Defining the mechanisms in lineage specification of progenitor cells in the regenerating adult liver

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text.

The data included in this text has not been submitted for any other degree or professional qualification, nor does it exceed the word limit of 100,000 words set by the College of Medicine and Veterinary Medicine.

Luke Boulter

Abstract

During hepatic disease the liver has the unrivalled ability to regenerate, by activating mature hepatocytes which can divide and thereby reconstitute the functional liver mass. However in the context of chronic hepatocellular disease the liver can regenerate from an endogenous population of hepatic progenitor cells (HPCs). The mechanisms which are involved in the activation and differentiation of these HPCs is not fully understood.

To investigate whether there is a differential signalling requirement in HPCs acquiring a biliary versus hepatocellular fate we established in the laboratory two models of chronic liver damage and regeneration, one of which causes hepatocellular death, and results in infiltrating HPCs regenerating hepatocytes, and a second which causes biliary blockage and death, resulting in biliary regeneration.

Here we describe how during biliary regeneration the Notch signalling pathway is highly expressed and activated. HPCs cells are consistently associated with a myofibroblast niche which expressed the ligand Jagged-1 at high levels. We have modulated the Notch signalling pathway in both a co-culture system and our models *in vivo* to demonstrate that Notch signalling is important in the specification of biliary cells, and that inhibition of this pathway both *in vitro* and *in vivo* results in the abrogation of biliary commitment.

During hepatocellular regeneration we have found that the negative repressor of Notch signalling Numb is highly expressed in tandem with a low expression of the Notch pathway. We suggest that Wnt signalling maintains Numb within these HPCs at a high level and that this, along with stimulation of a hepatocellular programme allows HPCs to exit from a biliary fate and assume a hepatocellular phenotype. Finally we have found that macrophage ingestion of debris promotes the expression of Wnt, and that ablation of these cells results in a phenotypic switch between HPCs assuming a hepatocellular fate and a biliary one.

“I don't want to achieve immortality through my work... I want to achieve it through not dying.”

Woody Allen 1935 -

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List of Abbreviations

3H, Tritiated Thymidine

aSMA, a Smooth Muscle Actin

AAF, 2-N-acetylaminofluorene

Alk Phos, Alkaline Phosphatase

ALT, Alanine Aminotransferase

AST, Aspartate Aminotransferase

BDL, Bile duct ligation

BM, Bone marrow

BMSC, bone marrow stem cell

BrdU, 5-bromo-2-deoxyuridine

CCl4, Carbon tetrachloride

CDE, Choline deficient ethionine supplemented

cDNA, complementary DNA

CK, Cytokeratin

CYP, Cytochrome

DAB, Diaminobenzidine

DAPI, 4',6-diamidino-2-phenylindole

DDC, Diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate

Dlk, Delta Like Kinase

DMSO, Dimethyl sulfoxide

DPPIV, Dipeptidyl Peptidase IV

EdU, 5-ethynyl-2'-deoxyuridine

ECM, extracellular matrix

EGF, Epidermal Growth Factor

EpCam, Epithelial Cell Adhesion Molecule

ES, Embryonic Stem

FACS, Flow Activated Cell Sorting

FAH-/-, Fumarylacetoacetate Hydrolase knockout

FCS, Foetal Calf Serum

FGF, Fibroblast growth factor

FITC, Fluorescein isothiocyanate

Fn14, Fibroblast growth factor-inducible 14

Fz, Frizzled

gDNA, genomic DNA

GFP, Green Fluorescent Protein

gGT, g-Glutamyltransferase

HRP, Horse Radish Peroxidase

HBV, Hepatitis B Virus

HCC, Hepatocellular carcinoma

HCV, Hepatitis C Virus

HGF, Hepatocyte Growth Factor

Hh, Hegdehog

HPC, Hepatic Progenitor Cell

IFN, Interferon

IL, Interleukin

i.p., intraperitoneal

is, induced pluripotent stem

i.v., intravenous

LIF, Leukaemia Inhibitor Factor

LIFR, LIF receptor

MMP, metalloproteinase

NPC, Non Parenchymal Cell

OC, Oval Cell

OSM, Oncostatin M

PanCK, Pancytokeratin

PBS, Phosphate Buffered Saline

PCR, Polymerase Chain Reaction

PH, Partial hepatectomy

PPIA, peptidylprolyl isomerase A

qPCR Quantitative PCR

rm, recombinant mouse

RT-PCR, Reverse Transcription PCR

SDF-1, Stromal cell derived factor 1

SEM, Standard Error of the Mean

T3, triodothyronine

TGF, Transforming Growth Factor

TIMP, Tissue Inhibitor of Metalloproteinase

TNF, Tumour Necrosis Factor

TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

TWEAK, TNF-like weak induce of apoptosis

uPA, urokinase-type plasminogen activator

Wnt, Wingless-int

WT, Wild Type

Chapter 1 - Introduction

The Global Burden of Liver Disease

Chronic liver disease is associated with high morbidity and mortality worldwide. The WHO ascribed a DALY of 37,760/100,000 people in 2004[1] and in the UK liver disease is the 5th biggest health burden; whilst the top four are decreasing liver disease is increasing exponentially[2]. Currently alcohol and chronic viral hepatitis serve as the main drivers of this disease and it is predicted that in excess of 3% of the global population are living with liver disease, with regions in which viral hepatitis is endemic within the population[3, 4]. The socio-economic burden of liver disease is rising, and currently the only curative option for end-stage liver disease is liver transplantation however donor organ availability has remained static whilst the requirement for transplant has increased, and as such many patients die whilst waiting for suitable transplant tissue[5].

As the burden of liver disease is growing globally it is important to define the mechanisms which function during this pathological process and devise strategies which promote both liver repair and regeneration during chronic and acute damage. During these processes the liver mass is damaged, scar is deposited, and cells are activated in order to regenerate and restore the site of damage[6, 7]. Moreover regeneration of the adult liver is associated with a large degree of inflammation which may play an important role in appropriate cellular activation. These processes together should be considered during hepatic damage and regeneration as investigation into these mechanisms may identify novel targets and could deliver positive clinical outcomes during disease [8, 9]. A step towards such emerging therapy is to understand the mechanisms which function during regeneration in chronic liver

disease and identify novel pathways which could be targeted in a therapeutic manner[10]. Further to the potential clinical impact of studying regeneration in the liver, this system provides a unique model of solid organ regeneration with a definite contribution from both mature parenchymal tissues as well as adult hepatic progenitor cells (HPCs). Critically therefore, control of hepatic regeneration may define specific cellular mechanisms underpinning adult progenitor cell proliferation and lineage specification in other solid organs.

Adult Liver Architecture

The adult liver is the largest mature organ of the body, and has a central role in homeostasis and metabolism of numerous compounds including many pharmacological drugs. The adult liver consists of sheets of parenchymal epithelium known as hepatocytes, which are the key metabolic and enzymatic site of the liver (Figure 1.1). These sheets are surrounded not by a basement membrane, but by a heavily fenestrated endothelial layer known as the hepatic sinusoidal endothelium[11]. Closely associated with the sinusoids are numerous phagocytic monocytes, such as macrophages. The sinusoids are separated from the hepatocytes by the space of Dissé which contains ECM components and stellate cells which in their resting state express intermediate filament Desmin and GFAP[12]. However in response to disease Stellate cells differentiate into activated Myofibroblasts, up regulate alpha-smooth muscle actin (αSMA), which can be used to make these activated cells. Myofibroblasts are responsible for the *de novo* synthesis of collagen rich extra cellular matrix during protracted injury; and are as such a key cellular playing during hepatocellular scarring[13].

The liver parenchyma can be divided into functional subunits known as acini which consist of both portal and venous circulation, as well as terminal braches of the biliary tree. The acini lie within periportal Zone-1 of the hepatic parenchyma and are associated with portal blood flow however a nutrient and oxygen gradient is established across the parenchymal hepatocytes from a high oxygen tension in Zone-1 to a low oxygen tension in Zone-3 which is anatomically located close to the central vein. In the adult liver this nutritional and oxygen gradient is maintained through portal and venous blood flow and is responsible for maintenance of zonation across the hepatic parenchyma. Reversal of blood flow results in reversal of zonation within the parenchyma and as a result the functional regions of the liver are lost or poorly defined. Interestingly during ontogeny non-circulating factors are required for correct liver zonation including ECM deposition, and Wnt signalling[14, 15] indicating that the ontogeny of liver zonation exists differently to the maintenance of hepatic zonation in the adult liver.

The adult liver is infiltrated with a large network of bifurcating ducts which consist of polarised epithelium known as cholangiocytes. These vessels begin at the bile canaliculi; small vessels formed between the membranes of hepatocytes[16]. Canaliculi filter into the canals of Herring – the terminal branches of the biliary tree which lead into larger bile ducts and ultimately exit into the duodenum. The biliary epithelium is capable of both bile salt and water transport[17-19]and maintains the flow of bile within biliary tree, as such the biliary network is critical for hepatic homeostasis.

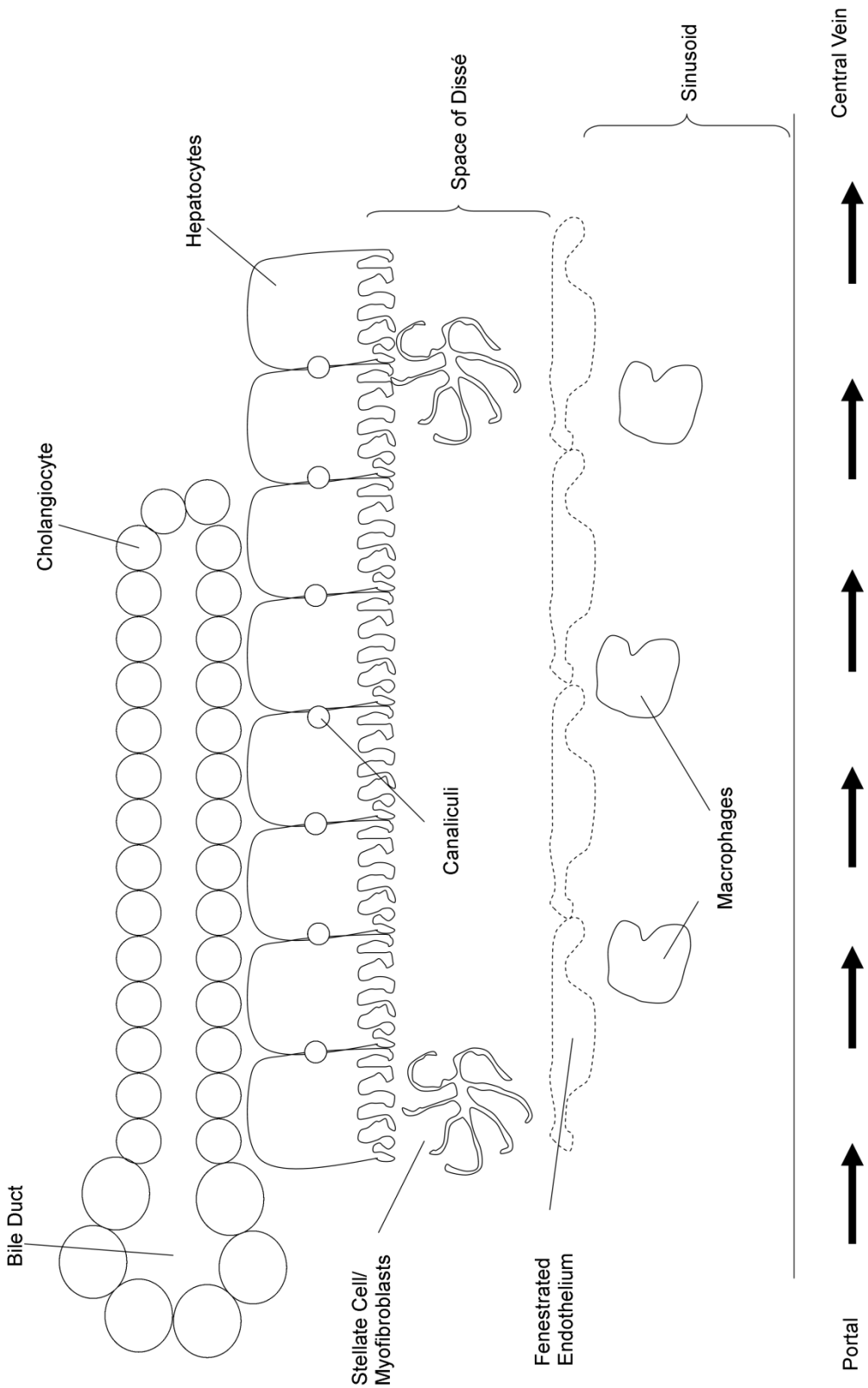

Figure 1.1 - The micro-architecture of the liver

Figure 1.1 – The micro architecture of the liver

The adult liver consists of two main epithelial cell types, cholangiocytes and hepatocytes which are organised into the bile ducts and hepatocyte plates respectively. Between plates of hepatocytes are layers of fenestrated endothelium which are the constituent components of the sinusoids. Residing within the sinusoids are macrophages, other monocytic and dentritic cells. The hepatic sinusoids are adjacent to the space of disse. Stellate cells and during disease, myofibroblasts occur within these spaces, and are responsible for the *de novo* synthesis of extra cellular matrix during normal turnover and during disease. This complex cellular and acellular environment is fundamentally dynamic, and during both disease establishment and epithelial regeneration the different hepatic cell types must interact to correctly resolve damage and repair the hepatic epithelium.

Regeneration in the Adult liver

Due to the primary function of the liver it is subject to both acute and chronic injury, as such the liver must be able to tolerate these damaging processes. To cope with these events the liver has evolved a two-tier regenerative mechanism by which it can replenish both the parenchymal hepatocytes and biliary cholangiocytes in response to damage[7].

During normal homeostasis the adult liver has a basal proliferative rate of approx 0.5% of hepatocytes in cycle at any one time; furthermore the parenchyma is remarkably quiescent with a cell life of 200-300 days[20]. Compared to tissues such as the intestine or testis, the liver is remarkably quiescent; however a healthy adult liver can regenerate completely from a partial hepatectomy of up to 75% and can activate mature hepatocytes and cholangiocytes to regenerate the complete liver within seven days[21]. Furthermore post transplant the liver has a remarkable homeostatic plasticity which allows it to grow or shrink depending on the body size of the recipient, again highlighting diverse activation of proliferation or apoptosis depending on the homeostatic requirement of the liver[22, 23].

In many tissues, including brain, skin, testis, intestine and bone marrow cells are regenerated from a resident tissue stem cell[24-28]. These cells must satisfy certain characteristics: longevity, self-renewal, asymmetrical division and mulitpotentiality and translatability are all criteria which must be met in order for a cell to be classified as a true stem cell. The presence of a true stem cell in the liver is contested

in the literature. There is little compelling evidence in the field that the hepatic stem cell has been identified, and the field is muddied by multiple reports with insufficient or partial validation of "hepatic stem cell" phenotype[10, 29, 30]. What is known though is that there is a mature regenerative component in the liver, and an immature hepatic progenitor cell which is capable of regenerating both hepatocytes and cholangiocytes[31, 32] – which for the continuation of this work will be called a Hepatic Progenitor Cell (HPC). In rodents these progenitor cells are historically known as Oval Cells (OCs) and are likely to be a transit amplifying population, rather than a true stem cell population, and as such exhibit a remarkable degree of heterogeneity throughout the population[33].

Intriguingly the mature parenchymal components of the liver fulfil some "stem cell" criteria. Large scale hepatic resection results in the mitotic activation of hepatocytes and cellular compensatory hypertrophy which within two cell divisions which can regenerate the hepatic parenchyma[34]. Importantly it appears that these proliferating hepatocytes from donor animals result in clonal patches when transplanted into a recipient liver. The ability of hepatocytes to divide and repopulate the liver was initially demonstrated by the incorporation of adenoviral β-galactosidase which was administered a limited dilution, and was only then incorporated into a small subset of hepatocytes. The transgene would then be replicated and separated into daughter hepatocytes during mitosis; this identifies these cells as products of labelled parenchymal hepatocytes, and demonstrated patches of hepatocytes can be regenerated in a clonal manner from a single labelled hepatocyte source[35].

Further to this work multiple groups have used the fumarylacetoacetate hydrolase (FAH) knockout mouse, as well as the urokinase plasminogen activator (uPA) transgenic mouse to investigate the regenerative potential of hepatocytes. These studies demonstrated that hepatocytes from a donor animal are capable of cellular engraftment into the damaged liver akin to haematopoietic stem cells in the bone marrow, but moreover are capable of proliferation in a metabolically deficient liver, and as such have a selection advantage over the host hepatocytes. Furthermore this work showed that the replenished wild-type hepatocytes can be isolated from this initial recipient, and will engraft into a second recipient for up to 69 serial transplantations, as such hepatocytes have an almost limitless replication potential [36, 37]. This work has been continued extensively by Grompe and colleges who have demonstrated that donor hepatocytes regenerate the recipient parenchyma in a clonally derived manner, but do however rely on the strong selection advantage of being in a metabolically deficient host environment[38] as such hepatocytes may be described as "facultative stem cells" (Figure 1.2).

Serial transplantation of hepatocytes does not define them as a stem cell. However in Dipeptidyl Peptidase IV (DPPIV) knockout mice wild-type donor hepatocytes have been shown to be bipotent, and reconstitute both hepatocytes during PHx and retrosine treatment and the biliary epithelium during bile-duct ligation (BDL)[39]. It should be noted with this study that the purity of the initial cell population may have been contaminated with biliary epithelial cells and as such highlights the need for means of clonal analysis during hepatocellular regeneration[40], never the less the study does highlight the plasticity of hepatocytes, and their potential as a bipotent

source of cells for liver disease. One great limitation of hepatocyte therapy for liver disease is their availability which often requires a cadaver as hepatocytes are notoriously difficult to expand *in vitro*[41]. One response to this has been to utilise ES and iPS cells directing these cells into hepatocyte-like cells[42-44]; which could in theory generate large numbers of patient specific hepatocytes for transplantation – these techniques are however still in their infancy[45] and as such the current focus of much research is investigating how to manipulate the liver's normal regenerative function. It is well known that certain cytokines such as T3 and HGF are for example able to mobilise hepatocytes and in healthy liver result in hepatomegly[22]; these compounds may also be effective during hepatocellular disease to enhance parenchymal restoration[22].

As previously discussed during biliary damage cholangiocytes are also activated and can proliferate to restore the biliary tree; however the mechanisms by which this is achieved is not as extensively studied as during hepatocyte regeneration. Recent work has shown that cholangiocyte serotonin can remodel the biliary tree in adult liver, further more EBP50 and the cholangiocytic exosome have been implicated in the activation and proliferation of cholangiocytes in response to damage[46, 47]. Furthermore the double-stranded RNA-activated protein kinase (PKR) is not typically expressed in quiescent bile ducts, however it is up-regulated during biliary proliferation in Primary Biliary Cirrhosis[48]. These proliferative stimuli are balanced by a novel, and atypical mechanism of apoptosis[49, 50] – suggesting that the cholangiocytic epithelium is highly specialised, and certainly worth further study by the field.

During chronic disease a second regenerative HPC compartment is classically described as becoming activated when hepatocyte and cholangiocyte regeneration is either impaired or indeed overwhelmed; however more recent work suggests that this progenitor cell compartment may contribute to the normal homeostasis of the adult liver and as such may be much more active than initially realised[51]. Sox9 is expressed throughout the bile duct and within the HPC population. Using a conditional LacZ reporter combined with the Sox9-CreER[51] Furuyama *et al.* demonstrated that even when the liver is not damaged the hepatocytes are regenerated from a Sox9 positive population – of course it is not known whether dividing hepatocytes go through a phase of Sox9 positivity; but this work does, for the first time investigate the possibility that hepatocellular and HPC regeneration are not as discrete as previously understood.

HPC mediated liver regeneration

Adult HPCs are well described as facultative progenitor cells which (until recently) have only been described as undergoing any significant expansion in response to liver damage. HPCs are scarce in the undamaged liver, however are apparent in high numbers during chronic damage or disease in both rodents and humans. Initially described in the early 20th century HPCs have a basophilic cytoplasm and high nuclear to cytoplasmic ratio[52, 53]. HPCs are now understood to be crucial during the chronic regenerative response.

Classically HPCs have been induced using surgical resection and inhibition of hepatocyte regeneration using 2-N-acetylaminoflourene (AAF) or Retrosine; these works were initially successful in rats and later have been translated into mice. This results in the activation and proliferation of the HPC population and results in the rapid restoration of normal liver architecture within a matter of days[31, 54]. Carbon-tetrachloride (CCl_4) has also been used to induce fibrosis and a modest HPC response[30, 55, 56]. These models have been extensively used in the exploration of HPC origin and the proliferation capability of HPCs[55], however both of these well described models are well suited to rats which have a greater propensity to develop end-stage liver disease, and are obviously large enough to operate on. There are though some disadvantages to the use of CCl_4 and PHx with hepatocyte inhibition. During both CCl_4 and surgical resection large components of the biliary tree and parenchyma are removed HPCs must contribute to the regeneration of both epithelial components. As such it is difficult to use this model to elucidate the differential regulation of HPC differentiation into hepatocytes and cholangiocytes. Dietary models have become increasingly popular as means of targeted hepatocellular or biliary damage and as such may allow investigation of this fate decision.

The Choline Deficient Ethionine supplemented (CDE) diet has been used to great effect in the induction of hepatocellular damage and regeneration[57-60]. Initially described as a carcinogenic model in rats, the CDE diet has been adapted for use in mouse. Ethionine is an ethyl analogue of methionine and is responsible for the inhibition of SAM (S-adenosyl-L methionine) synthetase enzyme, as the effect of ethionine can be prevented through administration of excess methionine, loss of

choline; a methionine precursor resulted in a more aggressive response to the ethionine treatment and as such resulted in the proliferation of HPCs throughout the liver. Furthermore chronic administration of the CDE regime results in progressively increasing numbers of HPCs which contribute ultimately to the establishment of HCC in mouse[61, 62].

In opposition to the CDE diet, which can be observed as a model of hepatocellular damage and regeneration is the 3, 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) model of biliary disease[63]. The DDC diet results in the accumulation of porphyrin aggregates within the biliary tree, this results in the blockade of the ducts and activates the HPCs, requiring them to regenerative the biliary tree – although the exact mechanism by which this damage and regeneration occurs in unknown. HPCs arising in this model evolve only close to the portal tract, and do not form columns into the parenchyma. Rather they evolve as distinct ductular vessels, which have poorly defined lumen. Chronic administration of DDC results not only in the establishment of biliary disease, but also in secondary necrosis of hepatocytes, and as such may, in the chronic situation provide a more appropriate model of human biliary disorders where hepatocellular necrosis is common place[64-66].

As multiple models of HPC activation are established in the field recent work has focused on the origin of HPCs. Work by Thorgeirsson and colleagues initially identified that HPCs arise from the terminal ducts or the biliary tree[54, 67], which is now understood to be where the putative liver stem cell resides[68-70]. In these studies,

and others, label retaining cells displaying a spectrum of differentiation were observed within the parenchyma and are also found within regenerative nodules suggesting that during parenchymal regeneration at least the liver 'streams' from portal tract to central vein producing clonally derived patches of HPC-derived parenchyma [71-73]. Experiments involving tracing cells with tritiated thymidine combined with mature cholangiocyte markers demonstrated that as well as deriving hepatocytes these HPCs could differentiate into biliary epithelium, suggesting that these cells are capable of differentiation into both mature hepatic epithelial cell types[31, 32] (Figure 1.2).

Despite the clear existence of bipotent HPC population the debate over their origin had been contested for several decades. One of the main limitations that the HPC field suffers is the lack of uniformity in what different groups call an HPC. The most accepted marker for HPCs *in vivo* is A6, which identifies HPCs though an as yet unknown epitope, however pan-cytokeratin has been shown to almost exclusively co-localise with this antigen[74]. Similarly EpCam[75] and Dlk1[33, 76] have been described as markers of the HPC population during regeneration. These are wide-spectrum molecules, and as such there has been an effort to identify novel and predicted markers of the HPC population which could give some insight into lineage hierarchy and differentiation capability of HPCs (similar to the situation in the haematopoietic system) however the search for these markers has resulted in a confusion of papers and for a while the field as a whole.

The HPC and Haematopoietic Stem Cell (HSC) share common markers, such as sca-1 c-kit, CD34 and Thy-1 [7, 29, 30, 77](although Thy-1 has recently been shown to be expressed in associated myofibroblasts, and not HPCs[78]). As many HPC markers are also found on haematopoietic stem cells opinion arose that HPCs may be bone marrow (BM) derived[79, 80]. These works are now generally discredited and the observations were likely to be a result of monocyte-hepatocyte cell fusion[81, 82]. There remains some recent work looking at BM differentiation into hepatocytes which, despite being convincing, is un-physiological and solely an *in vitro* phenomenon and has yet to be extensively tested *in vivo* models of disease[83, 84]. In recent years the consensus has shifted to a belief that the HPCs are prominently of hepatic lineage, however the identity of the hepatic stem cell remains elusive, and transgenic models which can be used for lineage tracing in the liver are still in their infancy[85-87]

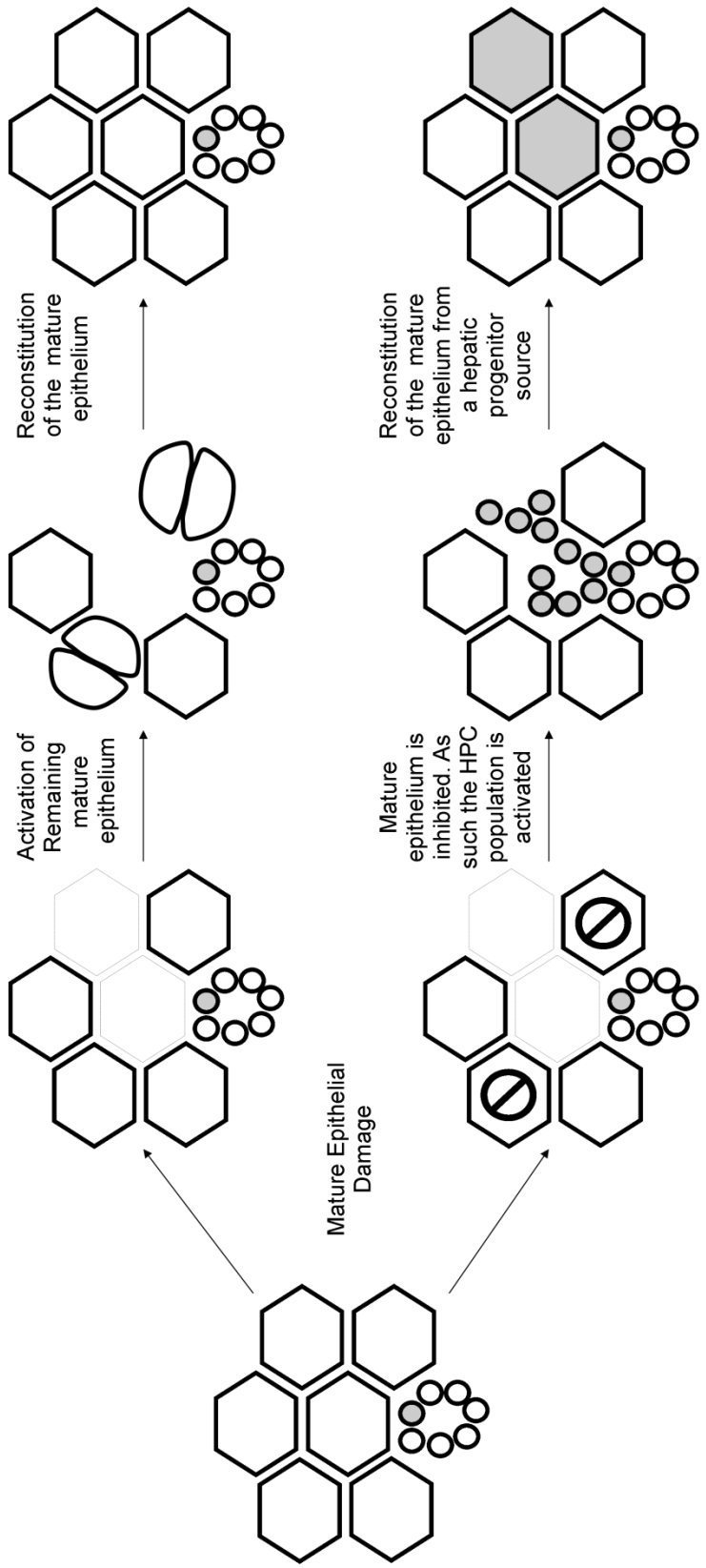

Figure 1.2 – The liver demonstrates a two-tier mechanism of epithelial regeneration

Figure 1.2 – The liver demonstrates a two-tier mechanism of epithelial regeneration

The liver has a two tier mechanism of regeneration. During acute parenchymal damage apoptotic hepatocytes and cholangiocytes are cleared though macrophage phagocytosis. Mature hepatocytes are then activated and are capable of multiple divisions to replenish the parenchyma. During chronic damage, where hepatocyte regeneration in overwhelmed or impaired a second population of hepatic progenitor cells is activated, where they proliferate and then migrate to the site of injury. Here they differentiate into mature epithelial cell types, depending on the type of epithelial damage and signals which are present.

The Hepatic Niche, and its Contribution to Liver Regeneration

During regeneration of other adult organs from progenitor cells a niche consisting of both a cellular and matrix component has is necessary for the correct regulation and maintenance of progenitor cell phenotype[88, 89]. However exploration of how a multicellular hepatic niche affects HPC proliferation and differentiation remains under-studied, and as such on-going investigation into what cellular factors contribute to maintaining HPCs is apparent in the literature[6, 70, 90].

Importantly though there is an HPC niche which is formed during hepatic damage which is well conserved between species, and as such appears to be critical in appropriate activation, proliferation and differentiation of HPCs[91] . During normal liver homeostasis this niche does not exist and as such may provide an insight into what cellular and a-cellular components are required for appropriate activation of HPCs *in vivo.* The HPC niche could also act as a putative therapeutic target through which the HPCs can be modulated *in situ*.

The Hepatic niche consists primarily of two inflammatory cell types Hepatic Stellate Cells/Myofibroblasts and resident macrophages, or Kupffer cells[90, 92]. The stellate cell is strongly implicated in the deposition of new matrix, but can also mediate matrix degradation by expression of TIMPs, which regulate MMP activity, commonly expressed by macrophages throughout the damaged liver[93]. Increasingly we understand that the dynamic turnover of matrix is necessary for correct activation of HPCs. The deposition of new matrix has recently been described as playing a role

in the initial activation of HPCs. Interestingly using the Col1a1(r/r) mouse, which is unable to degrade collagen 1 has a severely abrogated HPC reaction during regeneration of parenchymal damage[94]. Interestingly HPCs are closely associated with a laminin rich sheath which surrounds HPCs during regeneration, suggesting that the *de novo* production of certain matrix may influence the maintenance and proliferation of HPCs *in vivo*. Curiously the laminin sheath which abuts the HPCs is perforated. These pores allow the Stellate Cells to extend fenestrations through these gaps and directly contact the HPCs suggesting that they are not simply present to modify matrix, but rather they could be directly influencing the HPCs though cell-cell interactions[70].

More recent data that suggests that some of the cell types that were previously thought to be composites of the niche may actually be a sub-population of HPCs in their own right and are capable of contributing to epithelial regeneration through transdifferentiation[78, 95]. Stellate cells may be novel population of bipotent hepatic progenitors. As using a GFAP-GFP reporter there is notable expression of GFP cells in the parenchyma, which co-label with hepatocyte markers after damage has resolved and the $GFAP^+$ myofibroblasts have regressed – the authors suggested that these GFP^+ cells which co-expressed GFAP can transit into the parenchyma and become mature hepatocytes, however the authors did not investigate whether there is a developmental window in which HPCs are GFAP positive and as such the GFP positive parenchyma could arise from a classical epithelial HPC source and not through transdifferentiation of the mesenchymal component of the liver.

The hepatic macrophage (Kupffer cells) also appears critical during regeneration of damage. During fibrosis macrophages migrate to the site of injury where they phagocytose post apoptotic debris. Furthermore they generate numerous enzymes and cytokines which are responsible for the destruction of matrix but perhaps more importantly are implicated in the recruitment of other secondary cell types, which can have a pro-fibrotic or pro-resolution phenotype[92, 96]. During hepatocellular damage which results in HPC activation macrophages are known to migrate to these regenerative sites and as such may act to stimulate or influence the HPCs, certainly in models of other tissue injury macrophages are critical for the mobilisation of progenitor cells and are needed for the correct restoration of the epithelial architecture. In kidney for example regeneration of podocytes requires macrophage derived signals in order to regenerate appropriately – the loss of macrophages confers a reduction in renal repair[97, 98]. Tissue macrophages must by definition be influenced by their local environment, where epithelial cells will be dying and fibrotic matrix will be both synthesised and destroyed. The specific mechanisms by which macrophages can sense their damaged environment and appropriately stimulate the macrophage to produce Wnt ligand is unknown, however there is likely to be a common pathway between the point of phagocytosis and activation of gene transcription. The damaged epithelium may liberate TGF-β, which induces phagocytosis of debris by macrophages[99]. The act of phagocytosis could stimulate gene change of these macrophages and allow them to adopt a pro-repair phenotype[100, 101], however this has not been extensively explored, and has not been addressed during regeneration of the liver from progenitor cells.

It is unsurprising then that during hepatocellular regeneration from HPCs, where a dynamic niche is involved that there are complex arrays of signals which allow proliferation of HPCs, and some which may control and influence the correct differentiation of HPCs. Regarding the latter very few of these pathways have been described in the adult, however there is an increasing body of evidence which examines those factors involved in the activation and proliferation of HPCs.

The HPC Response is mediated by a many factors.

During regeneration of the hepatocytes and cholangiocytes the HPC response should not be observed as a separate entity to inflammation, in fact many of the signalling molecules which are released during fibrosis have been shown to play an important role in the activation, proliferation and migration of these cells during liver regeneration. Anecdotal evidence suggests that the degree of regeneration from HPCs is proportional to the amount of injury, and as such the inflammatory milieu would be the most appropriate conduit between damage detection and hepatic restoration[7].

Transforming growth factor beta (TGF-β1) is a potent cytokine produced by both hepatic stellate cells and resident macrophages; furthermore it is sequestered in the ECM. During matrix remodelling TGF-β is liberated and as such can act upon HPCs and other cell types[13]. The TGF-β1 ligand is also processed into its activated state though interaction with inflammatory associated enzymes such as the Matrix Metalloproteinases (MMPs), suggesting that there is a system feedback between

inflammation and epithelial regeneration[93]. During regeneration there is a differential expression of TGF-β type I and type II receptors on Hepatocytes and HPCs which correlates with the inhibitory affects of TGF-β on the hepatocyte population[102]. Activation of the TGF-β type I receptor results in the sequential phosphorylation of Smad2 and Smad3 which the authors suggest are the downstream mediators of proliferative inhibition in hepatocytes. As such HPCs are able to proliferate in the presence of this inhibitory cue due to heavy glycosylation of the TGFβII receptor on HPCs, and as such are not restricted in the same manner as hepatocytes, however the authors fail to investigate by which mechanism the TGFβII receptor is glycosylated.

Another potent cytokine for the activation of HPCs is TNF-like weak inducer of apoptosis (TWEAK)[103], which through its receptor Fn14 induces proliferation of HPCs. In the absence of TWEAK either through ligand or Fn14 knockouts the HPC response is delayed or an attenuated HPC response is observed[103]. Further work demonstrated that activation of the Fn14 receptor results in the upregulation of NFκB and stimulated HPC proliferation in a dose dependent manner – interestingly this affect was inhibited though RNAi against the p50 NFκB subunit[104].

Other members of the TNF superfamily have been implicated in the activation of HPCs; however none to date are as potent as TWEAK, and cannot induce an HPC response independent of damage. TNFα for example is known to increase in the majority of chronic liver diseases and acts through the TNFR1 and TNFR2 receptors resulting in an increased rate of proliferation of HPCs *in vitro*. Knockout of the

TNFR1 results in a partial abrogation of HPC activation – but does not prevent the activation of HPCs completely[105, 106]. As such there is suggestion that other TNF family members such as Lymphotoxin-α (LT-α), Lymphotoxin-β (LT-β) and LIGHT (homologous to LT, inducible expression, competes with herpes simplex virus (HSV) glycoprotein D for HSV entry mediator (HVEM), a receptor expressed on T lymphocytes) could act via a further receptor for the maintained, but attenuated HPC proliferation[107].

Many inflammatory cytokines are known to activate the gp130 pathway though dimerisation of the gp130 receptor and activation of the JAK/STAT and MAPK pathways. Classical inflammatory signals act upon HPCs through this pathway including Oncostatin-M (OSM), Interleukin-6 (IL6) and Leukaemia Inhibitory Factor (LIF); of these compounds IL6 is the most extensively studied and as known to be important in the activation, proliferation and migration of HPCs both *in vitro* and *in vivo* where IL6 knock out results in a decreased HPC response via the heterodimerisation of IL6Rα and gp130[108]. Conversely if the gp130 receptor is constitutively activated, such as in the $gp130^{Y757F}$ mouse HPCs arise in the absence of damage[108, 109].

Finally the Interferon-γ (INFγ), cytokine has been shown to increase the mitotc index of HPCs when mice are fed a CDE diet *in vivo*. The same report does not suggest that this is a direct influence of INFγ on HPCs[105], rather this is an upstream affect which exacerbates the number of $\alpha\text{-SMA}^{+}$ myofibroblasts, but also alters the

recruitment of inflammatory cells and alters their cytokine expression in CDE livers altering the proliferation of residual hepatocytes[96]– all factors which contribute to HPC activation during disease.

The ever increasing body of data which investigates the activation of proliferation of HPCs sits in stark contrast to those studies which have attempted to delineate the mechanisms by which HPC differentiation is mediated. Indeed there is almost no work in the literature looking at the pathways and components which allow lineage separation of cholangiocytes and hepatocytes from a bipotent HPC in the adult.

Only very recently has one study arisen which looks at the differential presence of Notch and Wnt pathways during the regeneration from HPCs during biliary and hepatocellular regeneration during adult human disease[110]. This work was predominantly descriptive and as such offered no insight into the mechanism behind differentiation of HPCs into hepatocytes and cholangiocytes. Spee and colleagues found that during regeneration in human biliary diseases primary sclerosing cholangitis and primary biliary cirrhosis the Notch pathway is highly up-regulated in HPCs when compared to HPCs isolated from patients presenting with end-stage HCV infection. Furthermore this work demonstrated that the Wnt signalling pathway is upregulated in both chronic biliary and hepatocellular disease states – however these are end stage samples and say nothing of how the regenerative response is appropriately established. Furthermore during these phases of disease there is

invariably a degree of biliary and hepatocellular regeneration which may confuse any mechanistic investigation of HPC specification.

During hepatic ontogeny however the roles of the Notch and Wnt pathway have been well described[15, 111]. As regeneration appears, in some sense to recapitulate development, the mechanisms by which hepatoblasts and HPCs are specified may be similar and as such the role of Notch and Wnt signalling during hepatic ontogeny shall be discussed here, along with any work which alludes to a role for Notch and Wnt signalling during regeneration.

Notch Signalling and its importance in development, disease and maintenance.

Metazoan development is regulated by multiple pathways which are known to operate as networks in order to confer specific information and as such phenotype on a cell[112-114]. The Notch signalling pathway is classically described as a means by which a uniform group of cells can establish differences by signalling with one another and has been implicated in differentiation and proliferation in both the developing embryo and during adult cellular homeostasis and disease [115]

The Notch receptors and their ligands

The vertebrate Notch signalling pathway consists of four Notch receptors (Notch 1-4). These receptors are large, multi-domain single pass receptors which sit in the cell

membrane and receive signals from the DSL family of ligands[116]. All Notch receptors contain multiple EGFR-like repeats which are found on the extracellular domain of the cell surface receptor. These sequences are followed by cystine rich LNR domains and a transmembrane domain. On the inner surface of the cell membrane the Notch receptor has an interaction domain which is required for Notch's transcriptional function followed by ankyrin repeats and a PEST domain[117](Figure 1.3).

Notch receptor is cleaved to form its active isoform in the endoplasmic reticulum by a furin-like convertase (S1 cleavage), and is then trafficked to the cell surface as an inactive receptor[118]. Upon ligand binding the receptor is cleaved by an ADAM-family metalloproteinase which is also membrane bound (S2 cleavage) [119, 120]. This cleavage facilitates subsequent Notch activation. The product of S2 cleavage is rapidly processed by the γ-secretase containing complex presinillin, this cleavage (S3) liberates the cytoplasmic domain of Notch receptor from its membrane anchor and allows it to translocate into the nucleus[121, 122].

The activation of Notch receptor requires an appropriate ligand. These can be classified into two families depending on their sequence homology to the *Drosophila* homologues Delta and Serrate, the latter is known as Jagged in vertebrates[123]. There are 3 Delta (Dll) homologues in vertebrates Dll1, 3 and 4. Also there are two homologues of *Drosophila* Serrate, known as Jagged 1 (Jag1) and Jagged 2 (Jag2). As this work will focus on vertebrate signalling Jagged will be used throughout. Both

the Delta and Jagged ligands have large sequences of EGFR-like repeats on the extracellular surface; however contain a degenerate N-terminal EGFR, known as the DSL domain; and is required for ligand function in invertebrates[117]. Furthermore the Jagged family of ligands contains a cystine rich region which may act to modulate ligand receptor interactions, and explain why binding of these divergent ligands to a common receptor could induce multiple phenotypes (Figure 1.3).

Figure 1.3 – The canonical Notch signalling pathway

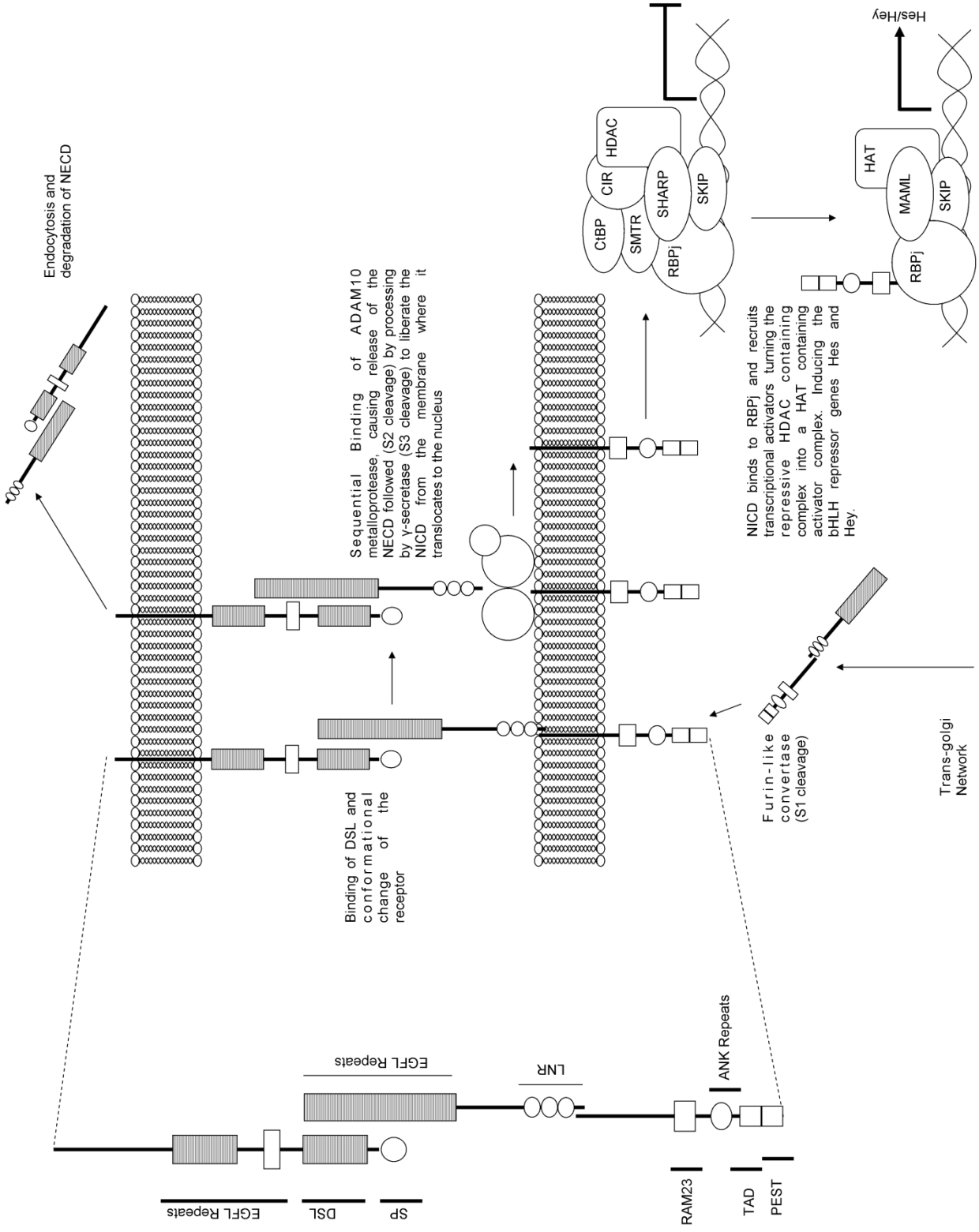

Figure 1.3 – The canonical Notch signalling pathway

The Notch receptor is a multi-domain single-span receptor which is found associated with the cell membrane. Upon binding with a Jagged or Delta ligand which is found on an adjacent cell the Notch receptor undergoes a conformational change and sequential cleavage events. These cleavage events result in the translocation of the Notch intra-cellular domain to the nucleus, where it complexes with a transcriptional repressor complex. The association of the Notch intracellular domain with this complex results in its dissociation and the recruitment of a transcriptional activation complex. This complex results in the transcription of Hes/Hey gene family which act to repress the transcription of early lineage genes.

Notch Signalling is driven by mechanical changes at the cell membrane.

As previously discussed the Notch receptor is sequentially cleaved by ADAM-10 metalloprotease and γ-secretase to liberate the N^{icd} domain[124]. Notch receptor however is not spontaneously cleaved and requires the interaction with Notch ligand, subsequent conformational change and processing of the Notch receptor. The mechanism of this processing has been intensely studied as there are multiple mutants which gives a striking phenotype similar to Notch receptor mutants implicating these molecules in the correct processing of Notch receptor[125-128].

The *Drosophila* Notch ligand Delta had a complex internal localisation pattern. Being detected as expected on the cell surface, but was also present in intracellular vesicles during certain stages of development. Further investigation elucidated that the Delta ligand undergoes endocytosis and it is this mechanism which is required to facilitate subsequent activation of the Notch receptor[125, 126]. Using the temperature sensitive Delta allele Dl^{RF} Parks and colleagues showed that this protein resides on the cell surface in a constitutive manner, and yet acts as a loss of function mutant. Similarly the same group also identified delta mutants which are described as 'trafficking-defective' – these too show a phenotype similar to Notch mutants leading to the hypothesis that removal of Delta from the cell surface membrane is critical for its role as a signalling ligand, and that loss of endocytic function results in a loss of Notch pathway activation[125].

Using the development of the *Drosophila* retina and primary wing vein it was shown that Delta binds to the Notch receptor. This ligand is trans-endocytosed along with the products of S2 cleavage. The remaining Notch receptor fraction is then available for subsequent activation via S3 cleavage. Flies that contain mutations in the third epidermal-like growth factor repeat (ELR) of Delta exhibit the ability to aggregate with S2 Notch expressing cells – that is to say there is appropriate binding of Notch Receptor and Delta ligand, however the integrity of the third ELR is compromised and as such there is a significant reduction in the endocytosis of Notch extra cellular domain (N^{ecd})[125] as such a Notch mutant phenotype is observed.

More recent studies implicate E3 ubiquitin ligases as playing a crucial role in Notch signalling. Neuralized (Neur) and Mindbomb (Mib) are two such ligases which are necessary for correct signalling by controlling the endocytotic activation of ligands. Mutants in these two proteins results in an increased level of ligand on the cell surface. In model systems such as *Drosophila* and Zebrafish, Neuralized and Mindbomb play a critical role[129, 130], with knockouts of these proteins causing a neuralized phenotype similar to that observed with Notch inhibition[131]. Furthermore specific regions of the Neuralized protein are required for correct endocytosis, and as such appropriate Notch signalling[132, 133]. Naïve and inactive ligand is rapidly endocytosed and trafficked to the proteosome for degradation. Ubiquitination of the Notch ligands activates them, and allows the recruitment of Epsin, which facilitates the endocytosis of ubiquitinated proteins into clatherin coated vesicles[134].

The endocytosis of receptor bound ligand facilitates a conformational change in the Notch receptor making it receptive to S2 processing[126]. Other mechanisms of ligand regulation play an important role in regulation of Notch signalling. Kuzbanian-like which cleaves the Delta ligand in *Drosophila* acts to negatively regulate activation of Notch. Knockouts of this protein exhibit ectopic Notch activation[120]

In *Drosophila*, Kuzbanian (Kuz), a homologue of the mammalian ADAM10 metalloprotease is required for correct processing of not only Notch receptor but also Notch ligand Delta[120]. This protein was originally identified in 1996 using *Drosophila* mosaics, as Kuz knockouts are embryonically lethal[135]. These flies exhibited clusters of sensory bristles – a typical phenotype of Notch mutants in *Drosophila*. This phenotype is characteristic of a lack of lateral inhibition. These sensory bristles are consistently Kuz negative suggesting that there is a cell autonomous need for Kuz so lateral ectodermal cells to be inhibited by the emerging neural cell[136]. Delta cleavage has more recently been identified as an endocytosis independent mechanism for down regulation of Notch signalling by regulation of active ligand even after it has been presented at the plasma membrane[137].

There is increasing evidence in the literature that mammalian Notch ligands can be sequentially processed in a similar way to both *Drosophila* delta and Notch[138-140]. Through a series of co-transfection studies Both mammalian Jagged and Delta were shown to undergo cleavage by the α-secretase ADAM-17 [139, 141]and these cleaved extra-cellular domains are then amenable for cleavage by the γ-secretase complex.

The cytoplasmic products of Jagged cleavage have NLS motifs which facilitate trafficking to the nucleus. Using a Jagged intracellular domain (J^{icd}) construct this cleavage product was shown to be enriched in the nuclei of Jagged expressing cells. The production of this novel signalling fragment acts competitively with N^{icd} upon ligand and receptor interaction, potentially acting as a buffer mechanism against aberrant Notch activation in ligand expressing cells[139, 140].

Notch signalling can also be regulated by a multitude of other mechanisms. Dll as mentioned is an activator of the Notch receptor. However there is increasing evidence that these typical ligands can also inhibit Notch receptor activation[142]. Recently atypical Notch ligands, which are structurally related to both Notch and Delta but are not involved in the facilitating the classical Notch cleavage events have been described[143]. These include Dlk1 and its homologue Dlk2[144]. Dlk1 can bind via the EGF repeats on both itself and Notch receptor can negatively regulate the normal action of Notch[143]. The Dlk1 family of atypical Notch ligands has been extensively characterised in adipogenesis. Known as pre-adipocyte factor 1 (Pref-1) in these tissues this protein is capable of modulating the differentiation potential of 3T3-L1 preadipocytes[145].

Appropriate Notch receptor activation depends on glycosylation

Ligand mediated activation of the Notch signalling pathway is not the only mechanism by which Notch signalling can be regulated. Notch receptor function is dependant, like many receptors on having the correct glycosylation pattern[146]. *O*-

fucosyl-transferase is necessary for the addition of the first fucose molecule the Notch receptor[128], these can then be extended by other members of the Fringe family of glycosyl-transferases to give a unique pattern which may be associated with Notch activation in specific tissue types, but also may affect receptor-ligand affinity and subsequent receptor activation[147]. The glycosylation pattern of the Notch receptor is critical in the adult liver, where absence of the molecules responsible for in appropriate Notch activity[148].

Notch signalling is mediated by CSL

Liberation of the N^{icd} domain as a result of the S3 cleavage event is the start of Notch mediated repression of differentiation. N^{icd} interacts with a CSL, (Su (H) in *Drosophila*, lag-1 in *C. elegans*, and RBP-Jk in Mammals). Notch target genes are normally repressed by CSL and co-repressors[149, 150]. Upon interaction with N^{icd} this transcriptional repressor is converted to a transcriptional activator. This switch from repression to activation facilitates the expression of Enhancer or split genes (E (spl)) or the Hairy/Enhancer of split (Hes) genes in mammals. These genes code for bHLH transcriptional repressors which act upon class II bHLH transcription factors, for example the Achaete-Scute (A-Sc) genes (known as HLH-3 in *C. elegans* and the MASH genes is mammals). Typically high levels of A-Sc genes are markers of directed differentiation. In mice high levels of MASH1 are associated with neuronal differentiation in the developing nervous system and adult Hippocampus[151].

The mechanism by which notch signalling can convert a complex of transcriptional repression into one of transcriptional activation if well described. In *Drosophila* the protein Hairless (H) [155]enables global repression of target genes via its co-factors. When Su (H) forms a complex with DNA it can recruit Hairless. Hairless can in turn recruit co-repressors of transcription such as CtBP[156]. This H-CtBP complex acts to recruit Histone Deacetylases (HDACs) which function to make chromatin transcriptionally inactive. Hairless also has the ability to recruit Groucho proteins, which act as long range transcriptional repressors[157]. These proteins are able to recruit HDACs and co-factors, which as before suppress transcription on a chromatin level[158]. This high level of transcriptional suppression in response to Notch signalling also exists in vertebrate systems of Notch signalling (Figure 1.3).

In mammals there is no known direct homologue of Hairless but global repression exists via co-repressors including SMRT and SHARP[159-161] which recruit CtBP or other global repressors including SKIP[127] and CBF-1 interacting co-repressor (CIR)[162]. Functionally Hes-1 can be observed as a homologue of Hairy (a binding partner to Su(H)) and as such Hes-1 can form a homodimer, which binds to the C or N box consensus DNA sites and can recruit the mammalian Groucho homologue TLE[163, 164]. As in *Drosophila* TLE recruits HDACs thus altering local chromatin structure preventing transcription from certain loci. The expression of Hes-1 can efficiently down-regulate its own expression through binding of the Orange domain to the Hes-1 promoter sequence[163]– thus preventing its own transcription and the repressive properties normally associated with the Hes genes

After the recruitment of N^{icd} and associated co-factors the CSL-N^{icd} complex is converted into a transcriptional activator[167]. This conversion facilitates the expression of the HES and HERP genes in mammals and Enhancer Split E (spl) in *Drosophila* these proteins block the downstream events of Notch signalling in a similar way to Hairless acting on the HES promoter upstream. HES and HERP recognise motifs in classes of bHLH transcription factors, many of which are implicated in the early specification events during differentiation as previously discussed.

Notch Signalling typically inhibits differentiation

Notch signalling has typically been shown to act as a repressor of differentiation in multiple cells systems. Classically this fate control, known as lateral inhibition has been studied in the development of *Drosophila*, *C. elegans and Xenopus* due to the ease by which they are manipulated.

At the level of patterning Notch signalling acts to amplify bias between adjacent cells where the levels of activator which drives differentiation is similar and only slight stochastic variations occur. The stochastic variation would cause an increase in Notch ligand in the signalling cell. This activates Notch receptor in the surrounding cells and resultantly suppresses the differentiation of these cells, or facilitates their transit along a default differentiation[168] in turn receptor-ligand balance between these two cell populations is amplified by a reduction in Notch receptor in the signalling

cell, and a decrease of ligand in the surrounding cells further driving these cells into divergent fates.

The simplest role of this patterning mechanism is to drive asymmetry between two cells which have the same or a similar potential known as lateral inhibition. When cells express similar levels of activator, stochastic variations will occur. When the levels of this activator are at critical level cells will adopt a certain fate. A random increase of activator such as Notch ligand in one cell leads to a relative decrease in pathway activation in this cell. The result of this in a two cell system is the repression of activator i.e. ligand in the adjacent, non-signalling cell whilst also facilitating the down regulation of pathway and up regulation of activator in the signalling cells, driving the phenotypic separation of these two, otherwise equipotent cells. The initial bias in these cells may not be entirely stochastic, but a result of a separate input, such as a morphogen gradient, however Notch signalling increases this initial bias to achieve a cellular difference further reviewed by Chitnis et al [168].

Lateral inhibition occurs in complex cellular environments - patterning of sensory bristles (macrochaetes) in many invertebrates is observed as the classical example of lateral inhibition within a cluster of cells. Macrochaetes arise from a proneural cluster of approximately 20 cells[169]. These cells all express proneural genes such as Achaete-Scute (A-Sc) complex genes, which promote the expression of neurogenic genes, as well as genes which inhibit cell-cell interaction. Inhibitory interactions

mediated by these neurogenic genes restricts expression of proneural genes to a single cell within the cluster[170-172].

Non-classical contributions to Notch signalling

Non-canonical notch signalling, that is to say Notch signalling which is not mediated by either CSL or DSL ligand can also dramatically influence cell behaviour, however has not been as extensively studied as the classical Notch signalling pathway.

Deltex is a protein initially identified in *Drosophila*, but is also implicated in Notch signalling in both Mouse and Human[173-175]. It was the first component of the pathway which was shown to reside in the cytoplasm, and the first to be associated with the Notch receptor intracellular region. The Ankyrin repeats found on the intracellular region of Notch are necessary and sufficient for Deltex binding[176]. More recent studies have implicated Deltex in being a positive regulator of Notch signalling, and an adaptor of the Notch Signalling pathway linking it with the Wnt signalling cascade[177].

Deltex may also play a crucial role in non-CSL signalling through associating Notch receptor with the Grb2 protein, which is classically implicated in receptor tyrosine kinase signalling. Similarly there is also evidence that *abl* cytoplasmic tyrosine kinase can interact with un-cleaved Notch receptor, to influence axonal growth and guidance in *Drosophila*.[178]

The importance of Deltex as a positive regulator of Notch signalling is further exemplified by its antagonistic counterpart the E3 Ubiquitin ligase, suppressor of Deltex (Su (dx)) in *Drosophila*, and Itch in mammals. Su(dx) acts to ubiquitinate N^{icd} at a point where it is membrane tethered targeting it for degradation[179]. Similar protein modifications by the E3 ligases on Notch receptor targets offer a plethora of regulatory events. N^{icd} which is localized to the nucleus is hyperphosphorylated, with at least one phosphorylation occurring upon entry into the nucleus. This event is crucial for the recruitment of the Sel-10 containing SCF which targets N^{icd} to degradation, specifically acting within the nucleus[180].

Further modifiers of the Notch signalling pathway have been described (summarised in Figure 1.4). Numb for example has been identified as a ubiquitin ligase which acts in *Drosophila*, mouse and human to prevent the ectopic activation of Notch receptor[181, 182]. The majority of functional work looking at Numb has been conducted in *Drosophila* where Numb acts to antagonize the Notch receptor in a dose dependent manner and results in a repression of a Hes1 reporter[183]. In mammalian cells Numb has been to shown to interact with the Itch (the mammalian suppressor of Deltex) and enhance ubiquitination of the Notch1 receptor[184]. Interestingly ablation of Numb does not prevent the endocytosis of Notch receptor, rather the receptor is recycled, however during Numb over expression the Notch1 receptor is endocytosed in clatherin containing vesicles and the receptor is targeted for destruction; and as such Notch1 is regulated by Numb mediated post-endosomal sorting[185].

The regulatory mechanism of Numb, as with many of the Notch signalling modifiers is complex, and involves the conversion of multiple pathways on Numb regulatory elements. Numb is regulated at the transcript level by Musashi-1 and this has been shown in liver cells to be modulated during differentiation[186, 187]; however the affect of this modulation on Numb was not demonstrated by the authors of this work, rather they focused on the role that a thermo reversible gelation polymer plays on HPC activation and Musashi-1 expression, there was no attempt to investigate the functional role for Musashi-1 and Numb interaction in these HPCs. Recently work looking into what are the key putative regulators of Numb demonstrated that there are conserved LEF/TCF binding domains upstream of the Numb gene, which are highly conserved in the Numb promoter region between species demonstrating that Numb is Wnt responsive[188]. Furthermore two recent papers have identified that in haematopoiesis and during zebrafish organiser restriction Wnt plays an important modulator role for Numb and associated protein Lnx-like[189, 190].

Figure 1.4 – The Notch pathway is heavily regulated through multiple interacting pathways

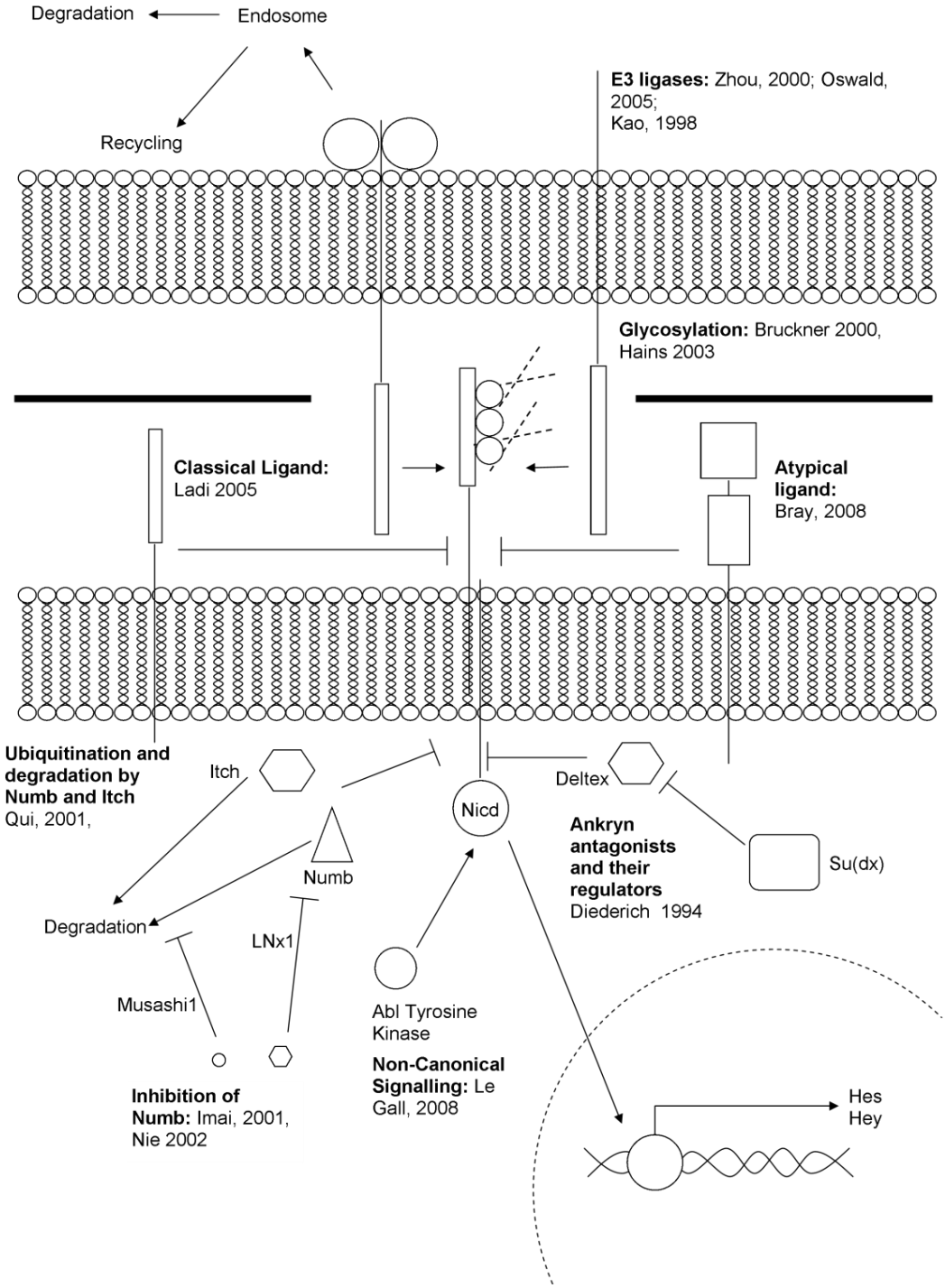

Figure 1.4 – The Notch pathway is heavily regulated through multiple interacting pathways.

The Notch signalling pathway is heavily regulated though multiple mechanisms. The receptor is targeted by multiple extracellular and intracellular proteins which affect the affinity of the receptor and ligands. Furthermore the activated receptor can be targeted for degradation through multiple cytoplasmic pathways which either positively or negatively regulate Notch receptor activity. The Notch ligands Jagged and Delta are also regulated at the protein level, and can also be targeted for degradation or protein recycling.

Notch Signalling, and its effect on cellular systems

The Notch signalling pathway is implicated in multiple tissue types, and at multiple developmental and postnatal stages. The functional role of Notch signalling has been widely explored both *in vitro*, and more recently, *in vivo* using transgenic technologies to monitor activation of the pathway, but also to "knock-out" or over express Notch receptor and pathway effectors as a means of demonstrating how Notch signalling regulates cellular and tissue homeostasis in multiple organ contexts.

Notch Signalling in Embryonic Stem (ES) Cells

Notch signalling has been implicated in the expansion and differentiation of multiple committed precursors and mature stem cells[191-193]. ES cells are derived from the inner-cell mass of the developing blastocyst. These cells are destined to become the embryo proper later in development and as such are capable of differentiating into all cell types if given the correct developmental cues. Human embryonic stem cells require close contact in order to grow efficiently[194]. In hES cells Notch2 is processed and sequentially cleaved to release the intracellular fragment. Using an siRNA against Notch2 cell growth was significantly inhibited and apoptosis in hES cultures was increased. This was confirmed using γ-secretase inhibitors where treated colonies were reduced in size as a result of cell cycle arrest and apoptosis, rather than due to spontaneous differentiation of ES cultures[195].

Notch signalling has been implicated in the proliferation as well as the differentiation of ES cells in several independent investigations[196, 197]. Down regulation of Notch

receptor and ligand in ES cells occurs when they are directed to differentiate into cardiomyocytes. Upon inhibition of the Notch signalling pathway contractile beating cells within the culture increased in frequency, this phenotype could be inhibited by the ectopic expression of N^{icd} in ES cells. These results indicate that entry into a mesodermal lineage can be inhibited by Notch signalling, a hypothesis which was corroborated by N^{icd} expressing cells showing a decrease in the mesoderm markers Brachyury and FGF-8[196]. Notch signalling and cardiac differentiation are mutually exclusive, in Embryoid Body (EB) experiments cells which have constitutive Notch signalling cannot form mesoderm, but express nestin, suggesting they are committed to a neurectodermal fate.

Notch Signalling in the Nervous System

Early in their characterisation Notch genes were identified as 'neural' genes. That is to say if any component were mutated the resulting neurogenic phenotype was observed. In *Drosophila* this phenotype is typified by an increase in Neurons at the expense of the surrounding epidermis[198, 199]. As previously discussed Notch signalling is required for lateral inhibition, and in *Drosophila* neuroblasts, the neural precursor signals to, and subsequently inhibits the proneural cells around it, facilitating its own progression along a neuronal route[200, 201]. This mechanism is conserved in vertebrates where a prospective neuron signals to other, surrounding neural tube cells. Through RPB-Jκ, a central component of the Notch signalling pathway, this prospective neuron activates Hes-5 and subsequently represses the transcription of early neural genes such as *Mash1* and *Neurogenin1/2*. This

suppression can be shown using RPB-Jκ$^{-/-}$ or Notch1$^{-/-}$ both of which demonstrate an increase these proneural genes, in the developing Neural tube[200].

Notch signalling is crucial for the correct specification of neural progenitors; however the expansion of these neural progenitor cells is required to have correct numbers of cells in the developing brain before patterning can proceed. Notch signalling is important for the transition between proliferation of NPCs and their commitment to a developmental fate[202]. The presumptive chick spinal cord develops in a rostro-caudal manner, relying on the generation of NPCs from the caudal plate. In this region cells express both high levels of Delta ligand and Notch receptor, which maintains a highly proliferative pool undifferentiated cells. Subsequent events lead to the delamination of these neural progenitors, and an increase in Delta ligand, which commits the signalling cell to a neural fate and the surrounding cells to an epidermal fate similar to that observed in other models[203]. A switch between this mutual activation (in the caudal plate) and lateral inhibition (in rostral neural tube) is induced through Notch mediated lateral inhibition. Cell autonomous Delta activity is implicated in the up-regulation of Tis21, an anti-proliferative gene which facilitates G1 arrest and exit from the cell cycle priming these cells for differentiation[202].

Despite the clear role of Notch signalling in the separation of a precursor cell from a group of equipotent progenitors the archetypal model of neurogenesis has also demonstrated that Notch signalling can also promote certain developmental outcomes in these multipotent systems. As Notch activation suppresses neural

differentiation it also promotes an astroglial fate in those neural precursors which are not destined to become neurons[204, 205]. In the classical signalling network Notch signalling maintains receptive cells in a naïve state, allowing them to be receptive to secondary signals and as such adopt a secondary fate.

Notch signalling induces neural stem cells to become neurons early and then switches to promote gliogenesis and a glial fate later through development of the central nervous system (CNS). Ge et al.[204] demonstrated that Notch signalling actively signals to NPCs in order to promote their expansion and differentiation into $GFAP^+$ glia. This is achieved through RBP-Jκ, and when these primitive cells are treated with a synthetic clustered Dll ligand gliogenesis is promoted. This phenotype is countered by application of γ-secretase – again showing that this is a direct action of Notch signalling. These data suggests a temporal separation of neurogenesis and gliogenesis during development. The authors suggest that this could be intrinsically linked to the JAK-STAT signalling pathway activated by LIF, which is inactive in NPCs taken early in neurogenesis, however is active, and responsive to LIF later on in NPCs[204].

Neurospheres are aggregations of neural cells which are produced in suspension from a single neural stem cell. Using these spheres as models of neural differentiation it has been shown that in this *in vitro* situation Notch signalling controls the differentiation of neurons and glia in temporally progressive signalling events. Using $Dll1^{lacz/lacz}$ knockout mice, which exhibit a greater propensity to generate neurons it

was possible to show that exposure to Jag^{ED} (a cleaved extra-cellular fragment of the Jag ligand) could significantly reduce neuronal differentiation after a short ligand exposure time, however glia require a longer exposure to the Jag^{ED} before they increase their representation within the neurospheres. These results combined implicate persistent Notch signalling in expansion and maturation of glial cells, but early and short exposure of neural progenitors to ligand is sufficient to restrict their commitment to the neural lineage[205].

Notch signalling in bone homeostasis

Notch signalling has more recently been implicated in bone remodelling and regeneration. The process of bone homeostasis is dynamic, and relies on the correct equilibrium between osteogenesis and osteodegradation. The production of a mouse model, where N^{icd} was under the control of the *Col1a1* promoter exhibited mice with an increase bone mass; consisting of immature woven bone in the marrow spaces[206]. Further analysis implicated an increase in early oesteoblasts, and a relative decrease in oesteoclasts. Excessive primitive bone was produced as a result of the oesteoblasts rather than a decrease in oesteoclastic activity as assessed by RANKL, M-CSF and osteoptegerin[206]

Transit into a late oesteoblast phenotype is restricted by excess Notch signalling as it directly binds to and suppresses the Runx2 gene. Similarly in C2C12 cells differentiated into oesteoblasts N^{icd} shows an enhancement in proliferation through *Sp7* whilst inhibiting their differentiation through Runx2. A separate study similarly

showed that removal of Notch signalling through deletion of Notch1 and Notch2, or through γ-secretase reduces the number of bone marrow stem cells by committing them to an oesteoblast lineage[207].

Conversely using the $Psen1^{f/f}$ $Psen2^{-/-}$ conditional double presinillin mutant mouse it was shown that a reduction in Notch signalling is implicated in the formation of Osteoporotic bones, which an increase in oesteoclastogenesis and increase in re-absorption of bone over bone formation[206].

Notch signalling in maintaining the intestinal epithelium

Endoderm patterning is important for the formation of multiple organ systems, such as the lungs, liver digestive tract and pancreas all of which arise from a primordial endodermal tube present soon after gastrulation[208]. The developing gut can be separated into three regions, the foregut, midgut and hindgut. The architecture of the gut is defined by signals from the overlying mesenchymal cells. The endodermal epithelium evaginates giving rise to villi and intervillus regions. The latter of which will become mucosal crypts in the adult[209].

In the adult intestinal crypts are the locations of intestinal stem cells. These cells can give rise to all cell types in the adult intestine, whilst at the same time are capable of self renewal. This is important in the intestine as epithelial turnover is constant and persists throughout adulthood. Using long-term label retention it is hypothesised

these ISCs are located approximately 4 cells up from the base of the intestinal crypts. Recently Lgr5/GPR49 has been described as specifically labelling intestinal stem cells and through use of the Lgr5-GFP-IRES-Cre ERT reporter mouse it has been demonstrated that these Lgr5 positive intestinal stem cells can mature into all intestinal epithelial cell lineages as well as undergoing self renewal[210]. The two stem cell populations Lgr5 and +4 are not mutually exclusive and may represent two separate to cooperative stem cell groups in the intestinal crypts[209].

Notch1 is the predominant Notch receptor found at the intestinal crypts. Along with it Jagged-1, Dll1 and Dll3 are also present. The importance of Notch signalling in proliferation and differentiation has been demonstrated in the gut. Using RBP-Jκ conditional knockout mice, or by treating mice with γ-secretase inhibitor secretory cells numbers are significantly expanded at the expense of enterocytes[211, 212]. These observations are corroborated in developing gut using the Hes-1$^{-/-}$ mouse, which shows aberrant regulation of endodermal endocrine cells in liver, gut and pancreas[213]. In a separate study the converse was also shown to be true, that in mutants where constitutive activation of Notch occurs there is depletion in secretory lineage cells and an increase in epithelial proliferation[214].

In the intestine Notch signalling acts in a typical manner, that is to say it functions to laterally inhibit cells from adopting a secretory cell fate. Atoh1 (also known as Math1 in mouse and Hath1 in humans) marks progenitors which are destined to become secretory and is implicated in the activation of several secretory cell specific

genes such as Neurogenin3 and Gfi-1. Atoh1 expression is negatively regulated by Hes-1 and RBP-Jκ knockout mice show an increase in Atoh1, and subsequent up-regulation of secretory cells[89, 153, 154].

Notch Signalling: A role for liver development

Notch signalling has been heavily implicated in both the development of the foetal liver and where inappropriate Notch signalling results in a retardation of hepatic phenotype. During development the ductal plate is located between the portal mesenchyme and the hepatic parenchyma as a continuous layer which undergoes focal dilations and gives rise to epithelial bile ducts some of which are incorporated into the portal mesenchyme[215]. Recent work has demonstrated that during hepatic ontogeny Jagged-1 supplied by the portal mesenchyme is necessary for correct patterning of the biliary tree. Ablation of the ligand Jagged-1 specifically in the portal mesenchyme results in a phenotype similar to that when Notch receptor is ablated transgenically (discussed extensively in the next section)[216]. Intriguingly recent data indicates that Notch-1 acts in a dose dependent manner to specify the branching and 3D structure of the portal tract[217]. Loss of the Notch1 receptor results in a retardation of the developing biliary tree however ectopic activation of Notch1 results in hyper-arborisation of the biliary structure, although it does not appear to affect the proliferation rate of the developing cholangiocytes, and as such acts permissively to pattern the biliary tree[217, 218].

Furthermore reiterative work in Zebrafish demonstrates that the Notch signalling pathway has a biphasic role during hepatic development. Initially to specify cholangiocytes from the bipotent hepatoblasts; but then latterly to select for the correct patterning of the biliary tree[218, 219]; this work has been corroborated in mouse where Notch, in tandem with BMP signalling is needed for specification of the biliary tree[220].

Delta-like1 (Dlk1) is a known marker of hepatoblasts in the developing liver[221]. However Dlk1 positive cells are also present in the CDE and DDC rat diet model of liver injury[222]. Dlk1, as discussed previously is a negative regulator of Notch signalling[143]. Hepatoblasts are foetal liver cell precursors and are known to become both hepatocytes, when located in the liver parenchyma, but can also become cholangiocytes when located in the portal Zone-1 regions. Over expression of N^{icd} in these cells this was shown to down regulate terminal markers of hepatocyte differentiation including G6Pase, tyrosine amino transferase, and carbanoylphosphate synthetase; this over expression also reduced levels of Albumin, a functional marker of Hepatocyte development[223]. When cultured in contact with laminin N^{icd} expressing hepatoblasts up regulate known cholangiocyte markers such as CK7, CK19 and HNF1β – suggesting a synergy between activated Notch and the Integrin signalling pathway in defining cholangiocyte and hepatocyte differentiation[224]. The link between Notch signalling and hepatic development in the developing liver is mediated by a plethora or liver-enriched transcription factors such as Hnfs and C/EBP, which are expressed in a highly stereotyped fashion and are

responsible for the correct specification of hepatocytes (involving Hnf1α and Hnf4α) and cholangiocytes (defined by Hnf6 and Hnf1β)[222, 225, 226].

Notch Signalling in the diseased liver

Alagilles syndrome results in the aberrant development of multiple organs including the heart, eye and is associated with severe cranio-facial malformations[219, 227]. Bile duct retardation is also characteristic of this condition, with sufferers often requiring a liver transplant as a result. In this situation the biliary tree is not correctly specified and as such the branches terminate prematurely, and fail to function correctly. Interestingly Alagilles patents are incapable of mounting an HPC response to damage, and as a result these individuals suffer from a lack of regeneration during disease, and increased scarring[228].

Alagilles syndrome is classically associated with a mutation in the *jag1* gene, which encodes the Notch ligand Jagged1[229]. Multiple mutations in this gene are implicated in an altered transcript which cannot function in a manner similar to the native protein. It is this haploinsufficiency which results in attenuated Jagged1 activation of Notch receptor. There are however, multiple cases of Alagilles patients who have normal *jag1* but who phenotypically appear to suffer from Alagilles. Current lines of study are also focusing on the presence of mutations in the Notch receptor[230]. In a mouse model of Alagilles mutations or heterozygous knock outs of *jag1* do not result in the typical hepatic and cardiac deficiencies commonly associated with Alagilles. It is only when a hypomorphic allele of Notch (in this case N2) is introduced that the

typical Alagilles phenotype is observed, suggesting that Alagilles is a heterogeneous disorder of the Notch signalling pathway[231, 232].

The specific action of certain Notch homologues reveals that Notch2 and not Notch1 is required for correct hepato-biliary specification[233]. Conditional liver specific knockouts of both Notch1 and Notch2 with Cre expressed under the Albumin (*Alb*) promoter have been reported. Conditional deletion of Notch1 and Notch2 resulted in retardation of the biliary network with cells rarely forming any tubular structures; these structures did appear later, however they were disorganised and the vessels do not appear to be incorporated into the portal mesenchyme. Notch2, but not Notch1 was indispensable for correct biliary development as demonstrated in single conditional knockouts. Notch1 displays none of the hyperplasia observed in the Notch2 single knockout, which were indistinguishable from the Notch1/Notch2 double mutant[233]. Intriguingly the AlbCreER transgenic mouse is widely used in these studies, giving widespread Cre expression in the liver, and may recombine any targets in the parenchyma as well as in the biliary tree; suggesting that in development these phenotypes could be induced though perturbed specification of the parenchyma rather than specific alteration of the developing bile ducts.

Notch signalling is clearly implicated in the development of normal liver and is a key mediator in congenital disorders of multiple organ systems. Is Notch then implicated in HPC mediated resolution of liver injury? In a partial hepatectomy model of liver injury and regeneration in the rat has shown post surgery that Notch1 levels decrease

for the initial 6 days. Jagged levels however increase significantly in the following hours before returning to normal by 12h. During this phase there is a considerable increase in N^{icd} into the nucleus of the remaining liver. There was enhanced expression of Jagged in both the biliary epithelium and periportal hepatocytes, where as Notch2 was up regulated strictly in the periportal hepatocytes[234, 235]. Furthermore recent work in human chronic and acute diseases has highlighted a difference in the expression of Notch pathway components when regenerating the biliary tree in primary sclerosing cholangitis, and primary biliary cirrhosis when compared to regeneration of the hepatocytes following infection with hepatitis c virus; where the notch signalling is down regulated[110].

HPCs are located in a multi-cellular niche as discussed above. The close physical relationship between the stellate cells, which protrude through the laminin sheath and the HPCs, implicates a potentially direct rather than a diffusible signal may be involved in the regulation of HPC differentiation *in vivo*. Notch signalling seems to be a likely candidate as in other systems where there is this close relationship between supportive stromal cells which constitute the niche and the progenitor cells.

The Wnt signalling pathway and its importance in development, disease and maintenance

The Wnt signalling pathway plays numerous and complex roles during foetal development, tissue homeostasis and disease progression[236, 237]. The pathway was originally identified by the *Drosophila* wingless (Wg) mutation[238, 239], and from this

a multifaceted protein pathway which is conserved between vertebrates and invertebrates has been described[240]. The Wnt signalling pathway is responsible in part for cellular proliferation, differentiation, apoptosis, survival and migration, and as such is regulated at multiple levels[241]. Furthermore the Wnt signalling pathway has been implicated in appropriate hepatic development and regeneration and as such may be a fundamental requirement for progenitor cell mediate regeneration of the hepatic parenchyma[242, 243].

The Wnt signalling pathway and its ligands

Both *Drosophila* Wg and vertebrate Wnt can operate through the dimerisation of two highly conserved receptor families known as Frizzled (Fzd)[244] and the LDL-receptor related proteins 5 and 6 (LRP5/6) – known as Arrow in the fly[245]. The mammalian genome contains 10 frizzled receptors, which contain striking sequence similarities, and as such probably act in a highly redundant manner to maintain the Wnt signalling cascade. LRP5/6 appears to have a partially redundant function, LRP6 is required for embryonic specification, and is sufficient for this role in the absence of LRP5[245] .

The Wnt receptors are known to activate two families of downstream pathways known as the canonical and non-canonical signalling pathways. The canonical signalling pathway, which is certainly the most extensively studied involves the dimerisation of the Fzd and LRP5/6 receptors and the modulation of gene transcription in a Ctnnb1 (β-catenin) dependent manner[241, 246, 247]. The non-canonical

pathways will not be extensively explored during this chapter, as they are by far less well described, and have not been implicated significantly in the specification and patterning of the vertebrate liver or its regeneration. Briefly, the Fzd receptor can bind a sub-family of non-transforming Wnt ligands, which induces either a heterotrimeric G-protein coupled receptor-type response, which involves the liberation of Ca^{2+} and activation of calcium dependent signalling processes, or can act though the Rho/Rac signalling pathway in order to maintain cell migration and polarity[248, 249].

The canonical Wnt signalling pathway requires the close association of Fzd and LRP proteins and formation of a hetero-dimer at the cell surface membrane in order to activate downstream events, this is achieved though the binding of anyone of a family of canonical Wnt ligands. Wnt ligands (of which there are 19 in vertebrates) are cystine rich proteins peptides with a secretory motif at the N-terminus[250]. The archetypal canonical Wnt ligand is Wnt3a, which has earned this position though it's efficient secretion, meaning that it has the most compete biological and chemical characterisation of all of the mammalian Wnt ligands, and correlates generally with what is known about the synthesis and secretion of Wg in *Drosophila* (Figure 1.5).

Wg and Wnt ligand is synthesised in the endoplasmic reticulum (ER), where it rapidly undergoes parmitoylation by the ER protein Porcupine[250, 251]. From here the Wnt ligand is transported through the Golgi to the cell surface in secretory vesicles, and is dependent on the presence of the Wntless (WIs). The WIs act to inhibit the

Retromer complex to allow loss of Wg/Wnt secretory vesicles. Studies in which WI has been ablated demonstrate that the Wg/Wnt containing secretory vesicles are degraded and recycled and as such there is a loss of Wnt signal produced in the extracellular space[252].

Secreted Wnt ligand binds to the Fzd and LRP5/6 extracellular domain, and as a result brings these two membrane proteins with functional proximity to one another. This change in location is sufficient for the activation of the Wnt signalling pathway, even in the absence of ligand, and results in the stabilisation of Ctnnb1, a critical mediator of the canonical Wnt signalling pathway[251, 253-255].

Activation of the canonical Wnt pathway stabilised Ctnnb1 and results in gene transcription.

Ctnnb1 is central to the transduction of the Wnt signalling pathway, and its persistence or degradation can be observed as the mechanism by which the canonical Wnt pathway functions. When the Wnt signalling pathway is in an off state, that is to say there is no dimerisation of the membrane receptors Fzd and LRP5/6, Ctnnb1 is degraded (Figure 1.5) .

The degradation of Ctnnb1 is by no means simple. In the absence of signal Axin, a major scaffold protein binds to GSK3, CK1α and Ctnnb1 which results in a phosphorylation complex with Ctnnb1 acting as the substrate[241], which is

sequentially phosphorylated initially by CK1α[256, 257] and then by GSK3 at multiple residues[258, 259]. Secondary phosphorylation by the GSK3 protein results in Ctnnb1 presenting a binding domain for the E3 ubiquitin ligase β-Trcp, and as a result these Ctnnb1 is ubiquitinated and targeted for destruction[260, 261]. This complex alone is sufficient for Ctnnb1 phosphorylation; however Axin acts in tandem with the APC scaffold protein, which can independently bind Ctnnb1 and facilitate its degradation. Interestingly CK1α and GSK3 also phosphorlyate both Axin and APC resulting in their increased affinity for one and other and resultantly causes an increased degradation of Ctnnb1, and removal from the cytoplasm[262, 263].

The formation of the Axin-APC destruction complex is highly robust. Seminal work in *Drosophila* demonstrated that in Axin null flies expressing truncated Axin proteins, lacking their APC-, GSK3- or Ctnnb1- binding domains were capable of a significant restoration of normal phenotype, again highlighting the requirement for a multivalent system which can continue function to a greater or lesser degree despite being mutated[264-267]. The net result of the destruction complex is that cytoplasmic and as such nuclear Ctnnb1 is not present and transcription of target genes cannot be achieved.

The result of the "Wnt-On" state, during which the Fzd and LRP5/6 receptors dimerise is that Ctnnb1 is the cytoplasm is protected from degradation. Wnt-induced phosphorylation of the LRP6 receptor is a critical event in the maintenance of Ctnnb1[268]. LRP5/6 and Arrow (the *Drosophila* equivalent of the LRPs) have five

PPPSPxS motifs which undergo a sequential dual phosphorylation, by GSK3 and as such bind Axin to the cytoplasmic surface of the plasma membrane. These PPPSPxS domains are necessary for the activation of Wnt signalling, and when expressed heterologously causes constitutive stabilisation of the Ctnnb1 protein[268-270]. The LRP5/6 receptor cannot be phosphorylated independent of binding or indeed close localisation of the Fzd co-receptor. Fzd binds a cytoplasmic scaffold protein known as Dishevelled (Dsh) which binds the Axin-GSK3 complex and sequesters it to the cell surface membrane, before LRP5/6 is phosphorylated and prior to the attachment of Axin to these phosphorylation motifs[269]. As such Fzd and LRP5/6 are synergistically required for the recruitment and long-term sequestration of the Axin-GSK3 complex, and also may establish a positive feedback loop which results in complete phosphorylation of the LRP5/6 receptor and then further recruitment and removal of Axin from the cytoplasm.

Translocation of Ctnnb1 to the nucleus results in TCF/LEF mediated transcription

During canonical Wnt signalling the levels of stabilised Ctnnb1 in the cytoplasm is reflected as an accumulation of un-phosphorylated Ctnnb1 in the cell nucleus. The mechanism by which Ctnnb1 translocates to the nucleus, and the means by which it is retained there is unknown, however Ctnnb1 does associate with the nuclear pore proteins through a nuclear localisation signal motif, and that this coupled with the association of Ctnnb1, transcription factors transcriptional co-activators may maintain Ctnnb1 in the nucleus[271]. Ctnnb1 may though be actively transported from the cytoplasm into the nucleus in a Rac1/JNK2 dependent manner, and as such the

activation of Wnt pathway targets may be much more complex that previously understood[272].

Once in the nucleus Ctnnb1 complexes with the TCF/LEF family of transcription factors, which function as the most downstream components of the Wnt signalling cascade[273]. The TCF/LEF transcription factor family contain a High Mobility Group domain through which these proteins can bind to DNA in the minor groove to a highly conserved consensus site. The binding of TCF/LEF transcription factors is not sufficient to initiate transcription (with the exception of LEF which contains a context-dependent activation domain and as such is transcriptionally competent when associated with the co-activator Aly), however the bend they induce in DNA means that co-transcription factors are able to access this site and initiate target gene transcription[274]. The TCF proteins frequently associate with Ctnnb1 in order to drive transcription, where TCF provides the DNA binding moiety and Ctnnb1 supplies the transcriptional ability, through the trans-activation domain at the C terminus of the Ctnnb1 protein, and may act though complexing with the TATA binding protein (TBP) in order to contact the transcriptional machinery[275]. Furthermore TCF-Ctnnb1 is known to bind with CREB binding protein, which is known to recruit histone acetyl transferases, and as such may make the region to which it is bond more responsive to other transcription factors[238, 276].

The TCF transcription factors are also mediators of transcriptional repression when the Wnt signalling pathway is off. Groucho (TLE and Grg in human and mouse

respectively) is a TCF associated co-repressor, which in the absence of Wnt signalling recruits histone deacetylases (HDAC) and prevents target gene transcription, and acts as a competitor for Ctnnb1[277, 278].

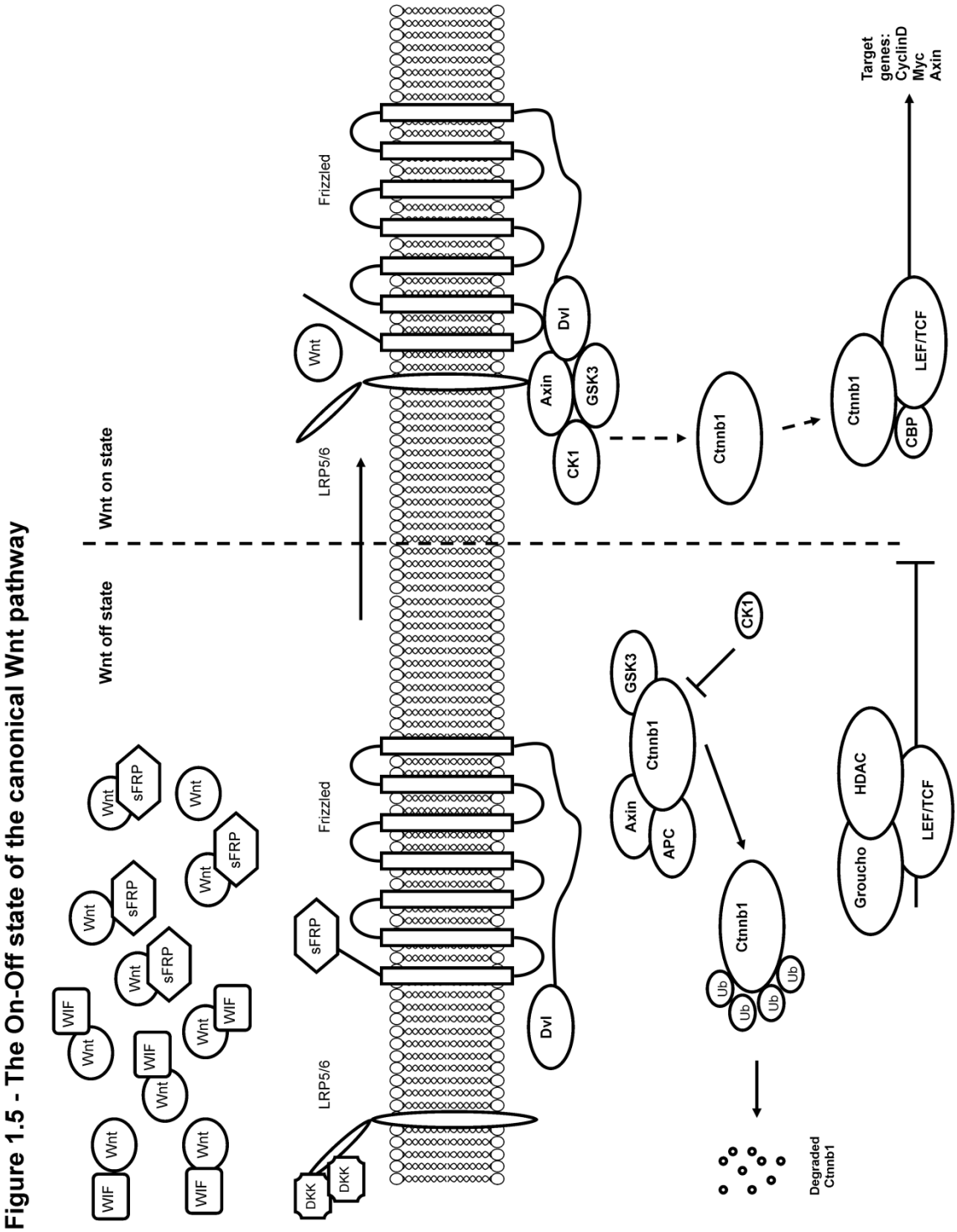

Figure 1.5 - The On-Off state of the canonical Wnt pathway

Figure 1.5 – On-Off activation of the Wnt signalling pathway.

The Wnt signalling pathway involves binding of the Fzd and Lrp protein families at the cell surface membrane in the presence of the Wnt ligand. In the "off-state" Ctnnb1 is phosphorylated, ubiquitinated and targeted for degradation by the destruction complex consisting of Axin, APC, GSK3 and CK1α. This results in repression of the canonical Wnt signalling pathway. Upon receptor dimerisation the LRP receptor is heavily phosphorylated and as a result the destruction complex is sequestered to the cell membrane, via Dishevelled. This results in the activation the Wnt pathway by allowing Ctnnb1 to translocate to the nucleus and convert the TCF/LEF mediated repressor complex containing Groucho and HDAC into a transcriptional activator, which induces multiple downstream genes. The Wnt signalling is classically repressed at the level of ligand and receptor through soluble receptor antagonists such as the sFRP and WIF1 or though interaction and modification with the receptor components, where sFRP binds to the Fzd receptor and the DKK family of repressors bind-to and inhibit the LRP receptor family.

The Wnt pathway is regulated though the expression of two families of receptor antagonists.

The Wnt-Ctnnb1 signalling pathway importantly regulated though signal binding, Ctnnb1 stability and through chromatin regulation; atop this pathway is a further level of fine-tuning and regulation which acts to restrict the effect of canonical Wnt signalling in certain cells, whilst allowing geographically close cells to be permissive to the signals.

The Dickkopf family of Wnt inhibitors bind specifically to the LRP5/6 receptor and as such are specific inhibitors of the canonical Wnt signalling cascade. They contain two cystine rich domains, Cys-1 and Cys-2 with the latter being highly conserved between family members. The classical Wnt antagonists can be divided into two functional classes the secreted Frizzled-related protein (sFRP) family which contains the sFRPs, WIF1 and Cerberus which generally though binding and sequestration of the secreted Wnt ligand. These sFRP proteins contain a cystine-rich domain, and as such can act as a decoy receptor. Furthermore the sFRP proteins can bind directly with Fzd, and as such become competitive inhibitors of Wnt ligand, as such preventing the assembly of the Fzd-LRP5/6 receptor complex. WIF1 on the other hand can only regulate a subset of the secreted ligands though its WIF domain (WD) of EGF-like repeats, and as such performs a similar function, but though a different mechanism.

The importance of Wnt signalling in development and disease

The Wnt signalling pathway is of critical importance in these cellular fate designations, and as such plays a critical role in the development and regeneration of multiple cellular systems. Here the role of Wnt-Ctnnb1 signalling during Neurogenesis and Neuronal repair as well as intestinal turnover will be discussed as the role for Wnt in these organ systems is well described and extensively studied. Less well defined is the role of Wnt signalling during hepatic ontogeny and repair; however there is emerging evidence which indicates that the Wnt signalling pathway is important for correct differentiation in both hepatic contexts.

The Role of Wnt Signalling during Neurogenesis

During Neurogenesis both the canonical and non-canonical Wnt signalling pathways have been implicated in correct specification of neurons, appropriate release of neurotransmitters, as well s maintenance of the glial cells populations within the brain[279].

The adult brain active Neurogenesis occurs within the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles of the forebrain. The putative neural stem cell resides within these two areas, and Wnt signalling has been implicated as an extracellular factor involved in their proliferation and specification[279, 280]. During development Wnt signalling is necessary for the expansion of progenitor cells which will ultimately contribute to the neural tube[281]. Moreover inhibition of GSK3, and as such activation of the canonical Wnt cascade resulted in the precocious differentiation of neural stem cells

into neurons, as did ectopic administration of Wnt7a[282]. More recent studies have found that the canonical Wnt signalling cascade is required for maintenance and selection of newborn neurons in the adult hippocampus, in a cell autonomous feedback loop which allows exit from a proliferative phase, and differentiation into mature hippocampal neurons[283].

The role of Wnt signalling during adult neurogenic diseases has also been highlighted. The induction of a dominant negative Wnt in the dentate gyrus for example reduces memory retention[284]. Furthermore suppression of the Disrupted in Schizophrenia-1 (DISC-1) gene which directly interacts with GSK3 resulted in a decreased proliferation of neuronal progenitor cells *in vitro* and *in vivo* again implicating the canonical Wnt cascade in the maintenance of neural phenotype in the adult[285]. NEUROD1, a key regulator in the specification of neuronal tissue during both embryogenesis and adult neural stem cell specification has been clearly demonstrated to be a direct target of the Wnt-Ctnnb1 signalling pathway and as such reiterates the need for an intact canonical Wnt cascade in the development and maintenance of neuronal cells[286].

As the neural stem cell compartment is relatively slow cycling, the requirement for canonical Wnt signalling within this cell population, and the phenotypes associated with aberrations in the Wnt signalling pathway may take time to manifest and may be subtle; in comparison the adult intestine has a high rate of cellular turnover and

has been used extensively to investigate the roles of Wnt signalling in tissue homeostasis and disease.

Wnt signalling is critical for Intestinal homeostasis

The importance of Wnt signalling in the murine intestine was initially described using the $APC^{Min/+}$ mouse which was generated as part of an ethylnitrosurea mutagen programme[287]. This mouse spontaneously develops frequent, small intestinal adenomas, and colonic polyps; indicating that the haplosuffiency of APC and ectopic activation of the Wnt signalling results in a phenotype similar to the human familial adenomatous polyposis. The use of transgenic tools during investigation of Wnt signalling in the intestine has, unsurprisingly, been powerful. Conditional deletion of functional APC regions using either the Villin-CreER or indeed the Cyp1A-Cre transgenic early on revealed that loss of Ctnnb1 degradation resulted in altered proliferation, differentiation and morphogenesis of the intestinal epithelium[288, 289]. Further to these observations stabilisation of Ctnnb1 in the intestine using a targeted exon3 mutation results in a recapitulation of the APC phenotype, and once again points to the critical role of the canonical Wnt signalling pathway in the maintenance of the intestinal epithelium[290, 291].

The recent description of the $Lgr5^{+}$ intestinal stem cell has brought about a new impetus, and generation of transgenic technologies to the intestinal field[210]. The Lgr5 stem cell population in the liver is capable of, from a single cell forming crypt like structures *in vitro*[292, 293]. Lineage tracing from this Lgr5 positive intestinal stem cells

demonstrates that all components of the intestinal epithelium are derived from this source, and that over time the collection of stem cells within the base of the intestine are progressively replaced by one stem cell, and as such the epithelium within a crypt is genetically identical[294, 295]. Using the Lgr5-Cre mouse Clevers and colleagues have demonstrated once again the importance of Wnt signalling in the maintenance and correct specification of intestinal stem cells. Loss of Wnt effector Achaete Scute-Like 2 (Ascl2) controls intestinal stem cell fate, and its ablation results in the loss of Lgr5 intestinal stem cells, as Ascl2 is a well described target of the Wnt pathway it is likely that modification of the Wnt signalling pathway in these progenitor cells is likely to affect their fate, and their proliferative potential[296-298]; Indeed in the fly Wg signalling is required to maintain *Drosophila* intestinal stem cells, highlighting the conserved, and ancient mechanisms by which intestinal tissue homeostasis occur[299].

The Lgr5-Cre mouse also allows tracing of crypt cells in intestinal cancers. When APC is conditionally deleted in the Lgr5 population, small polyps occur within the crypts, which develop over time and become large adenomas which protrude out into the intestinal lumen. Lgr5 positive intestinal stem cells do not migrate during this process, rather they remain at the crypt base, and contribute to the growing polyp as they would typically restore the intestinal epithelium[300]. Furthermore when the same APC transgenic was crossed with the tamoxifen inducible Ah-Cre, which recombines the transit amplifying population only small short lived polyps were observed, reflecting the finite potential of these transit amplifying cells and their contribution to polyposis in the intestine[300].

The importance of Wnt signalling within the intestinal niche is of critical importance, and again this is reflected in the role of Paneth cells in the maintenance of the intestinal stem cells, and crypt. Paneth cells migrate towards the regions of high Wnt expression within the intestine, and require Wnt signalling to ensure their maturation programme is correctly fulfilled[289, 301, 302].

As the intestine and liver are derived from a similar endodermal origin, the hepatic stem cell, although not yet defined, may respond in a similar fashion to the Lgr5 intestinal stem cell and have similar signalling requirements for its proliferation and differentiation. By investigating the HPC population, and the developing liver, it may be possible to garner information about what signalling pathways are required for hepatic regeneration, in a similar way to how Villin-CreER and Cyp1A-Cre studies informed understanding of the biology of the Lgr5 intestinal stem cell.

Wnt signalling during hepatic ontogeny

The mammalian liver develops from a region of the definitive foregut endoderm epithelium. During ontogeny the liver derives from the lateral domains of the endoderm, and the endothelial components of the liver such as the sinusoids, and vasculature derive from the ventral midline[303]. These endodermal movements occur early on in development, and in human the liver is specified by three weeks and in mouse within 8.5 days. During this period FGF and BMP signalling are necessary for initiation of the liver bud, and its migration[304]. Once the liver bud is specified there is a second phase of hepatic development and growth which is characterised though expansion and proliferation. There is conflicting evidence regarding the role of canonical Wnt signalling and its role in the correct patterning of the liver[305].

Repression of Wnt signalling, and Ctnnb1 stability is suppressed by sFRP5 and results in the specification of the liver from foregut endoderm, if Wnt repression is inhibited in the hind-gut endoderm, this region which classically contributes to the lower GI tract and intestine becomes an ectopic liver[306, 307]. There is emerging evidence however that the Wnt-Ctnnb1 axis is also activated during foregut specification, and required for promotion of hepatic specification by expression of Wnt2b by the mesoderm adjacent to the hepatic lobes[308].

Once the liver is specified from the for gut endoderm the Wnt-Ctnnb1 signalling pathway is required for expansion of the foetal hepatoblasts. Ablation of Ctnnb1 using developing liver explants and antisense morpholino studies, or indeed by

genetic ablation of Ctnnb1 in the developing mouse demonstrated a failure of liver growth and retardation of hepatoblast proliferation[309-311]. This phase of proliferation may be mediated though the activation of Cyclin D1, a known target of the Wnt signalling pathway[312]. Furthermore in embryos from APC knockout mice which demonstrated a high degree of biliary differentiation, however there is little to suggest a mechanism by this but may involve a temporal difference in Wnt signalling, priming hepatoblasts for subsequent specification through the Notch signalling pathway[313].

Ctnnb1 and the Wnt signalling pathway appears to directly drive hepatocyte differentiation and maturation too as when Ctnnb1 was conditionally knocked out using the Foxa3-Cre in hepatoblasts cells remained in a rudimentary phenotype with low expression of the hepatocellular enriched transcription factors Hnf4α and CEBP/α, did not express liver enzymes, and could not store glycogen[312]. Further to this postnatal liver growth requires intact Ctnnb1 in order to reach an appropriate volume. Adult murine livers where Ctnnb1 had been ablated using the αFP-enhancer-Albumin-promotor-Cre demonstrated that out to 12 weeks livers appeared immature on a cellular level and small compared to litter mates which had intact Ctnnb1[314, 315]. Further work has demonstrated that in Zebrafish there is a biphasic requirement for Wnt signalling, and that in $APC^{+/-}$ fish hepatomegly is commonplace and proliferation occurs are a greater rate[316]. *In vitro* hepatoblasts can be induced into mature hepatocytes using Dexamethasone, however treatment with sFRP3 results in the attenuation of hepatocyte differentiation, indicating once more that murine hepatoblasts require timed Wnt signalling in order to mature into functional

hepatocytes[317]. In extension to this, recent work has also demonstrated that not only is hepatocyte differentiation controlled by Wnt signalling, but hepatic zonation is also Wnt, but not c-Myc dependant, with aberrant metabolic zonation (or indeed failure of such) occurring as a result of canonical Wnt pathway ablation[14].

As such increasing evidence suggests that during ontogeny and shortly after birth the canonical Wnt signalling pathway is required for the initial establishment of the liver primordium, expansion of naive bipotent hepatoblasts, differentiation into mature epithelium, terminal differentiation into hepatocytes, but also metabolic patterning of the parenchyma. However during disease does the Wnt signalling pathway have a role in the expansion and differentiation of HPCs?

Wnt signalling during regeneration in the adult liver

During surgical resection levels of stabilised Ctnnb1 increase rapidly in the remaining liver; this occurs rapidly within minutes, and persists during regeneration[318]. Zebrafish and Mice with the $APC^{+/-}$ mutation, have an upregulation Ctnnb1 protein and increased proliferation in order to restore the hepatic parenchyma after partial hepatectomy[316]. Furthermore mice with Ctnnb1 loss in hepatocytes demonstrated a smaller liver after birth, but also showed that immediately after a partial hepatectomy, the rate of proliferation in hepatocytes was stunted for the first 2-3 days compared to WT and that only after this period was there an increase in the rate of hepatocyte proliferation, presumably due to residual Ctnnb1 accumulation reaching a threshold, or through a second pro-proliferative pathway[314]. Furthermore

in acute liver failure induced by overdose of acetaminophen in mice and a retrospective analysis of patents who had taken an overdose demonstrated a rapid inactivation of GSK3, and induction of Ctnnb1 stabilisation in those mice and patients which spontaneously regenerated following acute phase injury[319]. In both of these hepatocyte dependant regenerative contexts the requirement for Ctnnb1 is clear, and sufficient for rapid regeneration of the liver architecture, but may not be absolutely necessary for hepatocellular regeneration as the liver can regenerate in the absence of Ctnnb1.

Only a few studies have convincingly investigated the role of canonical Wnt signalling during chronic regeneration, however evidence is emerging in the literature that the Wnt-Ctnnb1 signalling cascade is important in the HPC mediated regeneration of liver in both human disease and animal models of such[110]. Monga and colleagues have demonstrated in a model of partial hepatectomy with hepatocyte injury that the nuclear localisation of Ctnnb1 is increased in HPCs, and that along with this GSK3 and WIF1 levels decrease in order to facilitate high levels of Wnt signal transduction[320]. Furthermore this group demonstrated that in the DDC dietary model of regeneration that Ctnnb1 deletion resulted in an attenuated proliferation of progenitor cells, however this study utilised a whole liver knockout of Ctnnb1, and as such the failure of regeneration may have been the result of altered hepatic environment, rather than a specific effect on the HPC population.

Further data has shown that during the DDC model HPCs are responsive to Wnt3a, and that this is capable of inducing cellular proliferation and de-phosphorylation of Ctnnb1, increasing its stability with the HPC population[321].

These two works are interesting as both use the DDC model which, is one of biliary fibrosis and sclerosing cholangitis, where Wnt signalling may be important for the initial propagation of these cells, however later on, when in DDC mice get secondary confluent necrosis of the hepatocytes there is no mention of how HPCs are able to reconstitute both the biliary epithelium and hepatocytes.

As such the field is limited. We do understand that during development both the Notch and Wnt signalling pathways act in unison, but also in opposition to correctly pattern the different cellular aspects of the liver, and that during tissue turnover in other organs Notch and Wnt signalling act to maintain tissue homeostasis in an appropriate fashion. However we have no functional evidence that the HPC in the liver is maintained by these two factors, or that they play any role in the maintenance and specification of HPCs.

Hypotheses and Aims

The Hypothesis for this work:

The Notch and Wnt signalling pathways play an important role in the specification of adult hepatic progenitor cells into hepatocytes and cholangiocytes.

I aim to test this hypothesis though:

1. Establishing two divergent models of liver regeneration, one which is hepatocyte specific, and the other which is cholangiocyte specific

2. Defining the activation status of both the Notch and Wnt pathways in both models of regeneration

3. Interrogate the Notch and Wnt Signalling pathways both *in vitro* and *in vivo* using small molecule inhibitors and cell depletion strategies.

Chapter 2 - Materials and Methods

Choline Deficient Ethionine Supplemented (CDE) and the (DDC) progenitor induction regime.

All mice were housed and cared for in accordance with UK Home Office Regulations with food supplied *ad libitum* in 12h light/dark cycles.

C57Bl6 strain animals were fed on a Choline Deficient diet (MP Biomedicals) for 18d. Animals were given sweetened water containing 0.15% DL-Ethionine (Sigma Aldrich) (w/v) as previously published[60]

S129S2 stain mice were fed Purina mouse food containing 0.1% w/w 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) for 18d with unaltered drinking water.

Fixation and Preservation of Liver Tissue

Mice were euthanized using CO_2 for 5mins.

Isolated liver tissue was excised and fixed in either buffered Formalin (10mls 37% formalin in 100mls PBS) or in Methacarn (70mls Methanol, 20mls Chloroform and 10mls Acetic Acid) over night. Fixed Tissues were then dehydrated and stored in 70% Ethanol and mounted in paraffin. Sections 4um thick were used for all immunohistochemistry

For frozen sections liver tissue was excised and mounted in OCT fixative. These samples were snap frozen in dry ice and 100% ethanol until the OCT had set.

Samples were stored at -80ºC. Frozen sections were cut at 10um thickness.

Immunohistochemistry of paraffin embedded liver tissue

For enzymatic detection:

Liver sections were dewaxed in xylene for 10m at room temperature and sequentially rehydrated through 100%, 75%, 60% ethanol for 5m each and then finally rehydrated in water for 10m. When antigen retrieval was required samples were antigen retrieved using Sodium Citrate pH6 or Tris-EDTA pH9 in a microwave indicated in Table 2.1. Proteinase K was used for 2m at 37ºC. Tissues were washed in PBS containing 0.05% Tween 20 for 5m. All further washes were performed using PBS alone.

Sections were incubated in 1% H_2O_2 for 20m to block native Peroxidase activity in the tissue. Sections were washed and mounted in Sequenza racks using PBS. Endogenous avadin and biotin were blocked using an avadin and biotin blocking kit (DAKO) for 15m each; sections were thoroughly washed between blocks. Non-specific binding of the secondary antibody was prevented using 10% species specific serum to block sections for 30m at room temperature. Sections were then incubated with primary antibody diluted in 10% species specific serum overnight at 4ºC. Primary antibodies are washed off using PBS and sections are incubated with biotinylated secondary antibody for 30m at room temperature. Secondary antibodies were washed off using PBS and sections are incubated for 30m with Vector RTU. Binding of the primary antibody is detected using DAB chromagen substrate for 3m followed by washing in water.

Sections were counterstained for 30s in Haematoxylin and dehydrated through graduated ethanol and into Xylene to dehydrate completely. Sections were mounted using Pertex hard set mounting medium and stored.

For Fluorescent Detection:

Liver sections were dewaxed in xylene for 10m at room temperature and sequentially rehydrated through 100%, 75%, 60% ethanol for 5m each and then finally rehydrated in water for 10m. When antigen retrieval was required samples were antigen retrieved using Sodium Citrate pH6 or Tris-EDTA pH9 in a microwave indicated in Table 2.1. Proteinase K was used for 2m at 37ºC. Tissues were washed in PBS containing 0.05% Tween 20 for 5m. All further washes were performed using PBS alone.

Sections were blocked using 10% species specific serum in PBS for 30m. Primary antibodies were diluted in 10% species specific serum over night at 4ºC. Sections were thoroughly washed and incubated with secondary antibody diluted in 10% species specific serum for 30m at room temperature in the dark. Sections were thoroughly washed and mounted in Vectashield containing DAPI.

Isolation and Culture of primary HPCs *in vitro*

Mice were euthanized using CO_2 for 5m. The livers were excised and minced in Leibovitz-15 medium containing Collagenase B and DNase I for 45m at 37ºC.

Digested liver was passed through a 40um cell strainer and spun at 50xg for 5m. Supernatant was removed to a clean tube and the 50xg spin was repeated twice more. The final supernatant was spun for 10m at 120xg. This cell pellet was re-suspended in Ammonium Chloride red cell lysis buffer on ice for 5m. Erythrocyte depleted cells were then pelleted and re-suspended in HPC medium:

HPC Medium:

50% Dulbecco's Modified Eagles Medium

50% Hams-F12 Nutrient Mix

10% FCS

50ug/ml Insulin

30ug/ml Hydrocortisone

5ml Sodium Pyruvate

500ul Gentamicin

Re-suspended cells were underlayed with a discontinuous gradient of 20%/50% Percoll diluted with PBS and centrifuged at 1400xg for 20m at 4ºC. Cells were collected from the 20%/50% Percoll interface and washed twice using culture medium. The resulting cells were pelleted by centrifuging at 120xg for 5m and resuspended with CD45R iMag magnetic beads (BD Biosciences), which were incubated for 20m at 4ºC. Bead labelled cells were removed using an iMag magnet for 15m at room temperature. CD45 depleted cells were re-suspended in culture medium containing 10% FCS. These cells were plated at $5x10^4$ cm^2 on tissue culture plastic which had been incubated with laminin for 2h at 37ºC.

Non-adherent cells were washed off 12h after initial plating with PBS. Adherent cells were plated with medium containing 10% FCS for 48h after which the amount of FCS was reduced to 5% for the remainder of time in culture to remove endothelial contamination.

Culture of the BMOL Progenitor Line

Cryopreserved BMOL cells were resuscitated into media described below. These cells were grown until 70% confluence and were then trypsinised and either passaged or re-plated for use.

BMOL growth Medium:

William's E Medium

5% Foetal Bovine Serum

2mM L-Glutamine

2.5ug/ml Fungizone

10U/ml Penicillin; 100ug/ml Streptomycin

30ng/ml IGF2

20ng/ml EGF

10ug/ml insulin

Growth of plasmids

All plasmids were propagated in DH5α strain *E. coli (*Invitrogen). 10ng of plasmid

was introduced to 50ul of DH5α and kept on ice for 30m followed by 1m heat shock in a 42ºC water bath followed by 2m incubation on ice. 0.5mls of S.O.C. medium was added to the cells and samples were shaken at 37ºC at 225rpm for 1h. Reactions were spread 1:50, 1:100 and 1:200 on LB agar plates containing 100ug/ml Ampicillin and were incubated overnight at 37ºC.

Distinct, single colonies were selected and transferred into 5mls of LB broth containing 100ug/ml Ampicillin and were shaken at 300rm for 8h at 37ºC. 50ul of starter culture was transferred to 25mls of LB broth containing 100ug/ml Ampicillin for 16h at 37ºC.

Cells were harvested by centrifugation at 6000 x g for 15m at 4ºC. Plasmid DNA (pDNA) was harvested using a MiniPrep kit (Qiagen). Briefly, bacterial cells were lysed using the buffer supplied. pDNA is sequentially precipitated using a range of buffers. The lysate is centrifuged at >20000 x g at 4ºC for 30m and the plasmid containing supernatant is removed. The supernatant is spun again for 15m and the plasmid containing supernatant is removed. The plasmid preparation is placed over a resin and washed to remove any contaminating proteins and carbohydrates. pDNA is eluted from the column and precipitated using isopropanol and pelleted at >15000 x g for 15m at 4ºC. Pellets are subsequently washed using ethanol, spun again and re-suspended in 200ul TE buffer pH8. The concentration of pDNA was determined using a Nanodrop (ThermoScientific).

Transfection of Notch constructs in the BMOL line and derivation of stably transfected lines

For both transient and stable transfections Qiagen Effectene reagent was used. 10^5 BMOL cells were plated and allowed to recover overnight. The following day 1ug of vector containing Nicd, GFP or Empty pDNA (kindly donated by Dr. Sally Lowell; MRC CRM Edinburgh) was combined with enhancer and Effectene as described in the manufacturer's instructions. Transfected cells were left in normal growth medium for 3 days and assayed for transgene expression. For stable transfectants cells were moved into medium containing 2ug/ml puromycin for 14d and colonies were picked and grown to give clonally derived lines which have stably incorporated Nicd, GFP or empty vectors.

Transfection of RNAi in the BMOL line

P22-24 BMOL cells were plated at 6 $x10^4$ cm^{-2} cells on tissue culture plastic and allowed to adhere overnight. The following day cells were transfected with 10nM siRNA against specific targets using 4ul of HiPerfect transfection reagents (Qiagen). A GFP tagged siRNA which has no homology to any murine gene was used as a positive control for transfection and served as a negative control for RNAi efficiency (Qiagen). Cells transfected with siRNA were assayed 6h – 96h after transfection to monitor loss of gene expression. A complete list of the siRNA used in this study can be found in table 3.

GSI inhibition of BMOL line and primary HPCs

Notch inhibitor N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycin e-1,1-

dimethylethyl ester (DAPT, also known as compound IX) was re-suspended in DMSO at a concentration of 25mM. This was diluted to a working concentration of 5uM in the appropriate culture medium.

Activation of the Wnt pathway using LiCl and Wnt3a

BMOL and primary cells were moved into medium containing 1% FCS and either 3uM LiCl (Gibco) or supplemented with 50ng/ml Wnt3a (Peprotech) for up to 5 days.

Isolation of RNA from liver tissue and cultured cells

Liver tissue was frozen in RNA protect (Qiagen) and stored at -80ºC. 200mg of tissue was homogenised in 500ul of TriReagent (Amersham). The homogenate was centrifuged at 12,000xg and removed to another clean tube. 100ul of chloroform was added to the homogenate and this was vortexed for 15s. Homogenate was centrifuged for 15m at 4ºC. The aqueous phase of the homogenate was removed to a clean tube to which 100ul of 70% RNase free ethanol was added. Samples were allowed to precipitate for 15m at room temperature and then were transferred to MiniPrep tubes (QIAGEN). Tubes were centrifuged at <8000g for 30s to bind RNA to the membrane. Samples were washed using 500ul RW1 wash buffer by centrifugation for <8000g. Samples were washed twice with RPE buffer by spinning for 1m at <8000g. RNA was dried by spinning for 2m at 13,000g. RNA was eluted in 30ul RNase free water. RNA concentration was determined using a Nanodrop from Thermofisher.

Reverse Transcription and qPCR

RNA from 10ng to 1ug was reverse transcribed using Quantitect reverse transcription kit from Qiagen. Genomic DNA was eliminated by incubating RNA samples with gDNase at 42ºC for 10m. Reverse Transcription of mRNA was achieved by incubation of template with reverse transcriptase and random hexamers at 42ºC for 30m. The reaction was quenched by heating the sample to 90ºC for 3m.

Generation of standard curves for qPCR:

cDNA from each sample was combined and this was diluted 1:10 with RNAse free water to give a starting concentration of 5ng/ul. Serial 1:1 dilutions were produced giving a final range of concentrations. Samples of cDNA were diluted 1:100 for analysis of gene expression (5.0, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 ng/ul)

SYBR Green- qPCR was performed using Qiagen SYBRfast master mix and Qiagen Quantitect primers. 2.5ul of diluted cDNA was mixed with 2.5ul of Qiagen Quantitect primers summarised in table 2 as well as 7.5ul of SYBR fast master mix. qPCR was conducted on an ABI 7500 fast. Primers are summarised in Table 2.2.

FACS isolation and characterisation of HPCs

Single cell suspensions were achieved as previously described in this chapter, and Hepatocytes were removed through centrifugation. The NPC population ($1x10^6$ for analysis 1×10^7 for sorting) was re-suspended in PBS containing 0.1%BSA and

1mg/ml Fc block for 30m. Cells were pelleted and re-suspended in blocking buffer containing Dlk1-FITC, EpCAM-648 or F4/80-APC at the appropriate concentration in the dark for 30m (summarised in table 2.1). Cells were washed and analysed on a FACS Calibur (Beaton Dickson) or sorted using either the FACS Diva or FACS Aria (both Beaton Dickson).

GSI blockage of Notch activation and BrdU lineage tracking *in vivo*

S129S2 mice were given 300ul of BrdU (Amersham) intraperitoneally (I.P.). For three days following mice were given 50mg/Kg DAPT dissolved in 1:9 ethanol: corn oil I.P. Finally mice were given EdU I.P. at a concentration of 50ug/g w/w.

Isolation of BM and culture of BMDMs

Male 8 week old mice were euthanized using CO_2 and the Tibia, Femur and Fibula were removed to PBS. After tissue has been removed from the bones the bone marrow was flushed out of the bone cavity using Glutamax DMEM/F12. Whole bone marrow was dissociated using a 21G needle to ensure a single cell suspension. Bone marrow was plated in low adhesion flasks with DMEM/F-12 with 10% FCS and 20% L929 conditioned medium (prepared in house)

In vivo macrophage depletion

Macrophages were depleted *in vivo* using repeat administration of clodronate liposomes (clodronateliposomes.org) that were kindly supplied by Dr. Nico van Rooijen. Briefly, liposomes were brought to room temperature and were injected into

the tail-vein at 200ul per 25g. During the study mice were given liposomal clodronate 3h before the start of damage, and then every three days throughout the time course of damage to ensure complete and persistent depletion of macrophages.

Isolation of primary hepatocytes and production of debris

Hepatocytes were isolated under terminal anaesthesia. Once sedated mice had their portal vein canulated and liver was perfused through the inferior vena cava for 100mls at 12.5mls/m with a EDTA containing perfusion medium (Gibco). After perfusion was complete, the liver was digested in situ using liver digestion medium (Gibco) for 10m at 12.5mls/m. The digested liver was dissected and dissociated in Williams E medium with 10% FCS, and kept on ice. Dissociated livers were passed through 100um cell strainers and spun at 50g for 5m. Hepatocytes were washed and spun a further three times using Hepatocyte Was Medium (Gibco), and were plated at $10^6/cm^{-2}$ on Collagen I coated dishes (Gibco). Isolated Hepatocytes were trypsinised and subject to 20 repeat freeze thaw cycles; hepatocytes were then sonicated to ensure disruption of any intact cells.

In vitro phagocytosis assay

Macrophages were plated at 10^5cells/cm^{-2} in DMEM/F12 and were allowed to attach overnight. The equivalent of 5 hepatocytes worth of debris was fed to macrophages for 18h, after which cells were extensively washed before analysis, this number was informed by previous studies in the lab of Prof. John Iredale which has used thymocytes to generate apoptotic material.

Statistical Analysis

Data was analysed for normal distribution using a D'Agostino and Pearson omnibus normality test. Parametric data was analysed using a two-tailed unpaired Students t-test. In instances where the n was too low to determine normal distribution or the data was non-parametric then a two-tailed Mann-Whitney test was used. In case where more than two groups were being considered, or where multiple variables were being considered then a one-way ANOVA or two-way ANOVA were used respectively.

Table 2.1: A complete list of primary Antibodies used during this work.

Epitope	Company	Code	Tissue, retrieval and Dilution	Secondary Antibody
Immunohistochemistry				
Notch1	Santa-Cruz	sc-6014	Frozen, 1/50	Anti-goat Alexa555
Jagged1	Santa-Cruz	sc-8303	Methacarn, 1/50	Anti-rabbit Alexa555
NUMB	Abcam	Ab14140	Formalin, Tris-EDTA pH9, 1/150	Anti-rabbit Biotnylated
αSMA	Sigma Aldrich	A5228	Formalin NaCt pH 6 1/3000	Anti-mouse Biotnylated
F-480	Abcam	Ab6640	Formalin PK 1/100	Anti-rat Biotnylated
pan-Cytokeratin	DAKO	Z0622	Formalin NaCt and PK 1/300	Anti-rabbit Biotnylated
Dlk-1	Abcam	Ab21682	Formalin NaCt 1/150	Anti-rabbit Biotnylated
EpCam	Abcam	Ab32392	Formalin NaCt 1/150	Anti-rabbit Biotnylated

Hnf4α	Santa-Cruz Biotechnology	sc-6556	Formalin NaCt 1/100	Anti-goat Biotnylated
Hnf1β	Santa-Cruz Biotechnology	sc-7411	Formalin NaCt 1/100	Anti-goat Biotnylated
Ctnnb1 (nuclear)	Transduction Laboratories	C19220	Formalin Tris-EDTA pH9 1/50	HRP polymer
Ctnnb1	Abcam	Ab2365	Frozen 1/100	Anti-rabbit Biotnylated
Ki67	Novocastra	Ki67p	Formalin Tris-EDTA pH9 1/500	Anti-rabbit Biotnylated
Flow Cytometry and Fluorescence Activated Cell Sorting (FACS)				
Dlk-1	Caltag	D187-4	NA	NA
F4/80	Caltag	MF48004	NA	NA
EpCam	Biolegend	118212	NA	NA
CD45	ebiosciences	12-0454	NA	NA

Table 2.2: Table of Primers used during this work.

Recognises	Company	Primer Code
Notch Pathway Components		
Notch1	Qiagen	QT00156982
Notch2	Qiagen	QT00153496
Notch3	Qiagen	QT01051729
Notch4	Qiagen	QT00135653
Jagged1	Qiagen	QT00115703
Jagged2	Qiagen	QT01043819
Dll1	Qiagen	QT00113239
Dll3	Qiagen	QT00113477
Dll4	Qiagen	QT01053598
Hes1	Qiagen	QT00313537
Hes5	Qiagen	QT00268044
HeyL	Qiagen	QT00128954
NUMB	Qiagen	QT00097328
Wnt Pathway		
Axin	Qiagen	QT00126539
Sox9	Qiagen	QT00163765

Myc	Qiagen	QT00096194
Twist1	Qiagen	QT00097223
Wnt3a	Qiagen	QT00250439
Wnt7a	Qiagen	QT00168812
Liver Specific Transcription Factors		
Hnf1α	Qiagen	QT00170975
Hnf1β	Qiagen	QT00103320
Hnf4α	Qiagen	QT00144739
Hnf6	Qiagen	QT00297815
Ggt	Qiagen	QT00297815

Chapter 3 - Establishing Models of Liver Regeneration

Introduction:

The ability of the hepatic parenchyma to regenerate from HPCs has been well described however the mechanisms by which HPCs assume a hepatocyte or biliary fate have not been addressed. Recent work has looked at the mechanism of proliferation and expansion of these progenitor cells and has highlighted the role of the HPC niche in this process[6, 90]. As a transient niche has been implicated in both the proliferation and differentiation of multiple progenitor types in other adult organ system I aimed to establish two models of regeneration in order to address fundamental questions in the biology of HPCs.

Two dietary models of liver damage and regeneration were established and characterised. The Choline Deficient Ethionine (CDE) supplemented model causes primary damage to hepatocytes whilst preventing the proliferation of any residual hepatocytes. The second model I established is the 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) model which acts principally through blockade of the bile ducts. This blockage results in periportal inflammation and rapid fibrosis throughout the portal tract and subsequent activation of HPCs.

The aim of this chapter is to describe how I established and characterised both the CDE and DDC models and demonstrate that they are appropriate for tools to study the mechanisms by which hepatocytes and cholangiocytes are specified from HPCs. I describe the effects of both the CDE and DDC models on mouse and undertake extensive characterisation of the niche surrounding the HPCs during regeneration. I

have also characterised the HPC population through immunohistochemistry and established *in vitro* culture system to investigate adult HPC regulation I also demonstrate a means by which to sort these HPC populations and analyse them for accepted HPC markers.

Characterisation of the CDE diet as a model of hepatocellular regeneration

The Choline-Deficient Ethionine supplemented diet (CDE) is well described as means of inducing primary hepatocyte damage though choline deficiency and prevention of hepatocyte division though Ethionine administration. The CDE regime was administered to adult animals from 0 days to 12 days (Figure 3.1a). During CDE administration animals lost weight for up to 5 days; this was followed by a period of normalisation and weight gain; however CDE treated animals did not reach equivalent weights of animals maintained on a normal diet with standard drinking water (Figure 3.1b).

Healthy adult liver has a small degree of apoptosis as healthy biliary ducts and hepatocytes die and are replaced by activation and division of mature cell types. This normal parenchymal turnover results in a low basal level of TUNEL staining throughout the adult liver (Figure 3.1c). The CDE diet induces hepatocyte apoptosis as demonstrated though a progressive increase in the levels of TUNEL positive hepatocytes throughout the adult parenchyma (Figures 3.1c).

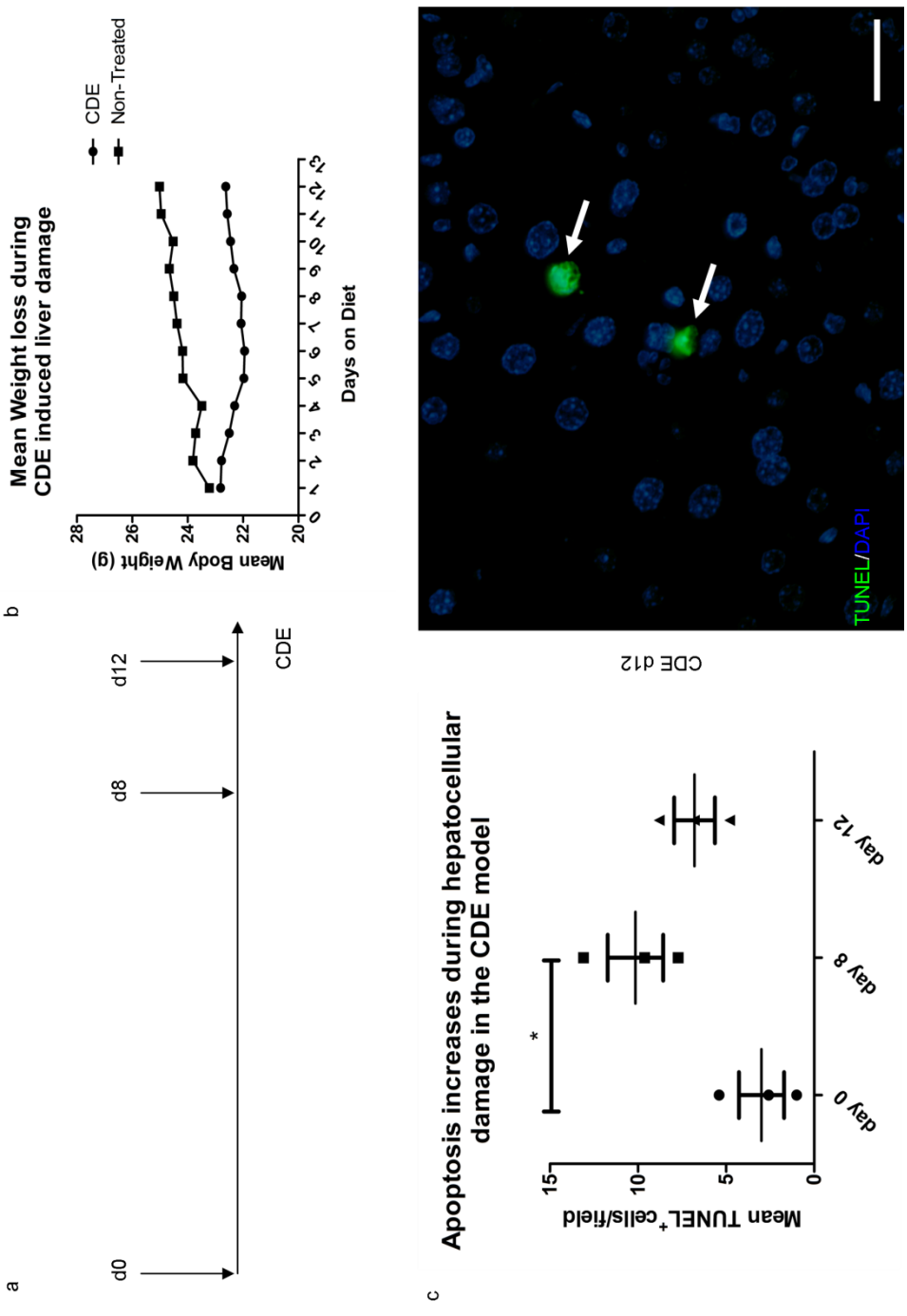

Figure 3.1 — The Choline-Deficient Ethionine (CDE) supplemented diet results in hepatocyte damage and HPC activation.

Figure 3.1 — The Choline-Deficient Ethionine (CDE) supplemented diet results in hepatocyte damage and HPC activation.

a. Adult mice were fed a choline-deficient diet supplemented with Ethionine drinking water for 12 days. Tissue was harvested at day 0, day 8 and day 12 for analysis. 12 days was chosen as a suitable time point based on previous investigation. **b.** During CDE administration mice rapidly lost weight out to day 5 followed by a period of stabilisation and minor weight gain compared to controls which as expected increased in weight as they aged. **c.** During the CDE regime I observe infrequent necrosis, but increasing levels of hepatocyte apoptosis as demonstrated by a marked increase in TUNEL positivity in the hepatic parenchyma (green nuclei, white arrows). This appears to be an early phase response as the level of apoptosis increases by day 8, but may have equalised by day 12 ($n=3$, $p<0.05$: Mann-Whitney U test). Scale bar = 50μm.

In healthy adult liver clearly defined portal tracts are found throughout the organ. These consist of venous and arterial vessels surrounded by well ordered hepatocytes and typically low numbers of biliary vessels (Figure 3.2a). During hepatocellular damage and regeneration induced by the CDE regime the hepatocyte parenchyma appears disordered with poorly arranged hepatocytes and an increased degree of inflammation around the portal tracts. After 12 days of CDE treatment hepatocytes appear both apoptotic and with observable secondary necrosis; in response to this there is an emergence of small cells from the terminal branches of the biliary tree (Figure 3.2 a-c, black arrows) which have a clear epithelial morphology and high nuclear to cytoplasmic ratio. Throughout the injury these cells emerge and infiltrate the hepatic parenchyma in small chord like structures, which can appear to differentiate into mature hepatocytes and as such reconstitute the damaged hepatic parenchyma.

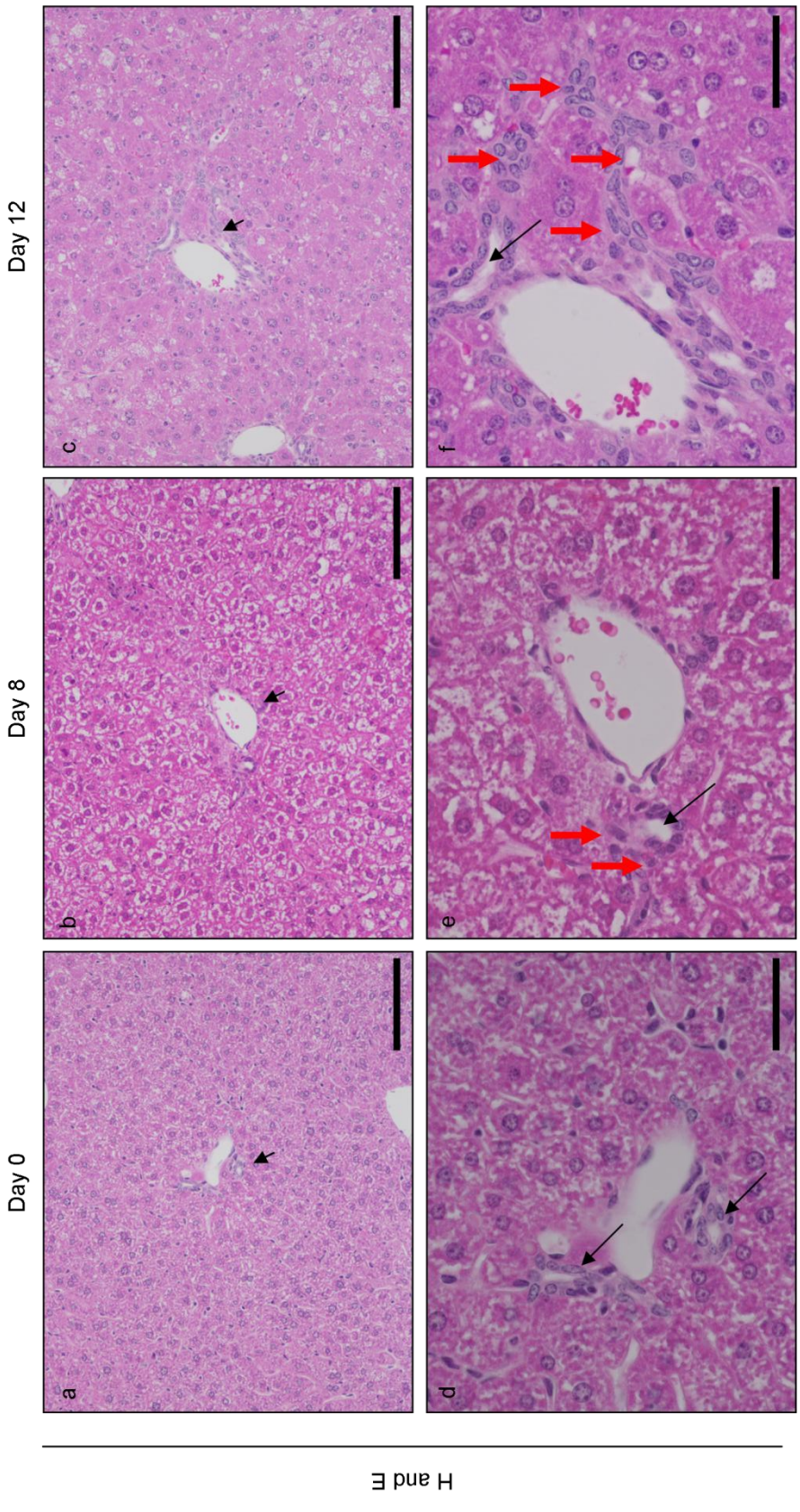

Figure 3.2 — The liver responds to CDE damage through HPC activation

Figure 3.2 — The Choline-Deficient Ethionine (CDE) supplemented diet results in hepatocyte damage and HPC activation.

a. In animals which had not been exposed to CDE a normal hepatic architecture was observed (day 0). **b.** By day 8 hepatocytes appear disorganised alongside a modest inflammatory infiltrate. **c.** At d12 there is considerable hepatocyte damage, considerable inflammation and also the emergence of a small population of cells with scant cytoplasm. (**a-c** arrows identify the portal tract in each image, scale bar = 200μm) **d.** Black arrows highlight a healthy bile duct with a clearly defined lumen and polarised epithelium in close proximity to the portal vein. **e.** At day 8 the emergence of small epithelial cells (red arrows) outwith the portal tract (black arrow) are detectable on histology, however they are infrequent **f.** By day 12 the portal tract appears disarranged, and chords of small epithelial cells are frequently found irradiating from the portal tracts (black arrow denotes a bile duct, red arrows denote HPCs).**d-f** Scale Bar = 50μm.

Hepatic Progenitor Cells arise in the CDE dietary model

As previously described chords of small progenitor-like cells emerge during the CDE model of hepatocyte regeneration. These small cells are positive for panCK, which detects the basic cytokeratins Krt7 and Krt19 – markers of the biliary epithelium and hepatic progenitor cells. In healthy liver panCK is localised to the cytoplasm of cholangiocytes which are the constituent cells of the biliary tree (Figure 3.3 a). After 8 days of CDE damage a population of panCK positive cells emerge in a similar pattern to progenitor cells observed in the H and E stains. These HPCs lack a ductular lumen, and as such are not proliferating bile ducts (Figure 3.3b). After 12 days of CDE administration panCK positive progenitor cells produce extensive cellular chords which extend to the sites of parenchymal damage. Here they terminate, and differentiate into hepatocytes (Figure 3.3c) at which point HPCs expand their cytoplasm and lose positivity for panCK. Quantification of panCK positivity in the liver shows a modest increase in HPC numbers by 8 days, however by 12 days there is a significant increase in the number of HPCs found thought out the hepatic parenchyma (Figure 3.3d; panCK day 8 vs. panCK day 12 $p<0.05$). The number of HPCs does not increase between 12 days and 28 days and decreases when CDE is removed (personal communication with Thomas Bird, Edinburgh)

Figure 3.3 — Hepatocellular damage in the CDE results in the emergence of a HPC population within the liver

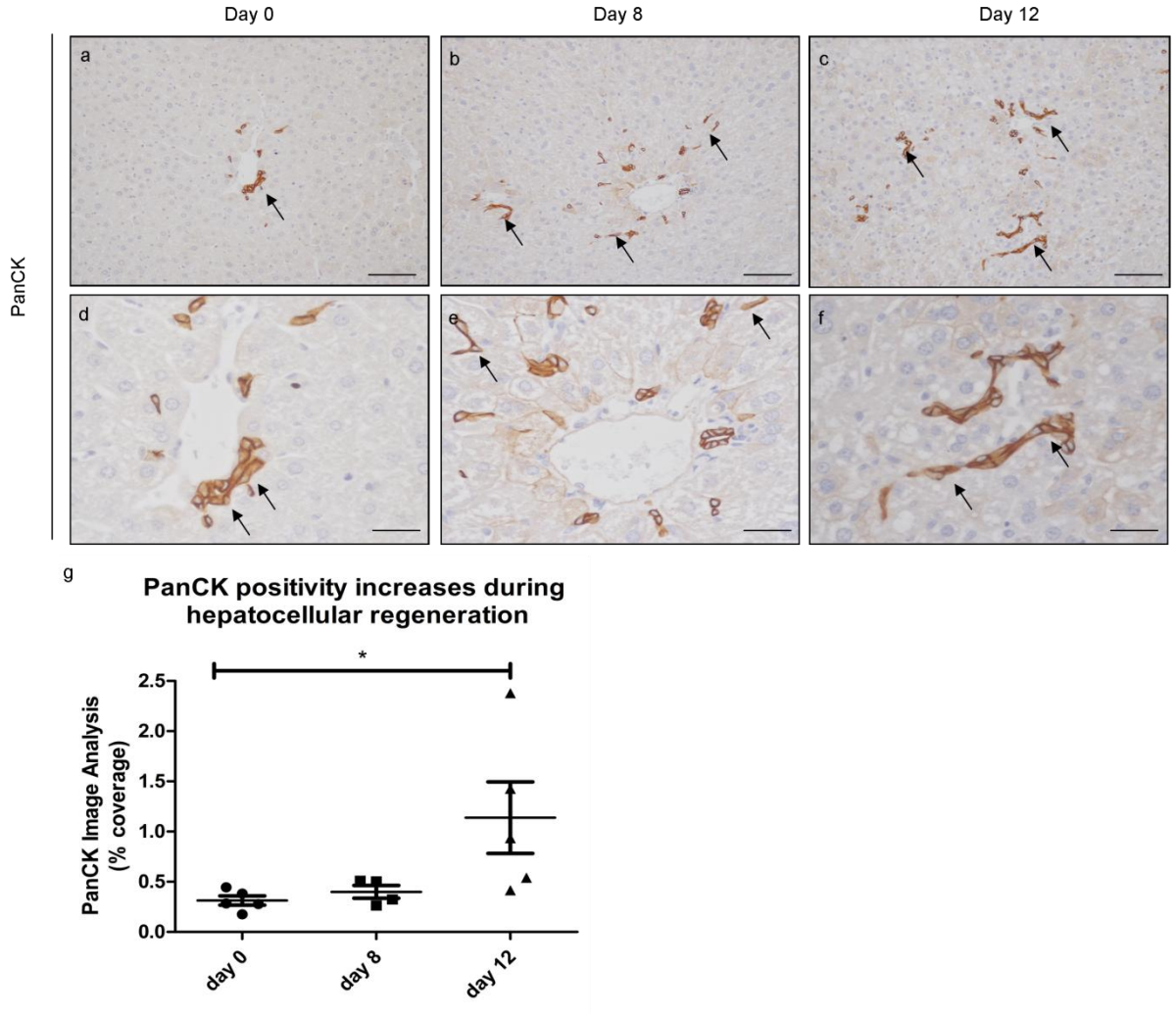

Figure 3.3 — Hepatocellular damage in the CDE results in the emergence of a HPC population within the liver

a, d. In healthy adult liver panCK predominantly identifies the biliary tree. This extends alongside the vasculature throughout the adult liver. **b, e.** 8 days into the CDE diet small panCK positive cells emerge from the portal tract and form thin migratory structures which stretch through the hepatic parenchyma. **c, f.** By day 12 these HPCs form long chords of cells which extend from the portal tract into the regions of damage. **f.** PanCK positive cells increase in frequency throughout the time course of CDE. By day 8 HPCs are becoming much more frequent around portal tracts **g.** however by day 12 there are a significantly higher number of HPCs in damaged livers (Day 0 0.31 ± 0.04 vs. Day 12 1.139 ± 0.3. (day 0 and 12 n=5, day 8 n=4, $p<0.05$ Mann-Whitney U test). **a-c** scale bar = 200µm, **d-f** scale bar = 50µm.

The HPC population can be identified using multiple markers

The phenotype of hepatic progenitor cells in the adult liver is not extensively categorised, and as such I qualified my initial observation of panCK positive HPCs by staining for two widely accepted foetal and HPC markers, Dlk1 and EpCam. During CDE treatment panCK positive HPCs express EpCam and Dlk1 (Figure 3.4a). Interestingly these markers are not universally expressed throughout the HPC population; panCK appears to be the most widely expressed of these markers, with extensive localisation to both the biliary tree and HPCs. EpCam and Dlk1 appear within subsets of these panCK positive cells, and demonstrate localisation with one and other as well as independent staining suggesting that the HPC population may not be as homogenous during the CDE diet as initially thought (Figure 3.4b).

Figure 3.4 — HPCs can be marked with numerous markers which identify both discrete and overlapping cell populations.

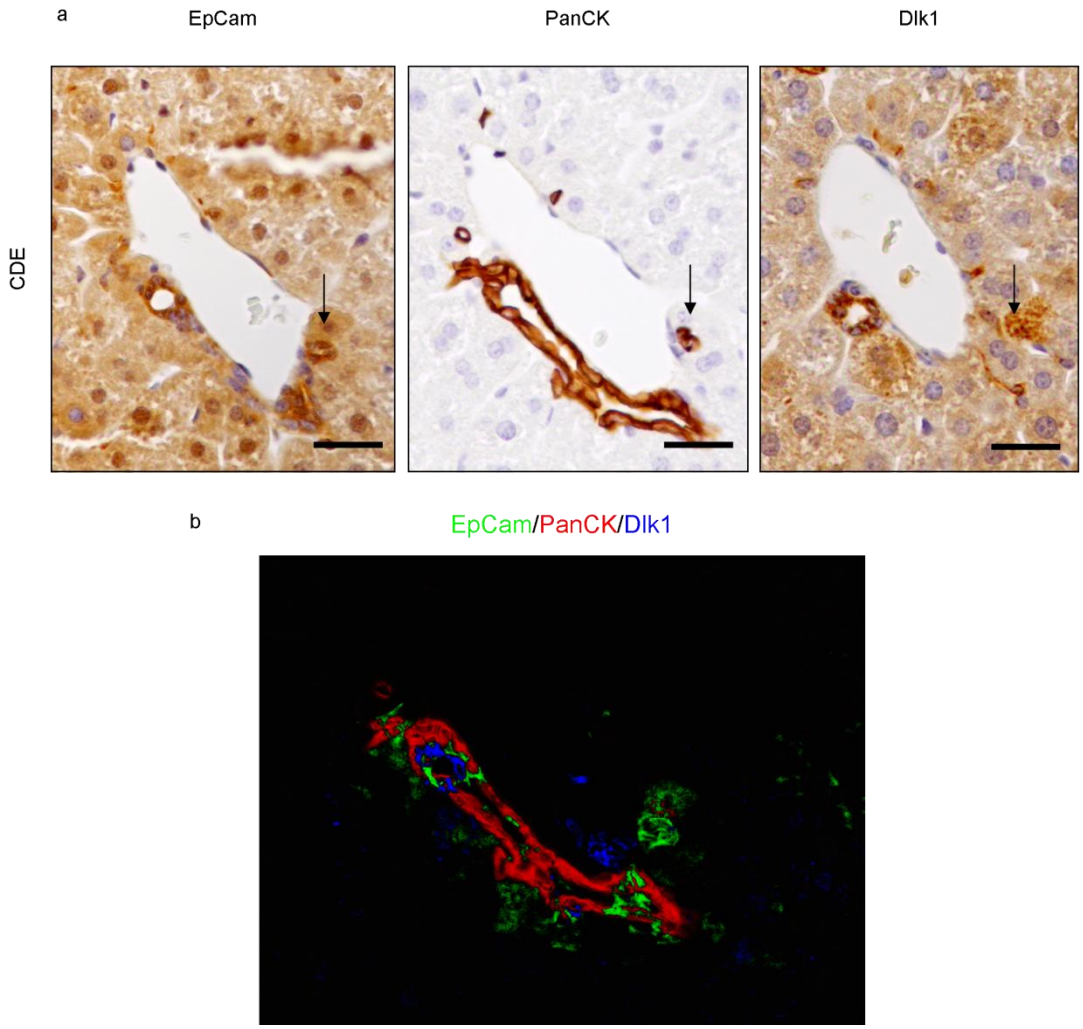

Figure 3.4 – HPCs can be marked with numerous markers which identify both discrete and overlapping populations.

a. HPCs can be identified with panCK, Dlk1 and EpCam. All three markers localise to the bile ducts of healthy animals and the HPC population of animals on the CDE diet **b.** When these expression domains of these three markers are artificially colours and digitally overlaid the three markers of HPCs co-localise in regions, and are found typically around the portal tract in HPC/ductular structures. Intriguingly there does appear to be a degree of heterogeneity, as panCK is much more widely expressed compared to EpCam and Dlk1.

Regeneration occurs on the back of inflammation during hepatocellular regeneration

During chronic human liver disease, the liver is exposed to chronic inflammation, with infiltration of multiple inflammatory cell types and the remodelling of matrix throughout the site of damage. It is increasingly accepted that these inflammatory processes are involved in the activation of HPCs in the adult liver, and as such may be involved in the specification of HPCs into mature hepatic epithelium; either hepatocytes or cholangiocytes.

Within the normal adult liver macrophages are found to lie in close proximity to hepatocytes (Figure 3.3a), and extend processes though the space of Disse. During the CDE regime macrophage migrate towards the site of regeneration and the portal tracts (Figure 3.3b, c). By day 12 there is a close association of macrophages and HPCs. The number of macrophages in the liver changes modestly after 12 days, however this increase is not as marked as one would expect in a chronic disease model; As such resident hepatic macrophages may migrate to the regions of regeneration (F4/80 day 0 vs. F4/80 day 12 $p<0.05$), and are observed clustering in Zone-1.

During chronic inflammation, fibrosis and cirrhosis it is well described that activation of resident hepatic stellate cells into myofibroblasts is an important factor in the deposition of new matrix and the formation of hepatic scaring[13]. Previously it has been shown that myofibroblasts form close associations with HPCs. During CDE

treatment I found that there is a progressive increase in the number of myofibroblasts (Figure 3.3h) throughout the hepatic parenchyma and that are frequently associated with HPCs. These cells are positive for αSMA at day 8 and day 12; whereas in the healthy liver, only the vessel walls are positive for αSMA. Myofibroblasts extend fine processes between mature hepatocytes to access the HPCs emerging from the portal tract (Figure 3.3d – f). The close association of both macrophages and myofibroblasts may play an important role in the maintenance of HPCs during progenitor mediated regeneration.

Figure 3.5 — CDE induces a HPC niche within the liver consisting predominantly of myofibroblasts and macrophages

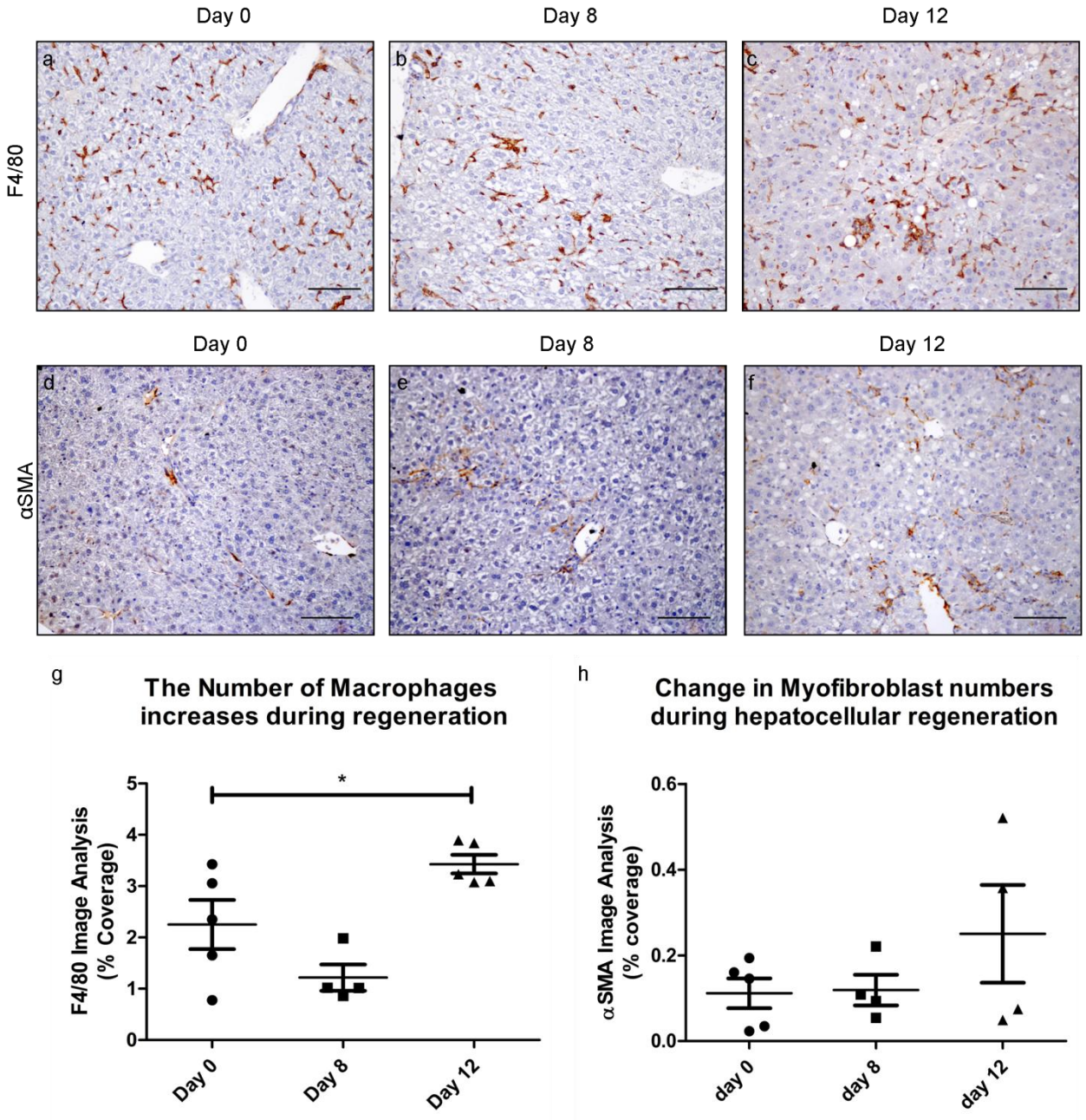

Figure 3.5 — CDE induces a HPC niche within the liver consisting predominantly of myofibroblasts and macrophages

a. In healthy liver F4/80 macrophages are distributed throughout the parenchyma. **b, c.** During hepatocyte regeneration from HPCs macrophages appear close to the portal tract, and in close proximity to the emerging HPCs. **d.** In healthy adult liver there is little presence of αSMA outwith the venous endothelia. **e, f.** During hepatocellular regeneration in the CDE diet there is a progressive increase in the presence of thin spindle like myofibroblasts which extend adjacent to proliferating HPCs. **g.** During regeneration of hepatocytes the number of macrophages changes significantly at 12 days (F4/80 D0 2.25 ± 0.47 vs. D12 3.42 ± 0.18) **h.** Similarly the numbers of myofibroblasts increases, but does not reach significance. (For all images scale bar = 200μm **g, h** day 0 and 12 n=5, day 8 n=4)

The 3, 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) model induces biliary inflammation and ductular regeneration

HPCs are able to regenerate both the hepatocellular parenchyma and the biliary epithelium. As I mean to investigate the mechanism by which these two divergent states are assumed from a bipotent cell population I established a model of ductular damage and regeneration.

3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) results in the accumulation of porphyrin within the biliary tree which ultimately causes biliary apoptosis and necrosis; the HPC population is therefore activated in order to regenerate the ductular epithelium. 0.1% DDC diet was administered to adult mice instead of normal food. Animals were sacrificed at day 0 - as a healthy control, and then at day 8, day 12 and day 18 to assess hepatic damage, progenitor response, and inflammatory response (Figure 3.6a).

Throughout the DDC regime animals were subject to aggressive weight loss, losing 20-30% of total body mass over 18 days (Figure 3.6b). After 18 days weights normalised, however animals became jaundiced. Within 8 days of DDC exposure hepatocytes appear relatively normal, although disorganised. More noticeably after 8 days of DDC small regions of inflammation are found surrounding the portal tracts (Figure 3.6c-j). This inflammatory infiltrate increases throughout the duration of injury and by 18 days large regions of inflammation are found surrounding the portal tracts. By 18 days there frequent biliary aggregates which can be observed

throughout the majority of ducts, extending throughout the biliary tree (Figure 3.6 f, h).

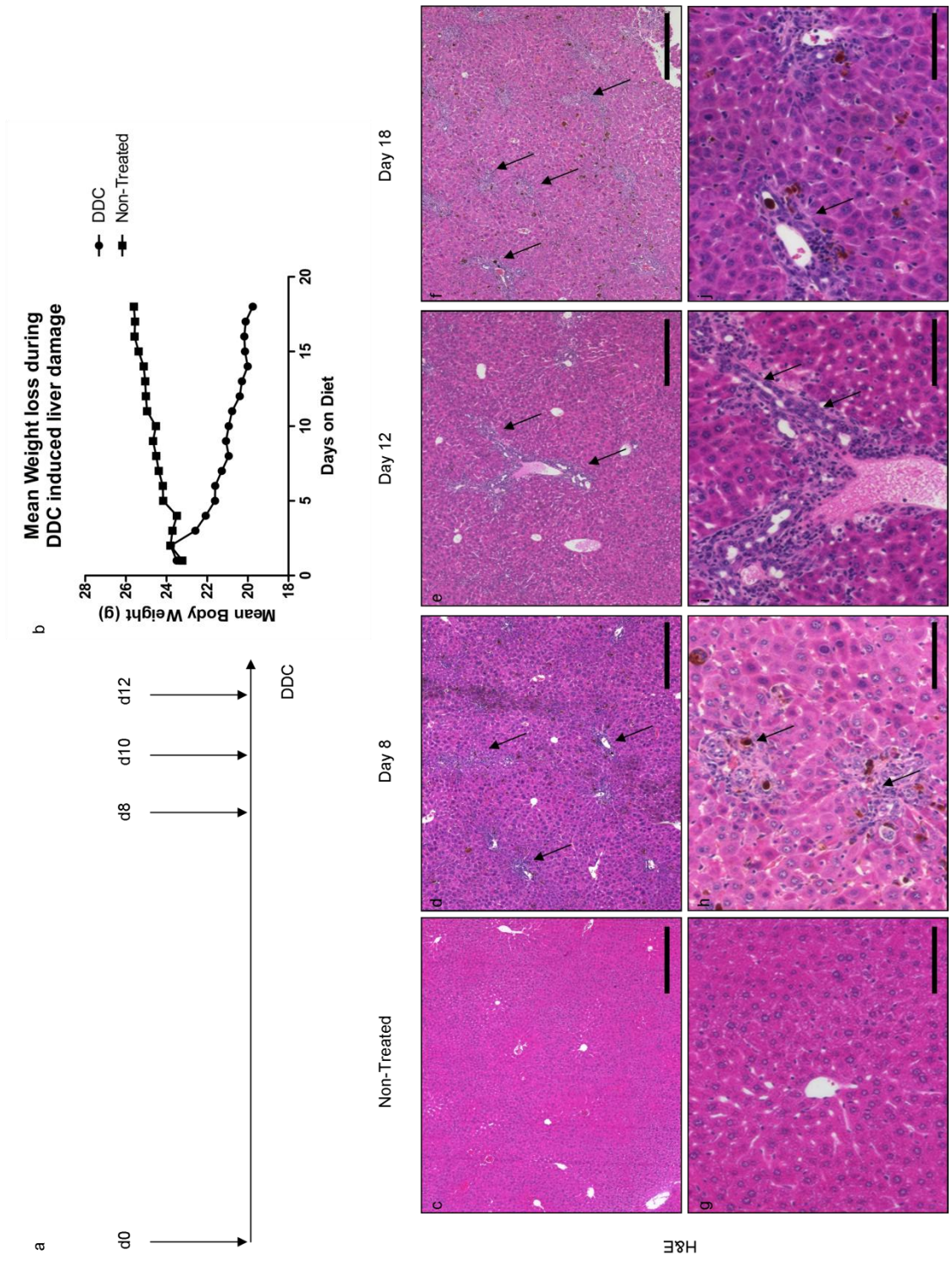

Figure 3.6 — Biliary damage is induced using the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) dietary model

Figure 3.6 — Biliary damage is induced using the 3, 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) dietary model

a. The DDC diet was administered for 18 days with normal drinking water; Animals were harvested at days 8, 12 and 18. **b.** Throughout the time course of DDC animals suffer from progressive weight loss which does not recover by 18 days (daily weights, n = 30-50, means plotted) . **c, g.** Throughout the course of damage animals move from having a normal hepatic architecture (d0) to acute portal inflammation by day 8 of DDC treatment (**d, h**) which gets progressively more aggressive throughout the regime out to day 12 (**e, i**) with large regions of infiltrating inflammatory cells and matrix deposition around the portal tracts by day 18 of DDC treatment (**f, j**).

The DDC model induces cell turnover through apoptosis and proliferation

The DDC model of biliary injury and regeneration unsurprisingly induces a large degree of apoptosis within the ductular epithelium which can be clearly visualised using TUNEL staining for DNA double strand breaks. (Figure 3.7 a, b) Throughout the duration of DDC TUNEL staining gradually increases; interestingly I found that hepatocytes are also apoptotic, indicating that during long term DDC there is a degree of secondary parenchymal damage in response to primary biliary death, and as such is representative of human cholestatic disease where secondary necrosis of the parenchyma is common.

H & E stains of the DDC model demonstrate the emergence of small HPC-like cells after 8 days, which form pseudo ducts surrounded by an inflammatory infiltrate. These new structures have a small, ill defined lumen, but do not migrate out into the parenchyma; rather they remain close to the portal tract and assume a more biliary phenotype. These structures are highly proliferative by 8 days as demonstrated by staining with Ki67 (Figure 3.7 c, d-f).

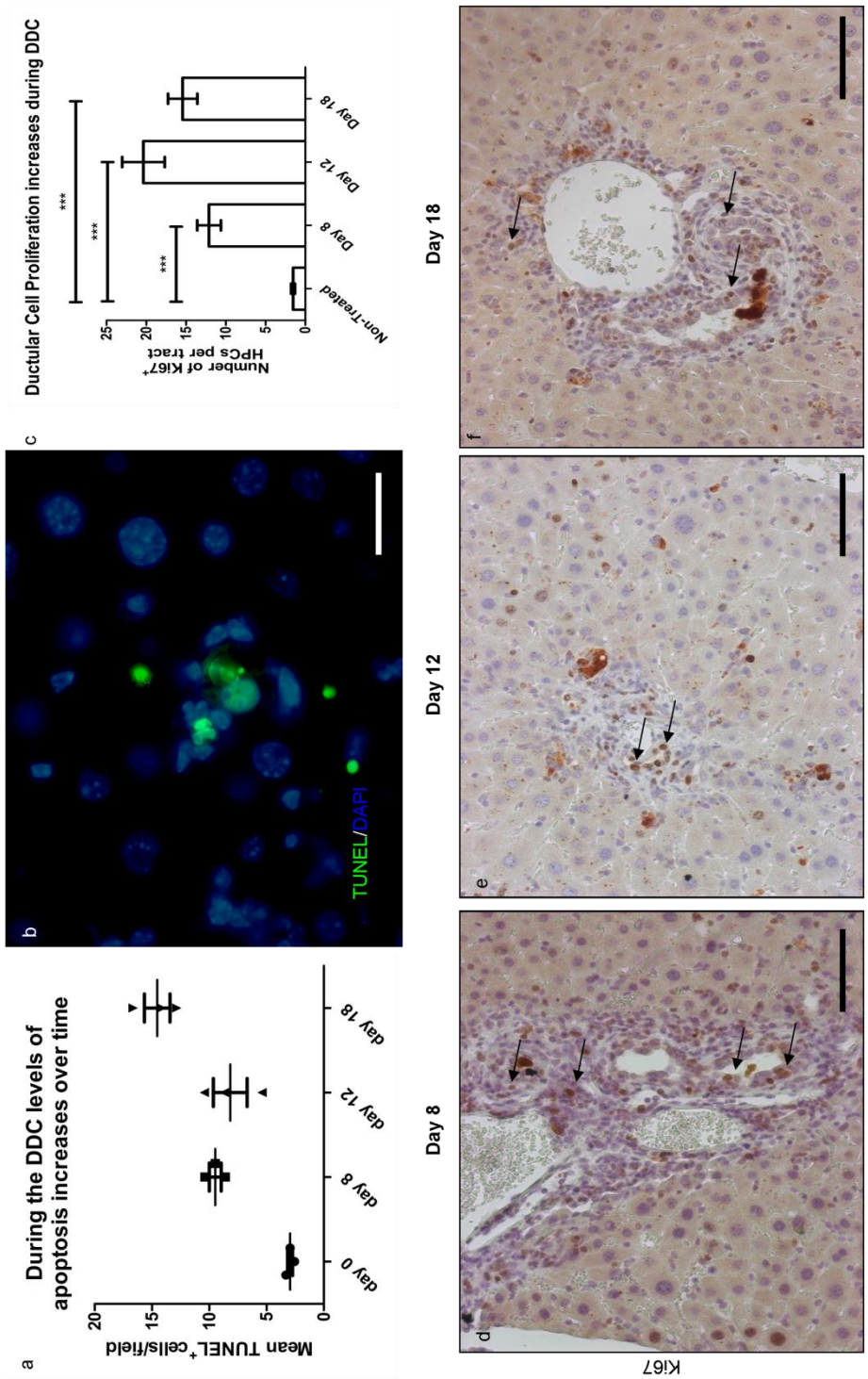

Figure 3.7 — The DDC model causes ductular apoptosis and proliferation.

Figure 3.7 — The DDC model causes ductular apoptosis and proliferation

a. During DDC there is an increase in hepatic injury, with progressively increasing levels of apoptosis as through increased TUNEL positivity when evaluated over 18 days. **b.** Demonstrates a representative cluster of apoptotic biliary cells induced by the DDC (TUNEL in green) **c.** In response to increased apoptosis HPCs are activated and proliferate. In the DDC model of liver damage there is a significant increase in the number of proliferating HPCs (Non-Treated 1.556 ± 0.24 vs. d8 12.13 ± 4.19, d12 20.38 ± 7.596 and d18 15.44 ± 5.52. For non-treated and day 18 n=9 for day 8 and day 12 n=8. *** p <0.001, a D'Agostino & Pearson omnibus normality test demonstrated that the data was normally distributed, as such a Student's t test was used to analyse these data.) **d-f** Increased proliferation can be visualised using Ki67 detection. Over 18 days the number of HPCs which are close to the portal tract, and positive for Ki67 increases (black arrows), of note however it is worth highlighting that there are proliferating inflammatory cells also surrounding the ductular structures.

The DDC model of biliary regeneration results in activation of HPCs

The resting liver has clearly defined panCK positive ducts with a wide lumen (Figure 3.8a). During treatment with DDC however, activation and proliferation of HPCs results in the rapid emergence of atypical ductular structures which remain close to the portal tracts as previously discussed. Similarly to the CDE ductular HPCs are positive for panCK. Throughout the time course of the DDC damage the network of panCK positive cells becomes increasing extensive and begins to develop an atypical lumen (Figure 3.8b-d).

The DDC model of regeneration is highly reproducible with similar injury responses between animals. Analysis of a small number of animals from each time point showed a remarkable homogeneity between animals on the DDC diet; at days 8, 12 and 18 animals have a similar mean number of luminal ducts per portal tract (Figure 3.8e). To confirm that there was indeed a ductular HPC response within DDC treated animals, I quantified panCK positive structures with a clearly defined lumen. These structures increase significantly by day 8 and continue to increase out to day 18 of damage (Figure 3.8f). The number of ducts per portal tract was compared to that of CDE. Following CDE diet HPCs have a much more “infiltrating phenotype with a ductular network similar to that of a non-treated animal.

As in the CDE model of hepatocyte regeneration DDC induced HPCs widely express panCK; however they also express EpCam and Dlk1 which co-localise within the ductular structures. This may indicate differences in the maturity of these cells *in vivo* (Figure 3.9 a, b).

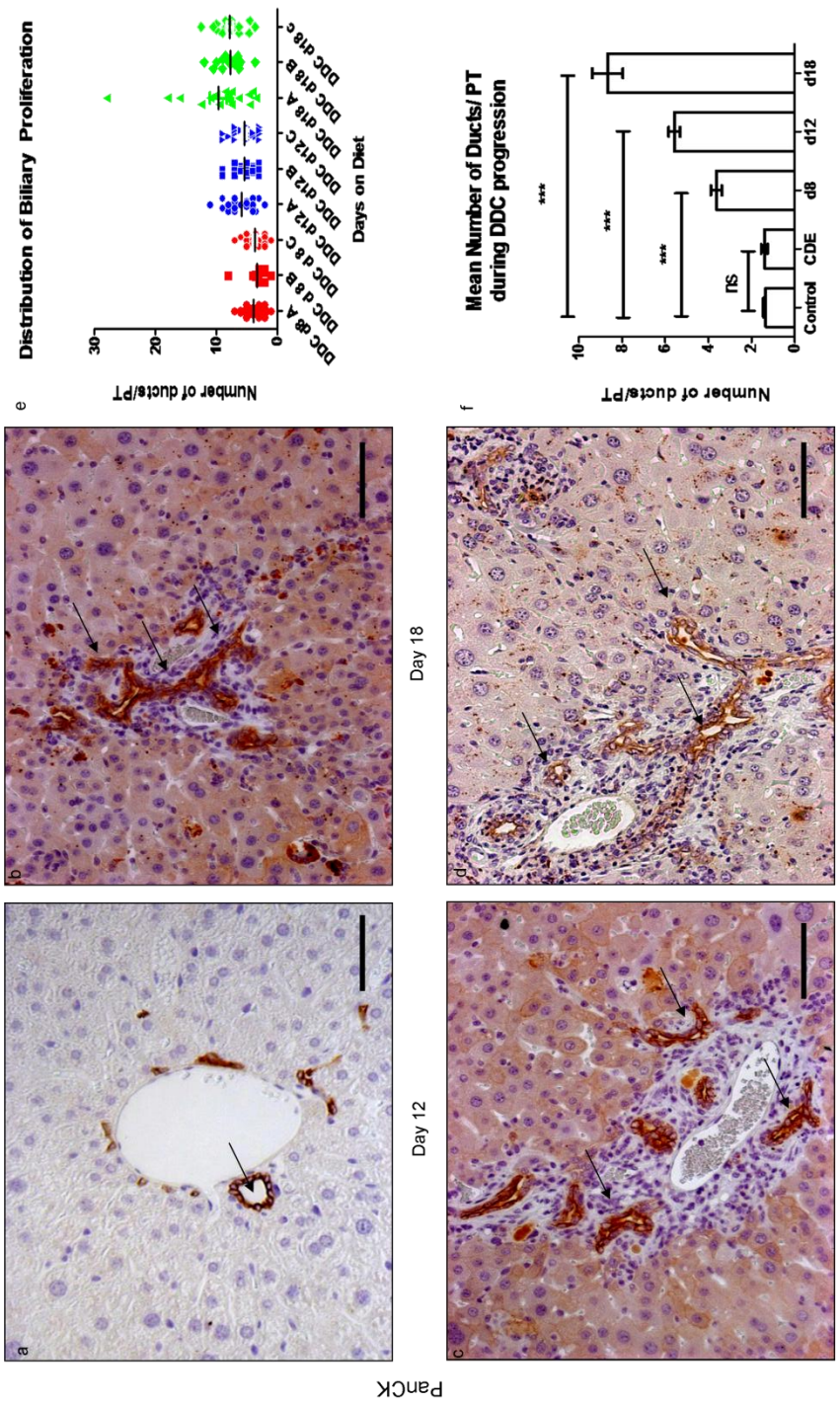

Figure 3.8 — The DDC regime induces a biliary progenitor response in adult mice.

Figure 3.8 — The DDC regime induces a biliary progenitor response in adult mice.

a. The healthy portal tract demonstrating a clear well defined portal tract (black arrow). **b.** During early DDC damage and regeneration small ductular structures appear from the portal tract and are associated with a modest inflammatory niche (black arrows denote the emergence of HPCs). **c, d** Throughout damage the number of atypical ductular structures increases associated with a sizable inflammatory infiltrate (black arrows). **e.** The DDC model is highly reproducible between animals with increasing numbers of ducts surrounding each portal tract the majority of which have poorly defined lumen (n=20 portal tracts counted in three independent animals per experiment). **f.** The numbers of ducts per portal tract increases throughout the damage time course (d0 0.40 ± 0.1 vs. d8 3.63 ± 0.25 vs. d12 5.58 ± 0.26 vs. d18 8.67 ± 0.7) n=4-6, ***$p<0.001$ using a Mann-Whitney U test

Figure 3.9 — The DDC diet activates a heterogeneous population of HPCs.

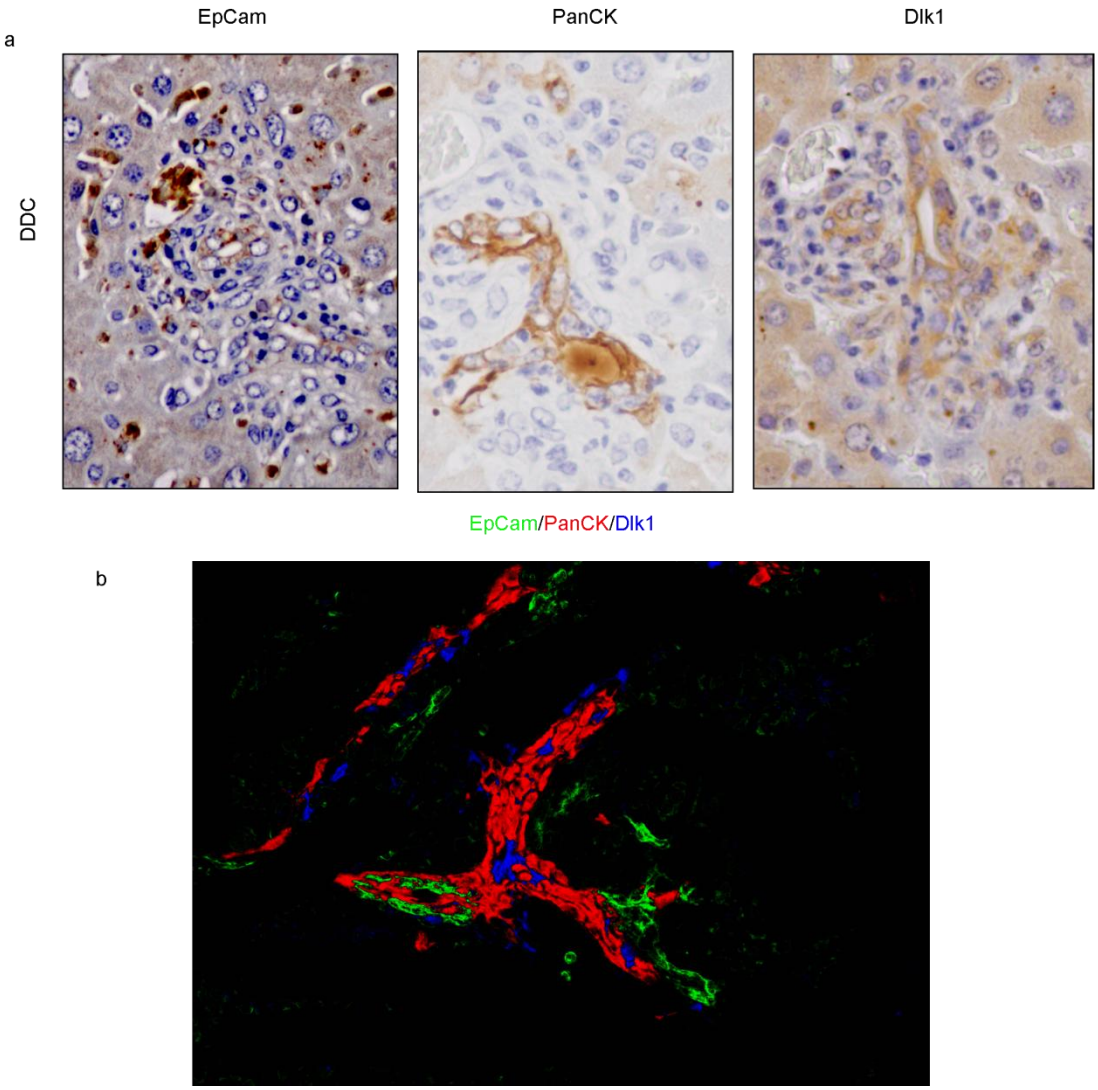

Figure 3.9 — The DDC diet activates a heterogeneous population of HPCs

a. During the DDC regime HPCs can be identified through their positivity for numerous antigens including EpCam, Dlk1 and panCK. These markers are expressed throughout the regenerative process. **b.** Using digital false colouring to represent EpCam in green, panCK in red, and Dlk1 in blue we stacked these images to look at co-localisation of the three markers, which despite having some regions of co-localisation also have exclusive expression domains, with panCK being most broadly expressed.

Localised peri-portal inflammation is associated with biliary regeneration

As with the CDE model of hepatocyte regeneration I find an impressive infiltration of inflammatory cells. Early in the DDC model there is a peri-portal association of HPCs with a thick band of small inflammatory cells. In healthy adult liver macrophages are typically localised throughout the parenchyma, however within 8 days these cells have migrated from the parenchyma and surround the emerging biliary ducts (Figure 3.10 a, b). This migration and association appears to increase over time with increased damage and regeneration. By 18 days of injury macrophages form dense cell layers around portal tracts (Figure 3.10 c, d). As in the CDE dietary model there is a very modest increase in the number of macrophages within the liver as a whole; however their distribution during damage and regeneration appears to be important in the restoration of normal biliary architecture and function (Figure 3.10 e).

The DDC model of biliary restoration has a much more aggressive activation of myofibroblasts which form direct associations with the emerging HPCs. Even at 8 days of DDC treatment there is a sizable sheath of myofibroblasts encircling each new duct. (Figure 6.10 g). The ensheathment of these ducts increases over time and by 18 days are many cell layers thick (Figure 3.6 f- i). During regeneration of the bile ducts αSMA positive myofibroblast numbers increase dramatically (Figure 3.10 j), but are infrequently found within the parenchyma – suggesting they may play an important role in the maintenance of ductular HPCs during chronic biliary disease and could play an important role in the regulation of HPC fate choice.

Figure 3.10 — The DDC diet is associated with an aggressive inflammatory response throughout the liver.

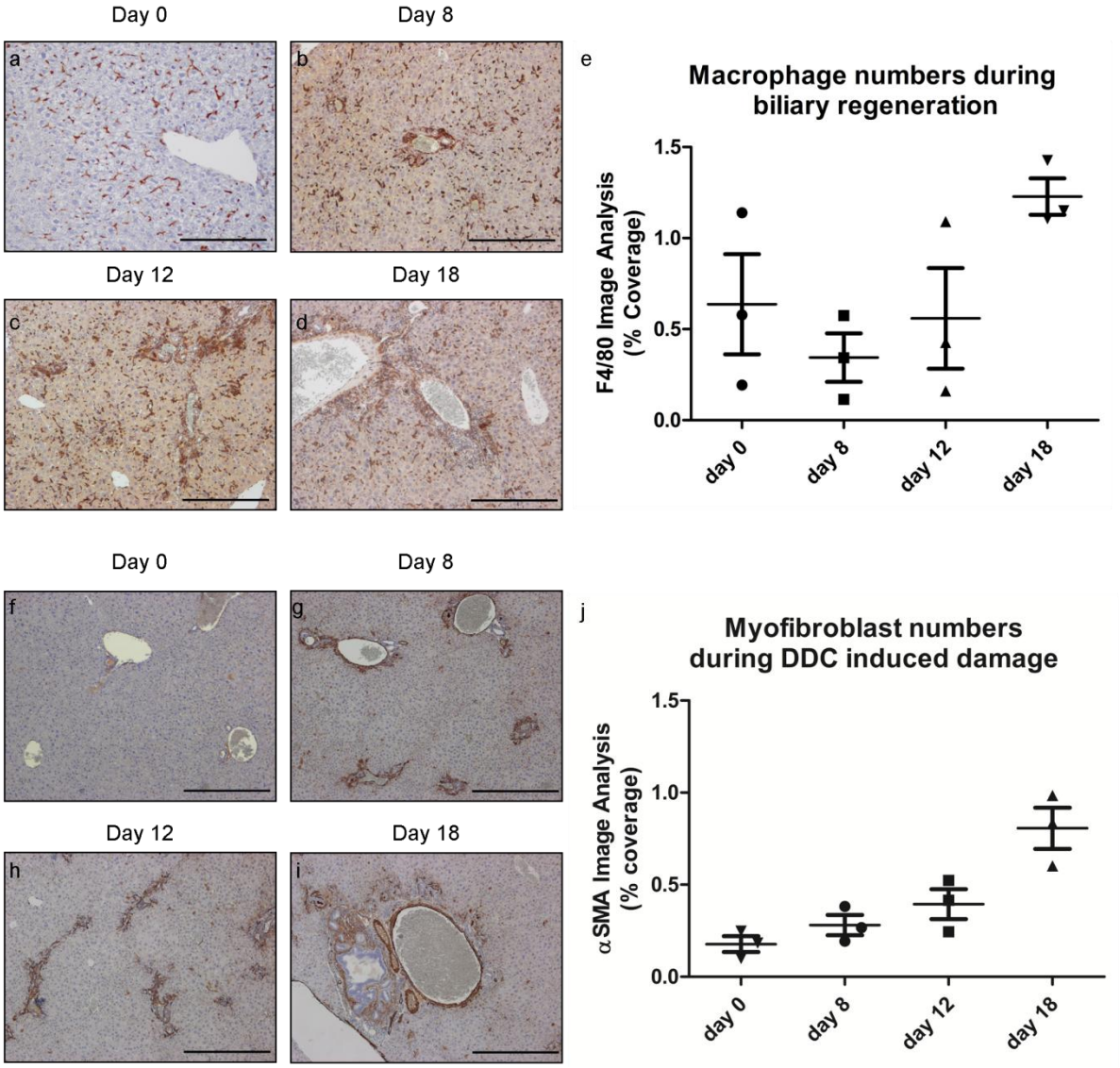

Figure 3.10 — The DDC diet is associated with an aggressive inflammatory response throughout the liver.

a. Macrophages are scattered throughout the parenchyma in healthy liver tissue, however very rapidly these cells migrate to the portal tracts. **b-d.** Over the DDC time course the association of Macrophages with HPCs increases by d18. **e.** The overall number of macrophages in the liver does not increase significantly. **f-i** During the DDC diet there is a progressive increase in the number of myofibroblasts which are closely associated with HPCs close to the portal tract. **j.** The overall number of myofibroblasts increases throughout the liver during regeneration. (n=3 for each time point. No significant difference with a Mann-Whitney U test. Scale Bars 400μm)

Hepatic Progenitor Cells can be isolated and cultured *in vitro*

To investigate the mechanisms of lineage specification I established a protocol by which HPCs could be isolated and cultured *in vitro*. Animals were treated with 12 days of either CDE or DDC. Whole livers were digested using an in situ perfusion protocol. Briefly, livers were perfused with an EDTA solution to remove blood, and chelate free metal ions. This was followed by a Collagenase and DNase digestion. The liver was removed and dissociated, and then further digested to ensure liberation of large cell numbers. Hepatocytes were removed from the cell suspension by slow centrifugation. The resulting non-parenchymal cells were separated though a density gradient, depleted for CD45 positive cells using MACS and plated on laminin (Figure 3.11 a). I assessed depletion of the CD45 positive cells from these cultures and found that there is an almost complete CD45 depletion following MACS (Figure 3.11 b). After 12 days of culture, HPCs proliferate and appear to have a cobble stone morphology with a high nuclear to cytoplasmic ratio, as described in the literature (Figure 3.11 c)

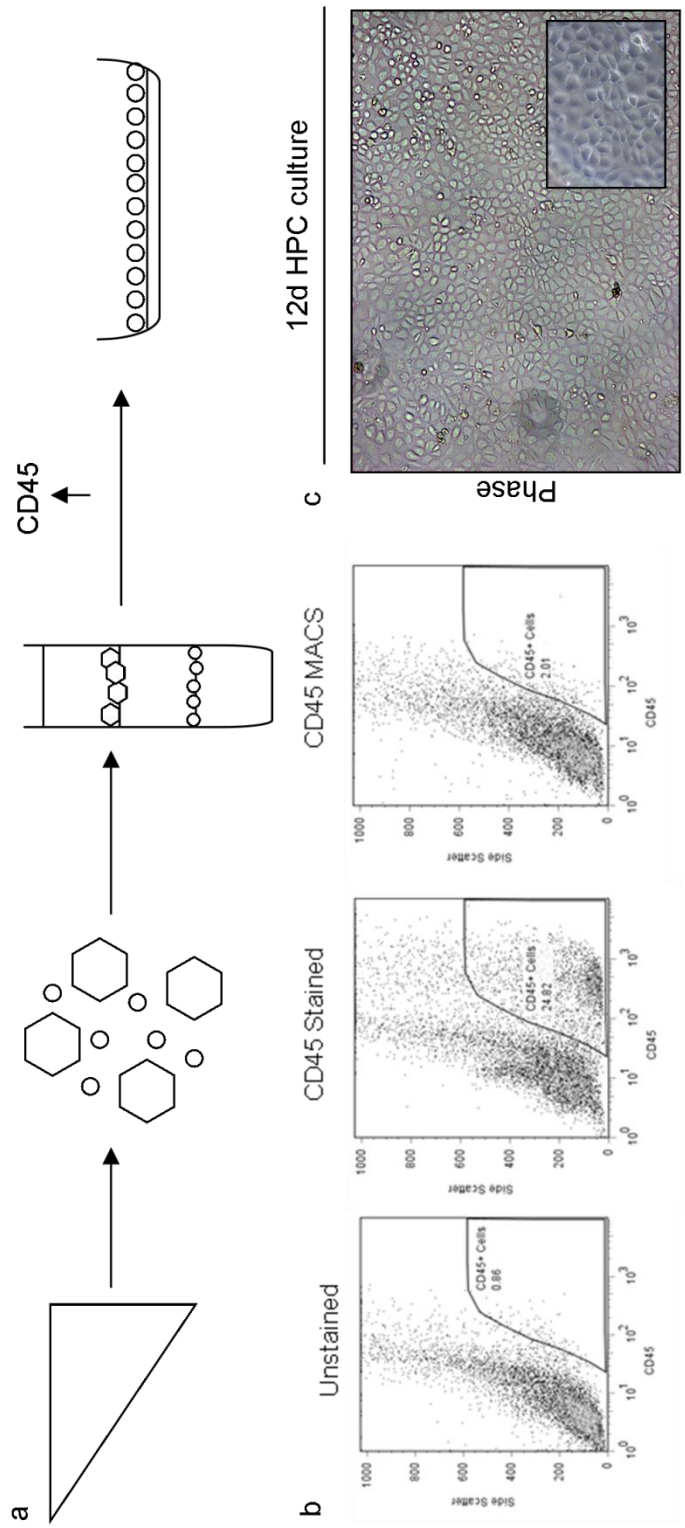

Figure 3.11 — Hepatic Progenitor Cells can be isolated and cultured in vitro

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Figure 3.11 — Hepatic Progenitor Cells can be isolated and cultured *in vitro*

a. Adult liver was digested into a single cell suspension and separated based on cell size using a density gradient. Small Cells were placed on Laminin and allowed to grow for 12 days before being used. **b.** To remove any circulating haematopoietic cells from the liver cell culture cells were purified by depletion of CD45 positive cells using MACS selection. **c.** HPC cultures regularly formed large densely packed colonies of morphological epithelial-like cells

Primary HPCs are positive for panCK after culture, which localises to fibres in the HPC cytoplasm (Figure 3.11 a). Primary HPC cultures express early hepatic transcription factors from the Hnf family. I observed expression of Hnf1α, Hnf4α, Hnf1β and Hnf6 at the level of transcript (Figure 3.11 b) within the epithelial population suggesting that this population has the ability to become both cholangiocytes and hepatocytes.

Further to this isolation and culture FACS for Dlk1 was established in the laboratory in order to isolate HPCs from animals undergoing liver damage without the need for *in vitro* cell culture which may alter the phenotype of the HPCs, and may result in spurious data. FACS isolation with Dlk1-FITC also offers a specific and 'clean' population of HPCs for gene expression analysis. Figure 3.11 c demonstrates a typical plot from CDE liver Dlk1 antibody; staining with which gave a clear population which could be sorted from other cell types, It should be noted that a FITC-Isotype control antibody gives a negligible shift in the FITC channel and as such a population which emerges with Dlk1-FITC could be sorted and correlated with the Dlk1 positive populations I observe *in vivo*.

Figure 3.12— Hepatic Progenitor Cells express hepatocyte enriched transcription factors and can be isolated ex-vivo for direct transcript analysis

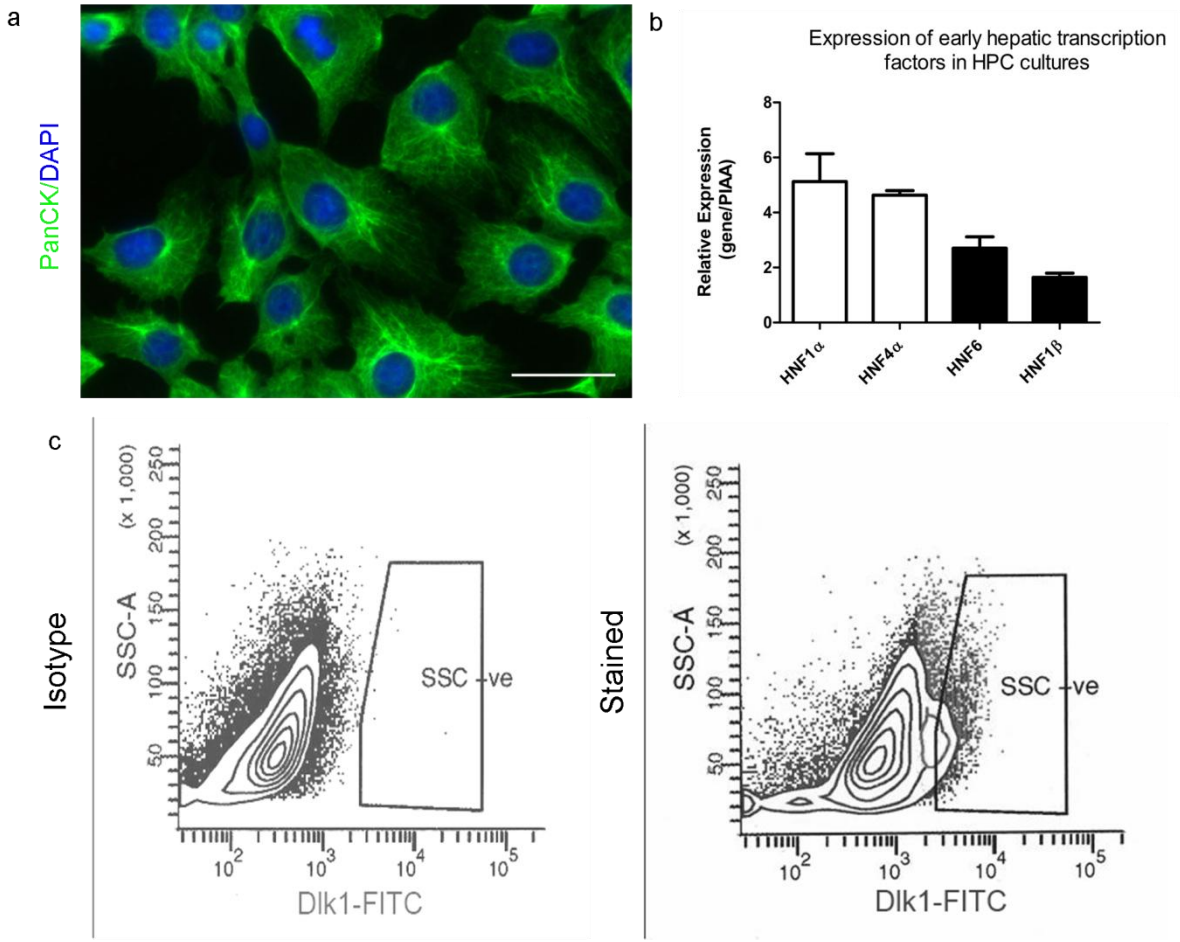


Figure 3.12 — Hepatic Progenitor Cells express hepatocyte enriched transcription factors and can be isolated ex-vivo for direct transcript analysis

a. Primary HPCs cultures express cytokeratins, which when detected with panCK demonstrates dense fibrils throughout the cytoplasm (green) scale bar = 20μm. **b.** Primary HPCs which have been cultured express liver enriched transcription factors Hnf1α, Hnf4α, Hnf6 and Hnf1β. **c.** HPCs can be isolated using FACS for Dlk1 from damaged liver. Isotype treated samples demonstrate very little shift in the FITC channel, however using a Dlk1-FITC a population of HPCs can be identified and sorted form the remaining cells within the population.

Discussion - CDE and DDC dietary models provide two diverse patterns of hepatic regeneration in adult liver

During chronic patterns of human liver disease both parenchymal, hepatocellular damage and biliary, cholangiocytic damage must be replaced by an endogenous population of HPCs. The mechanisms by which these diverse fates are regulated are currently understudied, and as a result it is impossible to manipulate these pathways in order to promote "healthy" regeneration and a positive clinical outcome.

Both the CDE and DDC models of hepatic damage and repair have been used previously to great success in induction of HPCs within the adult liver[60, 63]; however the difference in these disease models has not been addressed, and as such they have not been used to model diverse liver pathologies. This chapter examines how in both CDE and DDC there is localised damage to the hepatocytes and cholangiocytes respectively. In both these regenerative contexts I have found that there is an increase in HPCs alongside a concurrent increase in the macrophage and myofibroblast cell populations and that these classical inflammatory cells appear closely associated with HPCs and may be involved in the maintenance and specification of progenitor cells (Figure 3.13)[6]. This description may offer some insight into how adult HPCs are regulated.

Interestingly I found that HPCs express multiple markers, panCK, Dlk1 and EpCam; all of which have been classically associated with varying stages of hepatoblast development during ontogeny. During adult regeneration these markers associate with one another, however not all HPCs express these markers and as such may

indicate an inherent heterogeneity of HPCs during adult hepatic regeneration or may define degrees of naivety within the adult HPC population.

By utilising both the CDE and DDC models of hepatic regeneration, coupled with cell isolation and culture I can modulate components of varying signalling pathways to elucidate the fundamental mechanisms behind specification of adult HPCs.

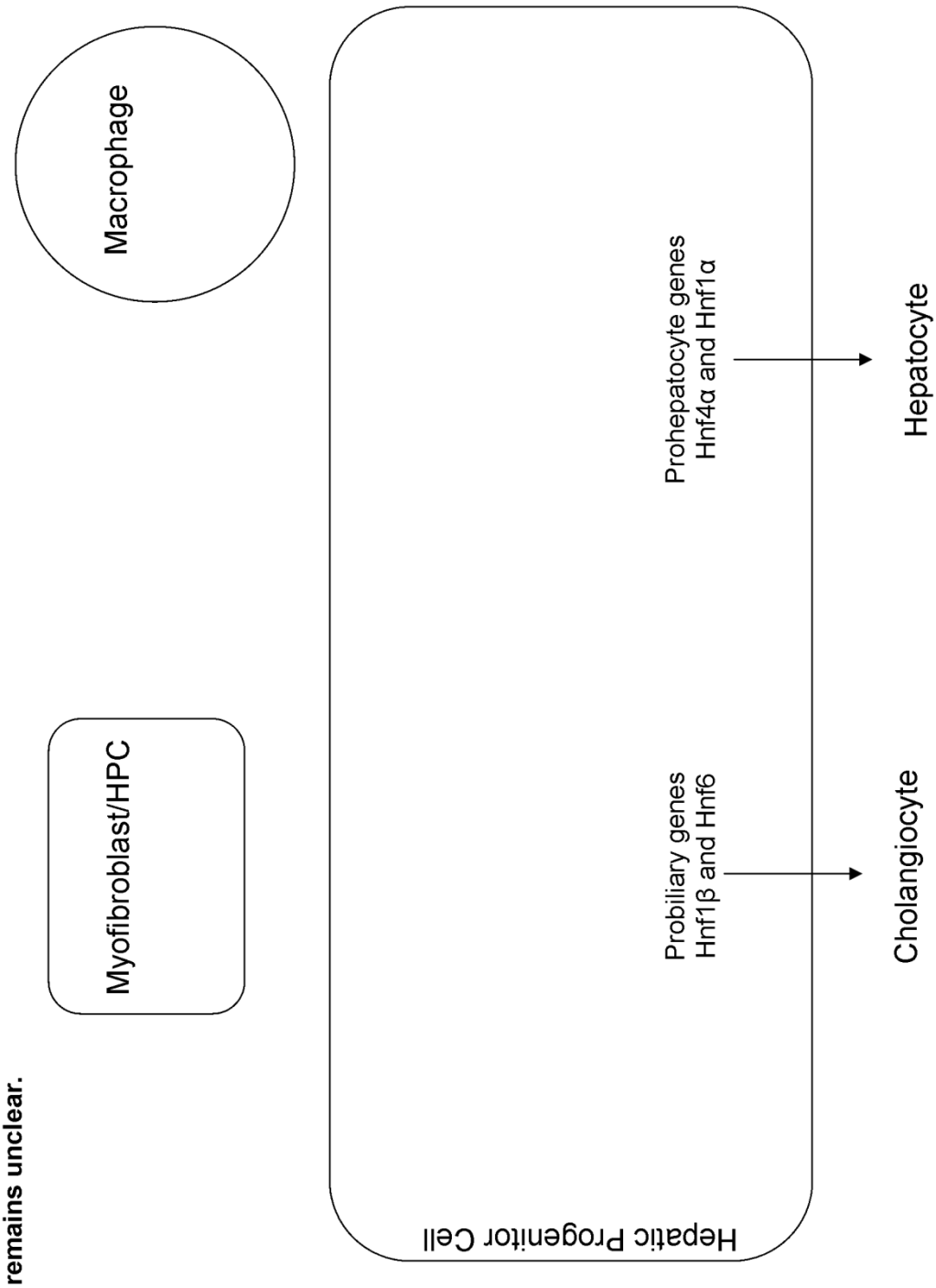


Figure 3.13— The adult HPC is associated with an inflammatory niche, but how its lineage is controlled remains unclear.

Figure 3.13— the adult HPC is associated with an inflammatory niche, but how its lineage is controlled remains unclear.

HPCs are found in two models of chronic liver disease—which have different regenerative patterns. In both of these systems HPCs are found closely associated with a niche which consists predominantly of macrophages and myofibroblasts. HPCs express early transcription factors which are associated with either a biliary or hepatocyte fate, however how these genes are controlled is yet to be ascertained, and the mechanisms upstream of these factors are not described.

Chapter 4 - Characterisation and Interrogation of the Notch pathway during hepatic regeneration

Introduction:

During hepatic ontogeny the Notch signalling pathway is required for correct patterning and specification of the developing biliary tree from embryonic hepatoblasts[220]. *In vivo* ablation of the Notch-2 receptor, or indeed ablation of RBPj in foetal hepatoblasts results in the retardation of biliary tree formation. Further to this over-expression of the Notch intra-cellular domain, which acts to constitutively activate the Notch signalling in these isolated hepatoblasts results in up regulation of early biliary lineage genes such as Hnf1β and Hnf6 and reduces expression of late hepatocyte makers such as Hnf4α and CEBP/α[222, 223]. During human development abrogation of the Notch signalling pathway, caused by mutation in Jagged-1 and/or Notch-2 results in the heterogeneous Notch pathway disorder Alagilles syndrome. Alagilles syndrome is typified by bile duct paucity as well as other congenital malformations. Interestingly sufferers of Alagilles syndrome fail to induce an HPC response in later life indicating that an intact Notch signalling pathway is needed for the emergence of HPCs[228].

The initial aim of this chapter is to characterise the Notch signalling pathway during both biliary and hepatocellular regeneration in the mouse and to understand whether there is likely to be an intact Notch signalling pathway during hepatic regeneration during chronic damage. This work led to the second aim discussed in this chapter which deals with interrogation and blockade of the Notch signalling pathway both *in vitro* and *in vivo*. Further to this I identify the endogenous mechanisms which may prevent inappropriate Notch activation during regeneration.

The Notch pathway is differentially activated during Biliary and Hepatocellular regeneration.

During regeneration of the adult liver HPCs differentially express components of the Notch signalling pathway depending on whether they are assuming a hepatocellular of biliary fate[322]. I have also investigated mouse models of hepatocellular and biliary regeneration (the CDE and DDC regimes respectively) to ascertain whether the Notch signalling pathway is differentially regulated in these contexts as this would offer an important tool in understanding the mechanism of HPC specification in two divergent hepatic disease phenotypes.

I have found that during liver regeneration in the mouse, $Dlk1^{+}$ isolated HPCs express Notch1 and Notch2 mRNA transcripts at high levels during regeneration of the bile ducts after DDC injury. In contrast to this I have also found that $Dlk1^{+}$ HPCs isolated during hepatocellular regeneration induced by the CDE regime express low levels of Notch1 and Notch2 (Figure 4.1a). These data suggest that my models may recapitulate high Notch state found during biliary regeneration in human PSC/PBC.

To further these initial observations I investigated the downstream targets of the Notch signalling pathway. Here I demonstrate that Hes1 expression differs only moderately between these two injury states, this is perhaps unsurprising as I know that Hes1 expression is downstream of other pathways which are potentially activated in murine $Dlk1^{+}$ HPCs. Instead I analysed expression of Hes5 and HeyL, both of which are more highly expressed at the mRNA level during DDC than during

CDE, once again indicating that during biliary regeneration the Notch signalling pathway is highly upregulated and highly activated (Figure 4.1a).

During biliary regeneration in the mouse I detected high transcript levels of both Notch1 and Notch2. To ascertain whether I observe both of these receptors expressed on the HPCs during biliary regeneration I used immunohistochemistry for these receptors, in tandem with EpCam (an alternative marker which co-localises with Dlk-1 during hepatic regeneration, observe results, chapter 3) to demonstrate their presence at the level of protein during biliary regeneration. I have found that during biliary regeneration Notch1 demonstrates consistent nuclear localisation within the $EpCam^{+}$ HPC population (Figure 1b), again indicating that this receptor may be cleaved and is translocating to the nucleus. However during hepatocellular regeneration I only observe infrequent Notch1 positive cells which co-stain with EpCam (Figure 4.1c). During both hepatocellular and biliary regeneration Notch-2 protein is undetectable within the HPC population, however can be faintly observed within the associated mesenchyme during biliary regeneration (data not shown). Of particular interest is that during human patterns of biliary and hepatocellular regeneration in PBC/PSC and HCV respectively in collaboration with Olivier Govaere and Tania Roskams, Leuven Belgium we find that there is a recapitulation of this paradigm where the Notch pathway is highly activated during biliary regeneration.

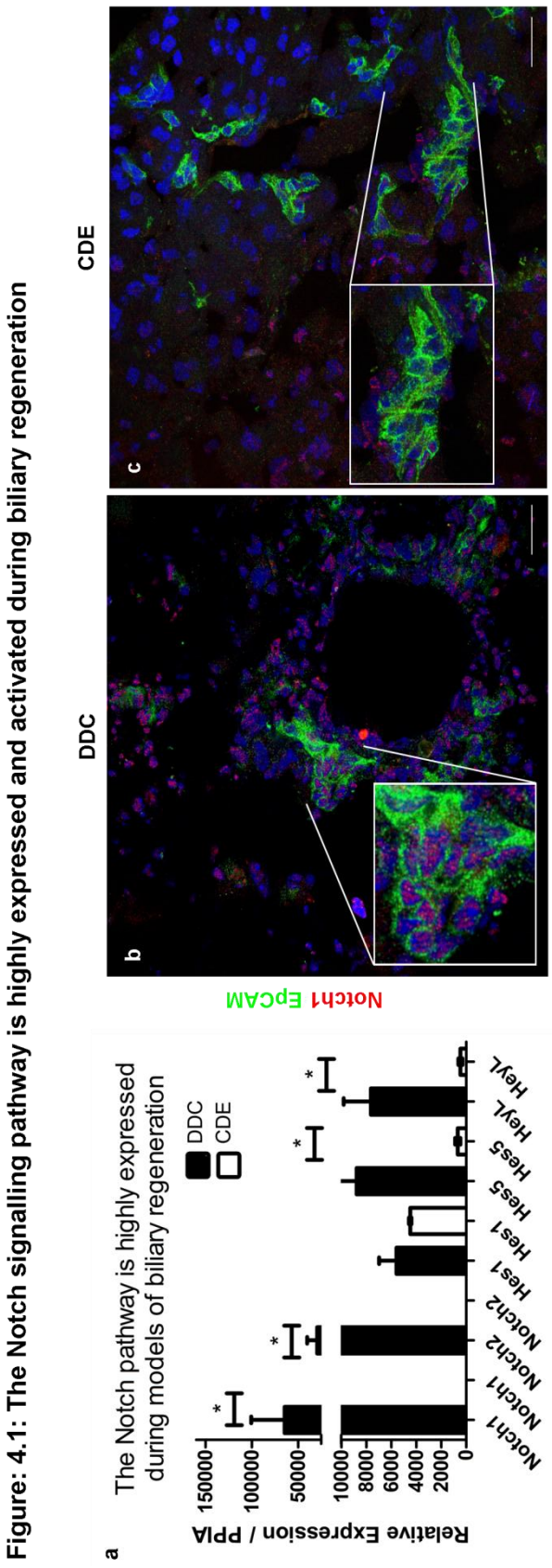


Figure: 4.1: The Notch signalling pathway is highly expressed and activated during biliary regeneration

Figure: 4.1: The Notch signalling pathway is highly expressed and activated during biliary regeneration

a. The CDE and DDC mouse models show differential expression of the receptors Notch1 (DDC: 64659 ± 36087 vs. CDE 12.78 ± 1,784) and Notch2 (DDC 29525 ± 10687 vs. CDE 2.015 ± 0.87) with high expression in HPCs isolated from DDC. Associated with these high levels of Notch receptor expression I find higher levels of Notch targets Hes1 (DDC 5595 ± 1391 vs. CDE 4491 ± 92.88), Hes5 (DDC 8785 ± 4266 vs. CDE 688.8 ± 211.4) and HeyL (DDC 7030 ± 2182 vs. 475.4 ± 166.3) expressed in DDC. For each group, DDC and CDE n=4, *$p<0.05$ according to a Mann-Whitney U test **b, c.** During biliary regeneration in the DDC I observe accumulation of Notch1 in the nucleus of EpCam positive HPCs, however during hepatocellular regeneration in the CDE I observe an absence of Notch1 within the HPC nucleus, this correlates with my data indicating a high Notch state during biliary repair, but not during hepatocellular regeneration. Scale bar = 100μm

During Biliary Regeneration Jagged1 is found in close association with Notch positive HPCs.

Activation of the Notch pathway is mediated through binding of a membrane tethered ligand from the Delta or Jagged families to the Notch receptor. During murine regeneration I find high transcript expression of Jagged1 during biliary regeneration after administration of DDC; with lower expression of this Jagged1 mRNA during CDE induced hepatocellular repair (Figure 4.2a). I further characterised the protein expression of Jagged1 in the mouse using immunohistochemistry for Jagged1, however rather than finding expression of the protein in the HPC population as described during human disease by Tania Roskams, and Olivier Govaere, Leuven Belgium, unpublished, personal communication, I found strong staining in the associated inflammatory infiltrate. To investigate this further I looked at two major consentient components of the inflammatory infiltrate myofibroblasts and macrophages (as described in the previous chapter) to ascertain whether these cells are the ligand expressing cells during regeneration.

Macrophages associated with murine biliary damage and restoration do not express the Notch ligand Jagged-1. F4/80^{+} cells surround the site of regeneration, however are infrequently found associated directly with the emerging HPC population (Figure 4.2b). There is rather, a population of cells directly adjacent to the HPCs which expresses Jagged1. I have found that αSMA positive myofibroblasts are often, but not always positive for Jagged1 suggesting some heterogeneity within the HPC associated myofibroblast population (Figure 4.1c). During murine biliary

regeneration these myofibroblasts form direct contact with HPCs and form sheaths around the emerging bile duct ranging from one cell layer to multiple cell layers which give an “onion-skin” phenotype around the duct.

During hepatocellular regeneration myofibroblasts can also form intimate associations with HPCs, however in this context I never found a αSMA positive myofibroblast which is positive for Jagged1 (Figure 4.1d) and as such conclude that as well as the Notch receptors being lowly expressed during hepatocellular repair that the cellular stereotypical niche is rather dynamic in the pathway components it expresses. It should be noted that again in human disease in collaboration with Olivier Govaere and Tania Roskams, Leuven, Belgium we find a higher levels of the Notch ligand JAGGED1; however this does not appear to localise to the inflammatory infiltrate, it is rather expressed by HPCs perhaps indication of a species difference during hepatic regeneration or could be reflective of disease progression.

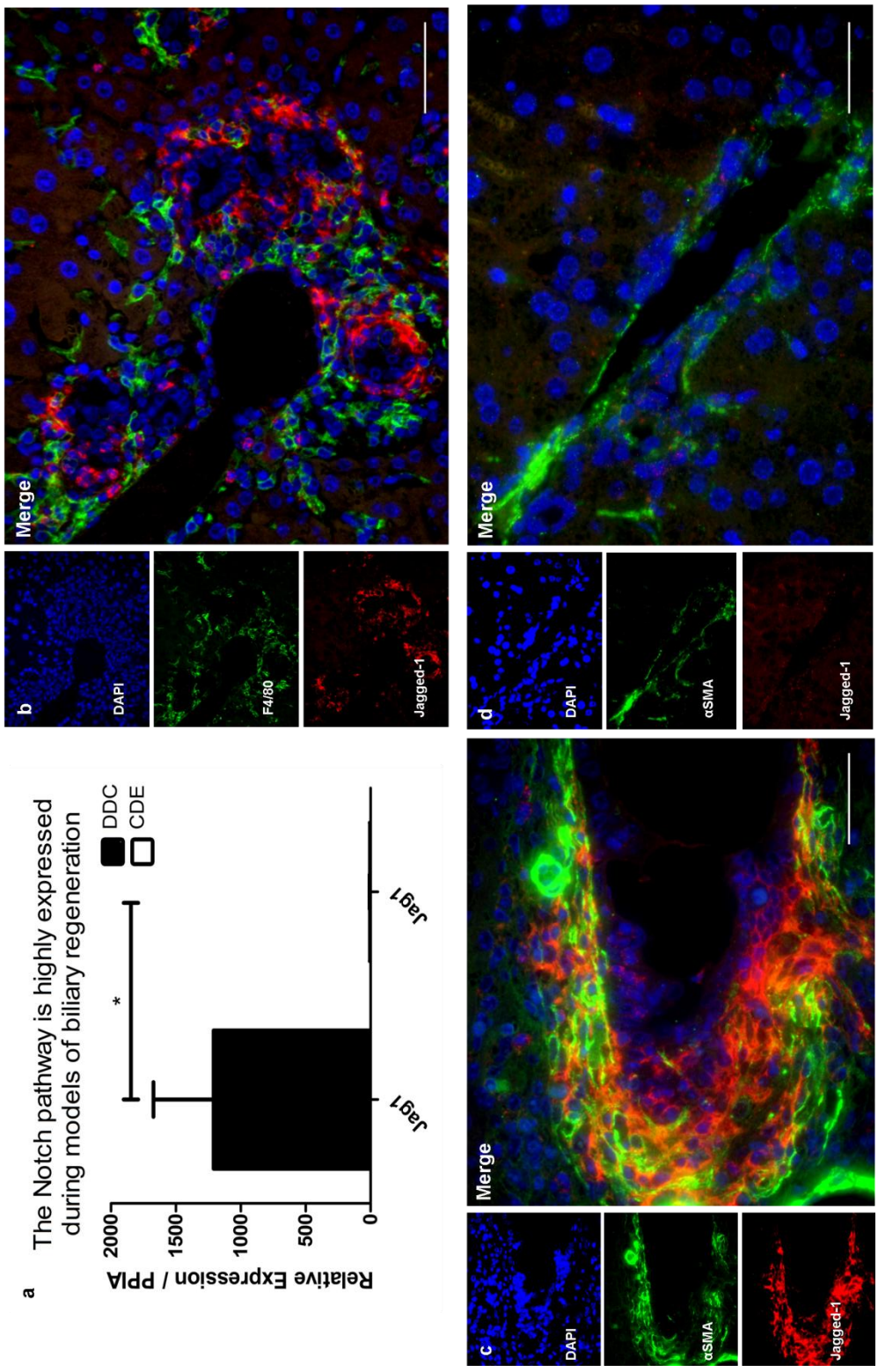


Figure: 4.2: The Notch ligand Jagged1 is highly expressed by myofibroblasts during biliary regeneration

Figure: 4.2: The Notch signalling pathway is highly expressed and activated during biliary regeneration

a. In mouse models CDE and DDC I find a differential expression of Jagged1 in isolated mHPCs, with negligible expression levels during hepatocellular repair (DDC 1211 ± 436.7 vs. CDE 6.153 ± 2.798.) n=4, *p<0.05 according to a Mann-Whitney U test **b.** In mouse Jagged1 (Red) shows a localisation which does not localise to the biliary epithelium similarly it is discrete from F4/80 positive macrophages (Green). **c.** During DDC Jagged1 co-localises with αSMA positive myofibroblasts, and form a sheath around the emerging HPCs which are regenerating bile ducts. **d.** During hepatocellular regeneration in the CDE regime αSMA positive cells can still form close associations with HPCs, however these myofibroblasts are Jagged1 negative during hepatocellular regeneration. Scale bar = 100μm

Inhibition of the Notch pathway *in vitro* attenuates expression of biliary genes.

To investigate the role of Notch signalling during specification of HPCs I isolated these cells and cultured them *in vitro*. These cells formed compacted epithelial colonies as previously described within the literature. These HPCs where co-cultured in contact with myofibroblasts under differentiation conditions for three days in the presence or absence of DAPT which prevents Notch signalling through inhibition of γ-secretase.

In vitro co-culture inhibition results in a reduction in biliary phenotype.

HPCs express the Notch1 receptor at the level of protein in HPCs when they have been isolated and cultured *in vitro* for up to 12 days (Figure 4.3a). *In vitro* cultured myofibroblasts express Jagged1 as protein on the cell surface (Figure4.3b). Having described that HPCs and myofibroblasts express receptor and ligand respectively I combined these cells *in vitro* in order to activate the Notch signalling pathway I analysed the effect of inhibiting this co-culture system using 10mM DAPT or DMSO vehicle alone (Figure 4.3c).

I found that during co-culture the Notch signalling pathway is activated as demonstrated through expression of Hes1, Hes5, Hey1 and HeyL. In HPCs alone I find only low expression of these pathway effector genes compared to when the HPCs are combined in co-culture with myofibroblasts (Figure 4.3 d and Figure 4.3 e). During DAPT treatment of these HPC/Myofibroblast co-cultures I demonstrate a

significant reduction in the expression of these Notch pathway effector genes, demonstrating efficient inhibition of γ-secretase and by extension the Notch signalling pathway (Figure 4.3e).

During treatment with DAPT I found a modest decrease of Hnf1β expression; a transcription factor classically associated with a biliary phenotype during hepatic ontogeny, and which has an adult expression pattern which is restricted to the bile duct. Along with this I also observe a significant reduction in the mRNA expression of a second biliary transcription factor Hnf6, and also in the expression of Gamma-glytamyl transpeptidase (Ggt), a clinical marker of biliary damage. During inhibition of γ-secretase *in vitro* I found no increase in the expression levels of HNF4α a transcription factor associated with terminal hepatocyte differentiation (Figure 4.3f); suggesting that inhibition of the Notch signalling pathway restricts a biliary phenotype; however blockade does not induce a hepatocyte phenotype within this co-culture system. Inhibition of the Notch signalling pathway importantly does not affect the expression of my housekeeping gene PPIA, or the proliferation of myofibroblasts *in vitro* assessed by monitoring cell density during the experiment. I can therefore extrapolate that the affect of DAPT on biliary fate is due to inhibition of the Notch pathway rather than through differences in gene normalisation or number of myofibroblasts.

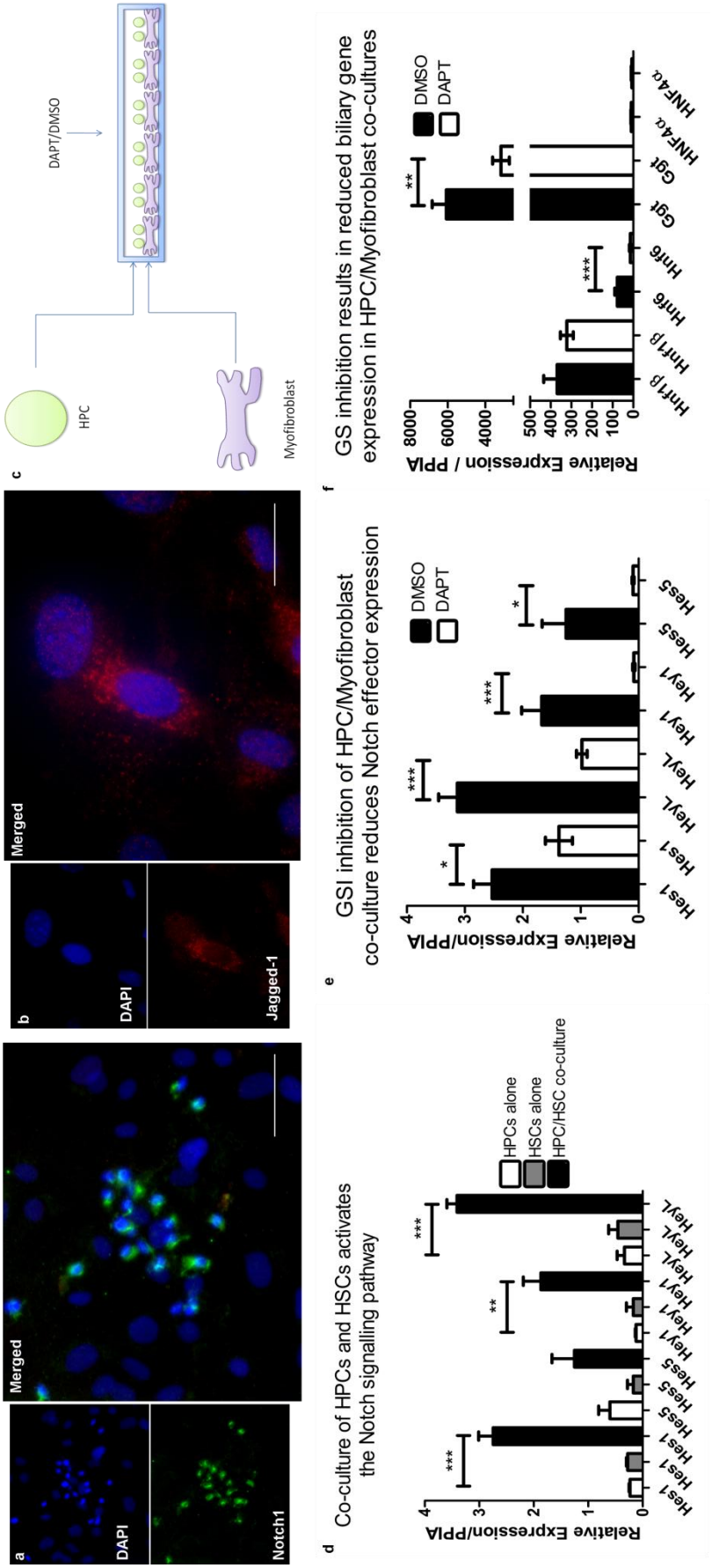


Figure 4.3: Inhibition of myofibroblasts/HPC co-cultures results in abrogated biliary gene expression:

Figure 4.3: Inhibition of myofibroblasts/HPC co-cultures results in abrogated biliary gene expression:

a. During culture of HPCs small colonies with epithelial 'cobble stone' morphology emerge in culture. These cells are small, have a high nuclear : cytoplasmic ratio and are positive for Notch1 receptor. **b.** Myofibroblasts isolated from healthy murine livers were activated by culture *in vitro*. Myofibroblasts are positive for αSMA (Red) which is typically fibrous. Cultured myofibroblasts are also positive for Jagged1 protein (Green) which is detected throughout the membrane and cytoplasm. **c.** Schematic representation of the co-culture experiment. HPCs and Myofibroblasts were cultured separately, and subsequently recombined *in vitro* in the presence of DAPT or vehicle (DMSO) alone. These cells were co-cultured for 3 days with repeat administration of DAPT on day 2. **d.** During co-culture of HSCs and HPCs Notch pathway effectors Hes-1 (HPC 0.2345 ± 0.017 vs. HSC 0.277 ± 0.025 vs. co-culture 2.749 ± 0.266), Hes-5 (HPC 0.604 ± 0.207 vs. HSC 0.174 ± 0.1078 vs. co-culture 1.354 ± 0.41), Hey1 (HPC 0.1236 ± 0.014 vs. HSC 0.1746 ± 0.128 vs. co-culture 1.871 ± 0.324) and HeyL (HPC 0.338 ± 0.131 vs. HSC 0.4592 ± 0.16 vs. co-culture 3.411 ± 0.187). **e.** Inhibition of HPC/Myofibroblast co-cultures with DAPT results in a reduction in Notch signalling pathway effectors Hes1 (DMSO 2.532 ± 0.32 vs. DAPT 1.377 ± 0.23), HeyL (DMSO 3.133 ± 0.3230 vs. DAPT 0.9829 ± 0.0906), Hey1 (DMSO 1.647 ± 0.3475 vs. DAPT 0.08625 ± 0.01981) and Hes5 (DMSO 1.254 ± 0.4185 vs. DAPT 0.983 ± 0.01693). **f.** In concordance with a reduction in Notch effector expression I find an abrogated expression of genes associated with the biliary lineage. I observe a modest reduction in the transcription factor Hnf1β (DMSO 369.8 ± 64.14 vs. DAPT 322.7 ± 31.28) however I observe a significant

reduction in the expression of Hnf6 (DMSO 77.94 ± 12.52 vs. DAPT 14.97 ± 4.57) and Ggt (DMSO 6082 ± 757.7 vs. DAPT 3169 ± 449.0). Interestingly during inhibition I observe promotion of the hepatocyte gene Hnf4α (DAPT 10.38 ± 1.338 vs. 8.039 ± 1.898). In all experiments n=4 repeated 3 times. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Data analysed using a Mann Whitney U test Scale bar = 10μm

Inhibition of gamma-secretase *in vivo* results in attenuated biliary regeneration without affecting fibrosis

During inhibition of the Notch signalling pathway in my co-culture system I demonstrate a reduction in biliary specification; to demonstrate that the Notch signalling pathway is required for biliary specification *in vivo* I inhibited γ-secretase *in vivo* using DAPT. Using the DDC diet to model biliary damage and regeneration for twelve days I administered a repeat dose of DAPT at 50mg/Kg daily for 3 days (Figure 4.4a). The physiological efficacy of DAPT administration was confirmed by the conversation of the small intestine into a secretory phenotype typified though high numbers of goblet cells as previously described in the literature (Figure 4.4b).

Figure 4.4: Inhibition of the Notch pathway *in vivo* induces intestinal conversion

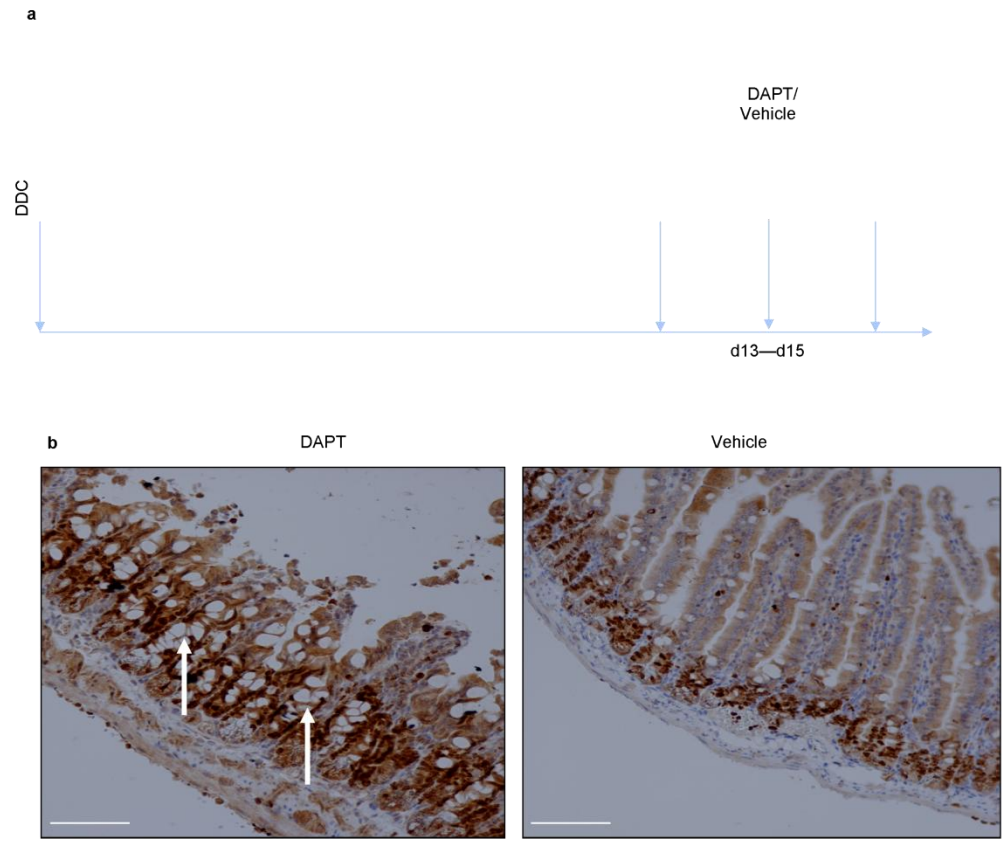


Figure 4.4: Gamma-Secretase inhibition *in vivo* results in a reduction of biliary phenotype

a. During biliary regeneration, where I have described HPCs as being in a Notch high state I have used DAPT given repeatedly between day 12 and day 15 in order to inhibit the cleavage of Notch receptor in the liver. **b.** As a positive control for γ-secretase inhibition I used small intestine and demonstrated that when treated with DAPT the intestinal epithelium assumes a more secretory phenotype when compared with control mice as described in the literature (white arrows in the DAPT treated animal highlight the large number of goblet cells). Scale bar = 100μm

Does Gamma-secretase inhibition affect proliferation and apoptosis in the regenerating adult liver?

DAPT acts as a general inhibitor of γ-secretase, and as such has off-targets affects on pathways other than those mediated through the Notch receptor. For example both Epidermal Growth Factor Receptor (EGFR) and amyloidal-beta are processed by γ-secretase, as well as numerous other membrane tethered proteins – and as such gamma-secretase inhibition may alter the survival of HPCs through deregulation of proliferation and/or apoptosis. As such I have investigated whether there is any change in the rate of proliferation or degree of apoptosis during DAPT treatment compared to Vehicle alone. I analysed the number of KI67 positive cells as a means of demonstrating changes in proliferation. As previously described I saw proliferation of HPCs, inflammatory cells and hepatocytes during DDC treatment. During γ-secretase inhibition I found that there was no detectable difference in the degree of proliferation when compared to vehicle treated alone (Figure 4.5a). Conversely I investigated whether administration of DAPT during biliary regeneration resulted in HPCs becoming apoptotic. I found no significant difference in the rate of biliary apoptosis between DAPT and control groups when TUNEL, suggesting that inhibition of the Notch signalling pathway does not result in increased apoptosis during biliary regeneration (Figure 4.5b).

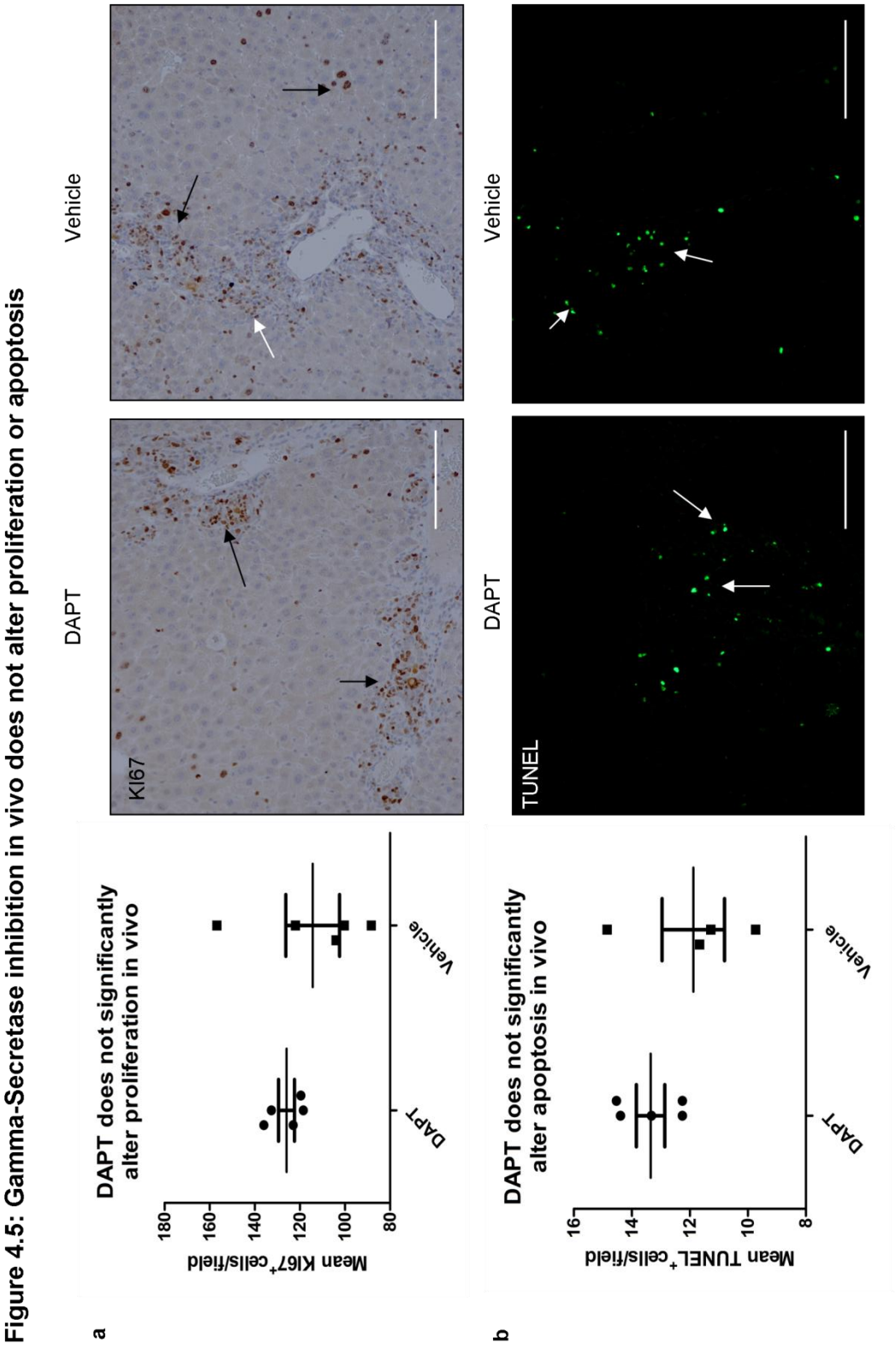


Figure 4.5: Gamma-Secretase inhibition in vivo does not alter proliferation or apoptosis

Figure 4.5: Gamma-Secretase inhibition *in vivo* does not alter proliferation or apoptosis

a. During DAPT administration the amount of proliferation is not significantly changed *in vivo* as assessed through quantification of KI67 positive cells. (DAPT 126.0 ±3.536 vs. Vehicle 114.3 ± 11.92). Black arrows denote positive, nuclear staining in HPCs **b.** DAPT administration does not significantly affect the rate of apoptosis during biliary regeneration (DAPT 13.36 ± 0.49 vs. DMSO 11.89 ± 1.075) as assessed through quantification of TUNEL positive cells in DAPT vs. Vehicle treated animals. For both groups n=5, data was analysed using a Mann-Whitney U test. Scale bar = 100μm.

Gamma-secretase inhibition results in a reduction of HPCs and reduced biliary gene expression

As I found no difference in the proliferation and apoptosis during DAPT administration I quantified the number of HPCs in animals treated with gamma-secretase inhibitor versus control animals. Inhibition of γ-secretase resulted in the significant reduction in the number of HPCs during biliary regeneration as demonstrated through quantification of $panCK^+$ cells in both DAPT and control livers (Figure 4.6a). To demonstrate that this reduction is due to a loss of biliary phenotype I isolated HPCs through a discontinuous Percoll gradient as inhibition of γ-secretase may have some off target affects on processing of other surface molecules such as Dlk1 and EpCam.

Inhibition of γ-secretase is associated with a reduction in the expression of Hes1 and Hes5, effectors of Notch signalling pathway; this loss of Hes expression indicates that in isolated cells the Notch pathway has been appropriately inhibited, although as the expression of these genes is not completely attenuated then I cannot assume complete inhibition of this pathway. The reduction in activation of the Notch signalling pathway is associated with a loss of biliary gene expression in DAPT treated animals vs. controls. I demonstrate a significant reduction in the mRNA expression of two transcription factors Hnf1β and Hnf6 which are both associated with commitment to an early biliary lineage during hepatic ontogeny (Figure 4.6b).

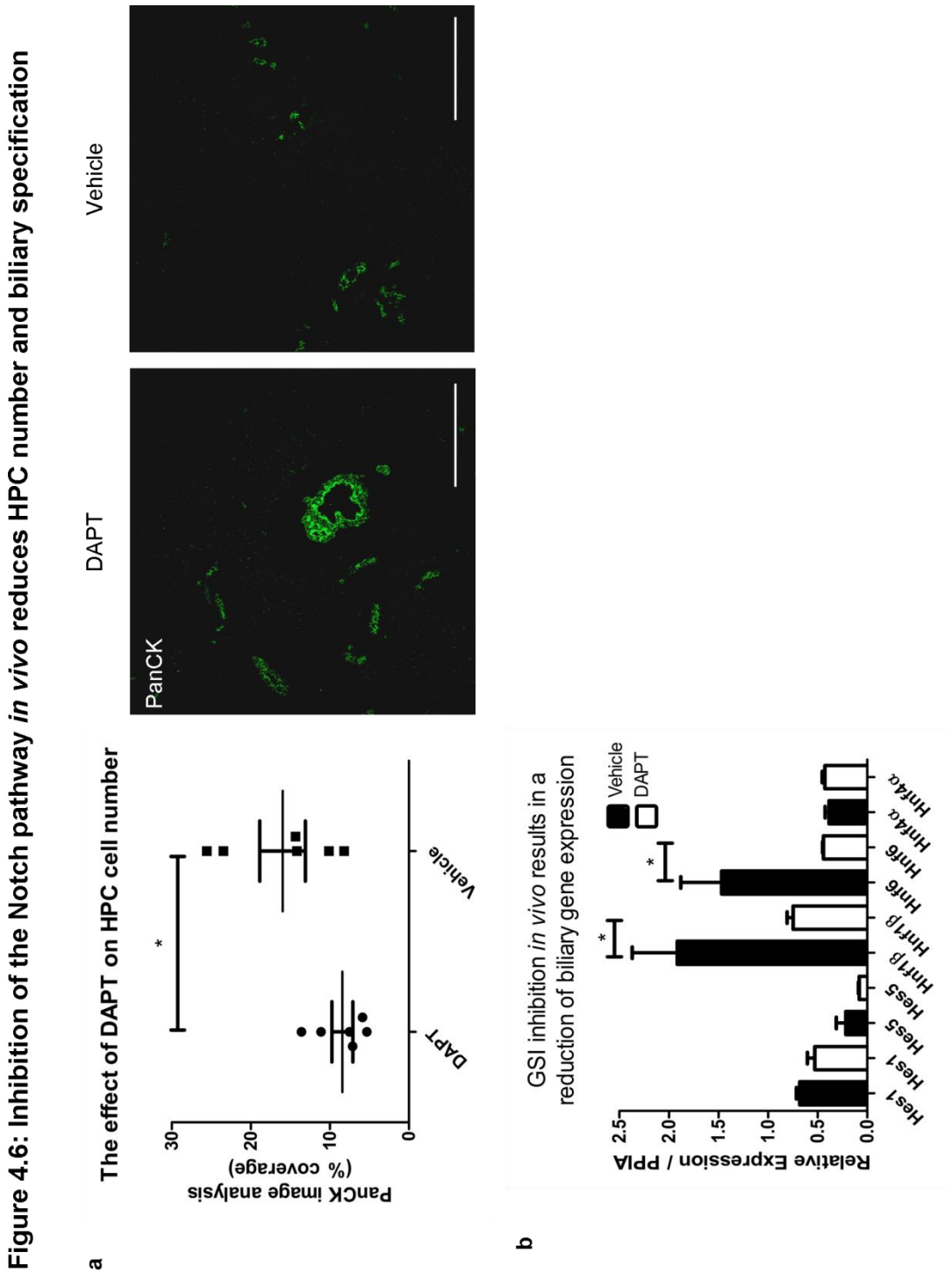


Figure 4.6: Inhibition of the Notch pathway *in vivo* reduces HPC number and biliary specification

Figure 4.6: Inhibition of the Notch pathway *in vivo* reduces HPC number and biliary specification

a. During DAPT administration I found that panCK HPC positivity throughout the liver was significantly decreased when compared to mice injected with vehicle alone (DAPT 8.437 ± 1.325 vs. Vehicle 16.00 ± 2.883).**b.** The decrease in panCK positive HPC number during biliary regeneration is associated with a decrease in Hes5 expression (DAPT 0.08 ± 0.008 vs. Vehicle 0.21 ± 0.09) as well as early biliary lineage genes Hnf1β (DAPT 0.749 ± 0.05 vs. Vehicle 1.918 ± 0.45) and Hnf6 (DAPT 0.41 ± 0.008 vs. Vehicle 1.47 ± 0.44). However I observe no change in the expression of the hepatocyte associated gene HNF4α (DAPT 0.42 ± 0.02 vs. Vehicle 0.386 ± 0.03). For both groups n=5, data was analysed using a Mann-Whitney U test. Scale bar = 100μm.

Inflammation and fibrosis are not affected by DAPT administration

Inhibition of γ-secretase and the Notch signalling pathway with DAPT may have some off target affects on other cell types within the liver, despite not observing expression of the Notch receptors throughout other cells, separate pathways may be affected. For example that CX3CL1/Fractalkine shedding by myofibroblasts in the liver affects chronic inflammation and fibrosis, as this shedding is mediated in part by γ-secretase[323], inhibition with DAPT could result in a reduction in fibrosis which results in a loss of biliary proliferation. To assess whether the change I observe in fate of HPCs is due to a change in the surrounding stroma I analysed the numbers of myofibroblasts and macrophages during biliary regeneration and during DAPT inhibition.

During the DDC regime I observe the emergence of portal fibrosis found, (as previously described in chapter 3). Repeated DAPT administration during DDC treatment and biliary regeneration does not significantly reduce the levels of portal fibrosis as demonstrated through image analysis of picrosirius red (PSR). Peri-portal PSR staining is still present surrounding the ductules and the distribution of this staining does not change with DAPT treatment (Figure 4.7 a).

PSR acts as a measure of total fibrillar collagen, and as such does not give any indication of whether during DAPT administration there is a change in the cellular components of the HPC niche. As I have previously described, macrophages and myofibroblasts are closely associated with the HPC reaction during biliary

regeneration and that in mouse Jagged1 is expressed by myofibroblasts; to this end I quantified αSMA and F4/80 to investigate whether γ-secretase inhibition resulted in a modulation of myofibroblasts and macrophages *in vivo*.

During biliary regeneration in either in the presence or absence of DAPT I found that the number of αSMA positive myofibroblasts remained constant during regeneration and that the spatial relationship of these cells to HPCs did not change compared to control vehicle only treated animals where myofibroblasts form close sheath-like associations with HPCs (Figure 4.7b). I made comparable observations when quantifying macrophages. As previously demonstrated these cells are found throughout the hepatic parenchyma, and also in close association with the regenerating bile ducts (Figure 4.7c). Administration of DAPT does not have any effect on the number or localisation of macrophages during biliary regeneration in the adult mouse.

Figure 4.7: Inhibition of gamma-secretase does not affect inflammation and fibrosis in vivo.

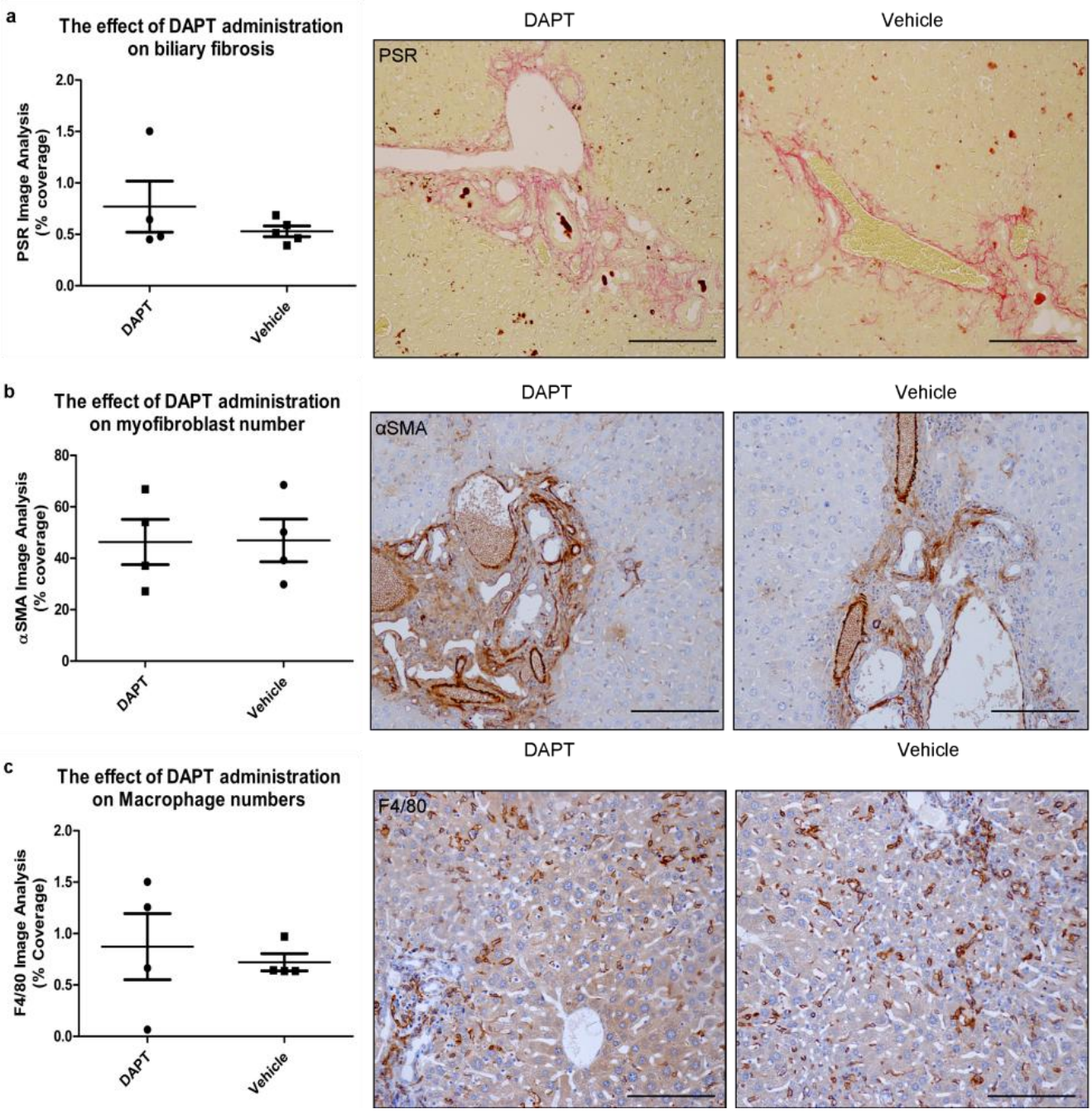


Figure 4.7: Inhibition of gamma-secretase does not affect inflammation and fibrosis *in vivo*.

a. During the course of DDC administration I observe a progressive increase in fibrillar collagen as detected by PSR. Treatment with DAPT does not cause a change in collagen deposition and fibrosis when compared to Vehicle alone (DAPT 0.76 ± 0.24 vs. Vehicle 0.529 ± 0.05). **b.** To demonstrate that γ-secretase inhibition does not alter the presence of myofibroblasts, which are closely associated with HPCs I stained for αSMA protein and quantified this, I found no significant difference in the numbers of myofibroblasts between those treated with γ-secretase inhibitor and vehicle alone (DAPT 46.94 ± 8.229 vs. Vehicle 46.30 ± 8.817).**c.** Similarly I observe no significant change in the amount of staining for F4/80 within these groups (DAPT 0.8721 ± 0.32 vs. Vehicle 0.655 ± 0.09). For each group n=4-5, data generated using image analysis of >30 adjacent, non-overlapping images in each case. Scale bar = 100μm.

During Hepatocellular Notch signalling is endogenously inhibited through expression of Numb

I have shown that during hepatocellular regeneration in my murine model CDE that components of the Notch signalling pathway are differentially expressed when compared with biliary regeneration in PBC/PSC and DDC We have confirmed this paradigm also exists within the context of human diseases such as PBC/PSC and HCV, in collaboration with Olivier Govaere and Tania Roskams, Leuven Belgium. I have also demonstrated that activation of the Notch signalling pathway is significantly abrogated during hepatocellular regeneration as effectors of the Notch signalling pathway, namely members of the Hes/Hey gene family are down regulated. Ectopic expression of the Nicd in hepatocytes, and as such constitutive activation of the Notch pathway results in the transdifferentiation of hepatocytes into CK19 expressing "biliary-like" cells[220] - This observations led me to ask whether there are any associated repressors of the Notch signalling pathway which may also act to suppress Notch signalling during hepatocellular regeneration, accounting for a second tier of Notch repression and as such a more complete prevention of aberrant Notch signalling.

Using my murine models of damage and regeneration I investigated whether I can find instances of Notch suppression during hepatocellular regeneration. Initial analysis of whole liver transcripts suggested that during hepatocellular regeneration induced with CDE, and biliary regeneration induced with DDC that there is a

difference in the levels of Numb during these divergent regenerative contexts (data not shown).

During normal hepatic homeostasis Numb is expressed within the bile ducts, however during biliary damage and regeneration isolated $Dlk1^{+}$ HPCs demonstrate a loss of Numb transcript (Figure 4.8 a). I confirmed the loss of Numb protein by staining a time course of DDC damaged livers. Numb protein is lost within the first 8 days of damage and this loss persists out to 18 days (Figure 4.5 b). During the DDC regime designed to give biliary damage I at times also observe some associated zone-one hepatocellular death; as such I also find very infrequent Numb positive HPCs during biliary regeneration – which I speculate remain in order to regenerate the hepatic parenchyma.

During CDE induced hepatocellular regeneration I find the converse to be true. As described previously the number of HPCs increases over time during CDE administration all of these HPCs are Numb positive throughout the time course I studied, suggesting that Notch signalling is inhibited during this process. Isolated HPCs demonstrated a clear persistence of Numb expression with a non-significant trend to up-regulation of Numb transcript expression (Figure 4.8 c) I also demonstrate that these cells stained strongly for Numb protein with definite cytoplasmic and membranous localisation; supporting the notion that during hepatocellular regeneration the Notch signalling pathway must be suppressed (Figure 4.8 d).

In collaboration with Olivier Govaere and Tania Roskams I investigated whether NUMB was important in HPCs during two patterns of human disease. In these two regenerative contexts I found that NUMB was indeed highly expressed in HCV, but expression of NUMB was repressed in the human diseases PSC and PBC – a context in which Notch signalling is activated.

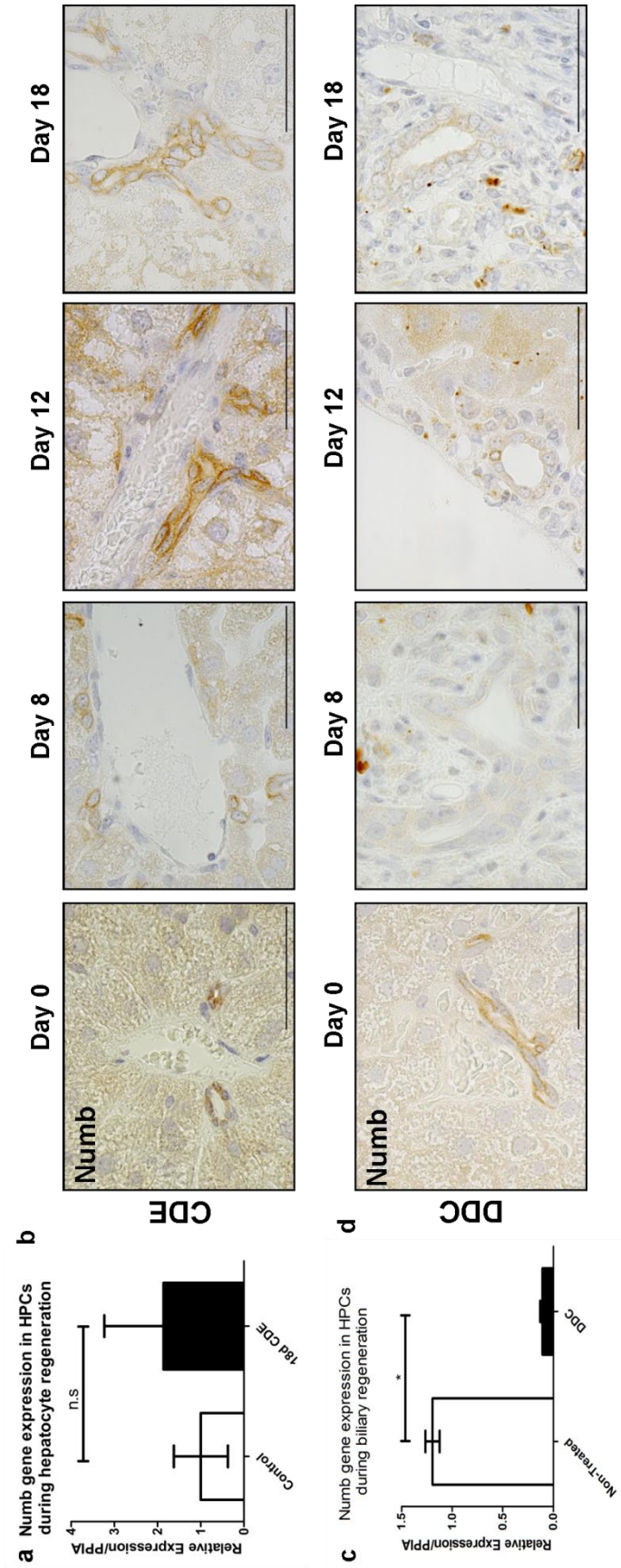


Figure 4.8: During Hepatocellular regeneration Numb is highly expressed, but is lost during biliary regeneration

Figure 4.8: During Hepatocellular regeneration Numb is highly expressed, but is lost during biliary regeneration

a. At 18 days this positivity was confirmed through analysis of Numb transcript which is present at high levels following 18 days of CDE injury (Control 1.00 ± 0.025 vs. CDE 1.866 ± 1.369) . **b.** During CDE induced hepatocellular regeneration I found that over 18 days Numb protein persists in the HPC population. **c.** The loss of Numb from HPCs was confirmed by analysing the expression of Numb transcript. In contrast to what I observe during hepatocellular regeneration, I find that during biliary repair there is a significant reduction in Numb gene expression. (Control 1.194 ± 0.070 vs. DDC 0.108 ± 0.022). **d.** During biliary regeneration the presence of Numb protein throughout the biliary regeneration is below levels of immunohistological detection, with only occasional Numb positive cells persisting (DDC, day 12). In each experimental group n=4, significant differences were demonstrated using Mann Whitney U test *$p<0.05$. Scale Bar = 100μm.

Discussion – Notch signalling is important for biliary regeneration but dispensable during hepatocyte repair

During hepatic regeneration I observe a cellular niche which is stereotypical as previously described by our group and others. However during divergent patterns of disease, damage and as such repair we observe a diverse signalling phenotype emerge in order that HPCs would correctly regenerate either biliary epithelium or hepatocytes.

This is certainly the case for both human and mouse where effectors of the Notch pathway, namely the Hes and Hey gene families are expressed to a greater degree during biliary regeneration. Interestingly our collaborator Tania Roskams finds that in human NOTCH2 appears to be the predominant receptor expressed on HPCs. This may not be surprising given the crucial role the role of NOTCH2 during hepatic ontogeny and the crucial role it plays during establishment of the biliary network.

Murine regeneration in typified by expression of the Notch1 receptor in HPCs. The presence of this Notch homologue may point to species variability in how the bile duct regenerates after damage; however the difference in Notch receptor may be down to the chronic nature of disease, where in human many of these pathologies have built up over decades in mouse these aetiologies take weeks, this too could explain why in chronic human disease I observe expression of the JAGGED1 ligand on the biliary epithelium, however during regeneration of the biliary tree in mouse I observe Jagged1 is expressed by the myofibroblasts surrounding the hepatic

progenitor cells; chronic diseases in human may eventually result in "niche independence", and as such allow the epithelium to operate independent of its surrounding microenvironment.

Inhibition of the Notch signalling pathway both *in vitro* and *in vivo* has severe consequences for biliary phenotype. In both these systems I have demonstrated that small molecule inhibitors targeted at Notch receptor processing restricts the expression of biliary genes in HPCs, and also abrogates the number of HPCs present during biliary regeneration. Reassuringly I have also shown that this reduction is not due to an off target affect which alters cell number, and that fibrosis is not reduced between in those mice given DAPT.

Critically I have identified that the Notch pathway is down regulated during regeneration of hepatocytes in a mouse model of hepatocellular regeneration CDE and during human hepatocellular disease (in collaboration with Olivier Govaere and Tania Roskams, Leuven Belgium). As well as there being a lack of Notch ligand (and indeed receptor expression in the mouse) and potential endogenous repression of the Notch signalling pathway through the ubiquitin ligase Numb it is possible to conclude that that there is a two tier repression of Notch signalling during hepatocellular repair (Figure 4.9).

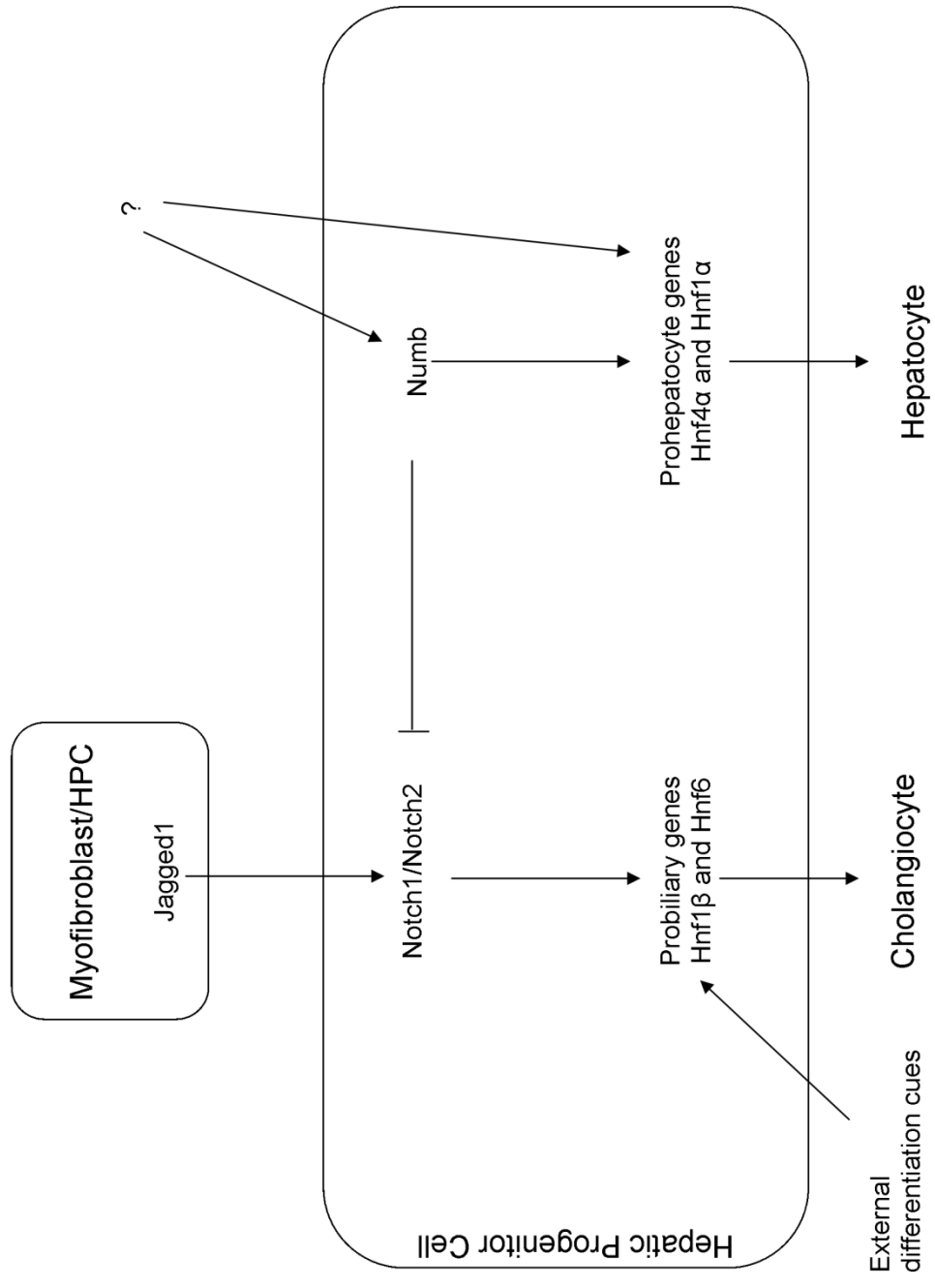


Figure 4.9— A schematic representation of Notch mediated regeneration in the adult liver

Figure 4.9— A schematic representation of Notch mediated regeneration in the adult liver

During biliary regeneration hepatic progenitor cells express high levels of Notch receptor, and the pathway is activated during biliary regeneration which acts through the Hes and Hey transcription factor family. This pathway is activated through stimulation by Jagged1 derived from the HPC population or a surrounding myofibroblast niche. Blockade of this pathway results in reduced cholangiocyte numbers *in vivo* and abrogated expression of early biliary transcription factors. During hepatocellular regeneration the Notch signalling pathway is expressed at low levels, however is also negatively regulated through high expression of Numb protein.

Chapter 5 - Wnt Signalling facilitates hepatocyte differentiation during liver regeneration

Introduction

During foetal development the Wnt pathway is important in specification of multiple different cells types. During hepatic ontogeny Wnt signalling is required for correct temporal regulation of hepatic differentiation. Early on in hepatic specification Wnt is required for correct budding and morphogenesis of what will become hepatic endoderm[304, 324]. During early hepatogenesis the role of Wnt signalling is disputed, with some contradictory data in the literature. During hepatocyte specification there are several reports of Wnt pathway inhibitor expression, such as Sfrp5 and calcinurin which interfere with Ctnnb1 signal and subsequently allow hepatic differentiation[307]. In contrast to this it has been demonstrated in Zebrafish that a homologue of Wnt2b, *Prometheus* is expressed in mesenchyme adjacent to the developing liver, and that abrogation of this signal results in a retardation of liver development; similarly when *Prometheus* is inhibited Prox1 and Hex, Ctnnb1 target genes, are down regulated. Initiation of hepatic development is not the only role played by the Wnt pathway. Later in hepatic development the liver must undergo zonation, where different regions of the parenchyma assume different functional traits[305]. This zonation is established through Wnt signalling, where loss of APC prevented peri-venous zonal restriction of Ctnnb1 activation and glutamine synthetase, but was rather found throughout the parenchymal zones 1-3 perturbing metabolic zonation[14].

Postnatal Wnt/Ctnnb1 signalling is also important in the growth and regeneration of the liver. Where over expression or stabilization of Ctnnb1 results in liver growth and hepatomegly. During hepatic regeneration Ctnnb1 levels rapidly increase after a partial hepatectomy (PHx) – mice lacking Ctnnb1 demonstrated an attenuated ability

to regenerate indicating the importance of Ctnnb1 activation during hepatocellular restoration[325].

This chapter aims to define the role of Wnt signalling in the hepatic progenitor response, and investigate whether interrogation of this pathway can induce a hepatocyte phenotype in HPCs. Interestingly I found that there is again a difference in Wnt/Ctnnb1 signalling between biliary and hepatocellular regeneration in the mouse, and that this may also correlate to what is observed in the human disease PSC/PBC and HCV. Macrophages are a rich source of canonical Wnt ligands and when they form close associations with HPCs then they can drive a hepatocellular phenotype. Ablation of Macrophages results in a reduction in Wnt/Ctnnb1 and as such suggests that this pathway, driven by macrophage derived Wnt is required for HPC specification into hepatocytes.

The Wnt signalling pathway is highly activated during hepatocellular regeneration but not during biliary regeneration

During both hepatocellular and biliary regeneration the Wnt pathway activation status of HPCs varies, ultimately depending on the fate of the HPC, and the context in which it is found.

During biliary regeneration in the mouse liver initiated by chronic DDC administration I found the emergence of HPCs as previously discussed. These HPCs

were found typically after 18 days of treatment to have low levels of Ctnnb1 in the nucleus of HPCs (Figure 5.1a). This observation correlates with HPCs found in human biliary diseases PBC/PSC where CTNNB1 protein is found to consistently localise to the HPC membrane and only infrequently is found localised to the cytoplasm and nucleus, personal communication Tania Roskams and Olivier Govaere.

I do however observe a different paradigm during hepatocellular regeneration in the mouse CDE model of hepatocyte regeneration. HPCs emerging from the portal tracts and have similar levels of staining for nuclear Ctnnb1 (Figure 5.1b). However using $Dlk1^{+}$ cells isolated from both CDE and DDC murine models I can demonstrate that there is a phenotypic difference in Wnt pathway target genes. During hepatocellular regeneration the Wnt pathway target genes Axin2, Myc, Sox9 and Twist are all more highly expressed during hepatocyte regeneration compared with biliary regeneration (Figure 5.1c) – suggesting that Ctnnb1 nuclear translocation activates the Wnt cascade.

Sox9 immunohistochemistry demonstrates that this variation in transcript level could be relayed to the amount of protein found in HPCs. During hepatocellular regeneration the Sox9 protein is nuclear and stains strongly, however the intensity of Sox9 staining during biliary regeneration is less, and appears more diffuse within the HPC nucleus (Figure 5.1d and Figure 5.1e), however further work would be required to validate the effects of this modulation.

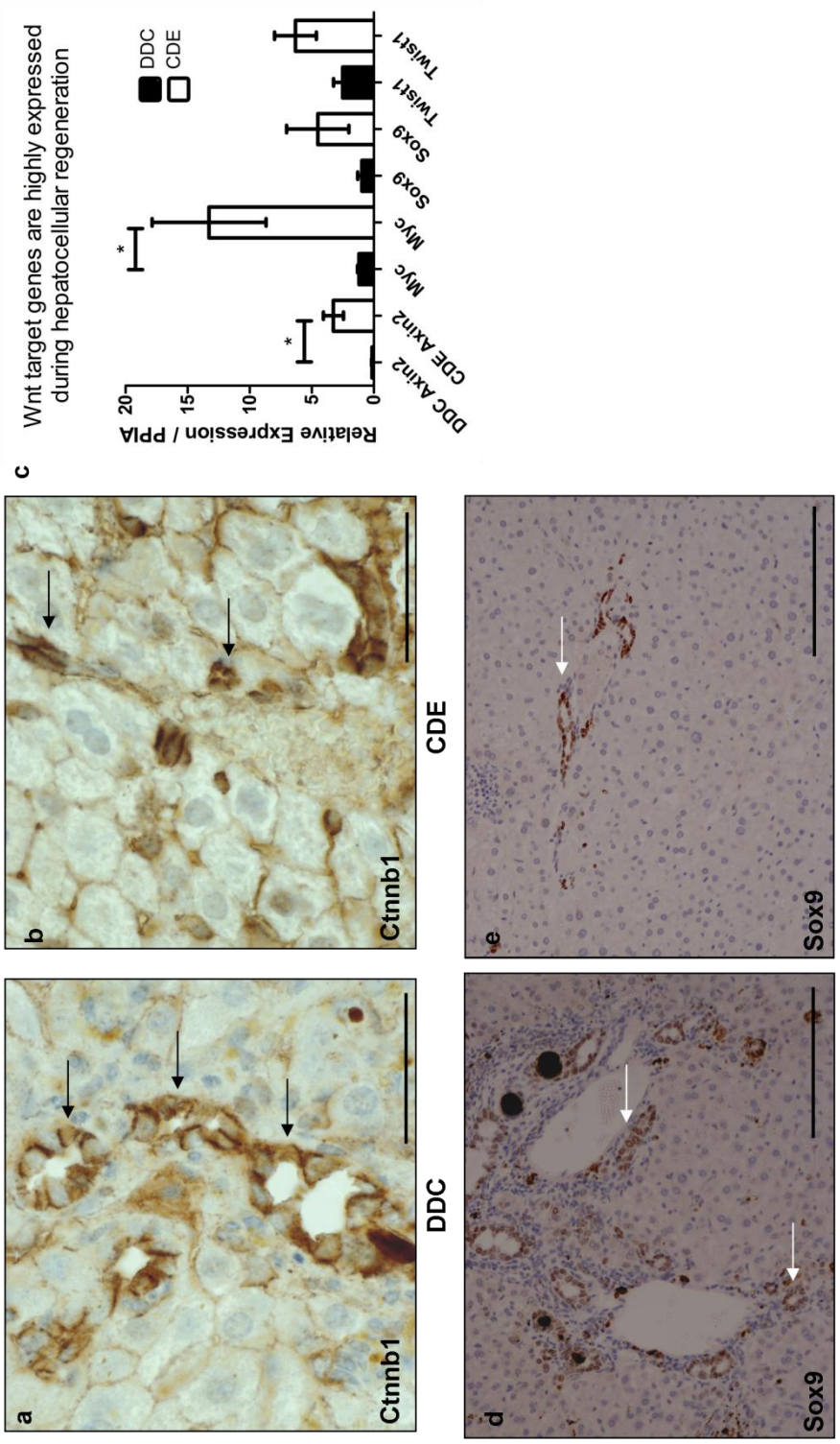


Figure 5.1 The Wnt/β-catenin pathway is activated during hepatocellular regeneration:

Figure 5.1 The Wnt/β-catenin pathway is activated during hepatocellular regeneration:

a During regeneration of the biliary tree I observe presence of β-catenin localised to the cell membrane of HPCs and hepatocytes. When looking for activated, phosphorylated I found none in the nucleus of HPCs regenerating the biliary tree. **b.** In contrast to this during CDE induced hepatocellular regeneration β-catenin is localised to the cell membrane and also is found within the cytoplasm and nucleus. **c**. Associated with varying localisation of β-catenin I found differential activation of the Wnt pathway as demonstrated through target gene expression Axin2 (CDE 3.270 ± 0.80 vs. DDC 0.1587 ± 0.36) and Myc (CDE 13.29 ± 4.59 vs. 1.209 ± 0.11) Sox9 (CDE 4.529 ± 2.50 vs. DDC 0.970 ± 0.33) and Twist1 (CDE 6.320 ± 1.684 vs. DDC 2.522 ± 0.735) were also more highly expressed during hepatocellular regeneration than during biliary restoration. **d.** During Biliary regeneration Sox9 is found within the nucleus of the HPCS, demonstrating that a well described Wnt target is found in these cells, **e.** Of interest is that during hepatocellular regeneration in the CDE the staining of HPCs appeared much more concentrated in HPC nucleus, again reiterating that Sox9 protein may be more highly expressed during hepatocellular regeneration;. In both CDE and DDC gene expression n=4, statistical significance was determined using a Mann Whitney U test *$p<0.05$. a, b Scale Bar = 100μm. D, e Scale Bar = 200μm

In vivo Wnt ligand is supplied by HPC associated macrophages, and is upregulated in response to phagocytosis of hepatocyte debris

I have demonstrated that during hepatocellular and biliary regeneration there is a divergent Wnt activation phenotype in HPCs undergoing biliary or hepatocellular regeneration. To examine this further I sought to identify and characterise the source of the Wnt ligand and describe how this Wnt ligand may be regulated.

Initial work (described in chapter 3) demonstrates that HPCs are found in a stereotypical cellular niche composed primarily of macrophages and myofibroblasts; however here I describe how the geographical distribution of these cellular components is variable depending on regenerative context. During murine biliary regeneration in the DDC model I found that HPCs are surrounded by F4/80 positive macrophages, however these inflammatory cells infrequently form contacts with the emerging HPCs as there is a cellular sheath of αSMA positive myofibroblasts surrounding the HPCs and preventing penetration of the macrophage to the site of regeneration (Figure 5.2a). As previously discussed this myofibroblast sheath is expressing high levels of the Notch ligand Jagged-1 and as such is involved in both positive signalling to the HPCs to assume a biliary fate, but also in preventing differentiation down a hepatocellular lineage by preventing the association of other cell types, namely the macrophages.

In contrast to this, during CDE, murine hepatocellular injury I describe a much more infiltrative niche where both myofibroblasts and macrophages are found in close

proximity to the HPCs, and as such can both influence the phenotype of the HPCs (Figure 5.2a).

As I found this geographical variation in the niche, and also know that there is phenotypic differences in myofibroblasts during regeneration I sought to identify whether macrophages themselves have a plastic phenotype between regenerative patterns. I isolated macrophages from the livers of mice undergoing both CDE induced hepatocellular regeneration, DDC biliary regeneration or age and stain matched healthy mice based on positivity for F4/80-PE. During hepatocellular regeneration in the CDE I found that compared to control animals, macrophages expressed high levels of Wnt3a, whilst during the DDC pattern of regeneration the expression of Wnt3a decreases relative to expression in macrophages isolated from control animals (Figure 5.2b).

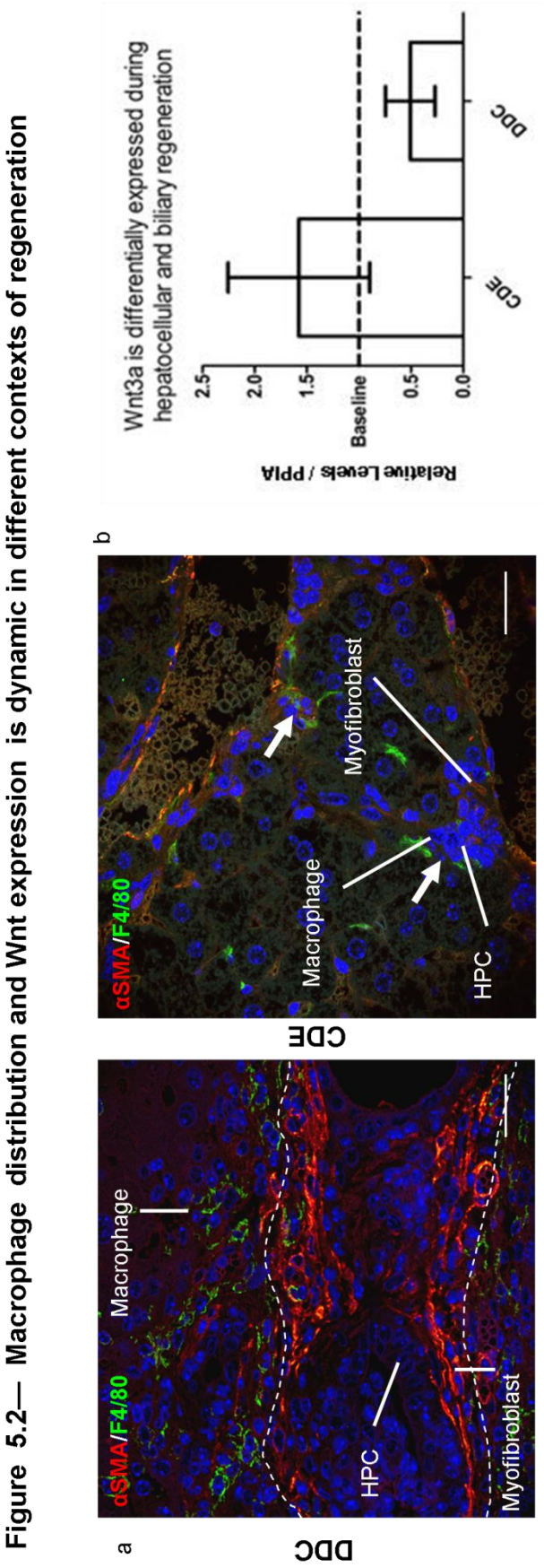


Figure 5.2— Macrophage distribution and Wnt expression is dynamic in different contexts of regeneration

Figure 5.2— Macrophage distribution and Wnt expression is dynamic in different contexts of regeneration

a. During regeneration Myofibroblasts and Macrophages are associated with HPCs, during DDC myofibroblasts closely associate with HPCs, however during CDE there is a more heterogeneous niche with both macrophages and myofibroblasts contacting HPCs. **b.** Macrophages isolated from both CDE and DDC demonstrate a divergent expression of the canonical Wnt3a ligand (CDE. 1.578 ± 0.67 vs. DDC 0.509 ± 0.23). For each image scale bar = 50μm. For gene expression F4/80 macrophages were isolated from CDE and Control and DDC and Control, n=4 for each. Students T test was performed, no significance were seen with a 95% confidence interval.

To investigate a potential mechanism for this Wnt status I isolated bone marrow (BM) from mice and differentiated it into bone marrow derived macrophages (BMDMs). I achieved approximately 80 – 85% F4/80 positivity during differentiation of BM into BMDMs (Figure 5.3a, FACS panels 1-4) – demonstrating a representative differentiation at 84%). F4/80 positive macrophages are grown in suspension. Once differentiated macrophages were removed from their differentiation conditions and plated on plastic where they rapidly attached. After 8 hours of attachment non-adherent cells were removed through washing

These macrophages were then fed with sonicated hepatocyte debris (Figure 5.4a). During the process of feeding macrophages ingested debris, as demonstrated through their large size and granulated cytoplasm (5.4 b-d). I found that those BMDMs plated in the absence of debris express levels of canonical ligands Wnt3a and Wnt7a, which have been implicated during hepatic ontogeny and also in the differentiation of ES cells into hepatocytes at levels barely detectable through qPCR, however after debris ingestion I found there to be a marked increase in the levels of Wnt3a and Wnt7a transcript within these macrophages (Figure 5.4e), whilst debris alone gave no signal.

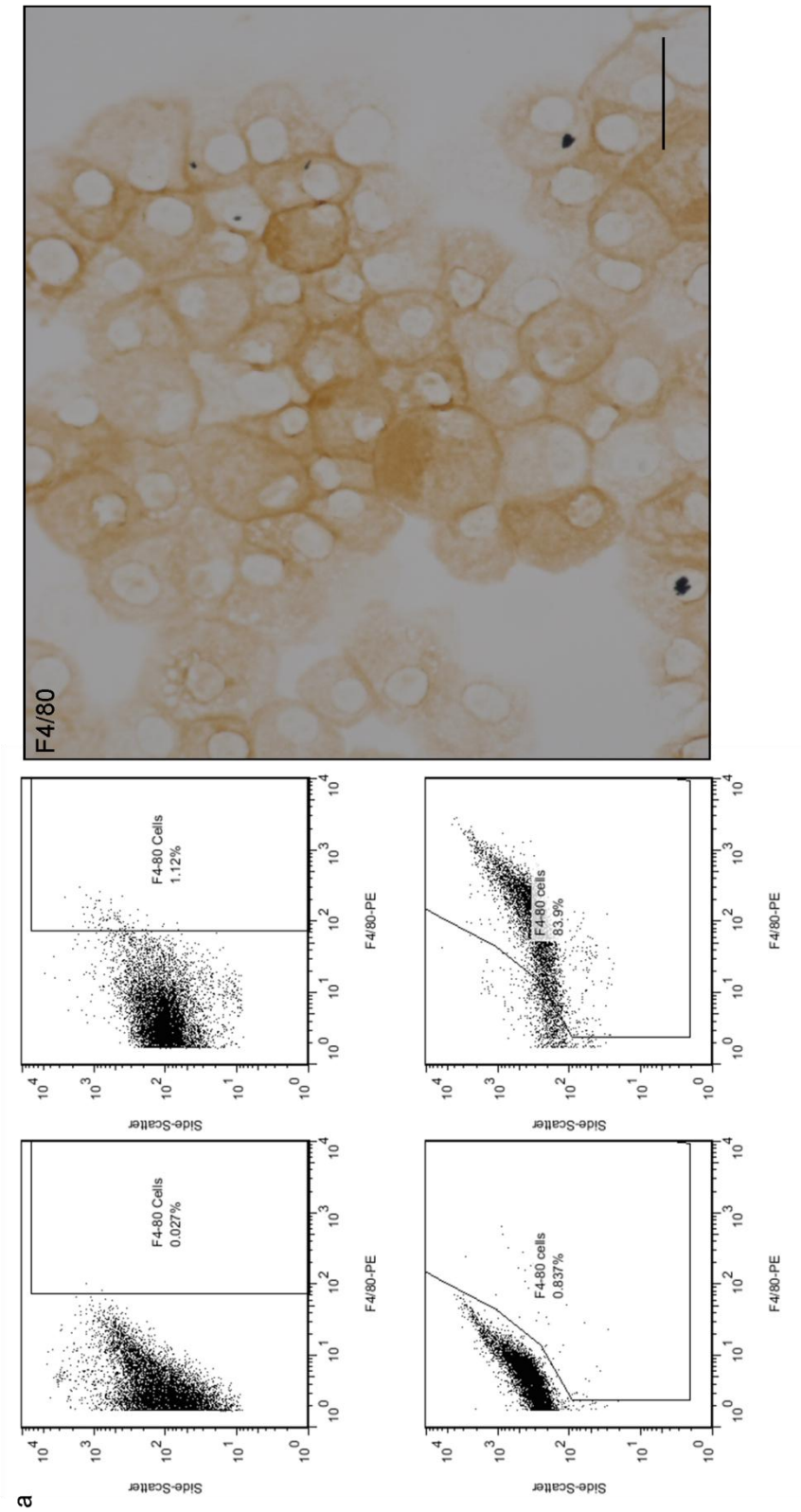
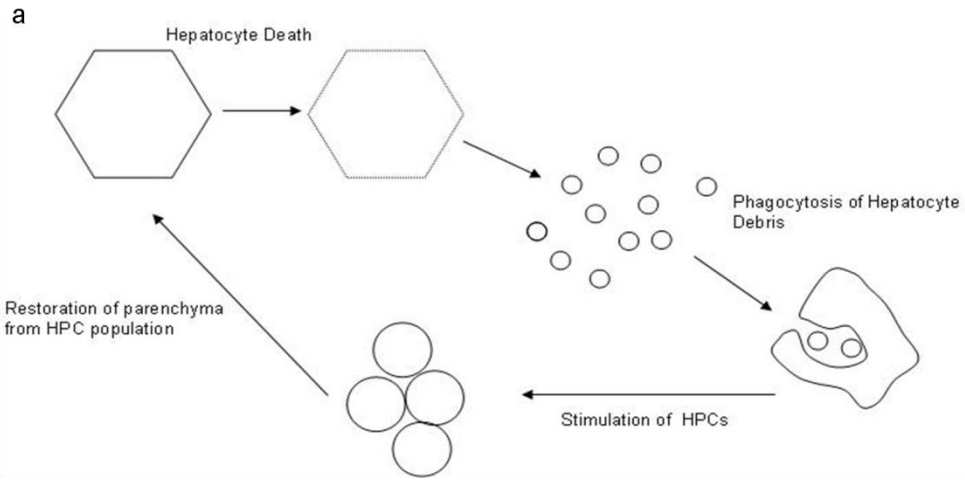


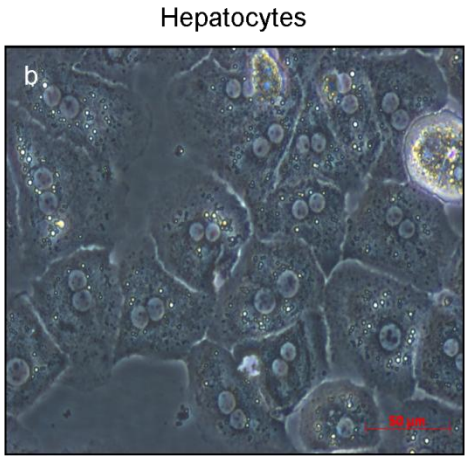
Figure 5.3— Bone Marrow can be differentiated into F4/80 positive macrophages *in vitro*

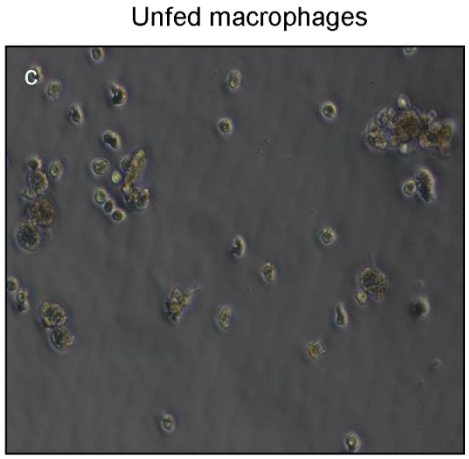
Figure 5.3— Bone Marrow can be differentiated into F4/80 positive macrophages in vitro.

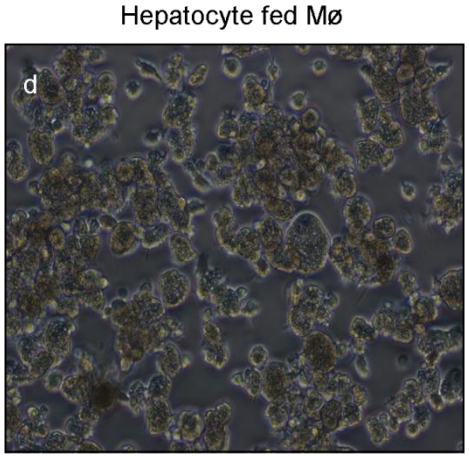
a. Isolated bone marrow was differentiated into F4/80 positive cells using L929 supplemented medium for 1 week. At day 0 unstained samples were negative for F4/80-PE as expected (top left panel), bone marrow stained for anti-F4/80-PE demonstrated approximately 1% of the bone marrow cells are F4/80 positive (upper right panel). After 7 days of culture the FSC/SSC profile of the differentiated bone marrow changes, unstained differentiated bone marrow remains excluded from the PE gate however differentiated bone marrow, stained for F4/80-PE demonstrates > 80% positivity for the epitope. The positivity of these cells was confirmed through cytospins and immunohistochemistry for F4/80. Scale Bar = 10μm

Figure 5.4— Debris fed macrophages up-regulate Wnt in vitro









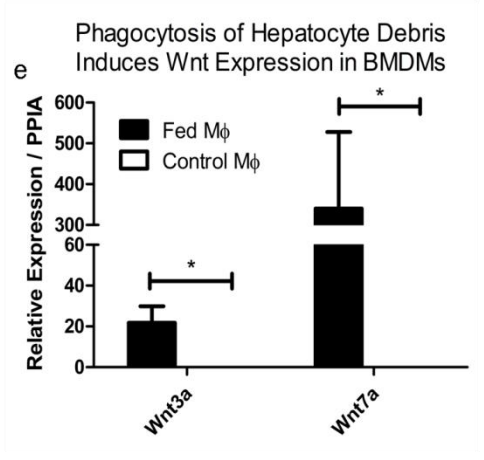


Figure 5.4— Debris fed macrophages up-regulate Wnt *in vitro*

Hepatocyte debris was fed to macrophages to ascertain whether ingestion induces the expression of Wnt ligand, briefly hepatocytes were isolated using a two step perfusion and digestion protocol followed by density centrifugation of the cells which pellet early due to their size. These cells were then sonicated and freeze thawed to ensure that they were sufficiently broken down. **b-d.** Isolated and sonicated hepatocytes were to macrophages. **e.** Macrophages fed hepatocytes expressed significantly higher levels of Wnt3a and Wnt7a when compared to unfed macrophages (Wnt3a; fed 21.79 ± 8.076 vs. unfed 0.0004 ± 0.0001, Wnt7a; fed 340.3 ± 187.4 vs. unfed 0.012 ± 0.006) on images scale bar = 50um, and all photomicrographs lacking a scale bar are at the same magnification. n=6 for fed vs. unfed macrophages. Analysis of data using a Mann Whitney U test, $p<0.05$.

Post-phagocytic macrophages can induce a Wnt mediated hepatocyte phenotype in HPCs *in vitro*.

Hepatic progenitor cells *in vivo* express high levels of Wnt target genes during hepatocellular regeneration, and this is associated with translocation of Ctnnb1 to the cytoplasm and nucleus of this HPCs.

To demonstrate that the canonical Wnt pathway can be stimulated in primary HPCs *in vitro* I treated cultures with recombinant Wnt3a (rWnt3a) or DMSO (Vehicle) alone. In control treated cells Ctnnb1 is found constantly associated with the cell surface membrane (Figure 5.3a); with only very faint cytoplasmic localisation. However during treatment with Wnt3a I found multiple clusters of cells which stain positive for both membranous and cytoplasmic Ctnnb1 indicating stabilisation of Ctnnb1 and translocation to the HPC nucleus activating the Wnt pathway (Figure 5.5a, Wnt3a, green cytoplasmic staining)

Interestingly I found that when I treated HPCs with rWnt3a I could detect modest but significant changes in the levels of Numb transcript and also the levels of Hnf4α transcript, both associated with the establishment of a hepatocellular phenotype (Figure 5.5b).

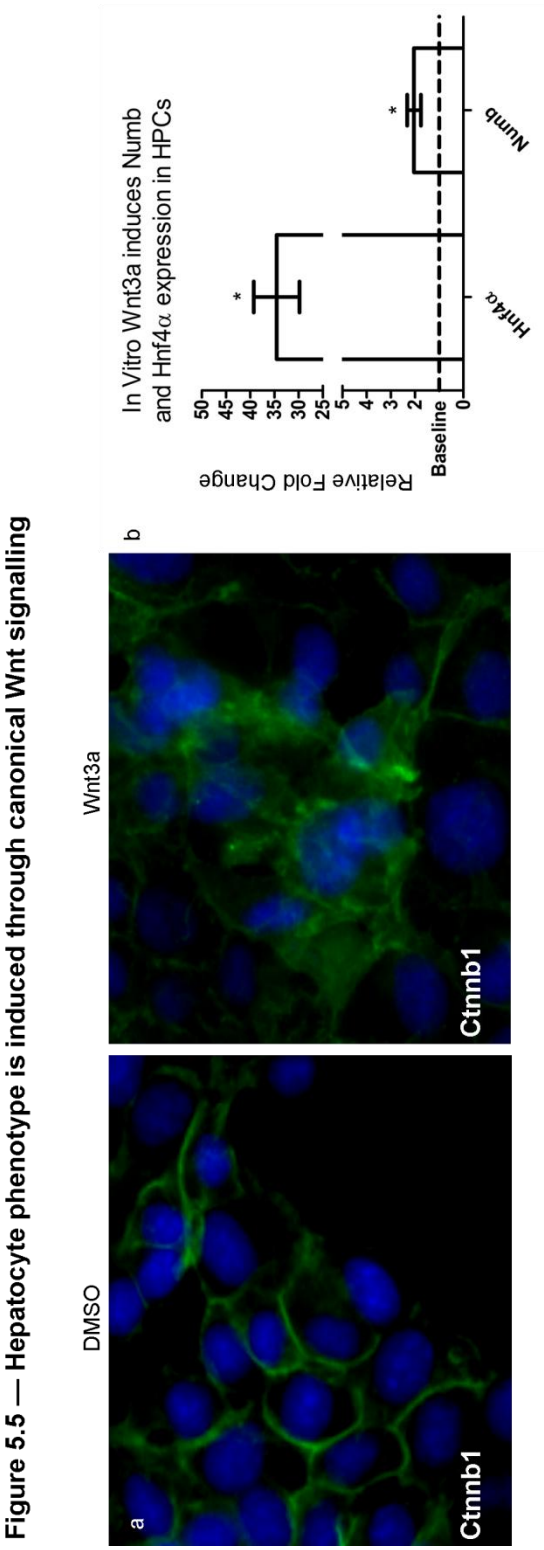


Figure 5.5 — Hepatocyte phenotype is induced through canonical Wnt signalling

Figure 5.5 — Hepatocyte phenotype is induced through canonical Wnt signalling

a. HPCs express Ctnnb1 which is predominantly localised to the cell membrane (green) however when treated with recombinant Wnt3a there is a shift in localization of Ctnnb1 from the membrane to the cytoplasm. **b.** Treatment with rWnt3a results in an increase in the expression of Hnf4α (35 fold induction) and Numb (2 fold induction). n=3, $p<0.05$ derived using a Mann Whitney U test

As I have shown primary HPCs *in vitro* are Wnt3a responsive I established a co-culture system between post-phagocytic macrophages expressing Wnt3a, and primary HPCs (Figure 5.6a). After the duration of co-culture I can demonstrate that the expression level of Wnt target genes increases compared with either macrophages or HPCs cultured alone (Figure 5.6b) – These data suggest that macrophage and HPC co-culture is capable of stimulating Wnt target genes, and hepatocyte phenotype *in vitro*, however this does not address the role of canonical Wnt signalling in this system or whether this co-culture can induce a hepatocyte phenotype.

I used my co-culture system in combination with Wnt Inhibitory Factor 1 (WIF-1) to investigate what role macrophage derived Wnt had on HPC specification *in vitro*. When I cultured HPC and Macrophage co-cultures in the presence of WIF-1 I found a significant abrogation of Wnt target genes Axin2, Myc, Twist1 and Sox9 (Figure 5.6c) . In tandem with this I found a decrease in expression of the early hepatocyte genes Hnf1α and Hnf4α, suggesting that early hepatocyte phenotype may be linked with Wnt signalling (Figure 5.6d). Unexpectedly during inhibition of the Wnt pathway with WIF1 I found a significant increase in expression of the early cholangiocyte genes Hnf1β and Hnf6 (Figure 5.6d), suggesting that in this context biliary differentiation is occurring at the expense of hepatocyte specification. I speculate that as these are primary co-cultures there may be contaminating cells such as myofibroblasts which in the presence of Wnt inhibition are alternatively specifying the HPCs into a biliary phenotype.

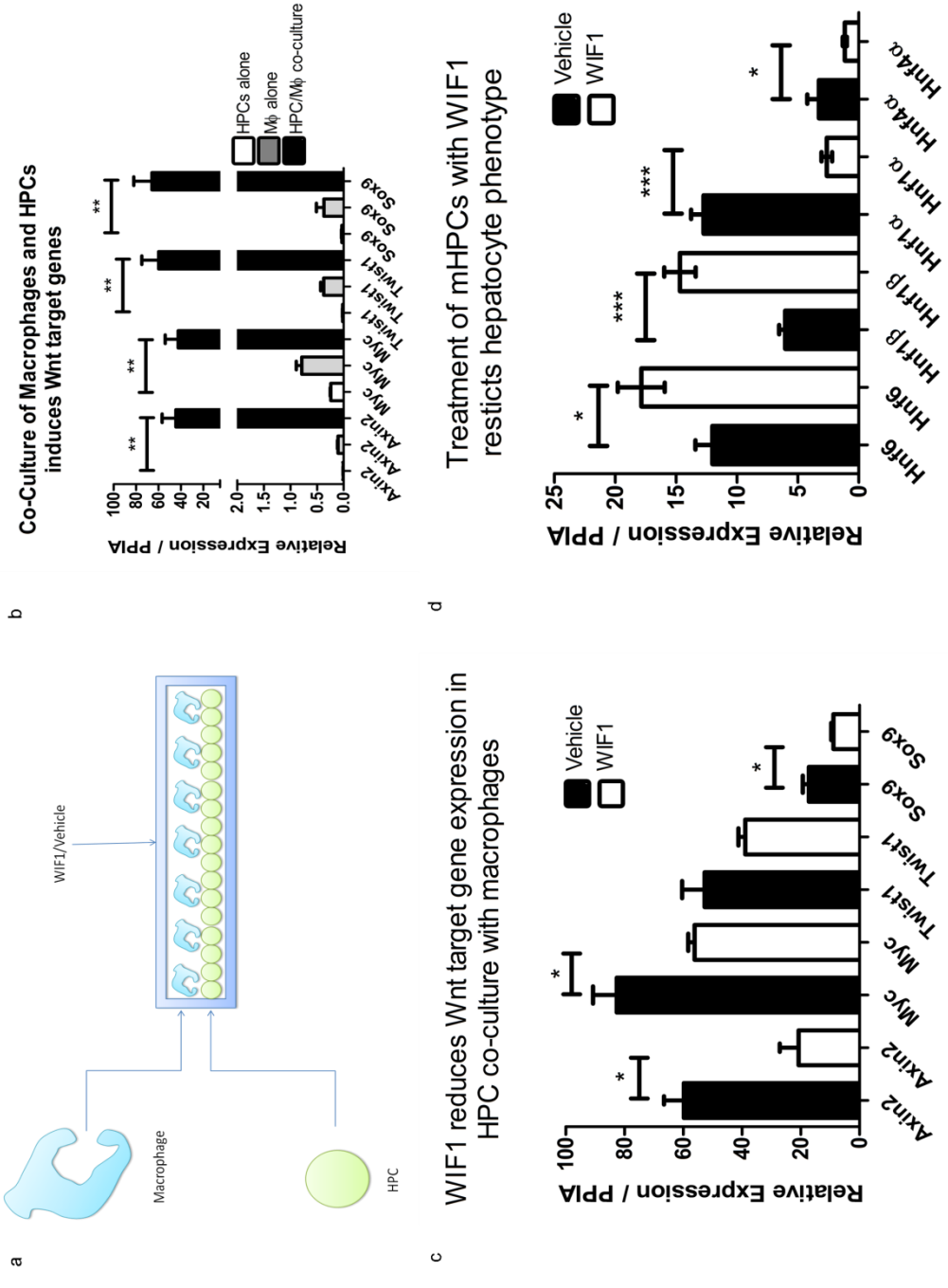


Figure 5.6 — In vitro perturbation of the Wnt pathway results in reduced hepatocyte specification and activation of the biliary differentiation programme.

Figure 5.6 — In vitro perturbation of the Wnt pathway results in reduced hepatocyte specification and activation of the biliary differentiation pathway

a. Macrophage and HPCs were co-cultured in the presence and absence of Wnt inhibition using WIF1. **b.** During macrophage co-culture Wnt target genes are more highly expressed when cells are combined than compared to HPCs and Macrophages alone (Axin2 HPC 0.008 ± 0.004, Mø 0.10 ± 0.01, HPC/Mø 44.52 ± 12.26; Myc HPC 0.242 ± 0.11, Mø 0.78 ± 0.10, HPC/Mø 42.45 ± 11.54); Twist1 HPC 0.01 ± 0.003, Mø 0.37 ± 0.05, HPC/Mø 59.80 ± 15.14; Sox9 HPC 0.03 ± 0.008, Mø 0.37 ± 0.13, HPC/Mø 65.84 ± 16.26) **c.** During inhibition with WIF1 Wnt pathway genes are down regulated (Axin2 vehicle 59.84 ± 6.8 vs. WIF1 20.75 ± 6.44; Myc vehicle 82.70 ± 8.05 vs. WIF1 56.21 ± 2.169; Twist1 vehicle 52.78 ± 7.63 vs. WIF1 38.96 ± 2.29; Sox9 vehicle 17.29 ± 7.63 vs. WIF1 38.96 ± 2.29) **d.** Inhibition of Wnt signalling results in abrogation of hepatocyte phenotype and induction of biliary genes (Hnf6, vehicle 12.04 ± 1.38 vs. WIF1 17.87 ± 1.9 0; Hnf1β vehicle 6.05 ± 0.50 vs. 14.70 ± 1.29; Hnf1α, vehicle 12.76 ± 1.02 vs. WIF1 2.639 ± 0.43; Hnf4α vehicle 3.28 ± 0.96 vs. 1.153 ± 0.15). n = 6 replicates, experiment repetition three times. *p<0.05, **p<0.01, ***p<0.001 derived from a Mann Whitney U test.

In vivo ablation of macrophages worsens hepatic function but has no affect on the cellular niche

As I have demonstrated Wnt signalling is an important factor in the specification of hepatic progenitor cells into a hepatocyte phenotype, and that inhibition of the Wnt signalling pathway *in vitro* results in attenuation of both Wnt target gene expression and early hepatocyte genes *in vitro*. To investigate the importance of Macrophages and by extension the role of Wnt signalling during hepatocellular regeneration I ablated macrophages using repeat administration of liposomal clodronate throughout the hepatocellular regenerative process (Figure 5.7a). Liposomal Clodronate is well described tool to ablate macrophages *in vivo*[326]. Briefly, clodronate containing liposomes are phagocytosed by macrophages where the toxic clodronate accumulates in the macrophage cytoplasm and ultimately induced apoptosis when it reaches a toxic threshold. Free clodronate is rapidly cleared from the circulation and cannot penetrate cell membranes without being loaded into a liposomal vector, and as such is inert to non-phagocytic cells.

During liposomal clodronate administration I found that there was a failure to degrade and clear dead and dying hepatocytes from the liver parenchyma, and that this resulted in regions of confluent necrosis within the liver parenchyma. Ablation of macrophages resulted in progressive weight loss and animals which received liposomal clodronate appeared less healthy than control counterparts. The ablation of hepatic macrophages was also associated with a worsening of liver function as demonstrated though elevated serum albumin (Figure 5.7c), AST (Figure 5.7d), ALT (Figure 5.7e) and Alkaline Phosphatase (Figure 5.7f) (data in collaboration with

Thomas Bird). As I found a change in liver function during macrophage ablation I investigated whether this was having an adverse effect on the formation multicellular HPC niches, and resulting in the failure to support HPCs.

Unsurprisingly I found that there was an almost complete ablation of F4/80 positive macrophages throughout the hepatic parenchyma compared with those animals which received vehicle alone (Figure 5.8a). However this did not result in a loss of myofibroblasts (Figure 5.8b) , and did not result in the increased apoptosis of HPCs (Figure 5.8c) demonstrating that macrophages are not required for the survival of either HPCs or indeed the myofibroblasts they are closely associated with.

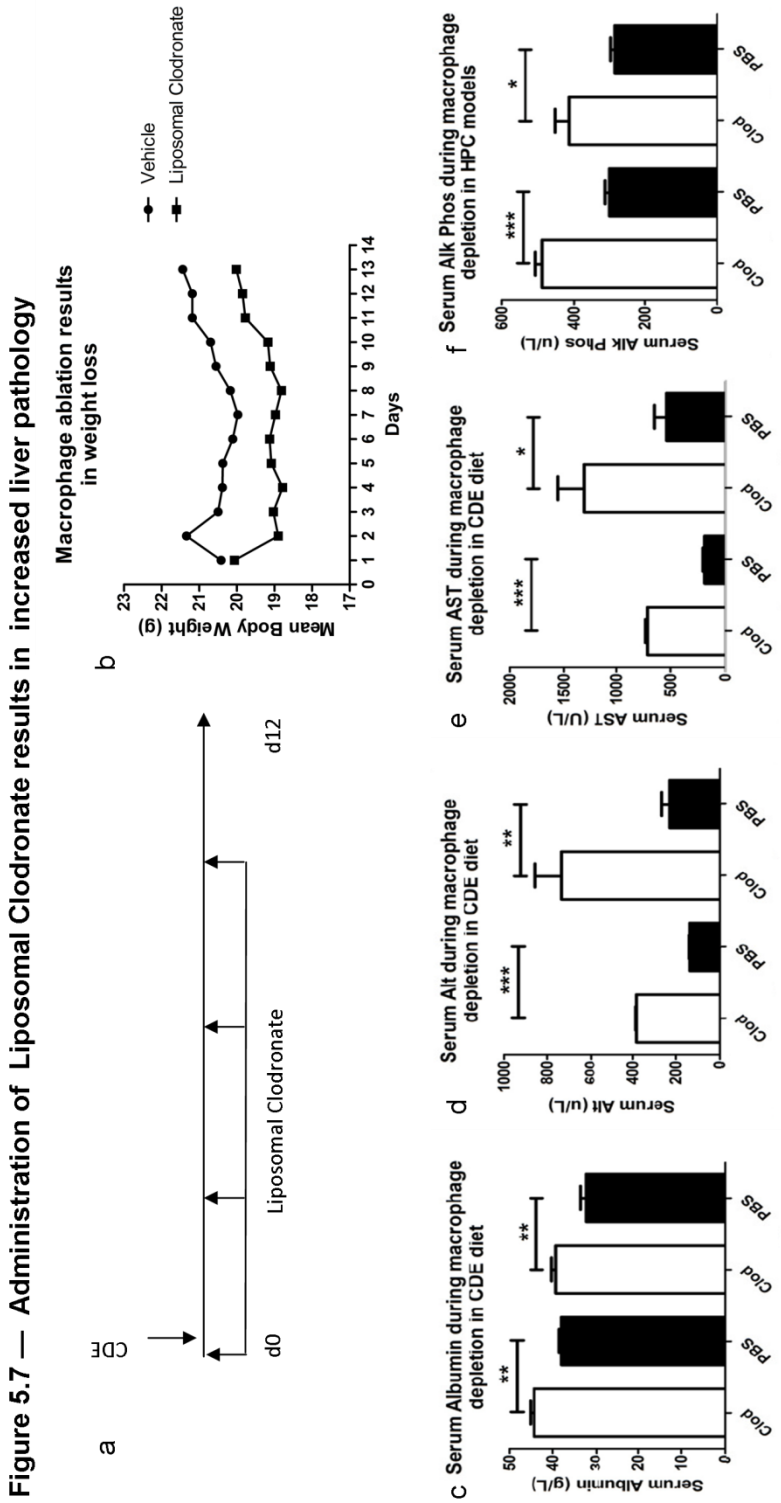


Figure 5.7 — Administration of Liposomal Clodronate results in increased liver pathology

Figure 5.7 — Administration of Liposomal Clodronate results in increased liver pathology

a. Liposomal Clodronate was administered IV every three days into animals receiving the hepatocellular damage from the CDE diet. **b.** Treatment with Liposomal Clodronate resulted in a greater weight loss than when treated with PBS alone. **c.** Albumin means 44.3 vs. 38.0 at day 6 and 39.32 vs. 32.41 at day 12. **d.** Alt 384 vs. 137 at day 6 and 736 vs. 228 at day 12. **e.** AST means 719 vs. 191 at day 6 and 1317 vs. 536 at day 12. **f.** Alk Phos mean 87 vs. 191 at day 6 and 414 vs. 132 at day 12. (LFT data in collaboration with Thomas Bird)

In vivo ablation of macrophages results in conversion of hepatocyte progenitors into a biliary phenotype.

During macrophage ablation during hepatocellular regeneration I was surprised to observe that there is change in the cellular phenotype of the liver. Indeed when I quantified HPCs in animals given liposomal clodronate versus those animals which had received vehicle alone there was no change in the number of HPCs. (Figure 5.8d), these experiments were conducted in collaboration with Thomas Bird, Edinburgh.

Further analysis of the HPCs during macrophage ablation demonstrated that rather than failing to proliferate, these HPCs fail to migrate in long extended chords as typically observed during hepatocellular regeneration (Figure 5.8e). During treatment with liposomal clodronate these cells form atypical ductular structures, with a poorly defined lumen and remain close to the portal tract – reminiscent of HPCs during ductular regeneration (Figure 5.8f *cf* DDC model of liver damage, Figure 3.5).

Figure 5.8— Liposomal Clodronate does not affect myofibroblasts and does affect HPC apoptosis.

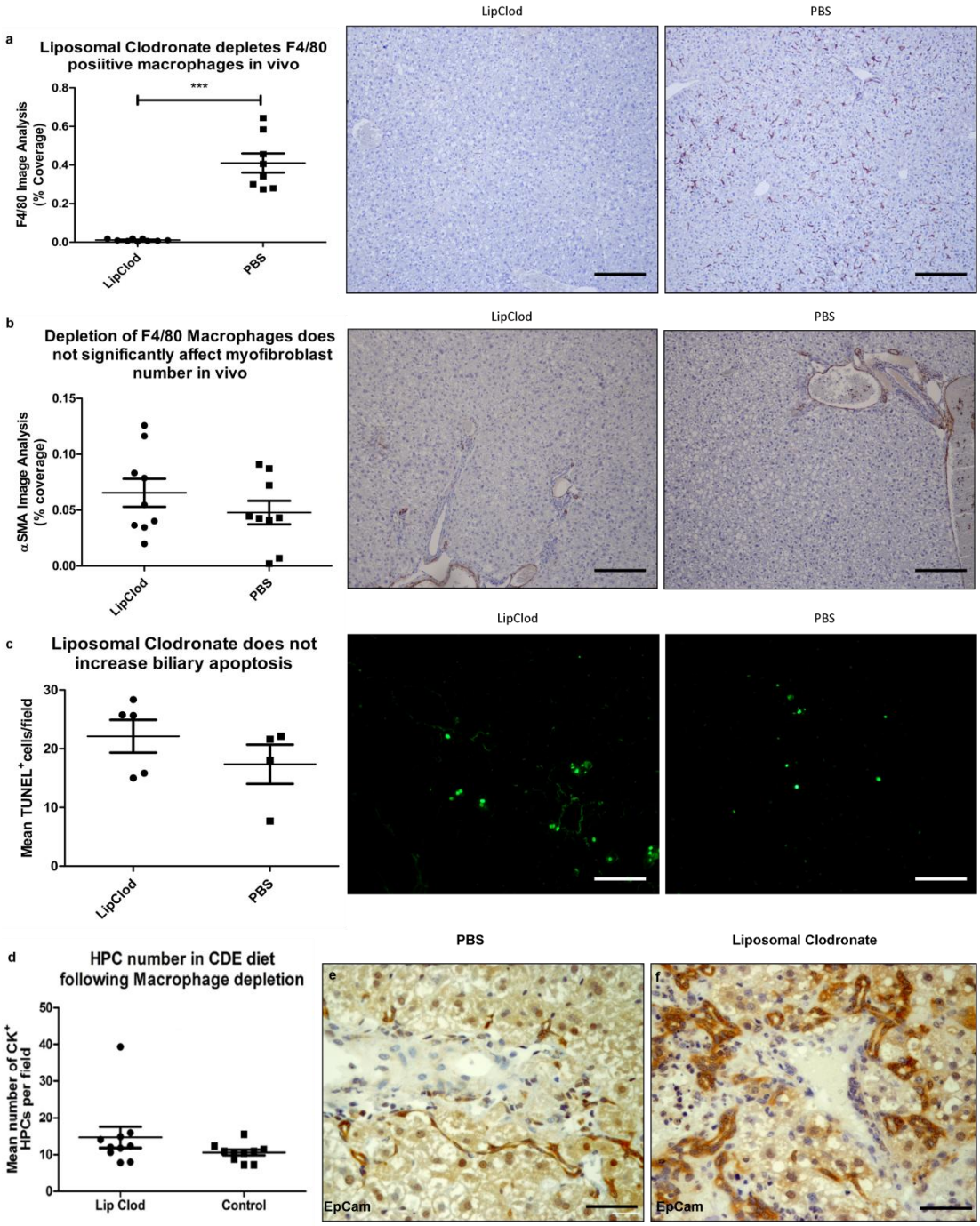


Figure 5.8— Liposomal Clodronate does not affect myofibroblasts and does not change HPC number.

a. During Liposomal Clodronate treatment macrophages are ablated and are barely detectable within hepatic tissue compared to animals treated with PBS alone (Lip Clod 0.01 ± 0.002 vs. PBS 0.41 ± 0.04). **b.** Myofibroblast numbers do not change during ablation of macrophages. **c.** In Liposomal Clodronate treated animals I observed apoptosis of macrophages throughout the parenchyma; however this is not associated with an increase in apoptosis of the biliary tree. **d.** Macrophage ablation during the CDE diet does not affect the number of HPCs detectable in the tissue. **e.** HPCs normally form thin, infiltrating cords which move into the parenchyma. However during macrophage ablation HPCs do not form these typical structures, but rather pseudo-ductule structures (**f**). N=8-9 for each group in each analysis. >30 adjacent, non-overlapping images were captured for each data point. Data points represent the mean numerical value of these images generated through pixel analysis. A D'Agostino and Pearson omnibus normality test was used to assess the distribution of the data. The data is normally distributed and as such analysed using a Student's t-test. Scale bar a-c = 400μm, d = 100μm.

Macrophage ablation results in the differentiation of HPCs into bile ducts

To investigate whether the failure of HPC migration and formation of ductular structures was due to a failure of Wnt signalling during macrophage ablation I looked at the localisation of Ctnnb1. In HPCs forming ductular structures I clearly observe the majority of Ctnnb1 is localised to the cell surface membrane (Figure 5.6a) and is not found in the cytoplasm and nucleus of the HPCs as normally observed during hepatocellular regeneration (Figure 5.6a), as such the Wnt pathway is abrogated. To confirm this finding I isolated HPCs from both liposomal clodronate and vehicle treated animals. In this situation I find a significant reduction in the Wnt pathway target genes Axin2, Myc, Twist1 and Sox9 (Figure 5.6b).

I have shown that reception of Wnt ligand in HPCs during macrophage ablation affects the morphological phenotype of HPCs and may cause a failure of HPC migration during regeneration. However to examine whether the ablation of macrophages had an effect on the specification of HPCs *in vivo* I assessed early hepatocellular gene expression. Here I found that ablation of macrophages during hepatocellular regeneration resulted in a reduced expression of hepatocyte specific transcription factors Hnf4α and Hnf1α. Interestingly I also observe a reduction in the expression of Numb transcript which may liberate Notch signalling in these HPCs and induce a biliary phenotype which I can defect here through increase expression of both Hnf1β and Hnf6. (Figure 5.6d).

To confirm that the ablation of macrophages during hepatocellular regeneration resulted in a conversation of hepatocyte progenitor cells into biliary progenitor cells I quantified the number of Hnf1β or Hnf4α positive HPCs. Here I demonstrate a significant increase in the number of Hnf1β positive cells which throughout hepatocellular regeneration form biliary structures. Furthermore the number of Hnf4α positive HPCs is decreased throughout regeneration during macrophage ablation.

Figure 5.9— Ablation of Macrophages results in a loss of Wnt signal and conversion of progenitors to a biliary phenotype.

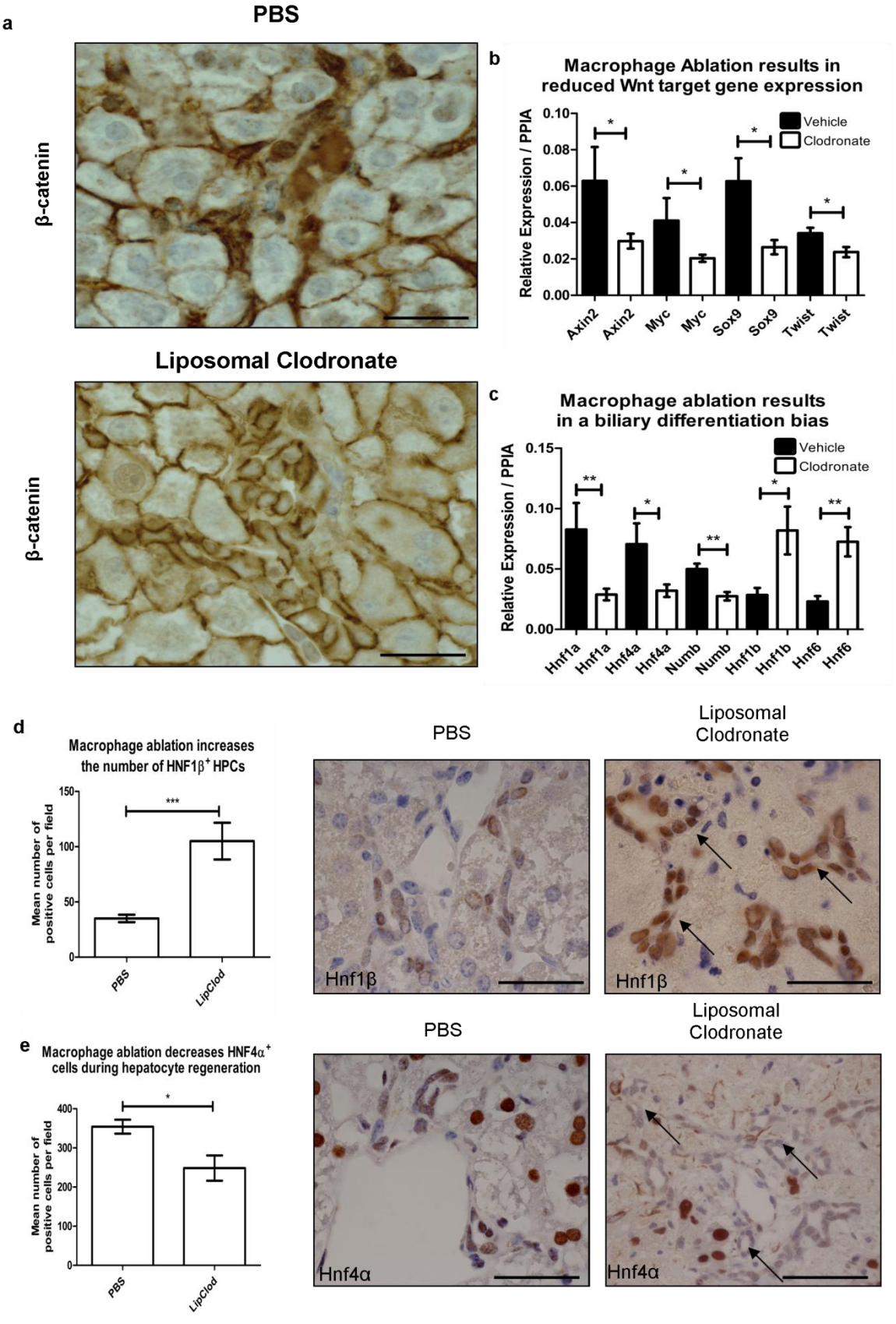


Figure 5.9 — Ablation of Macrophages results in a loss of Wnt signal and conversion of progenitors to a biliary phenotype.

a. Typically Ctnnb1 is found in the cytoplasm and nucleus of HPCs undergoing hepatocyte specification, however during macrophage ablation the majority of Ctnnb1 is localised to the cell surface membrane. **b.** Loss of cytoplasmic Ctnnb1 results in a reduction in Wnt target gene expression Axin2 (PBS 0.06 ± 0.01 vs. Lip Clod 0.02 ± 0.004) Myc (PBS 0.04 ± 0.01 vs. Lip Clod 0.02 ± 0.001) Sox9 (PBS 0.06 ± 0.01 vs. Lip Clod 0.02 ± 0.003) Twist1 (PBS 0.03 ± 0.002 vs. Lip Clod 0.02 ± 0.002) The reduction in HPC. **c.** During macrophage ablation HPCs lose early hepatocyte markers Hnf1α (PBS 0.08 ± 0.02 vs. Lip Clod 0.02 ± 0.004); Hnf4α (PBS 0.07 ± 0.01 vs. Lip Clod 0.03 ± 0.005) and Numb (PBS 0.04 ± 0.004 vs. 0.004) and increase the expression of early biliary genes (PBS 0.02 ± 0.005 vs. Lip Clod 0.08 ± 0.01) and Hnf6 (PBS 0.02 ± 0.004 and Lip Clod 0.07 ± 0.01) **d.** After macrophage ablation during hepatocellular regeneration there is a significant increase in the number of Hnf1β positive HPCs (black arrows). **e.** During hepatocellular regeneration ablation of macrophages results in a significant decrease in the number of Hnf4α, this is associated with clear loss of protein in the HPCs (black arrows). Scale Bar: a = 50μm, d, e = 100μm. n = 10 per group. *p<0.05, **p<0.01, ***p<0.001.

Discussion - Macrophage derived Wnt acts upon HPCs to induce a hepatocyte phenotype.

Hepatic ontogeny requires Wnt signalling at numerous junctures to achieve correct specification, maturation and biochemical zonation[15, 312, 314, 327]; similarly the initial proliferative stimulus for HPCs appears to include Wnt signalling. However the mechanism by which Wnt affects the fate of adult HPCs is as yet unknown.

During diverse patterns of injury such I found that there was diversity in Ctnnb1 staining, and that only during hepatocellular regeneration could I find consistent Ctnnb1 staining in the cytoplasm and nucleus of HPCs. During biliary regeneration I typically found localisation of Ctnnb1 at the cell surface membrane – suggesting that the canonical Wnt signalling pathway was not highly activated during this process. These data correlate with what is known in the field of hepatocyte differentiation from ES cells where correct terminal differentiation requires stimulation of the canonical Wnt pathway in a Wnt3a dependent mechanism. The diverse localisation of Ctnnb1 observed in two murine models of hepatocyte and biliary regeneration correlates this to a diverse expression of Wnt target genes, all of which show an increased expression during hepatocellular regeneration than compared to regeneration of the bile ducts. Interestingly it has been described in the literature that Wnt signalling is required for activation of the HPC population in the DDC biliary model[328]. This does not however lie in complete contrast to my observations, as Wnt is a potent mitogen for HPCs so is Tweak, as such the removal of the early Wnt signal may be compensated for over time by other mitogenic cues only once HPCs have expanded and later on in the life of the HPC population do I observe the

ultimate result of Wnt removal which is one of phenotype, rather than of proliferation.

It has been previously described in the Kidney that macrophage derived Wnt7a is an important mediator of renal epithelial repair, and that loss of this ligand results in abrogated re-epithelialisation of the glomerulus. During this work I have described how hepatic macrophages express Wnt3a in a modulated way – suggesting that the Wnt phenotype of the macrophage is important in eliciting the correct regenerative response. I have also discussed how the previously described stereotypical niche is highly dynamic, and is distributed differently between regenerative contexts in the mouse. During hepatocellular regeneration I found the cellular components of the niche to be much more heterogeneous, where both macrophages and myofibroblasts can form close associations with the HPCs. Conversely I describe how during biliary regeneration penetration of the macrophage to the site of regeneration is prevented by a sheath of myofibroblasts, preventing the delivery of paracrine signals from the macrophage.

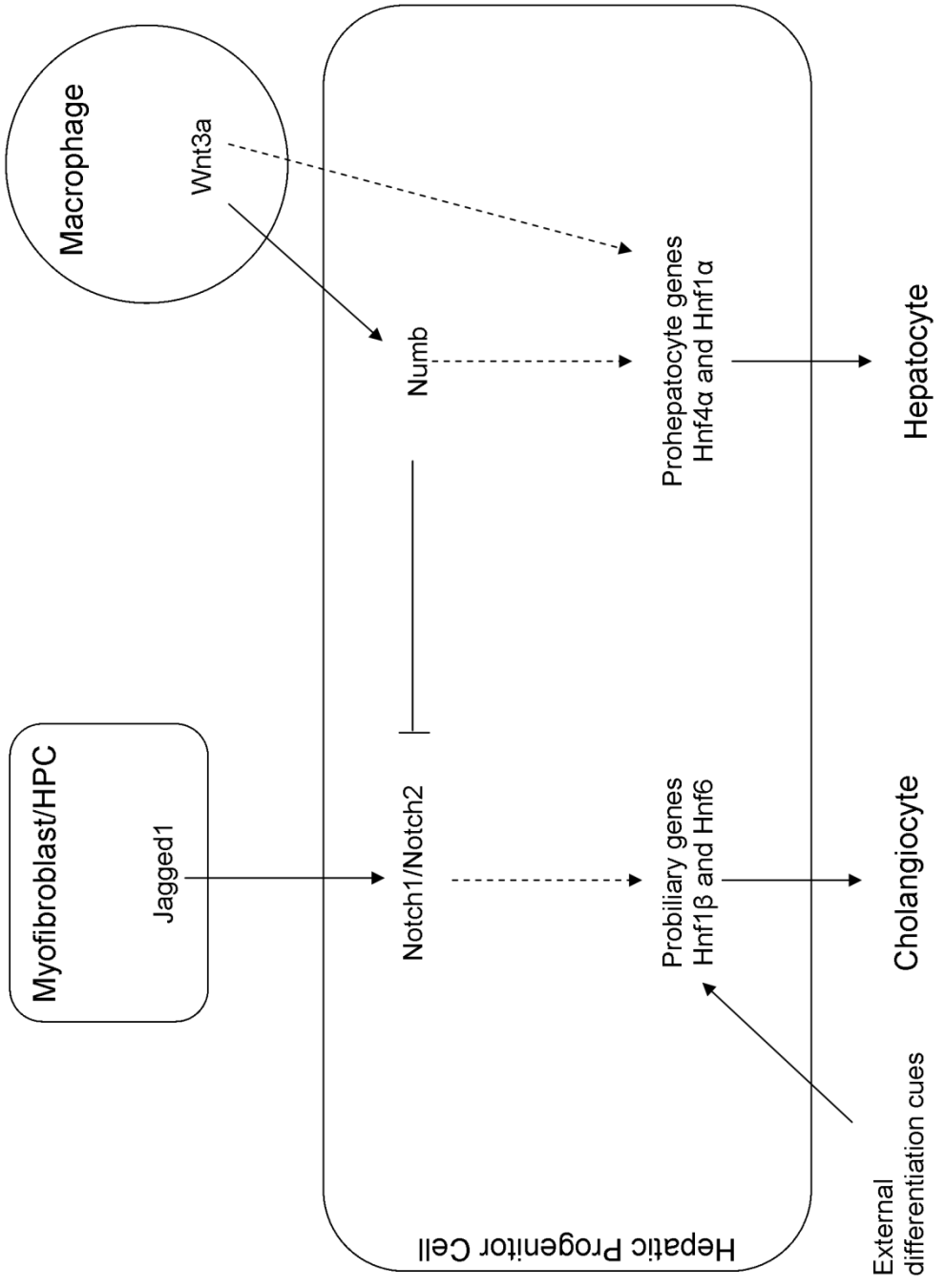


Figure 5.10— A schematic representation of Notch and Wnt mediated regeneration in the adult liver

Wnt Signalling facilitates hepatocyte differentiation during liver regeneration

Figure 5.10 — A schematic representation of Notch and Wnt mediated regeneration in the adult liver

During biliary regeneration hepatic progenitor cells express high levels of Notch receptor, and the pathway is activated during biliary regeneration which acts through the Hes and Hey transcription factor family. This pathway is activated through stimulation by Jagged1 derived from the HPC population or a surrounding myofibroblast niche. Blockade of this pathway results in reduced cholangiocyte numbers *in vivo* and abrogated expression of early biliary transcription factors. During hepatocellular regeneration the Notch signalling pathway is expressed at low levels, however is also negatively regulated through high expression of Numb protein.

Chapter 6 - Conclusions and Future Perspectives

The adult liver has a remarkable regenerative capability; however the health burden faced by the UK and Europe suggests that the normal regenerative capability of the adult human liver is being overwhelmed and as a result chronic liver disease is increasing throughout the western world[1]. In order to stem the progression of liver disease and its associated pathologies it is important to consider not only the gross pattern of disease, but also the molecular pathology of these diseases and how these factors are ultimately the driving force of both disease establishment, but also disease propagation. The adult liver offers a unique model of tissue regeneration in a solid organ, and could ultimately shed light on the regenerative mechanisms in other solid epithelial tissues which are subject to fibrosis and regeneration. Importantly the healthy liver does not demonstrate cellular turnover from progenitor cells[7]; rather it utilises activation of the mature epithelial cells to maintain its homeostatic turnover[36, 38]

Only during chronic disease does the liver rely on a population of progenitor cells to regenerate its epithelial components, and as such this organ offers a unique opportunity to evaluate the signals which are present in disease pathology, but not in healthy liver. Interrogation of these pathways to investigate what mechanisms are present to stimulate and control progenitor cell proliferation and differentiation, the latter of which is grossly under described within the current literature and as such may provide a novel insight into what pathways could be targeted to a therapeutic end.

During chronic regeneration of the adult liver diverse epithelial compartments must be regenerated in order for the liver mass to function correctly[18, 63, 90, 329].

The vast body of work in the literature which looks mechanisms of proliferation in HPCs[61, 102-104, 330], and to a much lesser degree the signals which govern their differentiation[304, 322, 331] infrequently distinguish between human disease aetiologies, and the pattern of disease induced in animal models. This has led the field to become muddied in its approach to elucidate the signalling pathways which are important in these processes.

The initial aim of this Thesis was to identify and establish within our laboratory divergent animal models which require regeneration of the epithelial compartments. To this end I have established and characterised the Choline Deficint Ethionine Supplemented (CDE) diet, and the 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) as models of hepatocyte and cholangiocyte regeneration from HPCs respectively, both of these models have been used in the literature with great success however have never been considered as separate patterns of hepatic disease[6, 60, 63, 104, 332]. Importantly establishing these two models in our lab confirmed our suspicions that despite HPC activation and the surrounding cellular and acellular niche being stereotypical in these models as described in human liver disease[90] the niche structure is variable depending on disease pathology. During hepatocyte regeneration the HPC niche is highly homogenous, with examples of both macrophages and myofibroblasts contacting the HPC and as such both cell types can exert some

influence on the HPCs, however during biliary regeneration in our DDC model we find that there is a florid myofibroblast niche surrounding the emerging HPCs, which prevents the penetration of macrophages into site of regeneration and as such prevents them interacting with and influencing the HPCs. These observations are salient as niche distribution will obviously affect the accessibility of both small molecules and larger cell based therapies for liver disease – fields which are emerging within the literature[8, 333, 334]. Moreover the variability in niche could be indicative of more subtle changes and differences in these disease patterns, a factor which I have defined more clearly during the course of this thesis.

The second aim of this work was to investigate whether or not there is a variation in the Notch signalling pathway during hepatocellular disease and epithelial regeneration. It is currently unknown what pathways are important in the lineage separation of HPCs during epithelial regeneration in this organ, as such we looked to the well described ontogeny of the liver to best inform which pathways could be important in the regeneration of the adult liver. This work demonstrates for the first time that during adult regeneration of the liver in diverse disease patterns hepatocytes or cholangiocytes require differential signalling cascades to be activated in order to assume the correct epithelial fate.

During definitive hepatic development Notch signalling is required for appropriate specification of the biliary tree[220, 226, 335-338]. This signalling pathway has been described during biliary disease but until this work the functional significance of

Notch signalling has not been investigated[228, 339-342]. It is interesting then that regeneration of the biliary tree from a progenitor source appears at least superficially, to resemble the initial specification and ontogeny of the bile ducts. In this work we demonstrate that there is a high upregulation and activation of the Notch signalling pathway during biliary regeneration; however during regeneration of hepatocytes from HPCs we see a restriction of the Notch signalling pathway in these cells[335].

To evaluate the functional importance of the Notch signalling pathway during biliary regeneration, we inhibited Notch pathway in a range of experimental systems to investigate the role of Notch signalling on biliary specification. During development abrogation of the Notch signalling pathway is associated with retardation of biliary specification and patterning, in humans this abrogation leads to Alagilles syndrome[232, 339, 341]. Using a co-culture system of Jagged-1 expressing myofibroblasts and primary HPCs I have shown that the Notch signalling pathway is activated and that this activation can be inhibited though treatment with DAPT which prevents the receptor processing required Notch pathway activation. In this co-culture system DAPT reduces the activation of the Notch signalling pathway. This pathway reduction is associated with the reduction in biliary phenotype as demonstrated though loss of biliary transcription factors such as Hnf1β and Hnf6.

Intriguingly during biliary regeneration I find that there is an establishment of mesenchymal stroma surrounding the HPC, this stromal component is redolent of the portal mesenchyme which has recently been described as the critical supplier of

Notch ligand Jagged-1 during development[216]. The administration of Notch pathway inhibitors *in vivo* results in the suppression of biliary phenotype though the loss of early biliary transcription factors. This results in the reduction of cholangiocytes in absolute terms though a mechanism which is not clear. What is clear however is that there is no change in the inflammatory niche during DAPT administration, and as such I suggest that the loss of biliary cells is not down to a lack of support from the surrounding myofibroblasts. Rather this DAPT treatment is affecting the proliferation and/or differentiation of the HPCs, potentially allowing them to enter a more hepatocyte like fate, however without a suitable lineage tracing model it is difficult to draw firm conclusions.

Further to describing the requirement of Notch signalling during biliary damage and regeneration I have found that the Notch regulator Numb is expressed both within the healthy bile duct and progenitor cells destined to become hepatocytes, however is lost during biliary regeneration (presumably to facilitate the function of activated Notch signalling). Interestingly suppression of the Notch pathway in the liver appears critical for the correct homeostasis of the liver. Ectopic activation of the Notch signalling pathway in the liver through generating mice with a haploinsufficiency in the Fringe family of genes resulted in biliary proliferation in adult mice indicating suggesting that Notch suppression is necessary to prevent aberrant proliferation of bile ducts[148].

Despite not completely understanding how Numb and Notch are interacting during biliary specification I have found that Numb is likely to be controlled, and maintained in the progenitor cells becoming hepatocytes through the canonical Wnt pathway – a mechanism which has been predicted in the literature, but few studies have addressed completely[188, 189]. To address the final aim of this work I have demonstrated that during hepatocyte regeneration from an HPC source the progenitor cells themselves express genes which are known to be upregulated in response to the canonical Wnt pathway, such as Axin2, Myc and Sox9 at higher levels than those progenitor cells undergoing biliary regeneration. This was coupled with the translocation of Ctnnb1 to the nucleus of these cells, supporting the notion that activation of the canonical Wnt pathway is important in the fate of these HPCs.

Recombinant Wnt3a treatment of progenitor cells *in vitro* resulted in the activation of the Wnt pathway and translocation of phosphorylated Ctnnb1 to the cytoplasm and nucleus of primary HPCs. Wnt3a is described in the literature as a mitogen for both ES cell derived hepatocytes, but also as a critical factor during hepatic ontogeny[317, 343]. This was associated with an increase in the expression of early hepatocyte gene Hnf4α, but also an increase in the expression of the Numb gene; indicating that canonical Wnt signalling may be crucial to exit biliary phenotype and differentiate into a hepatocyte. In other systems of epithelial restoration after damage the canonical Wnt signal has been shown to come from macrophages[98, 344]. Here I found that there is a spatial difference in the distribution of macrophages in the two patterns of hepatocellular and biliary disease. During regeneration of the bile ducts there is a thick bad of myofibroblasts and ECM which prevents the access of macrophages to

progenitor cells. During hepatocyte regeneration macrophages can form close associations with progenitor cells and express Wnt3a at higher levels than during biliary regeneration and as such activate the canonical Wnt pathway and influence hepatocellular phenotype.

As the role of Wnt signalling on the HPC population is scant within the literature we sought to co-culture Wnt expressing macrophages with HPCs *in vitro*. In this system Wnt pathway target genes were up-regulated when cells were combined compared with maintaining the macrophages and HPCs as separate populations. Associated with this co-culture was a high level of hepatocyte specific gene activation. When we inhibited the Wnt signalling pathway *in vitro* we found that as well as a reduction in the classical Wnt target genes we also found a reduction in the specification of hepatocytes from HPCs, a paradigm which reflects the requirement of Wnt signalling in the specification of hepatocytes from foetal liver progenitor cells. Intriguingly when we inhibited the Wnt signalling pathway in HPC/macrophage co-cultures we see an increase in the early biliary transcription factors. As it appears *in vivo* Wnt signalling is required to suppress Notch function via Numb then it could be likely that there is a liberation of biliary phenotype when the Wnt pathway and by extension Numb is repressed.

The identification of Wnt expression macrophages and their physical association with HPCs *in vivo* led us to ask whether these cells alone are important for the specification of HPCs from progenitor cells. Using liposomal clodronate we were

able to ablate the macrophage population in the regenerating adult liver[326, 345], and demonstrate that the HPCs acquired a more biliary phenotype, expressing higher levels of the cholangiocyte transcription factors than those animals which had intact macrophages. Moreover when we assessed the Wnt signalling pathway in HPCs from macrophage depleted animals and controls we found that there was a loss of Wnt pathway target expression, and also a loss of nuclear Ctnnb1 suggesting that there was a failure to activate the Wnt signalling cascade, and again highlighting this pathway as important in the appropriate specification of HPCs into hepatocytes.

Although the Wnt signalling pathway is perturbed through macrophage ablation, many other pathways will also be altered though this cellular ablation. As such future lines of investigation to confirm these phenotypes will be required. One possibility is to use an appropriate Cre line, such as a Krt19CreER[85] or Sox9-Cre ER[51] crossed with Ctnnb1 activating or knockout alleles. The use of transgenic systems in the field of liver disease historically has been slow, and as such small molecule or antibody approaches to Wnt pathway modulation could be employed. The recent publication of an LRP5/6 specific blocking antibody has raised the possibility of blocking the canonical Wnt pathway tightly and specifically, but without perturbation of any other signalling cascade[346]. To address whether Wnt signalling is required for hepatocyte specification from HPCs these blocking antibodies could prove useful.

My work also points to the role of phagocytosis of hepatocyte debris in the establishment of a high Wnt status within the macrophage population. Post

phagocytic macrophages express more Wnt3a than their un-fed counterparts. To investigate whether this is a phagocytosis specific effect this work is being explored further through the feeding of multiple cell types as well as non-biological substrates including latex beads and non-phagocytosed liposomes in the presence or absence of colchicine, an inhibitor of phagocytosis[347-349]. These data are in their infancy, and the work presented in this Thesis regarding the role of phagocytosis initiating the transcription and expression of Wnt is a tantalising suggestion as to how the inflammatory infiltrate is linked to the re-establishment of the correct epithelial components in a liver, or indeed numerous other tissues, as regeneration must function on a supply and demand basis.

Ultimately this work is one of the first descriptions of the mechanisms by which HPCs can assume a differential biliary or hepatocyte fate during regeneration of the damaged liver. Here we describe that there is a differential spatial and signalling requirements within a progenitor niche; a process which has been historically described as homogenous throughout disease patterns. The diverse phenotypes of these diseases are characterised in table 6.1 and demonstrates the diverse nature of these pathways during different disease states.

Furthermore this study describes a novel means by which Notch and Wnt signalling interact via Numb to establish correct lineage choice in the adult liver. These data demonstrate that appropriate fate, and as such regeneration may be achieved by environmental sensing though macrophages and that alteration of the inflammatory

milieu is critical in the correct specification of HPCs *in vivo*. Excitingly, but outwith the realms of this Thesis we have found in collaboration Prof. with Tania Roskams, Leuven that these data correlate with human diseases, and as such may represent an archetype in diverse hepatic regeneration, and as such may identify pathways which could be manipulated in order to modulate the outcome of adult liver disease.

Table 6.1 – A summary characterisation of hepatocellular and biliary regeneration in the liver.

Characteristics of hepatocellular and biliary progenitor cells	
DDC - Biliary	**CDE - Hepatocellular**
EpCam, Dlk1 and panCK positive	EpCam, Dlk1 and panCK positive
Found as ill defined ducts near the portal tract	Form thin infiltrating cords of cells which migrate into the parenchyma
Express Hnf1β and Hnf6	Express Hnf4α and Hnf1α
Are closely associated with myofibroblasts, but have macrophages surrounding this	Are found closely associated with both macrophages and myofibroblasts
Have a highly activated Notch pathway	Low levels of Notch pathway activation
Low expression of Numb	High levels of Numb expression
Low levels of Wnt expression and Wnt effector genes	High levels of Wnt expression and Wnt target genes

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Appendix 1 – Publications arising from this Thesis