td>
</tr>
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<td>MYT1</td>
<td>Myelin transcription factor 1</td>
</tr>
<tr>
<td>OCT4</td>
<td>octamer-binding transcription factor-4</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cooling temperature medium</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>OSM</td>
<td>oncostatin M</td>
</tr>
<tr>
<td>p</td>
<td>passage</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PIL</td>
<td>p53(^{-/-}) immortalised liver</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
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<tr>
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<td>reverse transcription-polymerase chain reaction</td>
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<td>sodium dodecyl sulphate–polyacrylamide gel electrophoresis</td>
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<td>SRY-box</td>
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</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
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<td>tyrosine aminotransferase</td>
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<tr>
<td>Thy-1</td>
<td>thymocyte differentiation antigen-1</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-like plasminogen activator</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless</td>
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Chapter 1

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GENERAL INTRODUCTION

1.0 Introduction

1.0.1 Liver and pancreas structure and function

In complex organisms, including mammals, the liver and pancreas co-ordinately control metabolism, producing hormones and enzymes which regulate blood glucose levels and support digestion. The liver is the primary site in the body for xenobiotic detoxification and nutrient processing, accepting blood via the portal vein laden with nutrients and secretions from the gut which are processed by hepatocytes. Hepatocytes constitute around 80% of the liver mass and secrete plasma proteins, synthesise amino acids and lipids into the liver sinusoids (the capillary system of the liver), and produce bile which is drained into bile canaliculi. From the canaliculi, bile drains into the terminal duct system, composed of cholangiocytes, and is transported to the small intestine to aid in digestion or to the gall bladder for storage (Figure 1.1). Uptake of glucose from the bloodstream into hepatocytes and its storage (in the form of glycogen), or release is controlled by hormone signalling from the endocrine pancreas.

The pancreas is composed of three distinct tissue types, exocrine, endocrine and ductal. The exocrine pancreas is composed of acinar cells, which produce nucleases, proteases, pancreatic amylase and lipases for digestion. Acinar cells form lobular structures, termed acini, which line the ductal network, giving the pancreas its alveolar-like appearance. The pancreatic duct system carries these exocrine secretions and drains into the duodenum via the main pancreatic duct, which interfaces with the ductal system exiting the liver. Interspersed throughout the acinar tissue are the endocrine ‘Islets of Langerhans’ (termed ‘islets’). Islets make up 1-2% of the total pancreatic weight in healthy adults and form spherical structures composed of five major cell types which produce specific endocrine hormones. These cell types are α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), P.P.-cells (pancreatic peptide) and ε-cells (ghrelin). The islets have a distinct structure with the majority of β-cells contained at the centre of the islet and the other cell types
dispersed around the periphery (Figure 1.1). Despite their relatively small volume, islets command up to 20% of the total pancreatic blood flow (Lifson et al., 1980), allowing for effective islet-mediated metabolic control exerted by hormone secretions of the five different islet cell types (Figure 1.1).

The β-cells constitute around 75% of the islet and produce the hormone insulin in response to high glucose levels. Glucose sensing takes place via the transport of glucose into the β-cell via the high affinity glucose transporter-2 (Glut-2). The α-cells comprise around 20% of the islet and release the hormone glucagon. In response to low blood glucose levels, glucagon is released into the bloodstream where it signals to the liver to upregulate glycogenolytic/gluconeogenic pathways to raise blood glucose levels. (Reviewed in Korc, 1993). Delta cells comprise up to 5% of the islet and are characterised by their production of the hormone somatostatin, which acts on the β-cells to inhibit insulin production. Making up less than 1% of islet cells are P.P-cells and ε-cells that make pancreatic polypeptide and ghrelin respectively. Pancreatic polypeptide is a paracrine hormone which inhibits acinar secretions whilst ghrelin exerts a positive effect on β-cell proliferation, survival and insulin release (Reviewed in Granata et al., 2010).
Figure 1.1. Spatial relationship and structural overview of the liver and pancreas. Exocrine secretions from the liver and pancreas drain via a shared ductal system into the duodenum to aid digestion. The liver is composed of hepatocytes which secrete bile into the bile canaliculi and process nutrients and secrete hormones into the adjacent sinusoidal blood vessels. Blood from the gut enters the liver via the portal vein (PV) and oxygenated blood via the hepatic artery (HA). Together, with the terminal bile ducts, these three structures form the ‘portal triad.’ Blood exits the liver via the central vein (CV). Liver-resident Stellate and Kupffer (macrophage) cells store fat and clear debris respectively. Pancreatic enzymes are produced by acinar cells that surround the ductal system and feed into the common bile duct connecting the liver, pancreas and duodenum. Endocrine hormones are produced by ‘Islet of Langerhans’ cells and are secreted into a rich network of islet-associated capillaries. Figure adapted from Burke and Tosh (2012).
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1.0.2 Diseases of the liver and pancreas

1.0.2.1 Diabetes Mellitus

Diabetes mellitus (DM) is the term which describes metabolic disorders where hyperglycaemia is observed due to inadequate insulin production, or impaired tissue responses to insulin signalling. Worldwide, ~285 million adults (6.4% of people aged 20 to 79) were affected by DM in 2010, and this number is projected to reach 439 million (7.7%) by 2030 (Shaw et al., 2010). DM can be broadly divided into two subtypes; Type 1 (T1DM), results from autoimmune destruction of islet β-cells and subsequent insulin-deficiency, whilst Type 2 (T2DM) results from inadequate insulin production from β-cells and tissue insulin resistance. T2DM is the major subtype of DM, affecting ~90% of DM patients. It is typically diagnosed during middle age (>40 years) and is associated with risk factors such as age, obesity and genetic predisposition. Conversely, T1DM onset typically occurs during puberty and affects 5-10% of all diabetes patients (Harjutsalo et al., 2008). Recent efforts to investigate genetic risk factors associated with T1DM have demonstrated the complexity of this disease, identifying over 50 risk-associated genetic regions via genetic linkage analyses (Barrett et al., 2009, Morahan et al., 2011).

Overall, DM is the 9th leading cause of global deaths (Lozano et al., 2012). Conventional treatment for T1DM involves administration of exogenous insulin and blood glucose monitoring, which does not achieve complete glucoregulation. Complications arising from inadequate blood glucose homeostasis include diseases such as retinopathy, nephropathy, neuropathy, cardiovascular disease and stroke, which result in significant reduction in lifespan and quality of life (extensively reviewed in Forbes and Cooper, 2013). Even with exogenous insulin treatment, half of all patients will develop diabetic complications after 30 years with the disease (Nathan et al., 2009). Since insulin release is glucose-dependent in β-cells, these outcomes may be improved with islet transplantation from cadaveric donors (Shapiro et al., 2000). Unfortunately, demand for transplant tissue far outweighs its supply.
1.0.2.2 Liver disease
Liver disease can also be divided into two major subtypes, acute and chronic, depending on the duration and progression of injury. Acute liver disease is most commonly caused by agents such as acetaminophen toxicity, drug-induced hepatotoxicity, acute viral hepatitis infection or autoimmune liver disease (Polson et al., 2005). The survival rates for such diseases are 48% prior-to, and 74% following liver transplantation (Bernal et al., 2013). In chronic disease, liver fibrosis develops during sustained injury and can progress to cirrhosis and hepatocellular carcinoma (HCC) and/or liver failure. Examples of fibrosis-causing agents include chronic hepatitis B and C infection, non-alcoholic fatty liver disease and genetic hemochromatosis. Liver cirrhosis and liver cancer are the 12th and 16th leading causes of global deaths respectively (Lozano et al., 2012), with most HCC cases occurring in the setting of established cirrhosis, with a median survival of 6-16 months if untreated (Bruix et al., 2005). Although treatment options such as antiviral therapy and weight reduction strategies can be effective, significant numbers of patients progress to end-stage liver disease and require orthotopic liver transplantation.

Although T1DM and liver disease are treatable through organ transplant, limited availability of donor organs and religious and/or economic reasons may restrict access to transplantation surgery (Johnson et al., 2014). Given the predicted rise in prevalence in liver disease and T1DM, alternative cellular sources are needed to meet the demand for transplant tissue. Stem/progenitor cells which can be expanded \textit{in vitro} to clinically useful proportions, and induced to form hepatocytes or \(\beta\)-cells represent a promising renewable source of cells for transplantation.

1.0.2.3 Progenitor-derived hepatocytes and \(\beta\)-cells
Because of their capacity to proliferate and form virtually any tissue type, stem/progenitor cells represent ideal cellular sources of surrogate hepatocytes and \(\beta\)-cells. Pluripotent embryonic stem cells (ESCs) can be derived from the
inner cell mass of blastocysts from pre-implantation embryos (Thomson et al., 1998). Alternatively, somatic cells can be reprogrammed to form induced pluripotent cells (iPSCs) via forced upregulation of the ‘pluripotency factors’ c-MYC, SOX2 OCT4 and KLF4 (Takahashi et al., 2007). Methods to derive liver and pancreas cells from pluripotent cell sources have involved attempts to recapitulate patterning signals of embryonic development in vitro to differentiate cells into hepatocytes or β-cells (Kroon et al., 2008, Si-Tayeb et al., 2010). Although much progress has been made towards producing clinically viable cells in vitro, concerns over both the potential tumourigenicity of pluripotent-derived cells (reviewed in Lee et al., 2013) and the ability to derive fully functional cells in vitro, still limit the translation of these technologies to the clinic.

Understanding the signals involved in patterning liver and pancreas domains, as well as promoting their proliferation and differentiation into functional tissue, is vitally important to develop efficient protocols to derive therapeutically viable cells. Additionally, since stem/progenitor cells and cancer cells share common pathways in activation and differentiation, the study of these processes also has application in chemotherapeutic targeting, particularly in the liver.

The ability to purify and culture organ-specific progenitor cell domains is fundamental in achieving this outcome. This introduction will explore what is currently known about the developmental programs of each organ, and how these programs are recapitulated during progenitor cell activation in disease and tumorigenesis. Finally, it will assess the current state and limitations in phenotyping liver and pancreas progenitor cells using monoclonal antibodies, and how these limitations may be overcome.
1.1 Liver and pancreas development

Because of their shared origins, the liver and pancreas are ideal organs to study in parallel. The following section of this review will examine the fundamental concepts of liver and pancreas development from primitive endoderm cells and is summarised in Figure 1.2.

1.1.1 Shared origins of the liver and pancreas

The liver and pancreas develop from a common pool of endodermal hepatopancreatic precursor cells which upregulate organ-specific gene expression cascades in response to Fibroblast Growth Factor (FGF) signals from the surrounding mesenchyme at E8.0-E8.5 in the mouse (Deutsch et al., 2001, Rossi et al., 2001, Serls et al., 2005). During this period, expression of key transcription factors such as the CAAT-enhancer binding protein family of transcription factors (C/EBPα, C/EBPβ), in concert with modulators of hepatic gene expression, Hepatocyte Nuclear Factor (Hnf)-4 and alpha fetoprotein (AFP), define the hepatic rudiment (Westmacott et al., 2006). Concurrent with C/EBP family expression in the liver primordium, pancreas development is initiated by the expression of the Pancreatic and Duodenal Homeobox 1 transcription factor (Pdx1) in the absence of FGF signalling (Gualdi et al., 1996), which initiates pancreas-specific gene expression (Jonsson et al., 1994, Offield et al., 1996). Organ-specific gene expression is further reinforced by the actions of Bone Morphogenetic Proteins (BMP), which suppress pancreatic gene expression (Rossi et al., 2001), and Retinoic acid (RA) signals that promote pancreatic specification but do not affect the liver (Chen et al., 2004, Martin et al., 2005, Molotkov et al., 2005). Suppression of Sonic Hedgehog (Shh) signalling by Activin-βB and FGF2 signalling from the notochord permits the formation of the dorsal pancreatic domain by promoting pancreas over stomach/duodenal fate (Apelqvist et al., 1997, Hebrok et al., 1998).

Following organ specification, the liver and pancreas undergo a period of expansion and morphogenesis. In the pancreas, this period is known as the
‘primary transition’ and involves the development of the pancreatic bud into a branched epithelium via a process of ‘branching morphogenesis’. Initially, the pancreatic primordium becomes stratified, before invading the surrounding mesenchyme and forming a ‘tree-like’ network of epithelial tubes (Villasenor et al., 2010). This process is driven by proliferative multipotent Carboxypeptidase-A1(Cpa1)+/Pdx1+ progenitor cells present at the distal tips of the branching epithelium (Zhou et al., 2007). In the liver, newly specified hepatoblasts delaminate and migrate through the basement membrane and invade the surrounding mesenchyme. The hepatoblasts continue to proliferate, forming distinct cords interspersed with mesenchyme which forms the hepatic sinusoids (Reviewed in Lemaigre, 2009). This process occurs under the control of the Hex homeobox protein and Prox1 (Sosa-Pineda et al., 2000), which along with Gata-6 promote the expression of albumin throughout the hepatic primordium (Gualdi et al., 1996, Bossard and Zaret, 1998). During this early expansion period, the transient upregulation of the Wingless (Wnt)/β-catenin pathway in the liver (Suksaweang et al., 2004, Tan et al., 2008) and Notch signalling pathway in the pancreas (Apelqvist et al., 1999, Jensen et al., 2000, Hald et al., 2003) promotes proliferation of organ-specific progenitors, whilst inhibiting premature differentiation.

1.1.2 Fate choices in liver and pancreas progenitor domains

As well as controlling expansion, Notch and Wnt/β-catenin signalling regulate cell lineage choices in the multipotent progenitors of the liver and pancreas. Notch signalling promotes ductal over non-ductal differentiation in both organs. If Notch signalling is activated in liver progenitors, ductal-specific genes are upregulated and hepatocytic gene expression is repressed (Tanimizu and Miyajima, 2004). During liver development, cells positive for the Notch ligand, Jagged-1, surround portal veins in close proximity to Notch2+ hepatoblasts that form cholangiocytes (Kodama et al., 2004, Tanimizu and Miyajima, 2004). Accordingly, duct formation is severely impaired in Notch-2 knockout mice but hepatocyte differentiation is not affected (Geisler et al., 2008).
Similarly, endocrine and ductal fates are determined in the pancreas by the interplay between Notch signalling and the islet-specific transcription factor Neurogenin-3 (Ngn3). This period from e13.5 to e17.5 is termed the ‘secondary transition’, and marks the time when islet progenitors are patterned into hormone-specific adult islet cell subtypes (Gradwohl et al., 2000, Schwitzgebel et al., 2000, Desgraz and Herrera, 2009). If Notch signalling is activated, endocrine-specific gene expression is suppressed and ductal cells form (Ahnfelt-Ronne et al., 2007). Conversely, upregulation of Ngn3 in islet progenitors promotes islet cell fate and reduction of competitive-inhibition of Notch signalling in adjacent ductal cells (Magenheim et al., 2011). Ngn3+ cells originate from Pdx1+ epithelium at e9.0 in the dorsal mouse pancreas and e10.5 in the ventral pancreas (Apelqvist et al., 1999, Gu et al., 2002). Ngn3 expression is transient during the secondary transition, peaking at e15.5 during islet progenitor expansion, then decreasing to be at low levels by e17.5 in the mouse, during which time islet lineages are determined (Apelqvist et al., 1999).

Hepatocyte and acinar cell differentiation are promoted by active Wnt/β-catenin signalling in the liver and pancreas. Targeted inactivation of Apc following hepatic specification leads to hypoplastic liver growth and embryonic lethality due to failure of hepatocyte differentiation. Meanwhile, cholangiocyte differentiation is unaffected and these cells form bile ducts when transplanted into normal recipients (Decaens et al., 2008). Knocking out β-catenin in the embryonic pancreas results in exocrine hypoplasia (Wells et al., 2007) and reduced acinar differentiation (Dessimoz et al., 2005, Wells et al., 2007).

1.1.3 Cell-type specific transcription factors

1.1.3.1 Gene expression networks for islet cell specification

The initiation of Ngn3 expression in islet progenitors promotes the upregulation of the islet developmental program (summarised in Figure 1.2), leading to the differentiation of functional adult islet cells (Juhl et al., 2008).
Pax4 and Pax6 are essential for endocrine development in duodenal, stomach and pancreatic cells (Larsson et al., 1998) and function to specify the β/δ-cell (Pax4) or α/PP-cell (Pax6) lineages in the pancreas. Evidence for this role comes from transgenic mouse studies. Pax4 mutant mice do not develop β- or δ-cells (Sosa-Pineda et al., 1997) and Pax4 knockout mice produce α-cells at the expense of the β/δ-cell lineage (Dohrmann et al., 2000). Pax6 knockout mice do not develop α-cells and display a perturbed islet structure (St-Onge et al., 1997). Additionally, Pax4/Pax6 double knockout mice do not develop any mature endocrine cells (Larsson et al., 1998). Nkx2.2 is regulated by Pax4, Ngn3 and Hnf1β (Wilson et al., 2003) and its inactivation leads to maturation arrest of β-cells (Sussel et al., 1998). Nkx6.1 expression is downstream of Nkx2.2 and is restricted to β-cells in neonatal development. Knockout of Nkx2.2/Nkx6.1 blocks β-cell differentiation (Sander et al., 2000).

In the adult pancreas, Pdx1 is restricted to endocrine cells and its downregulation is critical for ductal maturation and function (Deramaudt et al., 2006). In the islets, Pdx1 protein activates the downstream signalling cascade for proper endocrine development (Oliver-Krasinski et al., 2009) and in adult β-cells, is necessary for insulin gene transcription and β-cell function (Melloul, 2004, Babu et al., 2007). Islet-specific Pdx1 transcription is controlled by a combination of transcription factors including; Hnf1 (Gerrish et al., 2001), FoxA2 (Wu et al., 1997, Gerrish et al., 2000, Marshak et al., 2000), Pax6 (Samaras et al., 2002), Nkx2.2 (Van Velkinburgh et al., 2005), Hnf6 (Jacquemin et al., 2003), MafA (Vanhoose et al., 2008) and Pdx1 protein itself (Gerrish et al., 2001). MafA is a β-cell enriched transcription factor which acts to control Insulin (Zhao et al., 2005, Artner et al., 2008) and Pdx1 (Vanhoose et al., 2008) gene transcription, maintaining β-cell specific gene expression and function. Though initial studies found that Ngn3 is not expressed in adult pancreas (Apelqvist et al., 1999, Gradwohl et al., 2000), more recent studies have shown by RT-PCR, western blotting and fluorescent imaging that Ngn3 is both expressed and required for adult β-cell function (Wang et al., 2009). Furthermore, transgenic studies have demonstrated
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*Insulin* and *Ngn3* promoter driven fluorescent protein co-expression in the developing pancreas (Mellitzer et al., 2004, Hara et al., 2006).

### 1.1.3.2 Liver lineage determination

In addition to *C/EBPα*, *Hnf4* is a key regulator of adult hepatic genes (Parviz et al., 2003), binding to nearly half of transcriptionally active chromatin in human hepatocytes (Odom et al., 2004), and regulating a number of genes controlling hepatocyte maturation (Li et al., 2000). Furthermore, *Hnf4* overexpression in undifferentiated hepatoma cells promotes hepatocyte differentiation (Spath and Weiss, 1997). *Hnf6* is expressed in hepatoblasts and biliary epithelial cells, driving the cholangiocyte differentiation program following its upregulation in response to *C/EBPα* suppression (Yamasaki et al., 2006). Hnf6 regulates the correct timing and positioning of cholangiocyte differentiation, restricting bile duct formation to areas around the portal mesenchyme. Downstream of Hnf6, Hnf1β promotes biliary differentiation (Clotman et al., 2002). It is not until around e15.5 that the ductal plate starts to form in proximity to the hepatic sinusoids, which promote bile duct fate via Transforming Growth Factors (TGF’s), BMP’s and FGF signalling. Studies of hepatic explant cultures have shown BMP4 enhances FGF signalling to promote bile duct formation. FGF receptors are expressed in portal hepatoblasts and differentiate into cholangiocytes under the influence of mesenchymal bFGF and FGF7. Differentiation is enhanced by BMP4 which is also produced by liver mesenchymal cells (Yanai et al., 2008). Between e15.5 and e18.5, hepatocyte and cholangiocyte maturation occurs and a distinct ductular network forms (Reviewed in Lemaigre, 2009; Figure 1.2).
Figure 1.2. Lineage determination in the liver and pancreas. Endodermal precursors are patterned into liver or pancreas fates under the control of FGF and BMP signals from the mesenchyme. Liver and pancreas proliferation and fate choice is controlled by Wnt and Notch signalling, which divert the organ primordium away from ductal and towards parenchymal/islet lineages.
1.2 Liver and pancreas progenitors in regeneration

Under certain circumstances, progenitor cells in the liver and pancreas proliferate and differentiate to regenerate lost tissue. Understanding the mechanisms underlying these processes is important for identifying factors that could be used to derive functional cells for transplantation as well as mechanisms leading to progenitor cell tumorigenesis. In this section, we will explore the role of adult-derived stem cells in regenerative models in the liver and pancreas.

1.2.1 Liver progenitor cells

1.2.1.1 Progenitor cell mediated liver regeneration

The liver possesses a remarkable ability to regenerate via the division of remaining hepatocytes if acute injury, such as tissue resection or hepatocyte necrosis occurs. However, hepatocyte proliferation is impaired by chronic liver injuries, necessitating alternative pathways for liver regeneration. In response to signals from dying hepatocytes and immunomodulatory molecules associated with liver inflammation, a progenitor cell compartment is activated. Unlike hepatocytes, liver progenitor cells (LPCs) retain proliferative capacity and can differentiate to restore hepatocytes under severe injury conditions.

LPCs are a liver-resident population residing in the ‘Canals of Hering’, the interface between the hepatocytes canaliculi and biliary tree (Theise et al., 1999), and are characterised by their co-expression of hepatocyte and ductal markers (Germain et al., 1985, Jelnes et al., 2007) and ability to form both of these tissues (Alison, 2003, Wang et al., 2003). Proof of the regenerative capacity of LPCs during liver injury has come from lineage tracing studies in mouse models of chronic liver injury, which have demonstrated that LPCs arise from a subset of Sox9+ ductal cells that proliferate and differentiate to replace damaged hepatocytes (Furuyama et al., 2011, Espanol-Suner et al., 2011).
Early chronic liver injury is characterised by infiltration of inflammatory leukocytes which produce cytokines such as Tumour Necrosis Factor (TNF) (Knight et al., 2000, Viebahn et al., 2006) and Interferon (IFN)-γ (Akhurst et al., 2005) that promote LPC proliferation at the expense of hepatocyte division (Brooling et al., 2005). Macrophages, which function to clear cellular debris from the sites of injury, secrete TNF-like Weak Inducer of Apoptosis (TWEAK) (Viebahn et al., 2010), which signals via its receptor Fn14, present on LPCs, to promote their proliferation (Tirnitz-Parker et al., 2010). Transforming Growth Factor (TGF)-β is a suppressor of cell division. However, LPC's are significantly less sensitive to TGFβ-mediated growth inhibition compared to hepatocytes (Nguyen et al., 2007). Dying hepatocytes also support regeneration by releasing Hedgehog ligand (Jung et al., 2010) which signals via canonical Smoothened-dependent signalling to promote proliferation of primary-cilium positive LPCs (Grzelak et al., 2014). Thus, following hepatocyte injury and liver inflammation, a microenvironment develops which promotes LPC-mediated regeneration whilst suppressing hepatocyte proliferation.

The inflammatory reaction to chronic liver injury also initiates the fibrogenic wound healing response. This response is mediated by liver resident perisinusoidal Ito cells, also termed hepatic stellate cells (HSCs). In response to inflammatory signals, HSCs transition to an ‘activated’ state characterised by their expression of α-smooth muscle actin (αSMA) (Mederacke et al., 2013). Activated HSCs deposit extracellular matrix (ECM) proteins which act to shield the surrounding liver from diseased tissue, and modulate LPC biology. They also reinforce the wound healing and repair responses by recruiting inflammatory and progenitor cells to the site of injury via the expression of chemotactic factors such as intercellular adhesion molecule 1 (ICAM-1) and
regulated upon activation, normal T-cell expressed and secreted (RANTES)
(Ruddell et al., 2009).

If the hepatic insult is removed, fibrosis recedes and the structural and
functional integrity of the liver is restored. Conversely, continuing hepatic
insult reinforces the regenerative and fibrotic responses which become
pathological, progressing to liver cirrhosis and failure. Persistent stimulation
of LPCs by pro-proliferative/survival signals from inflammatory cells can give
rise to genetic and epigenetic mutations and subsequent neoplastic
transformation of LPCs resulting in the formation of hepatocellular
carcinoma (HCC) associated with chronic liver injury.

1.2.1.2 LPCs as a tumour precursor

Observations in chronic liver disease patients and rodent models of liver
disease have established LPCs as a potential HCC precursor cell. LPCs are
observed at all stages during chronic liver disease progression, regardless of
the underlying pathology, including chronic hepatitis B (HBV) and C virus
(HCV) infection (Lowes et al., 1999, Sobaniec-Lotowska et al., 2007), non-
alcoholic fatty liver disease (Nobili et al., 2012), alcoholic liver disease and
 genetic hemochromatosis (Lowes et al., 1999). LPC numbers increase with
disease severity in a number of different liver chronic liver injury pathologies
(Lowes et al., 1999, Clouston et al., 2005) and are observed in association with
human liver tumorigenic progression from preneoplastic lesions (Roskams et
al., 1996) to well-developed HCCs (Lee et al., 2005, Lee et al., 2006, Haybaeck
et al., 2009). Although these studies associate LPCs with the progression of
liver damage to tumorigenesis, several types of evidence from cell culture and
rodent modelling of chronic liver disease support the hypothesis that LPCs
represent a direct cellular precursor of HCC.

The mouse choline deficient, ethionine-supplemented (CDE) diet is a well-
established regime to induce hepatocyte damage, liver inflammation, fibrosis
and LPC activation in short-term studies (Akhurst et al., 2001) which progresses to neoplasia and HCC development in long-term studies (Knight et al., 2000). In this model, tumour incidence is reduced by inhibiting of LPCs by cytokine receptor knockout (Knight et al., 2000) or chemical inhibition (Knight et al., 2008) suggesting a contribution of LPCs to hepatocarcinogenesis. In transgenic mice with liver-specific doxycycline-controlled MYC oncogene expression, induction of MYC expression in liver cells gives rise to tumours in LPC-like cells which are dormant in non-induced tissue. Following MYC-induced carcinogenesis, these tumour cells differentiate and/or undergo apoptosis if inducible MYC oncogene expression is inactivated, providing evidence of a precursor cell-type within the liver that has tumour-forming potential (Shachaf et al., 2004).

*In vitro* modelling of HCC supports a progenitor cell origin of liver cancer. LPC lines have been isolated from p53-null mice fed a CDE diet which differ in their tumorigenic potential when injected into nude mice (Dumble et al., 2002) providing evidence that subpopulations of LPCs have the potential to give rise to tumours. Recently, it was demonstrated that cell lines can be generated from resected liver tissue surrounding human HCC containing cells positive for LPC markers. The cell lines derived were phenotypically similar to LPCs observed *in vivo* and gave rise to tumours in nude mice suggesting a precursor-product relationship between the LPCs observed *in vivo* and susceptibility to neoplastic transformation following culture immortalisation (Zhang et al., 2010).

Altogether, these studies provide a compelling argument that subpopulations of LPCs are capable of neoplastic transformation to form HCC in chronic liver disease and as such, represent an important chemotherapeutic target for HCC treatment.
1.2.1.3 LPCs as an expandable source of hepatocytes

Studies of LPCs in vitro have provided valuable information as to the mechanism underlying the conditions required for LPC expansion and differentiation. Many different liver progenitor cell lines have been derived from both adult and foetal sources of liver from human (Malhi et al., 2002), rat (Radaeva and Steinberg, 1995) and mouse (Dumble et al., 2002, Strick-Marchand and Weiss, 2002, Fougere-Deschatrette et al., 2006, Tirnitz-Parker et al., 2007). Studies utilising these cell lines have helped define conditions for hepatocyte differentiation of liver progenitors. Hepatocyte-like cells can be induced in foetal-derived cell lines through formation of cell clusters by culture on plates treated to prevent attachment, inducing cell to cell contact and upregulating hepatocyte genes (Strick-Marchand and Weiss, 2002). To date, the mechanisms underlying this differentiation have not been probed. However, the initiation of cell-to-cell contact and contact inhibition of mitosis has been shown to induce hepatocyte differentiation in adult-derived LPC lines, in combination with the glucocorticoid dexamethasone and factors supporting hepatocyte function such as insulin, transferrin and selenium (Tirnitz-Parker et al., 2007). Dexamethasone promotes hepatocyte differentiation of liver progenitors by upregulating hepatocyte-specific genes such as tyrosine aminotransferase (Yeoh et al., 1979a, Yeoh et al., 1979b, Shelly and Yeoh, 1991) and supports the expression and secretion of hepatocyte-specific proteins such as transferrin (Kraemer et al., 1986) and albumin (Yeoh et al., 1985).

Transplant studies with foetal and adult-derived LPCs have demonstrated their ability to functionally repopulate injured liver. Transplanted foetal LPCs expand in vivo before slowing their proliferation, differentiating to hepatocytes after four to six weeks (Cantz et al., 2003). Adult LPCs isolated from DDC-injured livers also repopulate the livers of fumarylacetoacetate hydrolase (FAH)-deficient mice with equal efficiency to hepatocytes (Wang et al., 2003). As well as primary LPCs, murine cell lines transplanted into Albumin-uroplasminogen (Alb-uPA) mice, in which hepatocytes undergo
repeated necrosis, are able to differentiate into hepatocytes and repair the damaged liver parenchyma (Strick-Marchand et al., 2004). LPCs derived from human foetal liver are also expandable in vitro and functionally repopulate the livers of immune deficient mice (Malhi et al., 2002), providing proof-of-principle for potential cell-based therapy for human liver disease using LPCs.

1.2.2 Models of β-cell regeneration in the adult pancreas

Cell tracing studies have revealed that in normal adult pancreas, β-cell turnover is very slow, and is mediated by endogenous β-cells rather than a progenitor cell. Furthermore, the expansion of β-cell mass between three and twelve months of age in the mouse is mediated by pre-existing β-cells (Dor et al., 2004). β-cells not only derive from pre-existing cells during neonatal development, but regenerate after partial pancreatectomy (Dor et al., 2004, Teta et al., 2007) and expansion of β-cell mass during pregnancy or after exendin-4 administration is also predominantly accomplished by the endogenous β-cells in the mouse and human (Teta et al., 2007, Meier et al., 2008). Under certain conditions, the pancreatic duct epithelium may serve as a progenitor cell compartment from which β-cells may be derived (Trivedi et al., 2001, Bonner-Weir et al., 2004, Lin et al., 2006, Oshima et al., 2007). In humans, CK19 cells co-expressing insulin and the proliferation marker Ki-67 have been observed in biopsies of pancreas tissue from T1D patients receiving pancreas transplants (Martin-Pagola et al., 2008) suggesting ductal to islet differentiation is not merely an in vitro phenomenon. Similarly, during partial ductal ligation in the mouse, pancreatic β-cell regeneration is partially mediated by a resident duct-derived progenitor cell expressing Ngn3 which can be expanded in vitro, and give rise to all islet cell types including glucose-responsive β-cells (Xu et al., 2008).
1.3 Liver and pancreas interconversions

1.3.1 Pancreas to liver conversions

Pancreas to liver transdifferentiation was first observed in rats fed a copper depleted diet for 8-10 weeks, after which copper deficiency was restored. This regime induced almost complete loss of pancreatic acinar tissue and concomitant pancreatic to hepatocytic metaplasia, with up to 60% of cells expressing albumin (Rao et al., 1988). Cells transdifferentiated using this regime exhibit a functional hepatocyte phenotype, making proteins including carbamyl phosphate synthetase-1, urate oxidase and activate β-oxidation pathways (Usuda et al., 1988). In vitro treatment of mouse and human pancreatic explants reveal that dexamethasone and/or FGF2 can initiate hepatic differentiation in these tissues (Horb et al., 2003, Sumitran-Holgersson et al., 2009). These studies indicate that the ‘default’ pancreatic program can still be overridden by signals conducive to hepatic differentiation to produce functional hepatocytes from pancreatic tissue well after the pancreatic lineage has been specified. In vitro studies of pancreatic to hepatic transdifferentiation reveal some of the molecular mechanisms underlying this phenomenon.

The pancreatic tumour cell line AR42J-B13 (B13) has been extensively studied for its ability to transdifferentiate to the hepatic lineage in response to dexamethasone treatment (Shen et al., 2000) and represents an ideal in vitro model to study this phenomenon. Dexamethasone treatment induces hepatic gene expression at the expense of Pdx1 in B13 cells and mouse foetal pancreatic explants (Shen et al., 2000, Shen et al., 2003). This phenotype is recapitulated when C/EBPβ is overexpressed in B13 cells, suggesting that promotion of hepatic over pancreatic fate is controlled by this transcription factor (Shen et al., 2000, Shen et al., 2003). Transdifferentiated cells are functional, expressing key enzymes that perform the detoxification functions of mature hepatocytes. However, the transdifferentiated phenotype is not
permanent, lasting up to 14 days after dexamethasone is removed from the culture medium (Tosh et al., 2002).

### 1.3.2 Liver to pancreas conversions

Because LPCs are readily expanded *in vitro*, and are therefore theoretically scalable to therapeutically beneficial numbers, they represent an exciting prospect for cell therapy applications. There is some evidence to suggest that under certain conditions, LPCs may be induced to give rise to insulin producing cells. Extrapancreatic insulin-positive cells, including liver cells, are a phenomenon observed in mouse models of Type 1 diabetes (Kojima et al., 2004, Vorobeychik et al., 2008). In the liver, these Insulin$^+$ cells co-localise with A6$^+$ cells indicating they may be of ductular and/or LPC origin (Vorobeychik et al., 2008). Indeed, if ductal/LPC expansion is induced in mice by administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) prior to streptozotocin-induced diabetes, functional, hepatic insulin producing cells develop which reverse the diabetes phenotype (Kim et al., 2007). These data suggest that LPCs can be diverted to pancreatic fates under high glucose conditions. This phenomenon can also be achieved by reprogramming hepatocytes using pancreatic transcription factors, whereby overexpression of *Pdx1* in the livers of diabetic mice gives rise to hepatic insulin-expressing cells (Ferber et al., 2000, Shternhall-Ron et al., 2007). This pancreatic phenotype is stable, even if *Pdx1* overexpression is transient (Ber et al., 2003, Horb et al., 2003) and can be recapitulated *in vitro* with LPC lines (Yang et al., 2002). Further studies have shown that *Ngn3* overexpression can also induce hepatic insulin expression and reverse experimentally induced diabetes through the emergence of periportal Insulin$^+$ clusters which morphologically resemble LPCs (YeChoor et al., 2009).
Several pathways exist to derive hepatocytes and β-cells from progenitor cell sources. Some types of liver or pancreas injury promote ductal-derived progenitor mediated regeneration in the liver and pancreas. Under certain conditions, such as high glucose, copper deficiency or by exogenous overexpression of lineage-specific transcription factors, liver and pancreas progenitors can be transdifferentiated to form mature cells of other organs. The mechanisms underlying in vivo differentiation/transdifferentiation are useful for informing protocols to develop mature liver and pancreas cells in vitro (e.g.: from pluripotent cells or LPC lines).
1.3.3 Summary
The identification of expandable progenitor cell subtypes from the liver and pancreas that are capable of differentiation to mature tissues is of vital importance for developing renewable cell sources for therapy. One of the biggest risks in transplantation of stem cell-derived tissues is the potential for progenitors to give rise to tumours. Therefore the ability to define the phenotypes of progenitor cells, including those with tumorigenic potential, is critical to develop cells for therapy. This would aid diagnosis of liver pathologies, and establishing a phenotype for tumorigenic cells would be beneficial for developing targeted chemotherapeutic drugs, particularly for HCC where LPCs have been shown to contribute to cancer formation. One potential avenue to achieve this goal is to define the cell surface phenotype of the progenitor cell system of the liver and pancreas with antibodies which enables cell populations to be isolated using antibody-based sorting techniques such as flow cytometry for further study. Current progress towards this goal will be reviewed in the next section.

1.4 Phenotyping hepatic and pancreatic progenitors

1.4.1 Surface markers of LPCs
The A6 monoclonal antibody was produced by immunising rats with a liver progenitor cell-enriched isolate from the livers of mice with induced chronic liver injury and it has since become the ‘gold standard’ antibody for identifying LPCs in mouse models of liver injury (Engelhardt et al., 1990, Bennoun et al., 1993, Preisegger et al., 1999, Petersen et al., 2003, Akhurst et al., 2005) and to characterise novel LPC lines (Fougere-Deschatrette et al., 2006, Tirnitz-Parker et al., 2007). Although an excellent antibody for immunoidentification of LPCs, the successful use of A6 for antibody-based isolation of LPCs has not been reported, presumably because the antigen does not survive the enzymatic digestion process used to disperse liver cells. Because of this, more recent research efforts have focused on defining new surface antigens on LPCs which are useful for identification and isolation of LPC subsets.
The majority of surface-reactive antibodies that have recently been defined on LPCs are co-expressed by ductal cells. These markers include EpCAM, CD24 and CD133 and have been used to enrich LPCs using antibody-based sorting techniques (Ochsner et al., 2007, Rountree et al., 2007, Okabe et al., 2009). Studies utilizing EpCAM and CD24 have shown that expandable bipotential progenitors are present in these preparations providing firm evidence of a liver-resident progenitor cell (Okabe et al., 2009, Qiu et al., 2011). Additionally, E-cadherin is strongly expressed on ducts and LPCs in chronically injured liver and weakly on hepatocytes (Ueberham et al., 2007). Few antibodies have been developed that recognize injured, but not normal liver. Recently, the novel cell surface marker Trop-2, a member of the GA733 gene family along with EpCAM (Linnenbach et al., 1993), was shown to co-stain EpCAM+ cells in DDC-injured livers, although the cell populations recognized by Trop-2 and EpCAM are identical (Okabe et al., 2009). Other surface markers of LPCs include the hematopoietic stem cell markers Sca-1 and CD34, which are expressed by a subset of A6+ cells in DDC-induced injury (Petersen et al., 2003) and Dlk/Pref-1 which also marks ductal cell subpopulations (Tanimizu et al., 2003, Tanimizu et al., 2004, Jelnes et al., 2007). However, when compared to the total CK19+ cells, Dlk/Pref-1+ cells are less proliferative, based on Ki67 immunoreactivity, suggesting that Dlk/Pref-1 cells may mark a subset of differentiating LPCs (Tanimizu et al., 2004).

More recently, a panel of monoclonal antibodies was generated by immunising rats with the liver non-parenchymal cells of mice fed a DDC diet. A number of antibodies were generated, representing a diverse resource for characterising LPCs. The ductal-specific MIC-1C3 antibody generated from this panel has proved useful for isolating LPCs (Dorrell et al., 2008). In combination with previously described antibodies, the use of MIC-1C3 antibody has helped progress towards distinguishing LPCs at the clonal level, in a similar fashion to landmark studies in the hematopoietic system. So far, significant enrichment of LPCs has been achieved by isolating a subset of duct cells, firstly by excluding hematopoietic cells and enriching progenitors using...
the MIC-1C3+/CD133+/CD26- subset of non-hematopoietic hepatic non-parenchymal cells (CD45+/Cd11b+/CD31+) to purify self-renewing cells capable of bipotential differentiation in vitro in up to 4% of isolated cells (Dorrell et al., 2011). Although this represents significant progress in identifying LPCs, there is still much to be done to define the surface phenotype of the liver stem cell for the purpose of purification at the clonal level.

1.4.2 Surface markers of islet progenitor cells

In the pancreas, studies of mechanisms underlying Ngn3+ islet precursor cell differentiation to β-cells have been carried out on primary tissue because of a lack of adequate cell line models. This has proved difficult because of the scarcity and transient nature of Ngn3+ islet progenitors in the developing pancreas (Villasenor et al., 2008). In order to study islet development properly, especially specific effects of factors, it would be optimal to have a pure fraction of Ngn3+ islet precursor cells. Unfortunately no specific marker or marker combination has been established to isolate Ngn3+ cells. CD133 is expressed on islet progenitors and has been used in combination with Cd49f or PDGFRβ to isolate these cells. Ngn3+ cells are enriched by sorting with CD133+/Cd49f
low population of e15.5 mouse pancreas of which 0.7% of cultured cells can be differentiated into insulin-expressing cells in vitro (Sugiyama et al., 2007). These markers can also be used to purify endocrine progenitors from human foetal pancreas (Sugiyama et al., 2007). Combining CD133 sorting with negative selection using PDGFRβ-antibodies on e13.5 pancreas achieves a significant enrichment of Ngn3+ cells which can differentiate into insulin producing cells in vitro and in vivo (Hori et al., 2008). These studies represent the current best efforts in enriching endocrine progenitors without genetic modification. However further studies are needed to uncover the antigenic phenotype of Ngn3+ cells distinct to ductal and neural progenitor cells.
1.5 Summary and project aims

The ability of researchers studying liver and pancreas development and regeneration has been hampered by a lack of antibodies to identify the common and lineage distinct progenitor cells that re-populate the respective tissues. Ideally, novel antibodies for this purpose would also be useful not only for identifying these progenitors, but for their isolation using antibody-based sorting techniques. Furthermore, identification of the antigens recognised by any new antibodies would potentiate the translation of isolation techniques of these cells from mouse models to human progenitors.

Accordingly, the aims of the research reported in this thesis are to:

1. Generate a panel of surface-reactive monoclonal antibodies against liver and pancreas cell populations and to identify potential markers of liver and pancreas progenitor cells.

2. Characterise the ability of novel antibodies to identify and isolate liver progenitor cell populations.

3. Characterise the ability of novel antibodies to identify and isolate pancreatic islet progenitor cell populations

4. Identify protein targets of putative markers and to determine whether human-equivalent antibodies can be used to isolate human islet and/or liver progenitor cells.
Chapter 2

Generation and characterisation of novel cell surface antibodies to mouse liver progenitor cells
2.1 ABSTRACT
Liver progenitor cells (LPCs) represent a heterogeneous precursor compartment that is enlarged during chronic liver injury and acts to restore the liver mass and function when hepatocyte-mediated regeneration is impaired. LPCs are implicated as cellular source of hepatocellular carcinoma associated with chronic liver injury. Therefore, understanding the mechanisms that underpin their differentiation and tumourigenicity is important for regenerative and cancer medicine. Here, we report the development of two new antibodies, WAM18 and WAM21, and describe their application in detecting and isolating mouse LPCs that will enable their characterisation. Cells recognized by WAM18 and WAM21 were significantly induced in the liver following CDE-induced injury, and dual immunofluorescent staining demonstrated that WAM18 and WAM21 marked highly similar cell populations to the ductal/LPC marker A6. We also observed overlapping staining with WAM18 and A6 in DDC-induced liver injury. However, WAM21 marked A6+ cells in addition to cells adjacent to the DDC-induced ductular reaction. An LPC line, PIL-4 was characterised with respect to WAM18 and WAM21 expression. WAM18+ and WAM21+ subsets of PIL-4 cells displayed features of bipotential progenitors, co-expressing hepatocyte and ductal markers. We interrogated microarray data from liver progenitor cell lines differentially expressing WAM18 and WAM21 antigens and identified two novel candidate surface markers of LPCs whose expression is induced in chronic liver injury. In this study, we show that our novel antibodies represent an ideal alternative to the A6 antibody, with the potential for use in antibody-based enrichment of LPCs.
2.2 INTRODUCTION

Conditions of severe and chronic liver injury inhibit hepatocyte-mediated liver regeneration, and induce the liver progenitor cell (LPC) compartment to proliferate and differentiate to address the hepatocyte deficit (Paku et al., 2001, Forbes et al., 2002). LPCs are characterised by their heterogeneous co-expression of markers associated with hepatocytes and biliary cells (Germain et al., 1985), and their ability to mature into either lineage (Evarts et al., 1989, Evarts et al., 1996, Strick-Marchand and Weiss, 2002, Strick-Marchand et al., 2004). Compelling evidence for this role has come from recent lineage tracing studies in mice using either the Sox9 or Osteopontin promoters to show that ductal-derived LPCs give rise to hepatocytes in chronic liver injury (Furuyama et al., 2011). Since the process of LPC proliferation and hepatocyte differentiation can be recapitulated in vitro (Strick-Marchand and Weiss, 2002, Tirnitz-Parker et al., 2007), and they can be transplanted and replenish injured rodent liver (Wang et al., 2003), LPCs represent a promising renewable cell-based alternative to organ transplantation to address some liver pathologies. In addition to their regenerative function, LPCs are also associated with liver carcinogenesis. Progenitors are observed at various stages of human liver tumorigenesis; in chronic liver injuries (Roskams et al., 1998, Lowes et al., 1999, Xiao et al., 2004), preneoplastic lesions (Roskams et al., 1996) and in hepatocellular carcinomas (HCC) (Lee et al., 2005, Lee et al., 2006). Furthermore, limiting LPC proliferation in mouse models of liver regeneration reduces tumour formation (Knight et al., 2000, Haybaeck et al., 2009). Thus, detailed studies of the processes regulating growth, differentiation and transformation of LPCs are an imperative for cancer and regenerative medicine.

Rodent models of diet-induced liver injury are useful to study the mechanisms regulating LPC biology during chronic liver injury. Commonly used models include the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Preisegger et al., 1999) and choline deficient-ethionine supplemented (CDE) diets (Akhurst
et al., 2001), which induce diverse LPC populations defined by intracellular marker expression (Jelnes et al., 2007). CDE-induced liver tumorigenesis is attenuated by impairing the progenitor cell response to chronic liver injury through cytokine-receptor knockout (Knight et al., 2000) or by inducing LPC apoptosis (Davies et al., 2006), providing an ideal model to study LPC-derived HCC. Availability of more markers of LPCs, especially of transformed LPCs, would greatly benefit studies of LPC transformation to HCC. Thus, generation of surface-reactive antibodies to purify and study different progenitor populations from complex cellular fractions would advance this cause.

Mouse LPCs are commonly identified by the antibody A6, which binds to an as-yet unidentified cell surface determinant present on ducts in normal liver (Faktor et al., 1990) and ductal cells as well as more centrally located LPCs in diet-induced chronic liver injury models (Preisegger et al., 1999, Akhurst et al., 2001). Subsets of A6$^+$ cells can be defined using antibodies against hematopoietic markers such as CD34, Sca-1 (Petersen et al., 2003) and the pan-hematopoietic marker CD45 (Petersen et al., 2003, Rountree et al., 2007). A6 is considered the ‘gold standard’ for immunohistological identification of liver progenitors. However, since its efficacy for antibody-based cell isolation has not been reported, new antibodies that stain A6$^+$ cells which could also be used for antibody-based isolation would be an invaluable tool for LPC biologists.

Recent efforts have focused on defining the surface marker phenotype of LPCs to permit their isolation and study. Some antibodies against surface antigens have been characterised and include Epithelial Cell Adhesion Molecule (EpCAM) and Trop2, which identify similar cell populations (Okabe et al., 2009); CD24 which stains a subset of CK19$^+/CD45^-$ cells (Ochsner et al., 2007); and CD133 which identifies bipotential progenitor cells in DDC-injured liver (Rountree et al., 2007). A recently described antibody panel generated against non-parenchymal cells from DDC livers marks various cell populations
induced in chronic liver injury (Dorrell et al., 2008). This panel includes the MIC-1C3 antibody, which can be used in combination with CD133 to isolate LPCs (Dorrell et al., 2011). Although these antibodies stain liver progenitor cell populations in DDC livers, they remain untested in other models of chronic liver injury such as the CDE diet. The significance of this model is underlined by the recent finding that only LPCs generated by feeding a CDE diet can be traced into hepatocytes (Espanol-Suner et al., 2012). Additionally, an antibody that can be used for cell purification and stains LPCs, that has been compared to A6 in different liver injury models, is not available.

Since the liver and pancreas derive from a single precursor cell type (Deutsch et al., 2001, Furuyama et al., 2011), share common developmental mechanisms (Zaret and Grompe, 2008) and even the potential for interconversions (Shen et al., 2000, Tosh et al., 2002), studying their progenitor domains in parallel is useful for developing novel techniques for deriving liver and pancreas tissue for transplantation. Thus, in this study we have generated a panel of monoclonal antibodies against liver and pancreas tissue. To overcome the aforementioned problems associated with identifying and isolating LPCs, we herein focus on a subset of antibodies from this panel which mark LPC surface antigens and describe their utility in identifying, isolating and characterising LPC populations in the CDE and DDC diet models of chronic liver injury and LPC lines.
2.3 MATERIALS AND METHODS

2.3.1 Production and screening of monoclonal antibodies
An eight week old Wistar rat was immunised with a mixture of foetal mouse liver and pancreas cells in Complete Freund’s Adjuvant (Difco) intra-peritoneally (i/p), followed by i/p boosts in Incomplete Freund’s Adjuvant at 4 and 8 weeks and an aqueous boost at 17 weeks. Spleen cells were collected and fused with the mouse myeloma line, Sp2/O (Sigma-Aldrich, Australia), using PEG 1500 (Roche, Australia) under conditions previously described (Morahan, 1982). Hybridomas were selected in HAT-supplemented medium (100 µM hypoxanthine, 0.4 µM aminopterin and 10 µM thymidine) and supernatants were screened by immunofluorescence on a variety of cell lines and tissues. These included the mouse embryonic stem cell line W9.5, bipotential murine embryonic liver (BMEL) cells, e13.5 mouse embryonic fibroblasts (MEF) and Sp2/0 cells. Supernatants recognising Sp2/0 cells were excluded from further analyses.

2.3.2 Liver cell culture
p53 Immortalised Liver (PIL) cell lines were maintained as described by Tirnitz-Parker and colleagues (Tirnitz-Parker et al., 2007). Primary cell isolates were cultured on collagen coated dishes in growth medium as described above. Cells were maintained in a 37 °C, 5% CO₂ incubator with 90% humidity.

2.3.3 CDE diet induction of liver damage
Mice were housed under specific pathogen free, temperature controlled conditions, with alternating 12h light-dark cycles. All procedures were performed according to guidelines set by the National Health and Medical Research Council of Australia under ethics approval from the University of Western Australia. Four week old male C57Bl/6 mice were fed choline deficient chow, supplemented with 0.165% ethionine (w/v) in the drinking water for two weeks as previously described (Akhurst et al., 2001, Tirnitz-
Age-matched control mice were fed normal laboratory chow and water. Liver damage was confirmed histological assessment and quantitation of serum alanine transaminase levels as previously described (Tirnitz-Parker et al., 2007). ALT level were elevated in all CDE-treated mice (Appendix figure A1.1).

2.3.4 Histology and immunofluorescence

Mice were anesthetised by i/p Avertin injection (2.5% 2,2,2 tribromoethanol, Sigma-Aldrich, 0.015 mL/g body weight) and livers were perfused with 20 mL PBS via the hepatic portal vein. Livers were embedded in OCT medium for cryosectioning and stored at -80 °C. OCT-embedded liver tissue from mice fed a DDC diet for 2, 4 or 6 weeks was kindly provided by Dr Belinda Knight (Fremantle Hospital, Fremantle, Western Australia). Cryosections (7 µm) were cut and fixed by exposure to RT acetone for 5 min. Sections were air dried and stored at -20 °C. Fixed cryosections were rehydrated by incubation in PBS for 5 mins and endogenous biotin was blocked using a biotin blocking kit (Dako, Denmark). Primary antibody labelling was performed overnight at 4 °C. Primary antibodies A6 (rat; 1:80) or CD45 (rat, 1:50, BD Biosciences) were revealed with FITC-conjugated goat anti-rat IgG (1:200, Vector Laboratories). Following secondary antibody labelling, sections were incubated with rat immunoglobulin for 1 h at RT to block non-specific binding of biotinylated primary antibodies to anti-rat secondary antibody. Biotinylated primary antibodies were incubated for 2 h at RT. Primary antibodies WAM18 (1:100) and WAM21 (1:100), which are described in section 2.4.1, were detected by incubation with Streptavidin-Cy3 (1:300, Amersham). Specificity of staining was confirmed with appropriate isotype control staining (Appendix figure A1.2). Slides were mounted with ProLong Gold Antifade mounting medium containing the nuclear stain 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Australia) and visualised by fluorescence microscopy. Single colour fluorescent microscope images were merged using ImageJ version 1.39u software for Windows (ImageJ Software, National
Institutes of Health, USA, available at: http://rsb.info.nih.gov/ij/). All colour corrections were applied equally to experimental and control samples.

2.3.5 Liver non-parenchymal cell (NPC) isolation
The liver NPCs of mice fed a CDE for two weeks were isolated using a modified version of a two-step collagenase digestion (Yaswen et al., 1984), incorporating low speed centrifugation to separate hepatocytes as previously described (Dorrell et al., 2008, Okabe et al., 2009), but not centrifugal elutriation steps. Mice were anesthetised by i/p injection of Avertin and livers were perfused with 20 mL PBS via the portal vein. Livers were diced and cells were dispersed by incubation in a 0.05% collagenase type I in PBS (Sigma–Aldrich; pH 7.6) at 37 °C for 20 min with gentle agitation. Cells were further dissociated by pipetting and filtered through a 40 μm cell strainer (BD Biosciences). Filtered cells were pelleted by centrifugation and resuspended in Williams’ E medium containing 2% FBS. The undigested clot on the strainer was subjected to further dissociation by incubation in a solution containing 0.1% collagenase type VIII (Sigma–Aldrich), 0.09% Pronase (Roche Diagnostics), 0.025% trypsin/0.01% EDTA (Invitrogen), 0.004% DNase (Sigma–Aldrich) in PBS for 30 min at 37 °C with gentle agitation. Cells were freed by pipetting and an equal volume of cold Williams’ E medium containing 2% FBS was added. The cell suspension was filtered through a 40 μm BD Falcon™ cell strainer (BD Biosciences) and washed three times in Williams’ E medium. Cells from the first and second digestions were combined and hepatocytes were pelleted by repeated centrifugation at 50 X g for 10 min at 4 °C. The supernatant containing NPCs were transferred to a fresh tube and cells were pelleted by centrifugation at 800 X g for 10 min at 4 °C. Cells were resuspended in flow cytometry buffer (0.5% FBS, 1 mM EDTA in PBS) prior to antibody labelling and flow cytometry.

2.3.6 Flow cytometry
Liver progenitor cell lines were dispersed into single cells with trypsin (0.05%, Gibco-BRL) and washed in FACS Buffer (Phosphate Buffered Saline/ 2%
CHAPTER 2 RESULTS

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FBS). Cells were incubated with 100 µL hybridoma supernatant for 20 min on ice. Antibodies were detected by incubation with anti-rat IgG-FITC (1:200, Vector labs, PIL cell lines) for 20 min on ice. Cells were washed and resuspended in FACS buffer containing Propidium Iodide (1 µg/mL) and analysed on a FACS-Canto flow cytometer (BD Biosciences). Positive antibody staining was compared to negative antibody isotype control staining.

Liver NPCs were filtered through a 40 µm BD Falcon™ cell strainer (BD Biosciences) and stained with primary antibodies. Antibodies used were, rat IgG2a-biotin (1:200), rat IgG2a-Alexa647 (1:100, eBioscience), WAM 18-biotin (1:200) or WAM 21-Alexa647 (1:100). Cells were washed twice and incubated with Streptavidin-Alexa750 (1:200, Invitrogen) for 10 min on ice. Cells were washed twice, counterstained with DAPI (1 µg/mL) and analysed on a FACS AriaII flow cytometer (BD Biosciences).

2.3.7 RNA Isolation and Reverse Transcription PCR

Cells were lysed and RNA was extracted in 1 mL TRIzol reagent (Invitrogen). RNA was treated with DNase I (Promega) to remove gDNA contamination and first strand cDNA was synthesised with random hexamer primers using Superscript III Reverse Transcriptase (Invitrogen). Negative control reactions for gene expression analysis were generated by omitting RT enzyme. Gene expression was analysed by RT-PCR amplification of cDNA preparations for 30 cycles using gene specific primers with Platinum Taq DNA polymerase (Invitrogen). All protocols were carried out according to standard manufacturer instructions. Primer sequences and annealing temperatures are listed in Appendix Table A1.1. RT-PCR products were resolved by electrophoresis through a 1.5% agarose gel containing ethidium bromide and visualised by UV illumination.

2.3.8 Interrogation of microarray data

Expression profiling of PIL-2 and PIL-4 cells, and CDE and control liver, was carried out using Affymetrix Mouse Genome 430 2.0 GeneChips according to manufacturer instructions (Affymetrix; http://www.affymetrix.com). Data
normalisation and quality control analyses have been previously described (Jellicoe et al., 2008). Heat maps of log-transformed data were generated using the web-based Genesifter® program (PerkinElmer). To generate candidate surface markers encoding WAM antigens, a ratio of PIL-2 to PIL-4 gene expression was generated by calculating log₂ (PIL-2/PIL-4). A list of 476 genes which were expressed 1.5-fold higher in PIL-2 cells compared to PIL-4 cells was generated. This gene list was further refined by gene ontology (GO) identification analysis using the Gene Ontology Enrichment Analysis Software Toolkit (GOEAST) (Zheng and Wang, 2008). Those genes which were deemed to be potential surface markers were chosen based on the following GO identifiers; GO:0016020 (membrane), GO:0044425 (membrane part), GO:00019867 (outer membrane).

2.3.9 Statistical Analyses
Plotted data represent the mean ± SEM of three separate samples. For each sample, antibody-marked cells were counted in at least 5 fields (200X magnification) of view per tissue and expressed as a percentage of DAPI⁺ cells. Unpaired t-tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Values were considered significantly different when p<0.05.
2.4 RESULTS

2.4.1 Production and screening of monoclonal antibodies

To develop novel antibodies that recognise mouse liver and pancreas progenitors, we produced a panel of 54 rat monoclonal antibodies which were raised against a mixture of BMEL cell lines and dispersed foetal pancreas cells, which we termed ‘Western Australian Monoclonal’ (WAM) antibodies. Screening to identify liver-specific antibodies are described herein. We initially screened hybridoma supernatants for their cell-type specificity using live, unfixed cells *in vitro*, to identify antibodies which would be useful for isolating live-cell populations. These cell types included embryonic stem cells (W9.5 cells), LPCs (BMEL), thymocytes and mouse embryonic fibroblasts (MEF). Hybridoma supernatants were screened against Sp2/0 mouse myeloma cells as a negative control. 16 WAM antibodies marked Sp2/0 cells and were excluded from further characterisation (data not shown). Of the remaining 38 WAM antibodies, 17 marked BMEL cells (Table 2.1). To find antibodies which would be useful for identifying liver cell populations, we assessed staining of these 17 antibodies on frozen sections of 2 wk CDE-injured liver. Although these antibodies stained LPC lines, labelling of cells in liver of CDE mice was inconsistent. WAM22 marked portal veins and emanating sinusoidal endothelial cells. WAM45 labelled blood vessels in addition to clusters of stellate-like cells extending from portal vasculature. Nine supernatants failed to label any cell populations in CDE livers. WAM18 and WAM21 stained numerous groups of small epithelial-like cells which extended from WAM+ portal areas into the liver parenchyma, suggesting that they may recognise LPCs *in vivo* (Figure 2.1). Accordingly, WAM18 and WAM21 were characterised further.
Table 2.1 Screening WAM antibody panel. Strength of staining on different cell types was visually determined. Strongly positive (++++), positive (++), weakly positive (+), negative (-). Cell types are a liver progenitor cell line BMEL (LPC), 2 wk CDE liver tissue (CDE), W9.5 embryonic stem cells (ESC), thymocytes (Thym), mouse embryonic fibroblasts (MEF) and Sp2/0 mouse myeloma cells.

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Figure 2.1 WAM antibody labelling in 2 week CDE liver. Immunofluorescent staining of cryosections (7 µm) of CDE diet-induced liver with WAM antibodies (green). WAM18 and WAM21 identified small epithelial-like cells around portal areas which formed clusters extending into the liver parenchyma. WAM22 marked blood vessels and thin cells resembling hepatic sinusoidal cells and WAM45 labelled stellate-like cells which extended from WAM45⁺ vessels. Nuclei were counterstained with DAPI (blue). Scale bars represent 100 µm.
2.4.2 WAM18 and WAM21 mark the ductular reaction to chronic liver injury

We assessed the capability of WAM18 and WAM21 to mark LPCs in the CDE and DDC diet-induced liver injury models. Figure 2.2 shows dual immunofluorescence labelling of our new antibodies compared with the established ductal/LPC marker A6 in normal and CDE-injured livers. WAM18 and WAM21 labelled a subset of A6+ cells in normal livers (Figure 2.2A). In livers with CDE-induced LPC response, we observed both an increase in the numbers of cells marked by WAM18 and WAM21 in addition to a high degree of overlap with A6+ cell populations (Figure 2.2B). This observation was supported by quantitative assessment of cell numbers that overlap with A6 in normal and CDE-injured livers (Figure 2.3). Cells marked by WAM18 and WAM21 were significantly induced in CDE-treated versus control livers (11-fold and 8-fold respectively). Overlapping staining of WAM18 or WAM21 with A6 was 40% and 45% in normal livers. Critically, the degree of overlap of WAM18 or WAM21 with A6 increased to 98% and 96% respectively in LPC induced CDE-injured livers.

In DDC-injured livers, WAM18 marked a subset of A6+ cells after 2 weeks of injury, and WAM21 staining was essentially similar to A6 immunoreactivity (Figure 2.4A.). However, from 4 weeks of treatment, WAM18 and WAM21 marked distinct cell populations when compared with A6. WAM18 and A6 marked virtually identical cell populations in 4 and 6 week DDC liver, whilst WAM21 identified A6+ cells as well as a subpopulation of cells that were A6- located adjacent to WAM21+/A6+ cells at 4 and 6 weeks of DDC treatment (Figures 2.4B and 2.4C.). To test the specificity of WAM antibodies for LPCs, we compared WAM antibody and CD45 immunospecificity in CDE and DDC treated livers. WAM18 did not mark CD45+ inflammatory cells in CDE or DDC-induced liver injury, confirming their specificity for LPCs induced by these diets (Figure 2.5A&B.). However, a small number of WAM21+ cells were also CD45+ in DDC-injured livers (Figure 2.5B.).
Figure 2.2 Comparison of WAM and A6 antibody labelling in normal and CDE liver. Immunofluorescent staining with the liver progenitor marker A6 (green) and WAMs (red) or WAM21 on cryosections (7 µm) of livers of mice fed (A) a normal chow diet for 2 weeks or (B) a CDE diet for 2 weeks. WAM18 and WAM21 localise to subpopulation of A6+ ductal cells in normal livers and co-localise with A6+ in CDE-induced livers. Nuclei were counterstained with DAPI (blue). Arrows indicate regions displayed by insets. Scale bars represent 100 µm.
Figure 2.3 Quantitation of LPC induction in the CDE model using WAM antibodies. The numbers of cells labelled with (A) WAM18 or (C) WAM21 significantly increased during CDE-induced chronic liver injury. The amount of co-staining of WAM antibodies with the LPC marker A6 was quantitated during LPC induction. Pie charts show the relative proportion of cells co-labelled with (B) WAM18 and A6, or (D) WAM21 and A6 increased in CDE-injured versus control livers (yellow segment). Bars represent mean ± SEM with n = 3. Statistical significance with respect to control diet is represented as *p<0.05 or **p<0.01.
Figure 2.4 Comparison of WAM and A6 antibody labelling in DDC liver. Immunofluorescent staining with the liver progenitor cell marker A6 (green) and WAMs (red) on cryosections (7 µm) of livers of mice fed a DDC diet for (A) 2 weeks (B) 4 weeks or (C) 6 weeks. WAM18 and A6 stain similar cell subsets at all timepoints. WAM21 stains a population of cells adjacent to A6+ cells that are not marked by A6 at 4 and 6 weeks of DDC treatment. Nuclei were counterstained with DAPI (blue). Arrows indicate regions displayed by insets. Scale bars represent 100 µm.
Figure 2.5 Comparison of WAM and CD45 antibody labelling in chronic liver injury. Immunofluorescent staining with the pan-hematopoietic maker CD45 (green) and WAMs (red) on cryosections (7 µm) of livers of mice fed a CDE diet for 2 weeks or a DDC diet for 6 weeks. (A) In 2 week CDE livers WAM18 and WAM21 positive cells are observed in close association with CD45⁺ inflammatory cells, but co-localisation of markers is not observed. (B) In 6 week DDC livers WAM18⁺ cells are CD45⁻. A small number of WAM21⁺ cells are CD45⁺ during DDC treatment. Nuclei were counterstained with DAPI (blue). Diagonal arrows indicate regions displayed by insets. Horizontal arrows mark WAM21⁺/CD45⁺ cell subsets. Scale bars represent 100 µm.
2.4.3 Cell sorting potential of WAM18 and WAM21

The applicability of WAM18 and WAM21 for antibody-based sorting of live cell populations was addressed by staining liver non-parenchymal cell (NPC) preparations from 2 week CDE livers. WAM18 and WAM21 marked 10% and 31% of total liver NPC’s from 2 week CDE livers respectively. Dual antibody staining of liver NPC’s with WAM18 and WAM21 showed that they defined two cell subsets that were WAM18+/WAM21+ and WAM18/WAM21+ (Figure 2.6A.). We purified these WAM-defined populations by FACs but were unable to propagate them in culture. We also used magnetic bead sorting to enrich WAM-defined cell subsets from normal livers, but were also unable to propagate these cells in vitro (Appendix Figure A1.3). Hence, we undertook further cell sorting studies to characterise subsets of WAM antibody-defined liver progenitor cell lines. Staining was assessed by flow cytometry on five p53 immortalised liver (PIL) cells. PIL-1, -2, -3 and -5 cells all expressed single positive WAM18 and WAM21 populations. However, PIL-4 contained two cell populations, positive and negative, defined by WAM18 or WAM21 staining (Figure 2.6B). We used this property to purify PIL-4 cell subsets based on WAM antibody expression and characterised gene expression in these populations. Expression of cytokeratins CK7 and CK18, the ductal gap junction protein Cx43 and the hepatocyte/progenitor transcription factor Hnf4α were enriched in WAM18 and WAM21-positive subpopulations of PIL-4 cells. Hnf1β and CK19 expression was uniform between different WAM18 and WAM21-defined PIL-4 cells. The hepatocyte marker Albumin was not expressed by PIL-4 cells (Figure 2.6C).
Figure 2.6 Defining liver progenitor cell subsets with WAM antibodies. (A) Staining of non-parenchymal cells from freshly isolated 2 week CDE liver shows WAM18 marks a subset of WAM21+ non-parenchymal cells. (B) Flow cytometric analysis of WAM antibody staining in PIL cell panel (grey) versus isotype control staining (white). (C) RT-PCR analysis of hepatocyte (Hep) and ductal (Duct) genes in WAM-defined PIL-4 cell populations. Expression in WAM-defined populations was compared to unsorted PIL-4 cells (US). E15 liver (e15) was used as a positive control for gene expression.
2.4.4 Identifying candidate genes encoding novel cell surface markers of LPCs

Since flow cytometric analysis shows that PIL-2 and PIL-4 cells differentially express WAM18 and WAM21 on their cell surface, we used this property to identify potential new surface markers of LPCs by comparing global gene expression between these two cell lines using a previously conducted microarray experiment (performed by Jasmine Low, University of Western Australia). We predicted that mRNA’s encoding WAM18 and WAM21 antigens would be expressed in PIL-2 cells in greater abundance than PIL-4 cells. RNA species that were expressed 1.5-fold higher in PIL-2 cells, and were deemed to be expressed on the cell surface by gene ontology classification were included in our analysis. Six putative LPC surface markers were identified; BMP binding endothelial regulator (Bmper), Cadherin 11 (Cdh11), Cdh17, Doublecortin-like and CAM kinase-like 1 (Dcamkl1), Insulin-like growth factor receptor 2 (Igf2R) and Slit homolog 2 (Slit2). To confirm this result, we assessed the expression of these markers, and the established LPC surface markers Cd24a, Cd34, E-cadherin, Glypican-3 and Dlk in WAM18 and WAM21-defined PIL-4 cells by RT-PCR analysis.

Of previously described surface markers of LPC’s, Cd34 and E-cadherin transcripts were enriched in WAM18+ and WAM21+ cells, whilst the expression of Cd24a was uniform between WAM-defined populations. Dlk, Glypican-3 and Slit-2 were not expressed in PIL-4 cells, and the expression of Dcamkl1 and Igf2R were not specific to WAM+ populations. Of our new candidate markers, Bmper, Cdh11 and Cdh17 expression was enriched in in WAM18+ and WAM21+ versus WAM antibody-negative cells (Figure 2.7A). To support cell line findings, we interrogated microarray data from CDE-induced injury. Of the newly identified markers, Bmper and Cdh17 were expressed at low levels in normal liver and were highly upregulated in 2 week CDE livers (18.8-fold and 96.9-fold respectively), as were the established markers Cd24a (14.7-fold), Glypican-3 (67.4-fold) and EpCAM (13.5-fold) (Figure 2.7B).
Figure 2.7 Screening for candidate genes encoding WAM18 and WAM21. (A) RT-PCR analysis of established LPC surface markers and putative surface genes encoding WAM18 and WAM21 antigens in WAM-defined PIL-4 cells. (B) Heatmap representation of microarray analysis of gene expression of candidate surface markers in 2 wk control versus CDE livers. The intensity of each colour denotes the standardised ratio between each value and the average expression of each gene across all samples. Red pixels correspond to an increased abundance of mRNA, green pixels denote decreased mRNA levels.
2.5 DISCUSSION

Development of antibodies against cell surface antigens of LPCs compatible with antibody-based isolation techniques are vital for characterising the regenerative and tumorigenic capacity of LPC subtypes in chronically injured liver. To increase the limited repertoire of antibodies that meet this criterion, we generated and characterised monoclonal antibodies recognising LPCs. We have produced two antibodies, WAM18 and WAM21, with significant value for *in vitro* and *in vivo* studies of LPCs.

The applicability of antibodies to enzyme-dissociation protocols for selecting non-parenchymal cells from whole livers has been an issue with previously described cell surface markers such as A6 (Engelhardt et al., 1990) and glypican-3 (Grozdanov et al., 2006). Additionally, EpCAM is downregulated in cultured LPCs (Okabe et al., 2009) preventing its use in LPC line model studies. Our new antibodies, WAM18 and WAM21, mark highly similar cell populations to the ‘gold standard’ LPC marker, A6, in CDE-induced livers, and WAM18 and A6 display an overlapping staining pattern in DDC-induced injury. Critically, we have shown that WAM18 and WAM21 are compatible with enzyme dissociation protocols used to disperse whole liver and cultured LPC lines. Thus these WAM antibodies are viable alternatives to A6 for immunoidentification in multiple chronic liver injury models, and additionally useful for isolation of chronic liver injury-induced and cultured progenitors.

We used this property to characterise subsets of the PIL-4 cells, which had previously been shown to heterogeneously express LPC markers including A6 (Dumble et al., 2002). We were able to purify a subset of PIL-4 cells which co-expressed ductal (*CK7, CK19, Hnf1β, Cx43*) and hepatocytic (*CK18* and *Hnf4α*) mRNA’s as assessed by RT-PCR. Since the co-expression of bi-lineage markers is a property of bipotential LPCs (Jelnes et al., 2007, Tirnitz-Parker et al., 2007), these studies provide evidence of the utility of WAM18 and
WAM21 in isolating LPC subsets from heterogeneous starting populations. Using WAM-antibody defined phenotypes in combination with previously generated microarray data, we identified new candidate LPC surface markers using LPC line models. Of these new markers present in PIL cell lines, Cadherin-17 and Bmper are promising candidates for future study as their mRNA's are also upregulated in CDE-induced liver injury.

We have demonstrated that our novel antibodies, WAM18 and WAM21; particularly WAM18 are useful reagents for identifying LPCs and they can be used to generate enriched LPC populations from whole liver. They are viable alternatives to A6 which has limited availability and cannot be used for cell purification.
Chapter 3

Definition, enrichment and development of pancreatic beta cell progenitors using a novel monoclonal antibody
3.1 ABSTRACT

*Aims:* Understanding the mechanisms regulating islet development is crucial in manipulating stem cells to produce viable beta cell surrogates for Type 1 Diabetes therapy. Progress to this goal can be achieved by studying islet progenitors isolated from the developing pancreas. However, few markers currently exist to isolate islet progenitors. Accordingly, we produced monoclonal antibodies that can define subsets of cells in the developing pancreas.

*Methods:* Monoclonal antibodies were raised against foetal mouse islet tissue and screened for their ability to bind to Ngn3-expressing pancreas cells from e15.5 double transgenic Ngn3-GFP*/RIP-dsRed mice. One antibody, WAM21, was characterised in detail for its ability to isolate putative islet progenitors which could differentiate to insulin-expressing cells in vitro.

*Results:* Staining islet-specific transgenic reporter mouse lines and gene expression analysis revealed developing islet progenitor cells are WAM21+. Culture of WAM21-sorted transgenic insulin-reporter foetal pancreas cells demonstrated the ability of WAM21+ islet progenitors to differentiate into insulin-expressing cells.

*Conclusions:* Our novel antibody, WAM21, identifies a subset of developing mouse pancreas cells which contains $Ngn3^+$ beta cell progenitors capable of differentiation to insulin expressing cells under defined culture conditions. The ability of WAM21 to enrich for beta cell progenitors will greatly aid studies of beta cell development for understanding islet development mechanisms.
3.2 INTRODUCTION

Type 1 Diabetes arises from immune-mediated destruction of insulin producing beta cells, resulting in chronic hyperglycaemia and risk of complications including diabetic retinopathy, nephropathy and vascular disease. Current therapies for Type 1 Diabetes include exogenous insulin administration, which does not effect complete glucoregulation and is associated with diabetic complications in the long-term, or transplant of cadaveric islet tissue (Shapiro et al., 2000), for which the demand far outweighs availability. Research efforts to alleviate this problem are focused on deriving beta cells from diverse stem cell sources. These sources include embryonic stem cells (Jiang et al., 2007, Kroon et al., 2008), pancreatic duct-derived progenitors (Bonner-Weir et al., 2004, Xu et al., 2008), and non-pancreatic tissue-derived stem cells such as liver progenitors (Yang et al., 2002, Ber et al., 2003, Kim et al., 2007, Yechoor et al., 2009). Though stem cells can be directed to produce insulin, current protocols are hampered by inefficiency of beta cell derivation and incomplete maturation to glucose-responsive cells. Consequently, *in vitro* studies of islet development are vital to define the mechanisms underlying beta cell differentiation, maturation and expansion for efficient derivation of clinically useful beta cell surrogates.

The pancreas develops from two endodermal buds; the ventral bud, which contacts the cardiac mesoderm neighbouring the liver primordium, and the dorsal bud which develops separately, adjacent to the septum transversum mesenchyme (Slack, 1995, Field et al., 2003). Mesenchymal signals such as fibroblast growth factors (Hebrok et al., 1998, Bhushan et al., 2001, Deutsch et al., 2001, Hart et al., 2003), bone morphogenetic proteins (Rossi et al., 2001) and retinoic acid (Kumar and Melton, 2003, Molotkov et al., 2005) induce the pancreatic domain to express the gene Pancreatic and Duodenal Homeobox 1 (*Pdx1*) (Wright et al., 1989, Jorgensen et al., 2007). This gene drives pancreatic-specific gene expression (Melloul, 2004, Babu et al., 2007, Oliver-Krasinski et al., 2009). All epithelial cell types present in the pancreas derive from the *Pdx1*+ epithelium (Wright et al., 1989, Burlison et al., 2008), which
undergoes branching morphogenesis and differentiates to form the ductal tree, endocrine and exocrine cells (Habener et al., 2005). Downstream of Pdx1, Neurogenin-3 (Ngn3) is transiently expressed in a subset of cells which form all islet cell types (Gradwohl et al., 2000, Schwitzgebel et al., 2000, Desgraz and Herrera, 2009). The Ngn3 transcription factor acts to inhibit Notch signalling (Apelqvist et al., 1999) thereby facilitating islet gene expression.

Currently, few cell surface markers are available to identify and isolate islet progenitor cells. One of the key problems is that candidate membrane proteins such as E-cadherin (Jiang et al., 2002, Sugiyama et al., 2007), CD133 and Cd49f (Sugiyama et al., 2007) are common to progenitors of islets, ducts and exocrine tissue. CD133 is expressed by Ngn3+ cells and carboxypeptidase-A+ acinar cells but not mature hormone-expressing islet cells (Sugiyama et al., 2007, Hori et al., 2008). Three distinct populations can be identified by antibodies to Cd49f, with the low-positive population containing Ngn3+ islet progenitors while the acinar cells are found in the Cd49f high-positive portion (Sugiyama et al., 2007). Given the shared origin of these three lineages, it is perhaps not surprising that no surface marker unique to Ngn3+ islet precursor cells has been identified.

Development of additional surface markers defining islet precursor cells would improve their isolation and aid in studies of beta cell differentiation. Thus, we generated a panel of monoclonal antibodies against pancreas tissue. In this study, we characterised the utility of antibodies in this panel to identify precursors in developing mouse pancreas capable of differentiate to insulin-producing cells under defined conditions in vitro.
3.3 MATERIALS AND METHODS

3.3.1 Production and screening of monoclonal antibodies

Wistar rats were immunised with e15.5 foetal pancreas cells and liver progenitor cells in Complete Freund's Adjuvant (Difco) intra-peritoneally (i/p), followed by i/p boosts in Incomplete Freund's Adjuvant at 4 and 8 weeks and an aqueous boost at 17 weeks. Spleen cells were collected and fused with the mouse myeloma line, Sp2/O (Sigma), using PEG 1500 (Roche) under conditions previously described (Morahan, 1982). Hybridomas were selected in HAT medium and supernatants were screened by immunofluorescence on foetal pancreas. Specificity for antigens expressed on foetal pancreas was assessed by staining of other tissues, such as liver and thymus.

3.3.2 Foetal pancreas isolation

Mouse embryos at e15.5 were dissected from the uterus and placenta and placed in a petri dish containing cold PBS prior to dissection of pancreata under sterile conditions. Embryonic pancreata were removed and stored in medium (high glucose DMEM (Invitrogen) with 10% FBS) until processing. Pooled pancreata were centrifuged briefly and the medium aspirated. Cells were dissociated as previously described (Jiang et al., 1999), using Dispase (BD Biosciences) instead of trypsin for 1 h at 37 °C. Animal experiments were performed according to the Australian Code of Practice for the care and use of scientific animals and ethics approval was obtained from the Animal Resources Centre, Perth and the University of Western Australia Animal Ethics Committee.

3.3.3 Cell sorting

For flow cytometric sorting, cells were resuspended in FACS Buffer (PBS/ 2% FBS) and stained with primary antibodies. Antibodies used were AlexaFluor 647-conjugated-WAM21 or rat IgG2a isotype control-AlexaFluor 647 (eBioscience). For dual antibody labelling, anti CD49f-PE (clone GoH3, BD Biosciences) and biotin-conjugated WAM21 antibody were used. Biotinylated
antibody was detected with Streptavidin-AlexaFluor 750 (Invitrogen). Cells were washed and resuspended in FACS buffer containing DAPI (1 µg/mL) and analysed on a FACSriaII flow cytometer (BD Biosciences). For magnetic bead sorting (MACS), cells were resuspended in MACS Buffer (PBS/ 0.5% FBS/ 1 mM EDTA) and stained with biotin-conjugated WAM21 antibody. After washing, cells were stained with Anti-Rat IgG MicroBeads or Anti-Biotin Multisort beads (Miltenyi Biotec). For fluorescent detection, cells were stained with Streptavidin-AlexaFluor 750 (Invitrogen). After washing, unlabelled cells were separated by passing the cells through an MS MiniMACS Column according to manufacturer instructions (Miltenyi Biotec). A small aliquot was stained with DAPI, and purity was assessed by flow cytometry.

3.3.4 Differentiation of WAM21+ cells
For in vitro differentiation, sorted cells were differentiated by culture in high glucose DMEM medium (Invitrogen) containing nicotinamide (10 mM, Sigma-Aldrich), growth factor reduced Matrigel™ (200 µg/mL, BD Biosciences) and B27 supplement (2X, Gibco). Cells were seeded at a density of 10^4 cells/well of a 96-well plate in triplicate. Differentiation was assessed by daily counting of cells that were MIP-GFP+ or RIP-dsRed+ (Ins-reporter+) over 5 to 7 days in a 37 °C, 10% CO_2 incubator with 90% humidity.

3.3.5 RNA Isolation and RT-PCR
Cells were lysed and RNA was extracted in 1 mL TRIzol reagent (Invitrogen). RNA was treated with DNase I (Promega) to remove gDNA contamination and first strand cDNA was synthesised with random hexamer primers using Superscript III Reverse Transcriptase (Invitrogen). Gene expression was analysed by RT-PCR amplification of cDNA preparations for 35 cycles using gene specific primers with Platinum Taq DNA polymerase (Invitrogen). All protocols were carried out according to manufacturer instructions. Primer sequences and annealing temperatures are listed in Appendix Table A2.
RT-PCR products were resolved by electrophoresis through a 1.5% agarose gel containing ethidium bromide and visualised UV light illumination.

3.3.6 Histology and immunofluorescence
Cryosections (7 µm) of OCT-embedded e15.5 foetal pancreas tissue were cut and fixed in acetone (-20 °C, 5 mins), air-dried and stored at -20 °C until use. Sections were rehydrated in PBS for 5 mins and non-specific binding was blocked by incubation in blocking buffer (5% FBS diluted in PBS) for 1 hour at room temperature. Labelling of primary antibodies was performed overnight at 4 °C. Antibodies used were WAM21-Biotin or Cd49f (BD Biosciences). Specificity of labelling was confirmed by a lack of labelling following incubation with IgG2a-Biotin or IgG2a isotype control antibodies (Appendix figure A2.1). Primary rat antibodies were detected with FITC-conjugated goat anti rat IgG (1:200, Vector) and biotinylated antibodies with Streptavidin-Cy3 (1:300, Amersham). Slides were mounted with ProLong Gold Antifade mounting medium containing the nuclear stain DAPI (Invitrogen) and visualised by fluorescence microscopy. Single colour fluorescent microscope images were merged using ImageJ version 1.39u software for Windows (ImageJ Software, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/).

3.3.7 Statistical Analyses
Plotted data represents the mean average ± SEM. Two-tailed, unpaired t-tests were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Values were considered significantly different for p<0.05.
3.4 RESULTS

3.4.1 Production and screening of hybridoma supernatants

In order to develop novel antibodies that recognise mouse liver and pancreas progenitors, we produced a panel of 54 rat monoclonal antibodies which were raised against dispersed mouse foetal pancreas cells. Hybridoma supernatants were initially screened to select appropriate specificities using various cell sources. These included embryonic stem cells (W9.5 cells), NIT-1 insulinoma cells (representing insulin-producing cells), and cryosections of e15.5 mouse pancreas. Supernatants were screened against thymocytes mouse embryonic fibroblasts (MEF) and Sp2/0 myeloma cells as negative controls. The selected hybridomas were designated with a ‘Western Australian Monoclonal’ (WAM) number. A total of 33 WAM antibodies marked pancreatic populations and were characterised further (Table 3.1). 16 antibodies were non-specific (data not shown) and were excluded from further characterisation.

To identify antibodies useful for isolating islet progenitors, we screened hybridoma supernatants on foetal pancreata isolated from dual transgenic mice (Ngn3-GFP/RIP-dsRed) that express GFP and dsRed under the control of the mouse Ngn3 and rat insulin promoters, respectively. By flow cytometry, four antibodies were found to be useful as positive selectors of Ngn3-GFP+ cells. The best of these was WAM21, which stained 17% of total cells, representing a potential 4.2-fold enrichment of Ngn3-GFP+ cells. Additionally, four antibodies identified populations of Ngn3-GFP- cells and were considered useful negative selectors of islet progenitors. The best of these was WAM316 which stained 43% of cells, a 1.9-fold enrichment of Ngn3-GFP- cells by negative selection (Table 3.2). WAM21 was the most promising antibody for isolating islet progenitors, so was selected for further characterisation.
Table 3.1. Screening WAM antibody panel. Strength of staining on different cell types was visually determined. Strongly positive (+++), positive (++), weakly positive (+), negative (-). Cell types are e15.5 foetal pancreas (Foetal panc.), NIT-1 insulinoma cells, W9.5 embryonic stem cells (ESC), thymocytes (Thym.), mouse embryonic fibroblasts (MEF) and Sp2/0 mouse myeloma cells.
<table>
<thead>
<tr>
<th>Category</th>
<th>WAM#</th>
<th>e15.5 cells detected (%)</th>
<th>Enrichment of Ngn3-GFP+ cells (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Enriching Abs</td>
<td>4</td>
<td>18</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>60</td>
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</tr>
<tr>
<td></td>
<td>280</td>
<td>65</td>
<td>1.2</td>
</tr>
<tr>
<td>Negative Enriching Abs</td>
<td>20</td>
<td>16</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>354</td>
<td>28</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 3.2. Screening supernatants from WAM hybridomas. Supernatants were evaluated for binding to cells from e15.5 pancreas of Ngn3-GFP/RIP-dsRed transgenic mice.

3.4.2 e15.5 pancreas WAM21+ cells are enriched for cells with islet progenitor traits

WAM21 was used to stain and sort cells from foetal pancreata from two different transgenic reporter strains: the above-mentioned Ngn3-GFP/RIP-dsRed mice and the MIP-GFP strain that expresses GFP under the control of the mouse insulin I promoter (Hara et al., 2003). In the former, WAM21 stained 28% of e15.5 pancreas cells; these included virtually all the Ngn3-GFP+ cells (Figure 3.1A, upper panel). The ability to mark foetal beta cells was assessed by FACS analysis of MIP-GFP foetal pancreata. WAM21 stained 29% of MIP-GFP pancreas, including cells in which the insulin promoter was active (i.e. GFP+ cells; Figure 3.1A, lower panel).
Figure 3.1. Identification of islet progenitors in developing pancreas. The ability of WAM21 to identify islet precursors was tested by flow cytometric analysis of WAM21 staining of cells from transgenic reporter mouse foetal pancreas. (A) WAM21 marked Ngn3-GFP+ and MIP-GFP+ pancreatic cells from Ngn3-GFP/RIP-dsRed and MIP-GFP mice, respectively. (B) RT-PCR analyses of genes expressed in islet cells, epithelial stem cells, neural/mesenchymal cells and ductal cells in WAM21− and WAM21+ e15.5 pancreas populations.

Gene expression analysis of sorted foetal pancreas subpopulations showed WAM21-defined populations demonstrated characteristics of islet progenitor cells. By RT-PCR, the WAM21+ population expressed Ngn3 and was enriched for cells expressing both insulin genes. Furthermore, WAM21+ cells contained all cells expressing Pdx1, as well as the beta cell precursor genes Pax4 and Nkx2.2, confirming that WAM21 could enrich for cells with an islet/beta cell progenitor phenotype. The WAM21+ population also expressed the acinar marker Ptf1a, progenitor cell markers E-cadherin and CD133. Cells expressing Cd24a, CK19 and the neural/mesenchymal marker PDGFRβ were not enriched in the WAM21+ population (Figure 3.1B).
3.4.3 WAM21+ e15.5 pancreas cells differentiate to Ins+ cells in vitro

Since WAM21+ cells had molecular characteristics of beta cell progenitors, we assessed the capacity of these putative progenitors to differentiate into Insulin-expressing cells in vitro (Figure 3.2). Developing MIP-GFP pancreata were separated into WAM21+ and WAM21- populations by FACS and seeded in defined culture medium. The number of GFP+ cells formed was assessed over 5 days. To calculate enrichment of islet progenitors, differentiation was compared to non-antibody enriched controls. It should be noted that MIP-GFP+ cells were excluded at the sorting stage, so that only those cells which differentiated in culture and activated the Ins1 promoter were detected. No MIP-GFP+ cells were observed on the day of plating. During differentiation, only single MIP-GFP+ cells were observed during the differentiation process, suggesting MIP-GFP+ cells did not arise by proliferation of progenitors (Figure 3.2A). WAM21-sorting significantly enriched progenitors which gave rise to MIP-GFP+ cells. Appearance of MIP-GFP+ cells peaked at 4-5 days post-plating (Figure 3.2B). In three independent experiments, WAM21+ cells differentiated at a frequency which was 3.2-fold higher than non-antibody enriched cells (Figure 3.2C). Conversely, WAM21- cells yielded very few GFP+ cells.

Gene expression in differentiated WAM21-defined populations was analysed by RT-PCR (Figure 3.2D). In addition to the production of MIP-GFP+ cells, culture in differentiation medium resulted in down-regulation of the progenitor cell transcripts Ngn3, Pax4 and Nkx2.2. Pdx1, which becomes restricted to beta cells during their development, was also down-regulated. Expression of both Insulin genes was increased. Loss of Ptf1a expression indicated that growth and/or differentiation of acinar cells were not supported by these culture conditions. No islet-specific gene expression was observed in cultures of sorted WAM21-negative cells. Together, these results demonstrated that WAM21-based sorting significantly enriched from islets those progenitor cells that could differentiate into Insulin-expressing cells.
Figure 3.2. Differentiation of WAM21-defined e15.5 MIP-GFP pancreas populations. Differentiation was assessed in MIP-GFP\(^{\text{neg}}\), WAM21\(^{\text{neg}}\) and WAM21\(^{\text{pos}}\) cells by observing MIP-GFP\(^{+}\) cells. (A) MIP-GFP\(^{+}\) cells at day 0 and day 4 of differentiation. (B) Differentiation dynamics of WAM21-defined populations compared to MIP-GFP\(^{-}\) cells show differentiation peak at 4-5 days. (C) Ratio of MIP-GFP\(^{+}\) cells formed after differentiation in WAM-defined foetal pancreas populations compared to MIP-GFP\(^{-}\) cells. (D) RT-PCR analyses of islet/β-cell markers post-differentiation in WAM21\(^{+}\) and WAM21\(^{\text{pos}}\) cells. Bars represent mean ± SEM (\(n=3\); **\(p<0.01\) compared to MIP-GFP\(^{-}\)cells). Scale bars represent 20 µm.
3.4.4 Further definition of WAM21+ progenitors with antibodies to Cd49f

As we had shown that WAM21+ foetal pancreas cells were of a mixed islet progenitor/acinar lineage by RT-PCR analysis, dual antibody sorting with an acinar cell marker could potentially be used to further purify islet progenitors in the WAM21+ subset. Cd49f was previously shown to mark Carboxypeptidase-A+ acinar cells (Sugiyama et al., 2007). We therefore assessed the potential of further enriching WAM21+ islet progenitor cells in combination with Cd49f. First, we compared the specificity of WAM21 and Cd49f. Immunostaining of e15.5 wild-type pancreas illustrated the similar but distinct staining pattern of these markers: WAM21 stained a subpopulation of pancreatic cells, strongly localising to the apical membrane of Cd49f low cells (Figure 3.3A). Cells which stained strongly for Cd49f also stained strongly for WAM21, and flow cytometric analysis of Cd49f and WAM21 staining of Ngn3-GFP/RIP-dsRed e15.5 pancreas confirmed that Ngn3-GFP+ cells were enriched in the WAM21+/Cd49f low subpopulation (Figure 3.3B). These results suggested that WAM21+ islet progenitors could be further enriched by their low Cd49f expression.

We further characterised WAM21/Cd49f-defined foetal pancreas populations by analysis of gene expression and in vitro differentiation to beta-like cells. Islet progenitors were purified in a two-step process. Magnetic bead-enriched WAM21+ progenitors were FACS-separated into Cd49f low and CD49f high populations. RIP-dsRed+ cells were excluded at the sorting stage so that subsequent analysis was restricted to newly formed beta cells (Figure 3.4A). Ngn3-GFP+ cells were enriched up to 38% in WAM21+/Cd49f low cells; this compared to 0.6% in the WAM21+/Cd49f high population (Figure 3.4B). The identity of cell populations defined by WAM21 and Cd49f was assessed by RT-PCR analysis of islet, ductal, acinar, neural and cell surface markers (Figure 3.4C). WAM21+/Cd49f low cells expressed a mixture of these markers including Pdx1, Ngn3, Pax4, Nkx2.2 insulin-1 and insulin-2 (islet lineage markers), CK19 (ductal/epithelial cells) and β3-tubulin (neural cells).
WAM21+/Cd49f<sup>high</sup> cells exclusively expressed Ptf1a (acinar), MAP2 and PDGFRβ (neural), and contained cells expressing Pdx1, Ngn3, Pax4, Nkx2.2, insulin-1, insulin-2 and CK19. Sorting WAM21<sup>+</sup> cells with Cd49f also further divided populations expressing mRNAs encoding the cell surface markers CD133, Cd24a and E-cadherin.

When we assessed the differentiation potential of these populations, only WAM21+/Cd49f<sup>low</sup> cells gave rise to significant numbers of RIP-dsRed<sup>+</sup> cells. We observed more Ngn3-GFP<sup>+</sup> cells in cultures of WAM21+/Cd49f<sup>low</sup> cells compared to those of non-antibody enriched cells. Over the first two days of culture, Ngn3-GFP expression was reduced to near-background levels (Figure 3.5A). RIP-dsRed<sup>+</sup> cells began to appear after 24h of culture and their numbers gradually increased, peaking at 4 days (Figure 3.5B). Conversely, WAM21+/Cd49f<sup>high</sup> foetal pancreas cells produced few RIP-dsRed<sup>+</sup> cells compared to non-antibody enriched cells (Figure 3.5B). At the peak of differentiation, WAM21+/Cd49f<sup>low</sup> cells differentiated at a frequency 11.5-fold higher than non-antibody enriched cells (Figure 3.5C).

RT-PCR analysis (Figure 3.5D) revealed that cultures of WAM21+/Cd49f<sup>low</sup> cells down-regulated Ngn3, Pax4 and Nkx2.2 and upregulated ins1, ins2 and Pdx1, confirming beta cell differentiation of enriched islet progenitors. These cells maintained expression of the neural markers β3-tubulin and a small amount of MAP2, indicating that neural cells were maintained in this culture. Intriguingly, in WAM21+/Cd49f<sup>high</sup> cells, neural markers were also down-regulated during culture but the neural-type insulin gene expression pattern was maintained; ins2 predominated over ins1 but Pdx1 was not present, indicative of a non-pancreatic epithelial phenotype (Figure 3.5D).
Figure 3.3. Comparison of WAM21 and Cd49f staining. (A) Immunofluorescent staining with the islet progenitor marker Cd49f and WAM21 on cryosections (7 µm) of wild-type e15.5 pancreata. Nuclei were counterstained with DAPI (blue). Scale bars represent 20 µm. (B) FACS analysis of populations of Cd49f and WAM21-defined e15.5 Ngn3-GFP/RIP-dsRed pancreata. Numbers of Ngn3-GFP+ cells were assessed in Cd49f<sup>low</sup>, WAM21+ and Cd49f<sup>low</sup>/WAM21+ populations.
Figure 3.4. Molecular characterisation of WAM21 and Cd49f-defined e15.5 pancreas cells. (A) Islet progenitors from e15.5 Ngn3-GFP/RIP-dsRed foetal pancreas were enriched by magnetic bead sorting with WAM21. WAM21+/RIP-dsRed cells were further separated into two Cd49f-defined populations (high and low) by FACS for subsequent RT-PCR and differentiation studies. (B) Ngn3-GFP+ islet precursor cells were enriched in WAM21+/Cd49f<sub>low</sub> cells, and depleted in WAM21+/Cd49f<sub>high</sub> cells. (C) RT-PCR analyses of genes expressed in islet cells, epithelial stem cells, mesenchymal, neural and ductal cells in WAM21+/Cd49f<sub>low</sub> and WAM21+/Cd49f<sub>high</sub> defined e15.5 pancreas.
Figure 3.5. Differentiation of WAM21/Cd49f-defined Ngn3-GFP/RIP-dsRed foetal pancreas populations. Differentiation was assessed in RIP-dsRed\(^{-}\), WAM21\(^{+}/Cd49f\(_{\text{low}}\) and WAM21\(^{+}/Cd49f\(_{\text{high}}\) cells by observing RIP-dsRed\(^{+}\) cells. (A) Ngn3-GFP/RIP-dsRed cells at day 0, 2 and day 4 of differentiation in Cd49f-defined WAM21\(^{+}\) cells. Images taken with GFP and dsRed filters were merged. (B) Beta-cell differentiation dynamics of Cd49f-defined WAM21\(^{+}\) populations. (C) Ratio of ins-reporter\(^{+}\) cells after differentiation in Cd49f-defined WAM21\(^{+}\) cells compared to RIP-dsRed\(^{-}\) cells. (D) RT-PCR analyses of islet/β-cell and neural markers post-differentiation in Cd49f-defined WAM21\(^{+}\) cells. Bars represent mean ± SEM (n=3, **p<0.01 compared to RIP-dsRed\(^{-}\) cells). Scale bars represent 20 µm.
3.5 DISCUSSION

Antibody-based sorting is a powerful method to isolate rare stem cells from heterogeneous populations for further study. Its usefulness for stem cell research was first demonstrated for the isolation of progenitor cells from the hematopoietic system (Spangrude et al., 1988) and more recently in the generation of new antibodies against cell surface determinants of liver progenitors (Dorrell et al., 2008b) and adult human pancreas (Dorrell et al., 2008a). Like these tissues, the developing pancreas is composed of many cell types of which progenitors comprise a small subset. A lack of surface markers defining islet progenitors has hampered efforts to identify and isolate these cells for further study. Although previous studies examined established antibodies for defining the foetal pancreatic progenitor cell surface (Sugiyama et al., 2007, Hori et al., 2008), we approached this problem differently. We generated a panel of monoclonal antibodies against pancreas progenitors. One marker, WAM21, proved most valuable for isolating and characterising foetal beta cell progenitors.

We demonstrated the effectiveness of WAM21-based sorting for isolating islet progenitors which gave rise to insulin-reporter+ cells in differentiation medium. WAM21 identified a subpopulation of CD133+ cells and could be used in combination with Cd49f to enrich islet progenitors significantly. CD133 was shown previously to mark non-committed islet precursors, excluding islet-hormone+ cells (Sugiyama et al., 2007). In contrast, we found that WAM21 identified a subpopulation of Insulin-expressing cells. Our studies suggest that both committed beta cell progenitors and primitive islet precursors are WAM21+. By using transgenically-labelled beta cells for visualising differentiation dynamics, we could exclude Ins-reporter+ cells at the sorting stage study, allowing study of only the progenitor cells. We achieved a differentiation rate of up to 5% ins-reporter+ cells from the dual antibody-sorted subset. In this system, ins-reporter+ cells did not develop in the absence of factors such as nicotinamide and glucose present in the differentiation medium (F.X. Jiang, unpublished data) suggesting that
fluorescently tagged cells derived over the culture period are progenitor-cell derived and dependent on specific factors for differentiation into Insulin+ cells. However, in order to understand the commitment and development of beta cells effectively, further delineation of the surface phenotype of all pancreatic lineages using monoclonal antibodies would be useful. In our studies, we provide promising candidate markers for future lineage definition studies in developing pancreas.

Unexpectedly, Ngn3 expression was observed in WAM21+/Cd49fhigh cells. A small number of Ngn3+ cells have been previously shown to arise from cultured Cd49fhigh cells (Sugiyama et al., 2007) though their differentiation to beta cells was not reported. The Ngn3+ cells observed in our Cd49fhigh population may be analogous to previously reported Ngn3+/Cd49fhigh cells as they naturally occur in vivo. When these cells were cultured under differentiation conditions, we observed Ins expression in the absence of Pdx1 expression. Although Pdx1 mRNA was present in this population prior to differentiation, its down-regulation in conjunction with loss of Ptf1a expression after culture suggests acinar-derived Pdx1 expression. Pdx1 is expressed upstream of both Ngn3 and Ins during pancreatic development, binding the Ngn3 promoter (Oliver-Krasinski et al., 2009) and is required for beta cell-specific Ins transcription, as well as beta cell function (Melloul, 2004, Babu et al., 2007) and becomes restricted to beta cells during development (Deramaudt et al., 2006). However, Ngn3 is also expressed in neural progenitors during brain development (Sommer et al., 1996). Ins genes are also expressed during neuronal development, with Ins2 expression predominating over Ins1 after e10.5 in the mouse (Deltour et al., 1993). WAM21+/Cd49fhigh cells express the neural markers MAP2 and PDGFRβ and exhibit a neural-type Ins2 over Ins1 expression pre- and post-differentiation. The absence of Pdx1 in cultured WAM21+/Cd49fhigh cells indicates non-islet transcription of neuroendocrine markers, and raises the potential for a neural cell origin of these transcripts.
Using antibodies that define beta cell precursors in conjunction with efficient models of differentiation will facilitate generation of better *in vitro* methods for producing beta cells for therapy. Our novel antibody, WAM21, is a versatile and effective surface-reactive antibody for identifying and isolating foetal beta cell progenitors which can be differentiated into $\text{Ins}^+$ cells *in vitro*. In other experiments, we also showed that WAM21 could also enrich liver precursor cells (MS in preparation, Chapter 2). This novel antibody will aid in studies of beta cell development, which in turn will allow for improved methods to derive beta cells from stem cells for Type 1 Diabetes treatment.
Chapter 4
Expression cloning a single cDNA encoding the WAM18 and WAM21 antigens
4.1 ABSTRACT

The ability to purify progenitor cells from complex organs is vital for studies of tissue regeneration and tumourigenesis. We have previously described two antibodies, WAM18 and WAM21, which are useful for isolation and characterisation of progenitor cell domains of the mouse liver and pancreas. To facilitate functional and translational studies, we sought to identify the antigens bound by these antibodies. A cDNA encoding the WAM18 and WAM21 antigens was cloned by immunoselection of COS-7 cells transfected with a cDNA library. Antibody inhibition studies demonstrated that WAM18 and WAM21 antibodies bind overlapping, but distinct, epitopes. Further characterisation of these epitopes showed that expression of WAM18 and WAM21 was cell type-specific in developing mouse brain and pancreas. Having identified a cDNA encoding WAM18 and WAM21, we carried out RNAi experiments, which demonstrated a role for this transcript in controlling liver progenitor cell proliferation in vitro. Finally, we showed that an antibody recognising a human WAM18/21 protein equivalent could be used to enrich human foetal islet pancreas cells with features of islet progenitors. These studies have enabled us to define a cDNA encoding WAM18 and WAM21 and suggest a role for this cDNA in regulating liver progenitor cell proliferation. Furthermore, we have shown the expression of the protein recognised by our antibodies on the cell surface of islet progenitors is conserved between mice and humans. This discovery of a novel marker of human islet progenitors will benefit human islet developmental studies to generate stem cell-derived beta cell surrogates for Type 1 Diabetes therapy.
4.2 INTRODUCTION

The liver and pancreas arise from a common bipotential precursor cell type residing within the developing endoderm (Deutsch et al., 2001). Although specific liver or pancreas-specific lineage gene cascades are initially induced by relative exposure to fibroblast growth factor signals from the septum transversum mesenchyme (Deutsch et al., 2001, Rossi et al., 2001, Serls et al., 2005) studies of their respective progenitor cell domains reveal striking similarities in mechanisms underlying their growth and differentiation. Signals such Wnt/β-catenin and Notch share common functions in both organs, controlling tissue-specific progenitor cell expansion and ductal versus non-ductal fate choices. Furthermore, under certain injury or tissue culture conditions, liver to pancreas (Ferber et al., 2000, Cao et al., 2004, Kojima et al., 2004, Kim et al., 2007, Shternhall-Ron et al., 2007) and pancreas to liver conversions are observed (Tosh et al., 2002, Shen et al., 2003, Westmacott et al., 2006, Tosh et al., 2007). Since the liver and pancreas share a common origin, develop under the control of similar pathways and have the potential to interconvert under appropriate conditions, they are ideal organs to study in parallel. Consequently, it is unsurprising that they also share cell surface markers defining their progenitor domains.

The majority of studies to develop new surface markers of liver and pancreas progenitors have focused on known antigens using established antibodies. Markers such as CD133 (Rountree et al., 2007, Sugiyama et al., 2007, Hori et al., 2008), Cd49f (Suzuki et al., 2000, Sugiyama et al., 2007) and E-cadherin (Jiang et al., 2002, Ueberham et al., 2007) are common to both hepatic and pancreatic progenitor cells. An approach that has been particularly useful in developing new antibodies for progenitor cell isolation is to raise antibodies against cell types of interest rather than known antigens. As opposed to targeting antibodies against known proteins or peptides, this approach generates antibodies against undefined cell surface proteins. Because relatively few tools exist for antibody based sorting of liver/pancreas
progenitors, more recent studies have focused on developing reagents which are useful for antibody-based enrichment of cells induced by chronic liver injury (Dorrell et al., 2008) and adult mouse pancreas cell subsets (Dorrell et al., 2011b). Using a similar approach, we have also described a panel of antibodies generated against developing liver and pancreas cells which mark progenitor cell domains from these organs (Chapter 2). This has led to the development of two antibodies, WAM18 and WAM21, which recognise liver progenitor cell lines and the ductular reaction induced by two unique chronic liver injury diets (Chapter 2). We have also shown WAM21-based cell sorting effectively enriches Ngn3-expressing islet precursors from foetal pancreas, which can be differentiated to insulin producing cells in vitro using defined conditions. This system represents a convenient in vitro model for studying beta cell development (Chapter 3). Critically, the antigens recognised by WAM18 and WAM21 remain stable following enzyme-mediated tissue dissociation protocols making them ideal tools for isolating progenitor cells from tissue isolates, a property not described for the ‘gold standard’ liver progenitor cell surface marker A6 (Faktor et al., 1990, Factor et al., 1994)

Although our newly developed WAM antibodies have demonstrated value in cell isolation and histological identification of hepatic and pancreatic progenitors, the determinants they recognise remain unknown. Knowledge of the antigens they mark would enable functional and translational studies. Accordingly, we have undertaken experiments in this study to establish the identity of WAM18 and WAM21, facilitating functional studies in a mouse liver progenitor cell line. Furthermore, we describe the use of a commercially available antibody recognising the equivalent human WAM18/WAM21 protein to purify putative islet progenitor cells from the developing human pancreas.
4.3 MATERIALS AND METHODS

4.3.1 Histology and immunofluorescence
Cryosections (7 µm) of OCT-embedded e15.5 foetal liver tissue were cut and fixed in acetone (-20 °C, 5 min), air-dried and stored at -20 °C until use. Sections were rehydrated in PBS for 5 min and endogenous biotin was blocked using a biotin blocking kit (Dako, Denmark). Non-specific binding was blocked by incubation in blocking buffer (5% FBS diluted in PBS) for 1 h at RT. Labelling of primary antibodies was performed overnight at 4 °C. Antibodies used were WAM18 or rat IgG2a isotype control. Primary rat antibodies were detected with FITC-conjugated goat anti rat IgG (1:200, Vector). After secondary antibody labelling, sections were incubated with rat immunoglobulin for 1 h at RT to block non-specific binding of biotinylated primary rat antibodies to anti-rat secondary antibody. Biotinylated primary antibodies were incubated for 2 h at RT. Antibodies used were biotin-conjugated WAM21 or IgG2a which were detected by incubation with Streptavidin-Cy3 (1:300, Amersham). Slides were mounted with ProLong Gold Antifade mounting medium containing the nuclear stain DAPI (Invitrogen) and visualised by fluorescence microscopy. Single colour fluorescent microscope images were merged using ImageJ version 1.39u software for Windows (ImageJ Software, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/).

4.3.2 Mammalian cell culture
COS-7 cells were maintained in high glucose DMEM (Invitrogen) supplemented with glutamine, penicillin/streptomycin (Gibco-BRL) and 10% FBS as previously described (Gluzman, 1981). NIT-1 insulinoma cells were maintained in high glucose DMEM supplemented with glutamine, penicillin/streptomycin, 0.02% bovine serum albumin (BSA) and 10% FBS as previously described (Hamaguchi et al., 1991). Bipotential Murine Oval Liver (BMOL) cells were maintained as previously described (Tirnitz-Parker et al., 2007). All cells were grown in a 37 °C incubator with 90% humidity. COS-7
and NIT-1 cells were maintained with a 10% CO₂ atmosphere. BMOL cells were maintained in a 5% CO₂ atmosphere.

### 4.3.3 Expression cloning cDNA clones encoding WAM antigens

Superscript® Mouse 13.5 Day Embryos cDNA was prepared according to manufacturer instructions (Invitrogen, Australia; cat. 10666-014). Library DNA was transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen, Australia). For expression cloning of WAM antigens from cDNA library plasmids, WAM18/21⁺ cells were selected by FACS 72 h post-transfection, replated and allowed to grow to confluence for 15 days to allow for stable integration of plasmids. After this period, cells were trypsinised and sorted with WAM18 and WAM21 a second time. WAM⁺ stably transfected cells were regrown to confluence prior to DNA extraction.

### 4.3.4 DNA purification and amplification of cloned sequences

WAM18⁺/WAM21⁺ COS-7 cells were lysed and gDNA purified using the QIAamp DNA Mini Kit according the manufacturers’ instructions (Qiagen). 100 ng of DNA was used for subsequent PCR amplification. Stably incorporated e13.5 cDNA library sequences were amplified using Platinum Taq DNA polymerase for 40 cycles, according to manufacturer instructions. Commercial primers specific for T7- and Sp6-RNA polymerase sequences flanking cDNA inserts were used (Promega, Australia). Prepared e13.5 cDNA library plasmid DNA was used as a positive control and adult mouse liver DNA was used as a negative control. PCR products were resolved by electrophoresis through a 1.5% agarose gel and visualised by ethidium bromide staining under ultraviolet light.

Specific PCR products were excised from the gel and purified using the QIAquick Gel Extraction Kit according to the manufacturers’ instructions (Qiagen, USA). Products were sequenced using labelled dideoxy-nucleotides by the Australian Genome Research Facility (AGRF, Perth, Western Australia) with Sp6 primers. Derived sequences were compared against
known sequences using the Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences alignments were generated using the Clustal Omega Multiple Sequence Alignment Program (http://www.ebi.ac.uk/Tools/msa/clustalo/) and edited using the Jalview program available at http://www.jalview.org/ (Waterhouse et al., 2009).

4.3.5 Foetal tissue isolation
Mouse embryos at e15.5 were removed from the uterus and dissected free of their placenta and placed in a petri dish containing cold PBS prior to dissection of pancreata, liver, brain or small intestine under sterile conditions. Tissues were stored in medium (high glucose DMEM (Invitrogen) with 10% FBS) until processing commenced. Tissues were incubated in 600 µL medium and 300 µL of Dispase (BD Biosciences) for 1 h at 37 °C with shaking at 400 rpm. The reaction was stopped by the addition of 500 µL of 12% BSA in PBS. Cells were dispersed by pipetting and pelleted by centrifugation at 800 x g for 5 min. Cells were incubated in 500 µL medium containing 1 µL DNase (Promega) for 10 min at room temperature. 1 mL of medium was added and the cells were centrifuged at 800 x g for 5 min at RT. Pelleted cells were resuspended in 10 mL medium and stored on ice until use.

Human foetal pancreata (14 to 18 weeks old) were dissociated by fine mincing with a scalpel blade and incubating with 500 µL Liberase TL (2.5 mg/mL; Roche, Australia) for 12 min at 37 °C. The reaction was stopped by the addition of 9 mL of neutralising buffer (HBSS with 10% FBS, 10 mM HEPES, 10 µg/mL DNase I). Cells were centrifuged at 300 X g for 5 min at RT, washed twice in neutralising buffer and filtered through a 70 µm cell strainer prior to antibody labelling. Human pancreata were prepared by Dr Erika Bosio and Emma Jamieson at the Centre for Diabetes Research, Western Australian Institute for Medical Research. Animal experiments were performed according to the Australian Code of Practice for the care and use of scientific
animals and ethics approval from the Animal Resources Centre, Perth. Human tissue was acquired with patient consent from King Edward Memorial Hospital (Perth, Western Australia) under ethics approval from the University of Western Australia.

4.3.6 Flow cytometry
Cells were resuspended in FACS Buffer (PBS/ 2% FBS) and stained with primary antibodies. Antibodies used were rat IgG2a WAM21-AlexaFluor-647, WAM18-biotin, rat M1/69 antibody (unconjugated; 1/100, eBioscience) rat IgG2a isotype control-AlexaFluor 647 (eBioscience), rat IgG2a WAM21-biotin, rat anti CD49f-PE (clone GoH3, BD Biosciences). Cells were washed and resuspended in FACS buffer containing DAPI (1 µg/mL) and analysed on a FACS AriaII flow cytometer (BD Biosciences). FACS plots were generated using FlowJo version 7.6 (TreeStar).

4.3.7 Treatment of COS-7 cells with glycosylation inhibitors
COS-7 cells overexpressing mouse Cd24a (pCMV-Cd24a; Origene, USA) were trypsinised and split into 6-well plates 24 h post-transfection. Growth medium was changed after 16 h and cells were treated with glycosylation inhibitors according to previously published protocols (Sohail et al., 2011). Tunicamycin (5 µg/mL; Sigma-Aldrich) and/or Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BGalNAc, 2 mM; Sigma-Aldrich). Control cells were treated with DMSO (final concentration 0.2%). Cells were incubated in glycosylation inhibitors after which time WAM18 and WAM21 staining was assessed by flow cytometry.

4.3.8 Antibody inhibition assay
Antibody blocking experiments were carried at similarly to previously described protocols (Alterman et al., 1990). NIT-1 cells were incubated with 2.5 µg/mL of unconjugated blocking antibodies for 30 min. After washing in FACS buffer, cells were incubated in 2.5 µg/mL Alexa647-conjugated rat IgG2a isotype control, WAM18 or WAM21 for 30 min. Cells were washed and resuspended in FACS wash buffer containing 1 µg/mL DAPI. The median
fluorescence intensity (MFI) was assessed and specific inhibition calculated using the following equation:

\[
\% \text{ Specific inhibition} = \frac{\text{Control block MFI} - \text{Antibody block MFI}}{\text{Control block MFI}} \times 100.
\]

### 4.3.9 Protein isolation, quantitation and Western blotting

Snap-frozen mouse thymus was pulverised using a mortar and pestle and resuspended in a lysis buffer containing Tris-HCl (20 mM, pH 8.0), 137 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1% (v/v) IGEPAL® CA-630 with a protease inhibitor cocktail (Promega, Australia). Lysates were freeze-thawed in liquid nitrogen three times, dispersed by pipetting and insoluble material removed by centrifugation at 16,000 X g at 4 °C for 15 min. Protein concentration was determined by Bio-Rad assay (Bio-Rad) against a BSA standard curve. For one-dimensional western blotting, 20 µg of protein was separated on a 12% SDS-polyacrylamide gel in running buffer containing SDS and β-mercaptoethanol. For non-denaturing conditions, SDS and β-mercaptoethanol were excluded. For two-dimensional Western blotting, proteins were first separated by isoelectric focussing then separated by SDS-PAGE and transferred to a PVDF membrane. Two-dimensional Western blotting analysis was carried out by Dr Nadine Dudek at the University of Melbourne, Australia.

Blotted proteins were washed in PBS/0.1%-Tween (PBS-T), blocked in 5% non-fat skim milk diluted in PBS-T for 1 h at RT. Primary rat antibodies were diluted 1:2000 in blocking buffer and incubated overnight at 4 °C. Following overnight incubation, membranes were washed once in PBS-T for 15 min, then three times for 5 min in PBS-T. Membranes were incubated with HRP-conjugated goat anti-rat IgG (1:5000, Abcam) for one hour at room temperature, washed once in PBS-T for 15 min, then three times for 5 min. Antibodies were detected with ECL plus reagent according to the
manufacturers’ instructions (Amersham) and visualised by film exposure (Kodak, Australia).

4.3.10 RNA interference in BMOL cells
BMOL cells were seeded at $10^5$ cells per well in a 6 well plate in growth medium and allowed to adhere overnight. The following day, medium was aspirated and 1 mL of OptiMEM medium (Invitrogen) containing 2% FBS without antibiotics was added to the cells. Plasmid shRNA constructs (Origene USA) were transfected using Lipofectamine 2000 according to the manufacturers’ instructions (Invitrogen, Australia). 24 hours after transfection, cells were trypsinised and split 1:5 into 3 wells of a 12 well plate in oval cell growth medium to generate 3 pools of transfected cells. After an additional 24 hours, medium was changed to oval cell growth medium containing Puromycin (1 µg/mL; Sigma Aldrich, Australia) for selection of stable transfectants. Cells were cultured in selection medium for 15 days after which time 2-3 colonies remained in each well. Cells in individual wells were expanded, mRNA assessed by qPCR to confirm gene knockdown and used for cell growth studies (Appendix figure A3.1).

4.3.11 Assessment of cell growth
BMOL cell growth was measured using the Cellavista™ system (Roche). Prior to performing experiments, a standard curve was generated to correlate cell numbers to measured well confluence (Appendix figure A3.2). To further validate the Cellavista™ system, Cellavista™ measurements were directly compared to the MTT assay (Appendix figure A3.2) which is commonly used to measure cell proliferation (Mosmann, 1983). BMOL cells were seeded at a density of 2000 cells per well in a 96 well plate in quadruplicate and incubated overnight to adhere. Plates were read in the Cellavista™ system twice daily for 6 days. Population doubling times were calculated in the log growth phase according to previously published protocols (Viebahn et al., 2006). Growth curves generated using Cellavista™ were fitted to an exponential curve of the
equation $y = a e^{gx}$, $R^2 > 0.96$ ($a$: constant; $g$: growth rate, $R^2$: correlation coefficient). Doubling time was defined as: doubling time = $\ln(2)/g$ and was calculated using GraphPad Prism (GraphPad Software, San Diego California USA, www.graphpad.com).

4.3.12 Analysis of gene expression

Cells were lysed and RNA was extracted in 1 mL TRIzol reagent (Invitrogen). RNA was treated with DNase I (Promega) to remove gDNA contamination and first strand cDNA was synthesised with random hexamer primers using Superscript III Reverse Transcriptase (Invitrogen). For semi-quantitative analysis of gene expression, RT-PCR amplification of cDNA preparations for 35 cycles using gene specific primers with Platinum Taq. DNA polymerase (Invitrogen) was carried out. All protocols were performed according to standard manufacturer instructions. Primer sequences and annealing temperatures are listed in Appendix table A3.1. RT-PCR products were resolved by electrophoresis through a 1.5% agarose gel containing ethidium bromide and visualised by ultraviolet light illumination.

4.3.13 Statistical analysis

Plotted data represent the mean ± SEM of three experiments. One way analysis of variance (ANOVA) with Tukey’s multiple comparison test, or paired t-test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Values were considered significantly different when p<0.05.
4.4. RESULTS

4.4.1 Expression cloning cDNA encoding WAM antigens

We had previously shown that WAM18 and WAM21 are expressed at the cell surface of liver (Chapter 2) and developing pancreas cells (Chapter 3), permitting antibody-based enrichment of live cell populations. Accordingly, we applied this property of our antibodies to expression clone potential cDNA’s from a mixed cDNA library encoding WAM18 and WAM21 antigens. To expedite this process, we utilised a commercially available e13.5 whole mouse cDNA library. Prior to our expression cloning experiments, we conducted immunofluorescence staining on e13.5 liver and found that both WAM18 and WAM21 identified similar populations of e13.5 liver cells (Figure 4.1A). Next, we overexpressed cDNA’s with flanking T7 and Sp6 sequences from a commercially available e13.5 whole mouse cDNA library in COS-7 cells and selected cells on the basis of WAM18 and WAM21 expression by FACS. To increase the initial pool of WAM-selected cells, we sorted with WAM18 and WAM21 antibodies at the same time. Seventy-two hours after cDNA library transfection, 3.1% of COS-7 cells were WAM18/21+ compared to isotype control staining (Figure 4.1B). These cells were purified, replated and expanded for 15 days to allow for stable integration of plasmid DNA. At the end of this period, 0.1% of cells were WAM18/21+ (Figure 4.1C). These cells were replated and expanded for DNA analysis of incorporated plasmids.
Figure 4.1. Expression cloning WAM18 and WAM21 antigens from a cDNA library. (A) Expression of WAM18 (green) and WAM21 (red) at e13.5 in mouse development was confirmed by immunofluorescent staining of e13.5 frozen liver sections. Merged image shows both antibodies recognise similar cell populations. Nuclei were counterstained with DAPI (blue). Scale bars represent 50 µm. An e13.5 cDNA library was overexpressed in COS-7 cells and WAM18/21+ cells were purified (B) 72 h post-transfection. These cells were replated and expanded for 15 days to allow stable incorporation of plasmids. (C) WAM18/21+ stably-transfected cells were re-purified. All staining was compared to rat IgG2a isotype control staining (control).
4.4.2 Establishing the identity of WAM18 and WAM21

After expansion and passage into two separate wells (‘pool 1’ and ‘pool 2’), cells were trypsinised and stained with either WAM18 or WAM21 antibodies prior to DNA extraction. WAM18 and WAM21 identified almost all cells compared to isotype control staining (Figure 4.2A). Genomic DNA was extracted from these cells, and a PCR was carried out to amplify stably transfected library DNA using T7 and Sp6 primers (which flanked cDNA inserts). Library plasmid DNA and normal mouse liver were used as positive and negative controls respectively. PCR amplification of e13.5 cDNA library DNA displayed multiple products shown by a ‘smear’ by agarose gel separation. A 2 kB insert was amplified from pool 1 and pool 2 DNA, which was not present in control liver DNA. (Figure 4.2B). These fragments were excised from the gel, purified and dideoxy-sequenced using Sp6 primer. A sequence which was 800 bp long was derived. A BLAST search revealed the identity of the cDNA encoding the WAM18 and WAM21 antigens as Cd24a. A comparison of the sequenced fragment and Cd24a demonstrated 100% identity between the two sequences (Figure 4.3).

To confirm this result, we amplified DNA using primers specific for muCd24a cDNA from the 2 kB T7/Sp6 PCR product from stably transfected COS-7 cells (Figure 4.4A). Additionally, we overexpressed a pCMV6-Cd24a expression cassette in COS-7 cells to confirm that WAM18 and WAM21 stained CD24 surface protein. A CD24 antibody (M1/69) was used as a positive control for CD24 protein expression. None of the antibodies assessed identified untransfected COS-7 cells. Cd24a-overexpressing COS-7 cells were M1/69+, WAM18+ and WAM21+ (Figure 4.4B). Altogether, these data confirmed the identity of the gene encoding WAM18 and WAM21 antigens is mouse Cd24a.
Figure 4.2. Isolation of a cDNA encoding WAM18 and WAM21. (A) Immunofluorescent staining of live cell populations of WAM-purified COS-7 cells immediately prior to DNA extraction shows WAM18 and WAM21 antigens are present on virtually all cells. DNA was extracted from these cells and inserts amplified using flanking T7 and Sp6 primer sequences. (B) Agarose gel electrophoresis and ethidium bromide staining of PCR using flanking primers shows a unique 2 kB fragment present in WAM18/21+ COS-7 cells which was extracted and purified from the agarose gel for further analysis (red box). Gel lanes are molecular weight marker (M), WAM18/21+ COS-7 cell DNA from pool 1 (1) and pool 2 (2), e13.5 mouse cDNA library (L), normal liver control (N) and water control (C).
Figure 4.3. Sequence alignment of Sp6-sequenced insert and the muCd24a cDNA. Gel purified 2 kbp insert amplified with T7 and Sp6 primers from WAM18/21+ was sequenced using Sp6 primers and di-deoxy nucleotides. The derived sequence was compared to the mouse Cd24a gene. Sequence alignment shows 100% identity between our WAM antibody-cloned product and muCd24a from bases 3 to 800 of Sp6-primed sequencing reaction.
Figure 4.4. Confirmation of WAM18 and WAM21 identity. (A) PCR using Cd24a-specific primers was carried out on T7/Sp6-amplified fragment from WAM18/21-selected COS-7 cells transfected with e13.5 whole mouse cDNA library. Lanes are molecular weight marker (M), pool 1 (1), pool 2 (2), cDNA library (L) and no template control (C). To confirm WAM18 and WAM21 identity, Cd24a was overexpressed in COS-7 cells. (B) WAM18 and WAM21 mark pCMV-Cd24a-transfected (black), but not non-transfected COS-7 cells (red). CD24 antibody M1/69 was used as a positive control. Rat isotype control antibody did mark transfected or non-transfected cells.
4.4.3 Biochemical characterisation of WAM18 and WAM21

Although WAM18 and WAM21 both recognise CD24 proteins, we had previously observed heterogeneity in the cells recognised by these antibodies in developing mouse pancreas and DDC-induced liver injury. Hence, we carried out studies to further clarify WAM-antibody epitope specificity. To determine whether WAM18 and WAM21 mark identical CD24 epitopes, we assessed the capacity of WAM18 and WAM21 to block the binding of one another in NIT-1 insulinoma cells. The blocking ability of the M1/69 antibody was also tested. Mean fluorescence intensity (MFI) was reduced when antibodies were self-blocked. M1/69 was able to block WAM18 binding and partially blocked WAM21 binding. However, when the ability of WAM18 to block WAM21, and vice-versa, was assessed, little change was observed in MFI of detection antibody staining suggesting that our WAM antibodies recognise distinct CD24 epitopes. (Figure 4.5A).

Immunoblotting of e15.5 liver and brain protein lysates revealed tissue-specific differences in protein sizes recognised by our antibodies. Proteins detected following denaturing or non-denaturing SDS-PAGE were of similar size, though bands were more intense under denaturing conditions. In liver, WAM21 appeared to mark a group of proteins whose sizes ranged from 30-37 kDa. Three faint WAM18+ bands could be visualised under denaturing conditions of comparable size in foetal liver. Interestingly, WAM18 and WAM21 appeared to differ in developing brain extracts. WAM21 marked a protein of approximately 32 kDa, whilst an additional smaller band of approximately 28 kDa was observed when WAM18 was used to probe the membrane (Figure 4.5B). We also assessed staining in adult thymus. Separation of proteins by size and isoelectric point revealed that WAM18 marked two proteins at 30 kDa and 60 kDa, whilst WAM21 preferentially identified the 30 kDa protein (Figure 4.5C).
Figure 4.5. WAM18 and WAM21 identify unique CD24 epitopes. (A) The ability of CD24 antibodies to block each other was assessed by flow cytometry. NIT-1 cells were pre-treated with isotype control or CD24 antibodies, then WAM antibody staining was assessed. Antibody specific inhibition was calculated by comparing antibody-specific blocking (‘antibody block’; blue) to isotype antibody blocking (‘control block’; red) and expressed as a percentage. WAM antibodies show differential blocking capacity. (B) Western blot analysis of WAM antibodies in e15.5 liver (L) and brain (B). Protein lysates were separated by non-denaturing (ND) or denaturing (D) SDS-PAGE. (C) Two-dimensional Western blot analysis of WAM18 and WAM21 in mouse thymus. Red arrows denote different spots (red boxes) identified by WAM antibodies.
The CD24 protein has been previously shown to be a heavily glycosylated molecule, containing multiple sites for N-linked and O-linked glycosylation (Ohl et al., 2003, Bleckmann et al., 2009). Since our antibodies marked distinct epitopes as characterised by antibody blocking and Western blotting experiments, we examined whether this distinction could be glycosylation dependent. We assayed WAM staining by flow cytometry following treatment of Cd24a-overexpressing COS-7 cells with inhibitors of N-linked (tunicamycin) or O-linked (BGalNAc) glycosylation. Treatment of COS-7 cells with tunicamycin significantly reduced cell surface binding of WAM18 and WAM21 compared to treatment with DMSO vehicle. Inhibition of O-linked glycosylation with BGalNAc did not affect WAM18 or WAM21 binding to the cell surface (Figure 4.6). These data suggest that the size and specificity of WAM18 and WAM21 on the cell surface may be due to N-linked glycosylation.
Figure 4.6. Effects of glycosylation inhibitors on WAM18 and WAM21 antibody surface labelling. COS-7 cells were transfected with pCMV-Cd24a and treated with glycosylation inhibitor of N-linked (Tunicamycin) and/or O-linked (BGalNAc) glycosylation for 72 h. (A) Flow cytometric analysis of WAM antibody staining on inhibitor-treated cells (blue) versus DMSO vehicle control (red). WAM staining was compared to isotype control (black). (B) Quantitation of glycosylation inhibitor effects on surface labelling of WAM18 and WAM21; Bars = mean ± SEM; n=3; *p<0.05, **p<0.01 compared to vehicle (DMSO).
4.4.4 Utility of WAM18 and WAM21 for investigating organ-specific CD24 biology

Although differences in CD24 expression have been noted in the brain, cellular subpopulations with respect to multiple CD24 antibody staining have not been assessed. Brain, pancreas, liver and intestine from e15.5 Ngn3-GFP/RIP-dsRed mice were stained with WAM18 and WAM21 antibodies and cell populations identified by these antibodies were compared by flow cytometric analysis. WAM18 identified virtually all cells in all tissue types assessed. Similarly, WAM21 also identified WAM18+ cells in foetal liver and intestine, but marked a subset of WAM18+ cells in foetal pancreas and brain. In contrast, WAM18 and WAM21 did not identify distinct populations of cells in foetal liver or intestine (Figure 4.7A). Since we had previously shown specificity of WAM21 for Ngn3-expressing islet progenitors (Chapter 3), we assessed the dual specificity of WAM18 and WAM21 for Ngn3-GFP+ neuroendocrine cells in developing pancreas, intestine and brain by flow cytometry. The Ngn3-GFP+ cell subset was gated and cellular distribution with respect to WAM18 and WAM21 immunoreactivity assessed. Ngn3-GFP+ cells in the developing intestine and pancreas were enriched in the WAM18+/WAM21+ cellular subset. However, most Ngn3-GFP+ cells in the developing brain were WAM18+/WAM21- (Figure 4.7B).

Having identified the cDNA encoding our WAM antibodies as Cd24a, we used this information to carry out functional and translational studies. Firstly, since WAM18 and WAM21 mark LPCs, we assessed whether knockdown of Cd24a mRNA could modify the growth of an LPC cell line BMOL. Secondly, since WAM18 and WAM21 mark mouse islet progenitor cells in developing pancreas, we assessed the suitability of an antibody against human CD24 to isolate islet progenitor cells from developing human pancreas.
Figure 4.7. WAM18 and WAM21 identify unique cell populations in e15.5 Ngn3-GFP/RIP-dsRed mouse neuroendocrine tissues. WAM18 and WAM21 staining was compared in developing mouse tissues. (A) WAM18 and WAM21 identify virtually identical populations of e15.5 liver and intestine. WAM21 marks a subpopulation of WAM18+ developing brain and pancreas. (B) Distribution of WAM+ cells was assessed in Ngn3-GFP+ neuroendocrine cells in e15.5 intestine, brain and pancreas. Intestinal and pancreatic Ngn3-GFP+ cells are WAM18+/WAM21+. Most brain Ngn3-GFP+ cells are WAM18+/WAM21-.
4.4.5 Inhibition of \textit{Cd24a} mRNA expression alters LPC growth

Since WAM18 and WAM21 mark LPCs (Chapter 2) and we had shown they recognise CD24 protein, we investigated whether modulation of \textit{Cd24a} expression could affect LPC biology. Specifically, we assessed whether RNAi of \textit{Cd24a} could affect the cell growth properties of a liver progenitor cell line BMOL using the novel Cellavista\textsuperscript{TM} system. Initially, to assess the precision of the Cellavista\textsuperscript{TM} system for measuring cell growth, we compared measurements of a standard curve of BMOL cells at varying densities to that of the routinely used MTT assay on the same set of cells. We found a high degree of correlation between cell numbers and absorbance readings derived using the MTT assay ($R^2 > 0.97$) as well as cell numbers and cell density measurements generated by Cellavista\textsuperscript{TM} in untransfected BMOL cells, or BMOL cells transfected with scrambled or \textit{Cd24a}-specific shRNA constructs ($R^2 > 0.98$; Appendix figure A3.2).

To determine whether inhibition of \textit{Cd24a} mRNA expression could affect the growth of BMOL cells, we assessed cell growth over 142 h in non-transfected (‘Parental’), and stably-transfected scrambled control shRNA (‘shScram’) or \textit{Cd24a}-targeting shRNA (sh\textit{Cd24a}) plasmids using the Cellavista\textsuperscript{TM} system. Visual assessment of well confluence after 78 h showed that Parental and shScram BMOL cells were actively expanding, whilst sh\textit{Cd24a} BMOL’s did not appear to have proliferated significantly (Figure 4.8A). Growth curves generated over 142 h showed that proliferation of sh\textit{Cd24a}-transfected cells was significantly decreased compared to both Parental and scrambled control shRNA transfected cells (Figure 4.8B). We achieved similar results using an MTT assay (Appendix figure A3.1B). Using the logarithmic growth phase of each cell condition, we calculated the population doubling time. Doubling time was significantly increased in sh\textit{Cd24a}-transfected cells (76±13.4 h) compared to shScram (38±1.7 h) and Parental BMOL cells (29±1.6 h; Figure 4.8C).
Figure 4.8. Effects of stable Cd24a knockdown on liver progenitor cell proliferation. Cell proliferation in BMOL cells that were untransfected (parental), or stably transfected with plasmids harbouring short hairpin sequences that were targeted against Cd24a (shCd24a) or a non-specific scrambled control sequence (shScram) was measured using Cellavista. (A) Thresholded microscope images used by Cellavista software for measuring cell confluence. (B) Cell confluence measurements over time in BMOL-T cells transfected with shRNA plasmid constructs. (C) Calculated cell doubling time of BMOL-T cells transfected with different shRNA plasmid constructs. Data represent mean ± SEM, n=3 Statistical significance of one way ANOVA with Tukey’s post-test is represented as *p<0.05 or not statistically significant (ns).
**4.4.6 Human foetal islet progenitors are defined by CD24 expression**

In Chapter 3, we showed that WAM21 could be used to significantly enrich mouse beta cell progenitors capable of differentiating into insulin reporter-positive beta-like cells. Flow cytometric assessment of a human liver cell line (BHAL; Zhang et al., 2010), and a human embryonic stem cell line (performed by Erika Bosio, Centre for Diabetes Research), demonstrated that our antibodies did not recognise an epitope on human cells. Hence, for studies on developing pancreas, we used a commercially available antibody recognising the human CD24 protein.

Since human islet progenitors had previously been shown to be enriched in a CD133+/CD49F<sup>low</sup> population (Sugiyama et al., 2007), we carried out dual immunolabelling with these markers and CD24 to determine whether our novel marker is redundant. FACS analysis of double antibody-labelled human foetal pancreas cell isolates demonstrated that CD24 marks unique subpopulations of CD49F<sup>low</sup> and CD133<sup>+</sup> cells (Figure 4.9A) demonstrating uniqueness of the populations recognised by CD24 compared to previously described antibodies. We then assessed key markers of islet cell development in CD24-defined populations of developing human pancreas. CD24<sup>+</sup> cells comprised a small subset of total foetal pancreas cells in two separate pancreata, recognising 2% and 5% of cells respectively. CD24-defined populations were purified by FACS and analysis of gene expression in these populations by RT-PCR showed that PDX1 and SOX9 mRNA expression were enriched in CD24<sup>+</sup> populations. CD24<sup>+</sup> populations contained virtually all of the cells expressing the islet progenitor transcripts NGN3, MYT1 and PAX4. Insulin-expression cells were enriched in the CD24<sup>+</sup> cell population. Lower levels of Insulin transcripts were detectable in CD24<sup>-</sup> cells (Figure 4.9B).
Figure 4.9. Human foetal islet progenitors are CD24-positive. (A) Comparison of CD24 staining of dispersed foetal human pancreas cells with established islet progenitor markers CD133 and CD49F demonstrates uniqueness of cellular populations recognised by CD24. (B) RT-PCR analysis of pancreatic/islet progenitor gene expression in CD24-defined populations of human foetal pancreas shows markers of islet progenitors are enriched in CD24+ cells in two separate experiments.
4.5 DISCUSSION

The generation of new antibodies recognising surface proteins is an important step in defining the phenotype of progenitor cell domains in the liver and pancreas in a way that permits their isolation. Consequently, recent research efforts have focussed on this goal leading to the development of novel antibodies against LPC subsets (Dorrell et al., 2008, Dorrell et al., 2011a) and pancreatic endocrine, acinar and ductal lineages (Dorrell et al., 2011b). Likewise, we have developed two novel monoclonal antibodies against liver and pancreatic progenitors, WAM18 and WAM21, which we have shown previously to identify liver and pancreatic beta cell progenitors. As these antibodies bind hitherto undefined antigens their application is limited to a tool for the isolation of mouse progenitor cells. To overcome this limitation, we used an expression cloning approach to show that the antigens recognised by WAM18 and WAM21 are encoded by the mouse \textit{Cd24a} gene. Using this information, we have extended our studies of liver and pancreas progenitors to assess the role of \textit{Cd24a} in LPC growth, and to establish the suitability of CD24 as a marker of human foetal islet progenitors.

The mouse CD24 protein is a small, 30 amino-acid protein (Kay et al., 1990) for which a number of monoclonal antibodies have been developed and mark a variety of proteins ranging from 28 to 55 kDa in developing mouse brain (Shirasawa et al., 1993), adult thymus (Rougon et al., 1991) and hematopoietic lineages (Kay et al., 1991, Hubbe and Altevogt, 1994). This difference in size is due to variations in N-linked and O-linked glycosylation which has been observed most notably in the mouse brain (Ohl et al., 2003, Bleckmann et al., 2009). Although these differences have been shown by Western blotting using the M1/69 antibody, which marks the core CD24 peptide (Alterman et al., 1990), the antibodies to determine whether these differences exist in different cell lineages have not been available since established CD24-specific monoclonal antibodies M1/69, J11d and B2A2...
mark an identical antigenic determinant (Alterman et al., 1990). Similarly, our antibody blocking studies suggest that WAM18 marks a similar epitope to M1/69. We had previously shown that WAM21 marked a unique populations of A6- and CD45+ cells in chronic liver injury which is not observed with ductal cells marked by WAM18 (Chapter 2), or the M1/69 antibody in DDC-induced injury (Ochsner et al., 2007). Additionally, we have observed that WAM21 marks a subset of e15.5 foetal mouse pancreas cells containing all beta cell progenitors (Chapter 3). In the current study, we have shown for the first time using dual antibody staining and flow cytometric analysis that CD24 heterogeneity is cell type-specific in developing brain and pancreas. Although different CD24 protein species have been known to exist, the development of WAM21 will enable more detailed studies of the role of CD24 cell populations of the foetal brain and pancreas, since cell subtypes of these organs can now be separated based on differential expression of CD24 antigens defined by WAM18 and WAM21.

Although CD24 has been shown to mark ductal cells in normal (Qiu et al., 2011) and DDC-injured livers (Ochsner et al., 2007), its biological function in LPCs is yet to be probed. We had previously shown that WAM18 and WAM21 are expressed in virtually all LPC lines and on A6+ cells induced in mouse models of chronic liver injury (Chapter 2). These observations suggest an important biological function for CD24 in regulating the progenitor cell response to liver injury. In the current study, we have shown reduced proliferation in BMOL cells following Cd24a knockdown using a stably expressed shRNA vector, providing evidence of a role for Cd24a in regulating LPC proliferation. Current research suggests that CD24 may be a potential target for anticancer therapies in liver tumours since it has been associated with poor prognosis in HCC (Yang et al., 2009) and cholangiocarcinomas in humans (Su et al., 2006, Agrawal et al., 2007), has been functionally associated with tumour characteristics such as invasion, migration and
adhesion in HCC cells (Zheng et al., 2011) and promotes HCC establishment via regulation of Nanog and STAT activation (Lee et al., 2011). Since LPCs are implicated as a potential tumorigenic source in hepatocellular carcinoma (HCC) associated with chronic liver injury, further studies on the role of CD24 in LPCs may provide valuable insight for hepatocellular carcinomas derived from progenitor cells during chronic liver injury.

To date, CD24 expression has not been examined in developing human pancreas. Currently, the only indication of CD24 as a marker of pancreatic epithelium in human cells is where it has been used as a selection marker for PDX1+ cells differentiated from human embryonic stem (hES) cell lines (Jiang et al., 2011). However, a more recent study found that CD24 protein was expressed at all stages of mouse and hES pancreatic differentiation in vitro (Naujok and Lenzen, 2012). Although the utility of CD24-based purification of committed pancreatic precursors in cell line studies is disputed, we have shown for the first time that CD24-based cell sorting can be used to enrich potential islet progenitor cells expressing NGN3, PDX1 and SOX9, from primary human foetal pancreas preparations. Expression data from this study, and previously generated by assessment of WAM21-defined developing mouse pancreas (Chapter 3), are consistent with the notion that CD24 marks PDX1-expressing epithelium. Examination of the dynamics of CD24 expression throughout pancreatic development in human and mouse tissue, particularly with respect to the different antigens marked by WAM18 and WAM21 in the mouse, would provide further insight into the role of this protein in defining the three major epithelial lineages of the pancreas. Importantly, CD24-based sorting of human foetal pancreas is compatible with previously described markers CD133 and CD49F (Sugiyama et al., 2007), and is valuable in defining cells at the NGN3+ islet precursor stage. Since one of the major hurdles of islet progenitor cell research is the definition of a definitive islet progenitor cell surface phenotype, this discovery will enable
further purification and more detailed study of human beta cell precursor cells.

CD24 is a complex molecule which exhibits multiple functions most likely due to its potential for multiple glycosylation patterns. Creation of antibodies recognising different epitopes may give further insight into biology of different cell types and the role of CD24 in diverse cellular processes. We have described two such antibodies which will be useful for researchers studying cellular subtypes defined by variations in CD24 epitopes from pancreas, brain, bone marrow, liver and thymus. We have shown a role for \textit{Cd24a} in LPC expansion, and in doing so provided the first data validating the use of the Cellavista\textsuperscript{TM} system to non-invasively measure cell growth of LPCs \textit{in vitro}. Finally, we have demonstrated that the expression of CD24 on the cell surface of foetal islet progenitors is conserved between mice and humans and as will be beneficial for studies of human pancreatic islet development.
Chapter 5

General Discussion
5.1 General discussion

Replacement of dysfunctional liver and pancreas tissue with exogenous sources has been a clinical reality since the first successful human transplants of these organs were performed in 1963 and 1967 respectively (Starzl et al., 1963, Kelly et al., 1967). In the last two decades, the establishment of techniques to isolate hepatocytes and islets from cadaveric tissue has enabled transplantation of cells as an alternative to orthotopic organ transplants. Hepatocyte transplants have been beneficial for patients suffering inherited metabolic diseases (Fox et al., 1998) and islet transplantation is currently the most effective strategy for limiting morbidity associated with T1DM, as glucose-sensitive insulin release from transplanted β-cells overcomes the blood glucose fluctuations associated with injectable insulin (Shapiro et al., 2000). The advent of liver transplantation has enabled significant extension of life for people with end-stage liver disease and liver failure. Although these treatments are successful, transplant of liver and pancreas tissue requires donor tissue. Unfortunately, the current shortage of donor organs dictates the need for alternative cellular sources for therapy (Johnson et al., 2014). Stem cells represent a promising and renewable resource to provide liver and islet cells for transplantation. As protocols to reprogram somatic cells into pluripotent cells that have the potential to form any virtually cell type have developed, autologous cell transplants are a foreseeable reality if certain hurdles can be overcome (Takahashi et al., 2007).

A major problem for liver biologists is the inability to form cells that are functionally equivalent to primary hepatocytes from pluripotent cell sources in vitro. Although these cells are extremely valuable for drug screening and disease modelling purposes, their translation to a clinically viable cell source is currently limited (Reviewed in Schwartz et al., 2014). Great progress has been made in deriving insulin producing cells (IPCs) from stem cells suggesting the goal of a stem cell-derived cell therapy for Type 1 Diabetes may be within reach. Recently, Pagliuca and colleagues developed a novel protocol for the differentiation of glucose responsive IPCs from one hES, and two hiPS cell lines with a differentiation efficiency of approximately 45%.
These cells function to a level that is similar to isolated human adult islets when transplanted into diabetic mice (Pagliuca et al., 2014). Although these studies represent excellent progress in translating the promise of stem cells to a clinical therapy, much work remains to improve efficiency of differentiation and in meeting safety requirements for therapeutic use. Since gene expression networks are conserved between stem/progenitor cells and tumours, the safety of stem cell-derived liver or pancreas cells for transplantation is a concern (Lee et al., 2013). To overcome these problems, a thorough understanding of liver and pancreas progenitor cell biology is an imperative for translating the promise of stem cell therapies in the laboratory to beneficial therapeutic outcomes for patients.

The phenotypic definition of liver and pancreas stem/progenitor cell compartments remain in its infancy. Currently, the benchmark approach for defining and isolating stem cells is the method applied in defining hematopoietic stem cells. Several decades of research have culminated in the definition of bone marrow-derived cell lineages using monoclonal antibodies recognising cell surface antigens (Spangrude et al., 1988, Baum et al., 1992). This has ultimately led to the definition of single cells capable of reconstituting the entire hematopoietic system (Krause et al., 2001). The liver progenitor and pancreatic islet progenitor compartments encompass a heterogeneous subset of cells. To date, the definitive stem/progenitor cell subtypes of each organ are yet to be defined at the single cell level (Jelnes et al., 2007, Desgraz and Herrera, 2009). A major limitation to these studies is the lack of cell surface markers defining liver and pancreas progenitor cell populations.

The availability of new markers would facilitate the isolation and characterisation and eventual generation of therapeutically functional cells from expandable stem cell sources. In light of these limitations, the overall
aim of the research described in this thesis was to generate and characterise monoclonal antibodies that recognise liver and pancreas progenitors.

Since the liver and pancreas share a common endodermal precursor, transcription factors networks for cell-type development and epithelial cell surface antigens, new surface markers developed for the liver may be useful in isolating progenitors from the pancreas and vice-versa. Hence, we produced a panel of monoclonal antibodies against liver and pancreas cells of foetal origin in order to characterise progenitor domains of these organs together (Chapter 2). This approach to generating cell surface antibodies against heterogeneous cell populations has previously been used successfully in developing surface antibodies such as the widely used LPC marker, A6 (Faktor et al., 1990) and more recently liver non-parenchymal cells from chronically injured liver (Dorrell et al., 2008) and adult mouse pancreas (Dorrell et al., 2011). Prior to the studies described in this thesis, this approach had not been used with developing liver or pancreas cells as an immunogenic source. Although antibodies against adult pancreas also mark developing pancreas populations (Dorrell et al., 2011), we reasoned that the use of foetal pancreas to produce antibodies would be more successful in producing antibodies against islet progenitors, since Ngn3\textsuperscript{+} cells are transiently present during pancreatic development (Schwitzgebel et al., 2000). Several antibodies from our panel co-identified developing liver and pancreas cells, reinforcing the notion that these progenitor domains share a similar phenotype and supporting our undertaking to study these organs in parallel (Chapter 2). After a rigorous screening process, two antibodies, WAM18 and WAM21 were further characterised.

WAM18 and WAM21 displayed a ductal/progenitor phenotype in liver (Chapter 2), and WAM21 a mixture of ductal/acinar/islet progenitor cells in developing pancreas (Chapter 3). This is similar to some established antibodies which display a ductal/progenitor cell specificity and mark hepatic/pancreatic populations such as CD133 (Hori et al., 2008, Rountree et
al., 2007, Sugiyama et al., 2007), Cd49f (Sugiyama et al., 2007, Suzuki et al., 2000, Tsuchiya et al., 2007) and E-cadherin (Jiang et al., 2002, Ueberham et al., 2007). Interestingly, none of these antibodies have specifically been raised against liver or pancreas tissue. For example, EpCAM (G8.8), which has been used to isolate LPCs (Okabe et al., 2009), was originally developed against thymus (Farr et al., 1991). The CD133 (13A4) marks liver (Rountree et al., 2007) and pancreas progenitors (Sugiyama et al., 2007, Hori et al., 2008), and was raised against mouse embryonic neural tissue (Weigmann et al., 1997). Cd49f (GoH3), which identifies pancreas progenitors (Sugiyama et al., 2007), was raised against mammary tissue (Sonnenberg et al., 1986). Likewise, the M1/69 antibody to CD24, which has been used to purify LPCs (Ochsner et al., 2007), was generated against spleen cells (Springer et al., 1978). Although these antibodies have been useful in isolating progenitors from the liver and pancreas, antibodies against tissue-specific epitopes of these glycoproteins may provide greater specificity of progenitors of these tissues.

The glycosylation of proteins, including those at the cell surface, have been shown to be cell type specific (An et al., 2012), and change during cell differentiation (Satomaa et al., 2009) and neoplastic transformation (Reis et al., 2010). Therefore, the development of antibodies against cell-type specific glycoproteins may prove beneficial in developing new tools for cell isolation. In this study we were able to develop WAM18 and WAM21 which mark overlapping, but distinct epitopes on the CD24 protein which are glycosylation-dependent (Chapter 4). Using these antibodies, we were able to demonstrate novel CD24+ cell types induced by chronic liver injury (Chapter 2) and present in developing pancreas (Chapter 3), developing brain and adult thymus (Chapter 4). This property was especially useful since WAM21 marked a subpopulation of Cd24a-expressing foetal pancreas cells which contained all β-cell precursors as assessed by Ngn3-GFP transgene expression and an in vitro differentiation assay (Chapter 3). These studies highlighted the specificity of the WAM21-CD24 antigen for the islet progenitor cell compartment which has enabled us to develop this antibody
as a useful tool for studies of islet development. Although we have shown our antibodies mark novel CD24 antigens, the biological reasons for their differences in cell type specificity remain unknown. In-depth epitope mapping of the glycodeterminants marked by WAM18 and WAM21 may provide further insight into the biological roles of this diverse protein and will be the subject of future studies.

One of the limitations of the work carried out in this thesis was the lack of significant numbers of antibodies which marked epithelial liver populations \textit{in vivo}. Although we initially produced 54 antibody-producing hybridomas which marked LPC lines, most did not stain liver tissue, or marked non-epithelial cell populations (Chapter 2). Comparatively, six of our antibodies marked transgenically-labelled islet progenitor cells and an additional four antibodies could be used as negative selection markers (Chapter 3). One explanation for this observation may the lack of cell surface antigen complexity of the initial liver-specific immunogenic source (immortalised BMEL cells), limiting the ability to produce antibodies marking rare cell populations induced by early chronic liver injury. This problem is paralleled in a previous study where expression profiling comparing undifferentiated and differentiated BMEL cells produced \textit{Cd24a} as a single candidate cell surface marker (Ochsner et al., 2007). Conversely, studies utilising primary liver cells have produced many antibodies against rare cell types (Dorrell et al., 2008). Our observations in the liver may be due to a combination of decreasing population complexity in addition to changes in gene expression associated with the process of cell line generation which will be discussed further in the following paragraph.

Although immortalised cell lines have been extremely valuable in studies of tumourigenicity (Dumble et al., 2002, Davies et al., 2006, Jellicoe et al., 2008), differentiation (Strick-Marchand and Weiss, 2002, Tirnitz-Parker et al., 2007) and in studying the biological effects of inflammatory cytokines on LPCs (Matthews et al., 2004, Akhurst et al., 2005, Yeoh et al., 2007, Tirnitz-
Parker et al., 2010), the process of cell line generation involves selection of a subpopulation of cells that are not entirely reflective of the complexity or nature of those seen in vivo. The ‘plate and wait’ method of spontaneous immortalisation used to generate LPC cell lines utilised in this study involves selecting for cell subpopulations which are able to bypass senescence and continue to proliferate indefinitely (Strick-Marchand and Weiss, 2002). Furthermore, cultured LPCs have been shown to downregulate expression of liver-specific markers such as albumin, transferrin, α-fetoprotein and tyrosine aminotransferase as cells are passaged (Dumble et al., 2002). Hence, the process of generating LPC lines excludes a significant proportion of the initial progenitor cell compartment through senescence, and the remaining cells become dissimilar to their in vivo counterparts as they are cultured. Despite this, antibodies marking LPC lines but not liver tissue may still prove useful in identifying novel liver cell types, since the process of cell line generation parallels some aspects of cellular transformation, an aspect of chronic liver injury not explored in this thesis. We will explore this idea further in the following paragraphs with a view to future studies using our antibodies.

The current framework for understanding the development of a cancer phenotype involves the acquisition of mutations which enable evasion of mechanisms regulating cell growth, such as reliance on growth factor stimulation, and sensitivity to regulators of cell proliferation and death (Hanahan and Weinberg, 2011). Pioneering work in a Retinoblastoma model has demonstrated the requirement of at least two aberrations in these control mechanisms for neoplastic transformation, termed the ‘two hit hypothesis’ of cancer (Knudson, 1971). This hypothesis holds true in liver cancer formation. Disruption of two tumour suppressor genes (TSGs) in combination, such as mTert and p53 (Farazi et al., 2006) and more recently the phosphatase and tensin homolog (Pten) with glucose-regulated protein 94 (Grp94) (Chen et al., 2014) accelerate liver tumorigenesis. Furthermore, studies in which overexpression of oncogenes or inhibition of TSGs in LPCs show that a combination of these genetic ‘hits’ results in acquisition of genetic
abnormalities consistent with HCC formation in resulting tumours (Zender et al., 2005).

The ability of LPC lines to bypass senescence, and proliferate indefinitely in culture, represents the acquisition of one of the two required traits for cell transformation. Since WAM antibodies were generated against immortalised LPCs, some of the antigens they recognise may be associated with tumorigenic progression associated with the development of an immortal phenotype. Accordingly, these antigens would not be present after two weeks of CDE diet-induced liver injury, as this timepoint represents the early stages of LPC expansion (Chapter 2). Since chronic liver injury-associated HCC arises after prolonged LPC activation which is supported by the inflammatory niche responding to liver injury (Roskams, 2006), antigens marked by WAM antibodies present on immortal LPC lines are more likely to be expressed as LPC transformation progresses during long term chronic injury. Thus, our WAM antibody panel may prove valuable in defining novel cell types induced during HCC formation in chronic liver injury in future studies.

5.2 Summary and conclusions
In this study, we have produced a panel of monoclonal antibodies which recognise progenitor cells of the liver and pancreas in addition to subsets of cells from diverse sources including thymus, brain and embryonic stem cells. Whilst the use of this antibody panel may have broader benefits for studying progenitor cells of these organs, we have focused on two antibodies, WAM18 and WAM21, which mark unique CD24 antigens on the cell surface of liver and pancreas progenitor cells. In further developing these antibodies we have demonstrated the following:
1. Our newly developed antibodies are effective alternative markers to A6, which is limited in supply, for identifying LPCs induced in mouse chronic liver injury models, with the additional benefit of applicability in antibody-based sorting of enzyme-dissociated tissues and cell lines.

2. WAM21 marks a subset of foetal pancreas cells which contains beta cell progenitors and is extremely useful for purifying these progenitors for studying islet development in vitro.

3. Our expression cloning approach using FACS selection to produce stably-expressing cells, rather than viral or serial transfection methods, provides a methodological framework that is technically simple and relatively fast in identifying the cDNA encoding antigens recognised by our antibody.

4. Identification of Cd24a as the gene encoding the WAM18 and WAM21 antigens has enabled us to advance CD24 as a novel marker of putative human islet progenitor cells.

The markers we have developed in this thesis are useful tools for researchers studying progenitor cells from the liver and pancreas in mouse models. Their use for isolation and characterisation of liver and pancreas progenitor cells will aid studies of the biology of liver and pancreas progenitors for understanding liver and pancreas disease, regeneration and in developing methods to derive mature cells for transplantation therapy.
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REFERENCES


REFERENCES


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References


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Guo, H., Tao, Y., Xue, Y., Jiang, N., Yao, L. & Liu, W. 2011. NDRG2 inhibits
hepatocellular carcinoma adhesion, migration and invasion by regulating


Zhou, Q., Law, A. C., Rajagopal, J., Anderson, W. J., Gray, P. A. & Melton, D.
Dev Cell, 13, 103-14.
Appendix I

Supporting and additional data from Chapter 2
A1.1 Appendix I Methods

A1.1.1 Cytopreparation of BMOL 1.2 cells

Cytospins were prepared by diluting $1 \times 10^4$ cells in 100 $\mu$L of ice cold PBS. Cells were centrifuged at 500 rpm for 5 min at RT onto microscope slides and fixed with methanol:acetone (1:1, -20°C) for 5 min. Slides were air-dried and stored at -20°C until use.

A1.1.2 Immunofluorescence

Liver cryosections (7 $\mu$m) were cut and fixed by exposure to RT acetone for 5 min. Sections were air dried and stored at -20°C until use. Fixed cryosections or cytospins were rehydrated by incubation in PBS for 5 min. Primary antibodies labelling was performed overnight at 4°C. Antibodies used were WAM18, WAM21 (rat; 1:200) and/or FITC-conjugated mouse anti-α-Smooth Muscle Actin (1:1000, Sigma). Rat antibodies were detected which were detected with Alexa 594-conjugated goat anti-rat IgG (1:200, Invitrogen) following incubation at room temperature for 1 h. Slides were mounted with ProLong Gold Antifade mounting medium containing the nuclear stain DAPI (Invitrogen, Australia) and visualised by fluorescence microscopy. Single colour fluorescent microscope images were merged using ImageJ version 1.39u software for Windows (ImageJ Software, National Institutes of Health, USA, available at: http://rsb.info.nih.gov/ij/). All colour corrections were applied equally to experimental and control samples.
## A1.2 Supplementary material

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Appendix Table A1.1 Mouse PCR Primer sequences and annealing temperatures (A. Temp.) used.
Appendix Figure A1.1 Assessment of liver damage in CDE diet. Haematoxylin and eosin staining of livers from mice fed a (A) control or (B) CDE diet for 2 weeks. Control livers display normal architecture. CDE livers display fat accumulation in hepatocytes and ductal expansion. (C) Assessment of serum ALT levels in control versus CDE fed mice. Serum ALT was increased in all CDE fed mice compared to control mice.
Appendix Figure A1.2 Immunofluorescence controls for dual rat antibody labelling. Specificity of antibody labelling on liver tissues was confirmed by incubating cryosections (7 µm) of 4 wk DDC-injured liver tissue with appropriate isotype control antibodies. WAM21-Biotin and WAM18 antibodies were used as positive controls.
Appendix Figure A1.3 Magnetic bead enrichment of WAM18 and WAM21-defined normal liver subsets. Magnetic bead sorting of normal liver non-parenchymal cells (NPC’s) with WAM antibodies. NPC’s from normal liver were stained with WAM18 or WAM21 and enriched using magnetic beads. Purity of fractions was assessed by flow cytometry. Cells were gated by size using forward scatter (FSC-A) and side scatter (SSC-A) profile, and live cells assessed by gating propidium iodide-negative cells. WAM-staining was then assessed in these live cell populations. Histogram overlays show that WAM-negative (black) and WAM-positive fractions were highly pure prior to culturing in liver progenitor cell growth medium.
Appendix Figure A1.4. Localisation of WAM antibody staining at the cell surface of BMOL1.2 LPCs. (A) Immunofluorescent staining with WAM18 or WAM21 (red) on BMOL1.2 compared to isotype control staining. (B) Confocal microscopy shows surface localisation of WAM antibody staining on BMOL1.2 cells. Nuclei were counterstained with DAPI (blue).
Appendix Figure A1.5. Comparison of WAM and α-Smooth Muscle Actin (αSMA) antibody labelling in chronic liver injury. Immunofluorescent staining with the activated stellate cell marker αSMA (green) and WAMs (red) on cryosections (7 µm) of livers of mice fed a DDC diet for 2 weeks. WAM18 and WAM21 positive cells are observed in close association with αSMA⁺ stellate cells, but co-localisation of markers is not observed. Nuclei were counterstained with DAPI (blue). Diagonal arrows indicate regions displayed by insets. Horizontal arrows mark WAM21⁺/CD45⁺ cell subsets. Scale bars represent 50 µm.
Appendix II

Supporting and additional data from Chapter 3
### Appendix A2.1 Supplementary material

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Appendix Table A2.1. Mouse PCR Primer sequences and annealing temperatures (A. Temp.) used.
Appendix Figure A2.1 Immunofluorescence controls for dual rat antibody labelling. Specificity of antibody labelling on foetal pancreas tissue was confirmed by incubating cryosections (7 µm) of e15.5 foetal pancreas tissue with appropriate isotype control antibodies. WAM21-Biotin and Cd49f antibodies were used as positive controls.
Appendix Figure A2.2. Enrichment and differentiation of WAM18-defined foetal pancreas cells. In vitro differentiation of WAM18-defined e15.5 MIP-GFP foetal pancreas was assessed. WAM18+ cells were separated by size by gating into FSC-low (low) and FSC-high (high) populations (FACS plots), plated into differentiation medium and the numbers of newly formed MIP-GFP+ cells were quantitated. (A) In the first experiment, WAM18+/FSC-low cells were enriched for beta cell progenitors. (B) In the second experiment WAM18+/FSC-high cells were enriched for beta cell progenitors.
Appendix Figure A2.3. Magnetic bead enrichment and differentiation of foetal pancreas cells with WAM21. (A) Efficiency of WAM21 enrichment assessed by FACS analysis after separation with (A) one or (B) two MiniMACS columns. (C) Beta cell differentiation dynamics of WAM21-defined populations compared to unsorted control. (D) Ratio of insulin-reporter+ cells formed after differentiation in WAM21-defined foetal pancreas populations compared to unsorted cells. Bars represent mean ± SEM (n=3; *p<0.05 compared to unsorted cells).
Appendix Figure A2.4. Immunocytochemical staining of differentiated WAM21\(^+\) MIP-GFP e15.5 foetal pancreas with islet markers. Comparison of MIP-GFP fluorescence and islet markers demonstrates that WAM21\(^+\) cells differentiated to MIP-GFP\(^+\) cells \textit{in vitro} (A) do not co-express Glucagon (α-cells), and (B) express nuclear Pdx1 (islet progenitor/β-cells). Scales bars represent 10 µm.
A2.2 Expression profiling WAM21\(^{+}\) foetal pancreas cells

A2.2.1 Methods

A2.2.1.1 In vivo incubation of WAM21\(^{+}\) foetal pancreas cells

WAM21\(^{+}\) e15.5 foetal pancreas cells were MACS-enriched as previously described (Chapter 3). 100 000 WAM21\(^{+}\) e15.5 foetal pancreas cells were embedded in 20 µL Matrigel\textsuperscript{TM}(BD Biosciences) containing Nicotinamide (10 mM, Sigma-Aldrich) and B27 supplement (2X, Gibco) and pipetted into 1 mm\(^{3}\) gelfoam to create an insert for transplant. Inserts were incubated at 37 °C for 20 min to allow Matrigel to solidify. Inserts containing cells were covered in high glucose DMEM medium (Invitrogen) and incubated overnight in a 37 °C, 10% CO\(_2\) incubator with 90% humidity.

The next day, gelfoam inserts containing WAM21\(^{+}\) foetal pancreas cells were transplanted into the left renal subcapsule of eight week old female NOD-SCID mice. Mice were housed under specific pathogen free, temperature controlled conditions, with alternating 12h light-dark cycles. All procedures were performed according to guidelines set by the National Health and Medical Research Council of Australia under ethics approval from the University of Western Australia. Transplant was performed by Dr Erika Bosio from the Centre for Diabetes Research at the Western Australian Institute for Medical Research. A surgical plane of anaesthesia was induced by i/p injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg). After shaving the left flank of the mouse, a small incision was made in the left back of the animal and the kidney was exposed on the outside of the body by applying pressure around the incision. A small cut to the kidney capsule was made by scraping a 23 gauge needle across the upper pole of the kidney. Gelfoam inserts containing foetal pancreas cells were inserted under the renal capsule, which was then heat-cauterised. The kidney was returned to the body and all incisions were sutured.
Appendix II

After 3 weeks, mice were anesthetized by i/p injection of Ketamine/Xylazine and euthanized by cervical dislocation. The left kidney was removed and the graft was dissected away from the kidney capsule and placed into 1 mL TRIzol (Invitrogen) for RNA extraction. For microarray analysis of gene expression, RNA was extracted from freshly isolated and in vivo incubated WAM21+ e15.5 foetal pancreas cells in 1mL TRIzol reagent (Invitrogen) and prepared for hybridization to Illumina Ref-8 arrays according to the manufacturer’s instructions (Illumina, San Diego, CA).

A2.2.2 Results

A2.2.2.1 Expression profiling WAM21+ foetal pancreas cells

In an attempt to define pathways regulating in vivo differentiation of islet progenitors, we transplanted enriched WAM21+ cells by magnetic bead sorting, and incubated these cells under the kidney capsule of NOD/SCID mice for a period of 3 weeks. We then extracted the in vivo incubated tissue and carried out microarray expression profiling to ascertain potential new pathways involved in islet development. Since we did not observe evidence of islet progenitor differentiation, we instead used this experiment to create a gene expression profile of WAM21-defined e15.5 foetal pancreas cells to determine which developmental lineages are enriched by WAM21-sorting. The in vivo incubated tissue was used as a baseline for non-islet gene expression.

384 microarray probes were greater than 5-fold higher in freshly isolated versus in vivo incubated tissue. We found many of these genes were acinar cell-specific enzymes including proteases and lipases, and also acinar-specific transcription factors Ptf1a and Mist1. Additionally, a number of transcription factors involved in islet development were also highly expressed in WAM21+ e15.5 foetal pancreas cells including Pdx1, and Ngn3, which we had previously observed by RT-PCR analysis, and more recently defined
transcription factors that are crucial for islet development Sox9, Rfx6, FoxA3 and Myt1. We also observed markers of committed islet progenitors of the beta/delta cell lineage Pax4, Nkx2.2 and Nkx6.1 supporting our differentiation studies showing that WAM21+ cells differentiate into beta-like cells. We did not observe enrichment of pathways associated with ductal differentiation, particularly upregulation of Notch pathway signalling (Appendix Figure A2.5).

Since there are limited cell surface markers available for enriching islet precursors, we carried out gene ontology (GO) profiling to determine if novel potential surface markers of pancreatic lineages were present in WAM21+ foetal pancreas cells. Three GO identifiers associated with cellular components, GO:0005911 (cell-cell junction; 11 transcripts), GO:00016324 (apical plasma membrane; 6 transcripts) and GO:00016323 (basolateral plasma membrane; 6 transcripts) defined transcripts encoding proteins expressed on the cell surface of WAM21+ cells. Of these markers, several genes encoding proteins involved in cell-to-cell contacts were enriched in WAM21+ cells including members of the claudin family of proteins, the transcript encoding the desmosome-associated protein desmoplakin and the gap junction protein connexin 36. Additionally, markers involved in cell-extracellular matrix interactions (β4-integrin, Cxadr), ion transport (Slc12a2) the glycoprotein mucin 1 and the previously identified surface marker of liver progenitor cells Dlk were all enriched in WAM21+ cells (Appendix Table A2.2).
Appendix Figure A2.5. Developmental hierarchy of gene expression in WAM21+ e15.5 foetal pancreas cells. Microarray analysis was carried out on WAM21+ e15.5 foetal pancreas. WAM21+ cells are enriched for acinar-specific transcription factors and enzymes, and islet precursor cells of the beta/delta cell lineage according to the schema of pancreatic development. Colour-coding represent fold upregulation of lineage-specific genes in freshly isolated versus in vivo incubated WAM21+ e15.5 foetal pancreas cells.
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Table A2.2. Surface markers enriched in WAM21+ foetal pancreas cells.
Appendix III

Supporting and additional data from Chapter 4
A3.1 Appendix III methods

A3.1.1 Relative quantitation of gene expression

RNA from BMOL cells was extracted and reverse transcribed according to previously described methods (4.3.12). *Cd24a* and β-actin gene expression was assessed using SensiMix qPCR mastermix according to manufacturer instructions (Bioline, Australia) in a Rotor Gene 2000 real-time PCR thermal cycler (Corbett). Primers and annealing temperatures are listed in supplementary table S4.1. No template controls (no reverse transcriptase and water) were used to control for DNA contaminants. Standard curves were generated using serial 10-fold dilutions of RT-PCR products for each gene assessed and used to calculate reaction efficiency (>90%). Relative gene expression was calculated using the comparative Cₜ method (Schmittgen and Livak, 2008). Data was normalised to β-actin expression and expressed as fold change over shScrambled control cells.

A3.1.2 MTT assay of cell proliferation

The MTT assay was performed as previously described (Mosmann, 1983). Cell viability is determined by the conversion of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL: Sigma, USA) to formazan, a purple precipitate. 10 µL of MTT solution was added to each well of a 96-well plate containing BMOL cells in 100 µL of growth medium, and incubated at 37°C for 4 hrs. Formazan, produced by cell mitochondria, was solubilised by the addition of 100 µL of solubilisation buffer (26.6% (w/v) SDS in 46.8% dimethylformamide; pH 4.7) and incubation for 2 h at 37°C. Optical density (O.D.) was measured at 595 nm and directly compared to well confluence measurements generated by the Cellavista™ system (Appendix figure A3.2).
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Appendix Table A3.1. PCR Primer sequences and annealing temperatures (A. Temp) used.
Appendix figure A3.1. Effect of stable Cd24a knockdown on BMOL cell growth. Cell proliferation in BMOL cells that were untransfected (Parental), or stably transfected with plasmids harbouring short hairpin sequences that were targeted against Cd24a (shCd24a) or a non-specific scrambled control sequence (shScrambled) was measured using MTT assay. (A) Stable knockdown of Cd24a mRNA was confirmed by qPCR. (B) BMOL cell numbers were indirectly measured using an MTT assay over 6 days. Cell numbers were significantly reduced in shCd24a-expressing BMOL cells after 5 days in culture. Data represent mean ± SEM, n=3 Statistical significance of paired t-test is represented as *p<0.05 or **p<0.01.
Appendix figure A3.2. Assay precision of the Cellavista™ system versus the MTT assay. BMOL cells were seeded at densities from 25000 cells to 500 cells per well, allowed to adhere for 16 h and well confluence was measured using Cellavista™. A strong linear correlation ($R^2 > 0.98$) between cell number and measured well confluence was observed in (A) untransfected BMOL cells or BMOL cells stably expressing (B) shScrambled or (C) shCd24a constructs. Measured wells were then measured using an MTT assay. A strong linear correlation ($R^2 > 0.97$) between well confluence measurements using the Cellavista™ and the established MTT assay was observed in (B) untransfected BMOL cells or BMOL cells stably expressing (D) shScrambled or (F) shCd24a constructs.