CELLULAR MECHANISMS OF MAMMALIAN LIVER REGENERATION

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Dedication

I would like to dedicate my thesis to my grandparents, Mr. and Mrs. N. Talitemjen Jamir and the late Mr. and Mrs. L. T. Yanger.

Both my grandfathers were government administrative officers in the early days of Indian independence from the British. They were assigned difficult tasks to open and establish civil administration in remote and dangerous frontiers within the young country. Armed with their strong sense of duty and courage, they accepted these postings that took them and their young families to faraway places, away from the comfort of home and loved ones, often at the risk of their own lives.

Despite the myriad limitations they faced, my grandmothers always made sure their children did not lack anything, and as a couple, sacrificed much and prioritized education for their children. My own parents mirrored this sentiment and thus two generations later, I am one of the many grandchildren who have reaped the fruits of my grandparents' foresight and labour. They have been my source of inspiration and I hope to be able to emulate their dedication in my own career and life.

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My friends and family have been an integral part of my happiness. I want to thank them for being my biggest cheerleaders. Whether near or far, the space and distance in between has not hindered their ability to encourage me and bring plenty of laughter and perspective into my life.

Last but not least, I would like to thank my parents, Toshimenla and Imtiwati Yanger. Words cannot adequately express my gratitude and awe of them. Their dedication, sacrifice and hard work in the interest of their children's personal pursuits never cease to amaze me. I am fortunate to have their love and support.

ABSTRACT

CELLULAR MECHANISMS OF MAMMALIAN LIVER REGENERATION

Kilangsungla Yanger

Ben Z. Stanger

The liver is an essential organ that aids in metabolic processes, protein synthesis and detoxification of harmful substances. As the centre for detoxification, the liver is able to compensate for this routine damage with its robust regenerative ability. All vertebrate livers, for example, can make up for tissue mass loss (via surgical excision of a portion of the liver) by replication of their differentiated cells within the remnant lobes. These differentiated cells include parenchymal cells such as the hepatocytes and biliary epithelial cells (BECs) and also non-parenchymal cells. Despite the proliferative capacity exhibited by hepatocytes, the mechanism for how the liver regenerates after toxin injuries is debated. The liver is thought to utilize facultative stem cells (FSCs) originating from the BECs, often referred to as "oval cells," for regeneration following toxin-based injury. However, the notion that oval cells act as stem cells has been based largely on *in vitro* studies and transplantation models; where lineage tracing has been employed, results have been conflicting. This thesis work employs multiple genetic tools to lineage trace the origin and contribution of various cell populations to liver regeneration *in vivo*. The findings reveal that contrary to stem cell-based models of regeneration, virtually all new hepatocytes come from pre-existing hepatocytes with no evidence of BECs

functioning as FSCs. Instead, hepatocyte lineage tracing reveals in addition to replication, they can function as FSCs. Upon perturbations including toxin injuries, they undergo a hepatocyte-to-BEC reprogramming process *in vivo*. Cellular reprogramming is the ability to interconvert distinct cell types with defined factors. This phenomenon has rarely been observed *in vivo* without exogenous factors. However, a detailed *in vivo* analysis reveals hepatocytes undergoing this cellular reprogramming process in a robust and step-wise manner involving both morphological and transcriptional changes. This hepatocyte-to-BEC reprogramming requires Notch signalling, similar to how the pathway functions in BEC-specification during liver development. These results provide direct evidence that mammalian regeneration prompts extensive and dramatic changes in cellular identity under various perturbations and thus can also serve as a cellular source for various diseases and potentially for therapy involving BEC paucity and dysfunction.

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1 INTRODUCTION[1]

[1] This chapter, containing excerpts with modifications, has been published: Yanger, K., Stanger, B.Z. *Facultative stem cells in liver and pancreas: fact and fancy. Dev Dyn*. 2011 Mar;240(3):521-9.

1.1 Overview of tissue regeneration and homeostasis

1.1.1 Stem cells

Stem cells are distinct from other mature cellular populations due to their unique ability to both self-renew (give rise to more stem cells) and differentiate into other cell types (Potten and Loeffler, 1990). The latter ability becomes more restricted as development progresses, resulting in a stem cell hierarchy based on the extent of potency (Slack, 2008). For instance, early on in development, cells from the inner cell mass of the blastocyst are considered to be pluripotent stem cells because they are able to give rise to all cell lineages except for extra embryonic tissues. With the onset of organogenesis later in development, stem cell potential becomes restricted as commitment to distinctive tissue-specific lineages occurs (Eckfeldt et al., 2005; Slack, 2008). An example of this is the male germline, in which the potential of self-renewing spermatogonial stem cells is limited to spermatogonia for the lifetime of a male organism (de Rooij, 2001). Adult tissues have two main mechanisms for replacing cells lost during routine cellular turnover. In some tissues, adult stem cells are the source of new cells throughout life, while other tissues are devoid of adult stem cells and maintain homeostasis through replication of existing cells. The skin, intestine, and blood are examples of tissues that continuously generate new cells from stem cells, while bone, kidney, and cartilage are examples of tissues in which stem cells play a limited, if any, role in normal organ homeostasis (Figure 1.1).

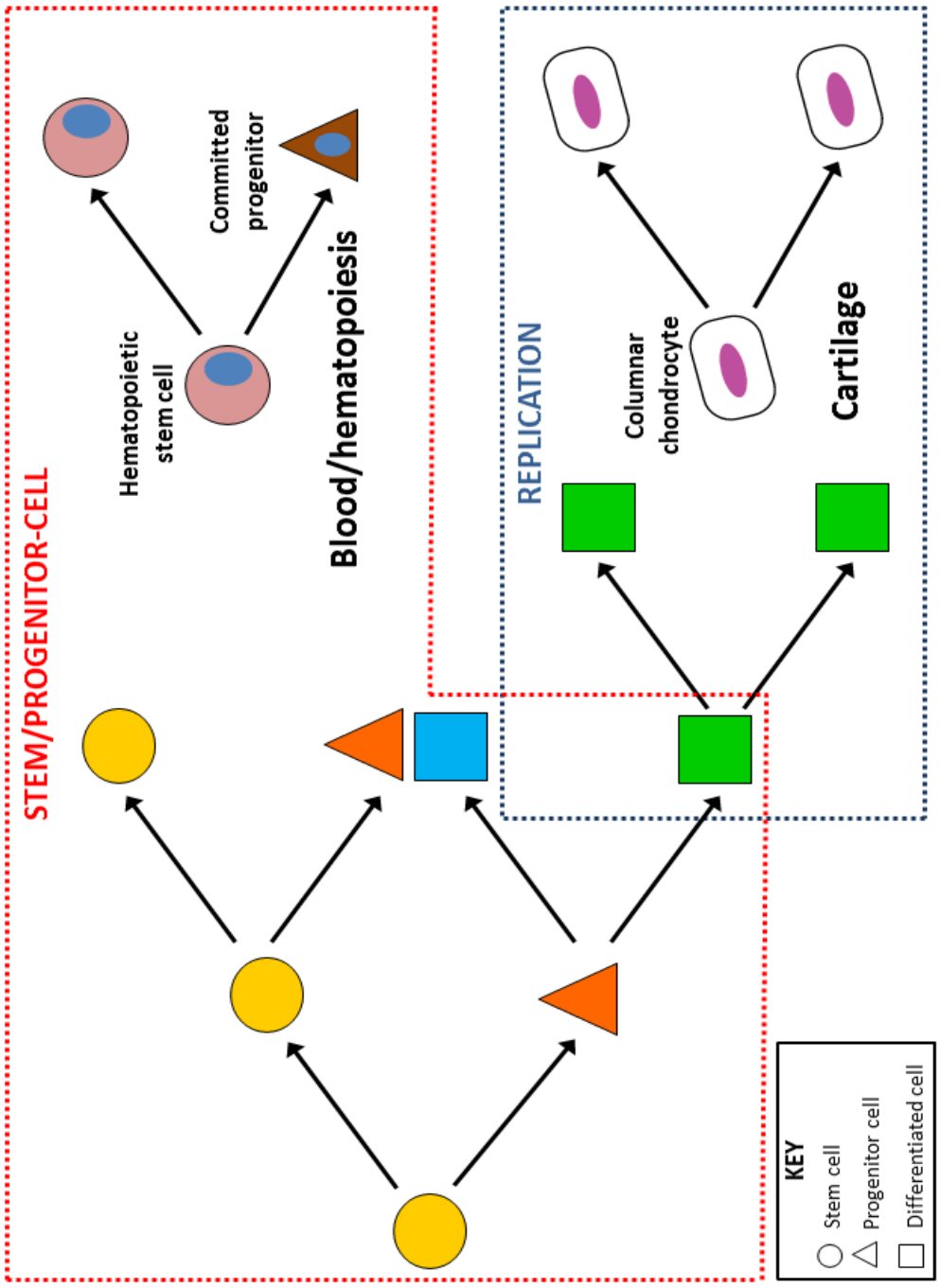

Figure 1.1 Mechanisms for maintaining homeostasis and regeneration

Figure 1.1 Two classical mechanisms for tissue homeostasis/regeneration involve differentiation of a stem/progenitor population (red box) or proliferation of differentiated cells (blue box). Hematopoietic stem cells have an apparently unlimited self-renewal capacity that enables them to continuously supply new blood cells, while cellular maintenance of the cartilage anlagen occurs by means of chondrocyte proliferation within the columnar region.

In contrast to normal tissue turnover, regeneration describes the process whereby new cells arise to replace those lost by injury. As with normal homeostasis, both stem cell-dependent and stem cell-independent mechanisms for regeneration are used by different tissues. However, under conditions of both homeostasis and injury, the relative balance between stem cell-dependent and – independent mechanisms of recovery has not been quantified. Thus, for most tissues, the relative degree to which stem cells contribute to tissue maintenance and regeneration remains undefined. The nature of the injury may also play a role in determining the recovery mechanism used by a given tissue. It has been postulated that following particular types of injury, a subset of differentiated cells can, in certain tissues, adopt a "stem cell-like" fate (Zipori, 2004). These cells have been termed facultative stem cells (FSCs) due to their ability to acquire multipotent qualities during conditions other than homeostasis, despite being initially unipotent (Figure 1.2). Such a potential blurs the stem cell-progeny paradigm that has been used by developmental biologists for decades. Thus, the biology of FSCs has relevance not only for tissue regeneration but could also serve to greatly inform our understanding of the multipotent or pluripotent state. Despite the potential importance of FSCs, the evidence supporting their existence is based mainly on *in vitro* models.

1.1.2 Stem cell assays

Historically, three major assays have been used to document stem cell activity: 1) clonogenic (*in vitro*) growth, 2) cellular transplantation, and 3) lineage tracing (Slack, 2006). Each technique has both advantages and limitations. For example, clonogenic

growth can provide evidence of self-renewal and multilineage differentiation. Moreover, as an *in vitro* culture system, clonogenic growth can be technically straightforward.

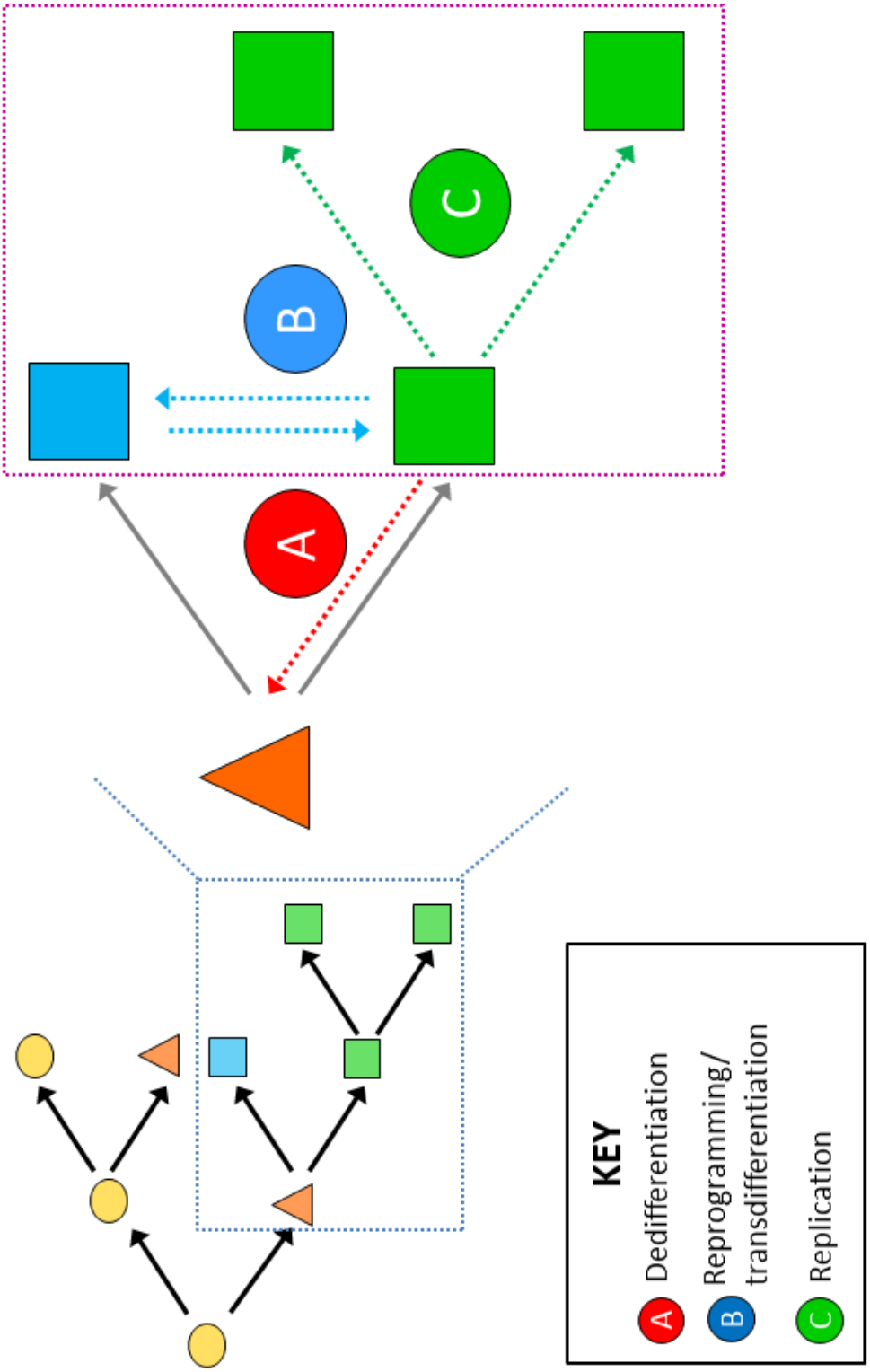

Figure 1.2 Schematic of alternative cellular mechanisms of regeneration

Figure 1.2 As opposed to a unidirectional hierarchy resulting in mature cells through stem-cell differentiation or replication (black arrows), other mechanisms could account for tissue restoration (blue box expanded). These putative mechanisms include both the FSC model and other alternatives. (A) A mature, differentiated cell (green square) could dedifferentiate and acquire a progenitor identity (orange triangle), thus functioning as a FSC. (B) Mature cells could undergo reprogramming, which would allow them to interchange/transdifferentiate into other differentiated cells. Such a mechanism could be difficult to distinguish from the dedifferentiation–redifferentiation model in (A) unless the fates of the differentiated cells were followed with precision. (C**)** Finally, simple replication of existing differentiated cells could account for restoration of tissue mass. In this scenario, putative FSCs could simply be "bystanders" and not formally contribute to the regenerated tissue.

However, such assays do not necessarily indicate "stemness" *in vivo*. Moreover, growth assays assume that the progeny of the putative stem cell are "stable" *in vitro*. This latter point is critical, because the appearance of multiple cell types in a colony arising from a single cell is commonly taken as evidence of multipotency, yet this interpretation would be incorrect if differentiated cells placed in culture have the capacity to interconvert or "transdifferentiate." Likewise, cell transplantation assays have been tremendously important in the identification and study of stem cells, particularly hematopoietic stem cells. However, transplantation assays can also be subject to confounding phenomena. One of the most important of these confounders is cell fusion, which can occur with many different types of cells and which can give a false impression regarding potency (Wagers and Weissman, 2004).

The use of *in vivo* lineage tracing is a key technique for determining the origin of new cells. The most commonly used technique for lineage tracing in a mouse is Cre-Lox technology, which permits labelling to occur in a cell-type-specific manner. Additionally, a variant of the Cre recombinase fused to a mutated oestrogen receptor (Cre-ER^{T2}) allows temporal control, labelling cells at a desired time point during development or adulthood. Through such genetic labelling, a cell's subsequent fate and that of its progeny can be followed, as genetic lineage labelling constitutes a heritable marking. Such labelling of putative stem cell populations allows for stringent testing of stem cell properties of self-renewal and pluripotency, and can provide insight into the cellular mechanisms of regeneration. However, Cre-Lox-based cell labelling uses the use of "tissue-specific" promoters to label cells, and thus the technique relies upon the specificity of such

promoters. Moreover, the inducible Cre-ER^{T2} variant requires tight regulation in order to avoid false labelling, particularly for FSC-studies. Hence, studies which use lineage labelling to determine whether stem cells contribute to homeostasis and/or regeneration are ultimately constrained by the specificity of the particular promoters and mouse strains used.

1.2 Modes of liver regeneration

1.2.1 Cellular mechanisms of liver regeneration after partial hepatectomy

For several decades, the liver has stood as the model organ for mammalian regenerative studies. The liver has multiple functions in normal physiology – including production of plasma proteins, synthesis of bile for fat emulsification, and detoxification, and liver failure is incompatible with life. The liver's regenerative ability is apparent when, after removing two-thirds of its mass (partial hepatectomy, PHx), the remnant third is able to grow back to its original size and restore normal function (Higgins, 1931). The liver consists of several cellular components – two epithelial cell types (hepatocytes and biliary epithelial cells, or BECs) and several "non-parenchymal cell" (endothelial cells, macrophage-like "Kuppfer cells," and fibroblasts) – organized into structures referred to as hepatic lobules. Hepatocytes comprise the vast majority of cells in the centre of the lobule, while BEC-lined bile ducts are found in regions known as portal tracts at the periphery of the lobule. Bile made by hepatocytes drains into the bile ducts through tubular structures known as the "Canals of Hering," which constitutes a transitional zone between hepatocytes and BECs (Figure 1.3)

The dominant cellular mechanism by which the liver regrows is proliferation (Figure 1.4 A) (Michalopoulos, 2007). In rats, normal liver mass is attained completely within 1 week while the mouse takes an additional week to do so. Studies using thymidine-H^3 incorporation during various phases of PHx recovery in rodents have

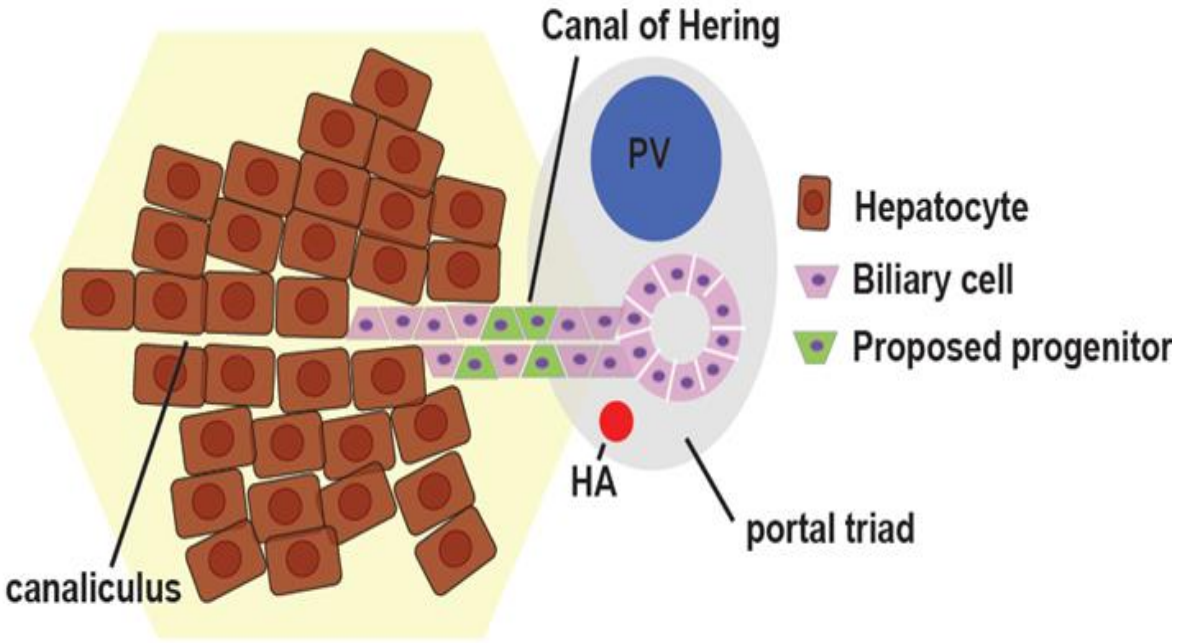

Figure 1.3 Schematic view of FSCs location in the liver

Figure 1.3 Schematic depiction of a portal tract in the liver. Bile made by hepatocytes drains into the canalicular space and subsequently through the Canals of Hering into the bile duct. The precursors of oval cells in the liver have been proposed to reside within the Canals of Hering, the transitional zone between hepatocytes and biliary cells. HA, hepatic artery; PV, portal vein.

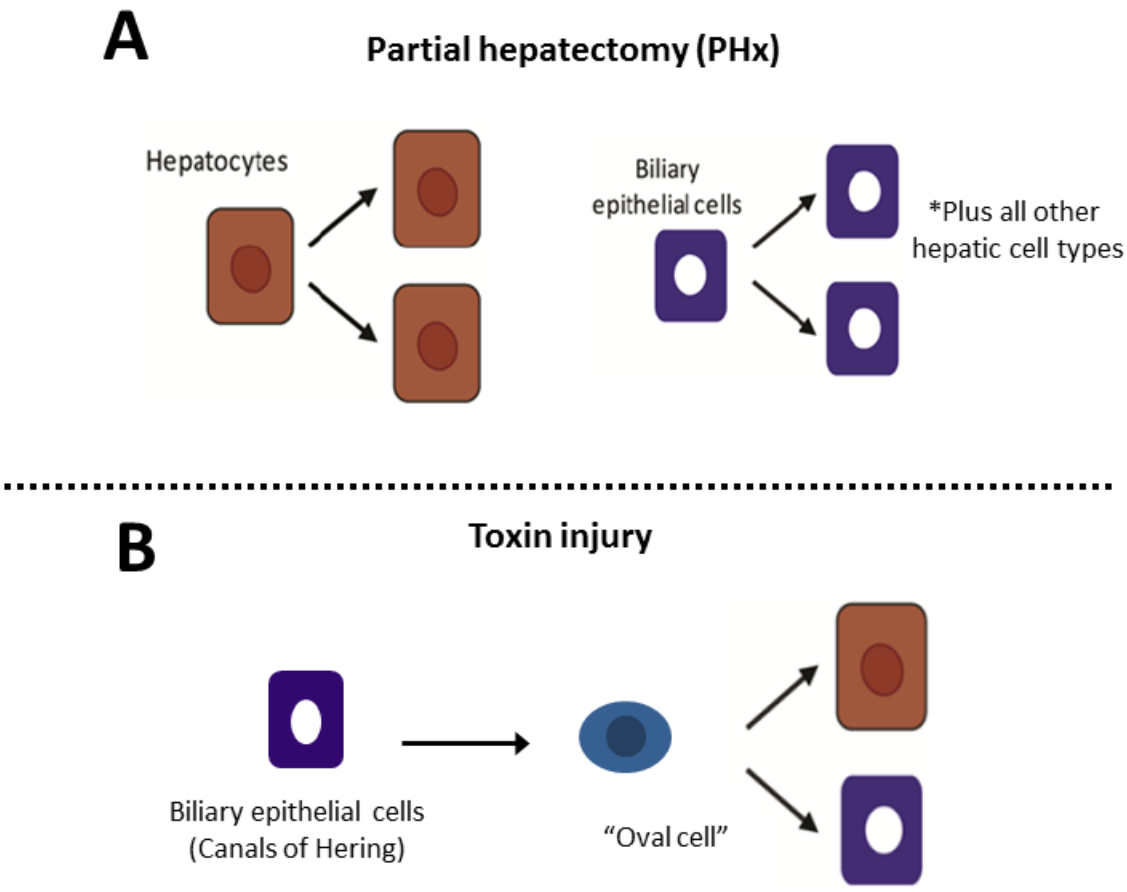

Figure 1.4 Mechanisms for maintaining homeostasis and regeneration in the liver

Figure 1.4 Following partial hepatectomy (PHx; A), differentiated liver cells undergo replication to make-up for the surgical loss of mass. In a toxin injury (B), a new cell type known as an “oval cell” is proposed to arise from BECs and function as a bipotent facultative stem cell (FSC).

demonstrated that cell replication following partial hepatectomy follows reproducible kinetics (Grisham, 1962). Hepatocytes constitute the first cell population to enter the cell cycle, followed by the biliary epithelial cells and then non-parenchymal cells (Michalopoulos and DeFrances, 1997; Taub, 2004).

In addition to cell proliferation, cellular hypertrophy has been shown to be another important regenerative mechanism dependent on the degree of injury and the state of the animal. Older mice have impaired liver regenerative capability compared to younger mice due to decreased proliferative ability (Iakova et al., 2003; Timchenko, 2009). However, this defect in ageing female mice is reversed during pregnancy. Older, pregnant mice utilize cellular hypertrophy instead of cell proliferation, to recover at rates comparable to its younger control animals (Gielchinsky et al., 2010). Additionally, the degree of the surgical excision has been shown to induce hypertrophy in addition to cell proliferation (Miyaoka et al., 2012).

1.2.2 Molecular mechanisms of liver regeneration after partial hepatectomy

The exact mechanism of how organs such as the liver 'senses' and controls its regenerative size is not known (Stanger, 2008). However, studies allude to both intrinsic and extrinsic signalling mechanisms being involved in PHx regeneration (Michalopoulos and DeFrances, 1997; Taub, 2004). For example, factors as diverse as interleukin-6 (IL-6) and the bile acid receptor, FXR, have been reported to be important (Cressman et al., 1996; Huang et al., 2006). Both IL-6 and FXR knockout mice after PHx display many anomalies during its regenerative process. Mortality rates during recovery are higher in IL-6 -/- and FXR -/- mice compared to their respective wildtype counterparts. Hepatocyte

replication appears to be compromised in both knockout animals as bromodeoxyuridine (BrdU) staining is greatly reduced in hepatocytes following injury. In IL-6 -/- animals this appears to be hepatocyte-specific as the nonparenchymal cells such as the endothelial and Kupffer cell proliferate normally and are comparable to wildtype livers (Cressman et al., 1996). This suggests that nonparenchymal cells do not need IL-6 for this process. However, mitogenic signals such as endothelial vascular growth factor (VEGF) are secreted by hepatocytes for sinusoidal endothelial cell proliferation in PHx recovery, thus revealing the existence of a paracrine relationship (Shimizu et al., 2001).

Many IL-6 -/- animals that recover after a PHx are jaundiced, have smaller and highly necrotic livers (Cressman et al., 1996). However, despite the IL-6 knockout, not all animals display these pockets of necrosis, potentially indicating the ability of the liver to compensate for this mutation. Indeed, wildtype hepatocytes have been shown to be able to clonally repopulate ~95% of the liver in urokinase-type plasminogen activator (uPA) transgenic mice (Sandgren et al., 1991). The replicative ability of hepatocytes has been displayed in serial transplantation studies, with calculations suggesting that a single hepatocyte is competent to give rise to many entire livers (Overturf et al., 1997).

Transplantation assays allude to species-specific intrinsic regenerative mechanisms. For example, rat hepatocytes transplanted into mice livers that undergo a PHx exhibit entry into the cell cycle sooner than mouse hepatocytes. Despite being in a mouse-specific cellular milieu, the rat hepatocytes label with BrdU within 24 hours like they do normally and not later at 32 hours like the mouse hepatocytes (Weglarz and

Sandgren, 2000). Thus hepatocytes display a certain degree of species-specific cell autonomous features.

1.2.3 Liver regeneration in toxin injury and origins of FSC model

Despite the robust regenerative capacity of hepatocytes, it is widely believed that an alternative mechanism for adult regeneration, involving FSCs, is used following certain types of liver injury (Figure 1.3). This hypothesis initially emerged from studies in which the rat liver was forced to regenerate following exposure to one of several hepatotoxic carcinogens. In rats, it is believed these cells emerge as a result of impaired hepatocyte replication, although in the mouse, counterparts of these cells still appear in injured livers despite the ability of hepatocytes to proliferate (Ghoshal et al., 1983; Wang et al., 2003a). Under such conditions, a distinctive histological picture was noted, characterized by the emergence of a heterogeneous population of small oval-shaped cells with biliary properties (Farber, 1956). Subsequent work revealed that cells with a similar appearance are observed in many or most models of hepatocarcinogenesis or toxin-induced injury (Lee et al., 2006; Roskams et al., 2003). These cells have been referred to by many different names including “ductular hepatocytes,” “intermediate hepatobiliary cells,” “atypical ductular proliferation,” or more commonly as “oval cells,” a term adopted from rodent studies (Factor et al., 1994; Gerber et al., 1983; Preisegger et al., 1999; Zhou et al., 2007). They are characterized by small size, an ovoid nucleus, scant cytoplasm, poorly defined lumen, and lack of basement membranes (Factor et al., 1994; Gerber et al., 1983; Preisegger et al., 1999). Although debated, oval cells are proposed to emerge from the BECs in the Canals of Hering (Dorrell and Grompe, 2005; Fausto and

Campbell, 2003) (Figure 1.2) and, after injury, are thought to interpose themselves between biliary ductules and so-called "intermediate hepatocytes," cells which share characteristics of both hepatocytes and oval cells (Factor et al., 1994; Preisegger et al., 1999). This transitory morphology and proximity of the hepatocytes to oval cells has been taken as evidence of differentiation of the latter into the former, and static visualization by light microscopy is consistent with that view (Factor et al., 1994).

1.2.4 *In vitro* experiments of FSC

Several *in vitro* experiments performed in the 1980s demonstrated that cultured oval cells could adopt either hepatocyte or biliary features depending upon culture conditions, a phenomenon similar to that observed with culture of fetal rat hepatocytes that have bona fide multipotent properties (Germain et al., 1988a; Germain et al., 1985; Germain et al., 1988b). Similar results were obtained by investigators who derived cell lines with "oval" cell properties (Tsao et al., 1984) and subsequently many such lines have been derived which are referred to as "liver epithelial cell lines" or BMEL cells ("bipotential mouse embryonic liver"; (Strick-Marchand et al., 2004)). Nevertheless, there is conflicting lineage-based evidence to confirm such ontological conclusions about bipotentiality (Taub, 2004; Zaret and Grompe, 2008). One of the difficulties in doing lineage tracing experiments with oval cells has been the lack of oval cell-specific markers. Although several features that distinguish oval cells from normal biliary epithelial cells have been reported (e.g.(Sirica et al., 1990)), immunohistologic studies with antibodies are unable to distinguish between oval cells and BECs (Fausto and Campbell, 2003). Importantly, oval cells represent a heterogeneous population that

includes both epithelial-derived and bone marrow-derived cells that exhibit distinct cell surface profiles (Dorrell et al., 2008; Okabe et al., 2009). Recent efforts have sought to identify novel cell-surface antibodies that are oval cell specific (Dorrell et al., 2008), but these and other studies have often yielded antibodies that also recognize normal BECs. Thus, at least based on immunostaining data, there is no compelling evidence that oval cells represent a distinct cell type that is not present in the normal liver.

1.2.5 Cell transplantation assays of FSC

Cell transplantation experiments provide another line of evidence in support of oval cell bipotentiality. Again, because of a paucity of markers, oval cell isolation for initial studies have been forced to rely on cell fractionation techniques based on the smaller size of the oval cell (Fausto and Campbell, 2003). Cells isolated in this fashion, although contaminated with others cells such as hematopoietic cells, can rescue fumarylacetoacetate hydrolase mutant (FAH-/-) mice as effectively as hepatocytes, indicating that this population has robust hepatocyte differentiation-reconstitution capacity (Wang et al., 2003a). This study, however, does not specifically address whether new biliary cells emerged from these transplanted cells. Lineage tracing studies also give support to the notion that nonhepatocytes can give rise to hepatocytes during toxin-mediated injury (Pichard et al., 2009).

During the late 1990s, it was proposed that bone marrow (BM) derived cells might serve as facultative stem cells for liver regeneration and might even give rise to oval cells (Petersen et al., 1999). However, subsequent studies showed that this phenomenon was not due to transdifferentiation but cell fusion (Alvarez-Dolado et al.,

2003; Wang et al., 2003b). This fusion was shown by transplanting Cre-expressing bone marrow cells to R26-LacZ reporter mice that require Cre recombination for LacZ expression. β-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) staining occurs in cells positive for LacZ. Hepatocytes in the liver were found to be β-Gal positive, signifying a recombination event with the fusion of a Cre-expressing and a R26-LacZ cell (Alvarez-Dolado et al., 2003). Another study using female donors and male recipients confirmed cell fusion using karyotype analysis. Hepatocytes in transplanted male animals were found to consist of XXXY and XXXXYY karyotypes (Wang et al., 2003b) . These cell fusion events occur with low frequency, but in the appropriate selective environment, hepatocytes generated by a fusion event are capable of significant expansion through simple replication. Thus, while there may be a small degree of cell-fusion occurring with bone marrow cells, the frequency and extent of this phenomenon is considered to be of non-physiological significance (Oertel and Shafritz, 2008; Wang et al., 2003a).

1.2.6 *In vivo* studies of FSC

Electron microscopy studies reveal that following injury with the hepatotoxin Dipin, a series of "transitional" cells – cells with ultrastructural features of both ductular cells and hepatocytes – were found (Factor et al., 1994). These cells were larger than oval cells but smaller than hepatocytes, had variable nucleocytoplasmic rations, mitochondrial content, and glycogen rosettes (Factor et al., 1994). Thus, at the ultrastructural level, oval cells do seem to comprise a heterogeneous population of cells, with some exhibiting intermediate features "between" hepatocytes and BECs.

One marker used for lineage tracing study which supports the view that oval cells can exhibit bipotentiality was the Foxl1 marker, in which Foxl1-Cre mice were used to label putative progenitor cells (Sackett et al., 2009). Foxl1 is a mesenchymal marker, and mice in which the Foxl1 lineage was labeled (using a lacZ lineage 'reporter') exhibited β-Gal activity in both hepatocytes and biliary cells following injury. This result does not distinguish between the possibility that a single bipotential progenitor cell was labeled vs. the alternative explanation that hepatocytes and biliary cells both independently activated the Foxl1 promoter (leading to cell labelling) upon injury, and additional experiments will be needed to resolve these issues.

More recent studies utilizing inducible-Cre promoters have been used to lineage trace oval cells *in vivo*, though they have also led to disparate results (Friedman and Kaestner, 2011). For instance, studies utilizing similar *Sox9-CreER* strains result in opposing findings, with one favouring a stem role for $Sox9^{+}$ BECs (Furuyama et al., 2011) while another did not find evidence of this (Carpentier et al., 2011). Cre-based labelling employing osteopontin (*Opn*) or *Lgr5*- inducible drivers have supported the idea of bipotent oval cells (Espanol-Suner et al., 2012; Huch et al., 2013). In both cases, inheritance of the label was found in hepatocytes, though in low amounts. However, other *in vivo* lineage tracing studies come to opposing conclusions, of which has been shown by other studies (Malato et al., 2011) and my own, which will be discussed in this work.

1.2.7 Summary

In summary, the liver is believed to have two mechanisms for regeneration depending upon the mechanism of injury (Figure 1.3). Following partial extirpation (PHx), remaining hepatocytes re-enter the cell cycle, undergoing one or two rounds of cell division resulting in a complete recovery of liver size. This cellular mechanism for regrowth is well established, and several of the signalling pathways that mediate this growth response have been identified (although the identity of the size "sensor" that informs the liver that regrowth is needed remains elusive). By contrast, injury with hepatotoxins results in the emergence of numerous small cells – most commonly referred to as oval cells – that arise in the portal tracts and make their way into the lobules. Cell transplantation studies and *in vitro* differentiation experiments suggest that these cells exhibit multipotency under these experimental conditions, but *in vivo* studies exhibit opposing views and thus definitive evidence that these cells act as multipotent progenitor cells remains deficient. Likewise, although these cells are commonly believed to arise from BECs within the Canals of Hering, their precise origins also remain uncertain. Such a cell type holds much promise for regenerative medicine in light of the acute shortage of transplantable donor livers (Karp, 2009) .

2 ADULT HEPATOCYTES ARE GENERATED BY SELF-DUPLICATION RATHER THAN STEM-CELL DIFFERENTIATION

2.1 Introduction

Tissue homeostasis in adult tissues is maintained via replication of differentiated cells or stem cell differentiation. In addition to these well-established paradigms, it has been proposed that select tissues contain a population of so-called "facultative stem cells." By definition, facultative stem cells (FSC) lack stem cell activity during normal tissue turnover but are thought to be recruited during specific types of injury to behave like stem cells (Yanger and Stanger, 2011). The mammalian liver is widely viewed as a paradigm for regeneration via FSC-mediated recovery. In response to various disease states and toxin-induced injuries, rodents and humans exhibit an accumulation of atypical ductal cells (ADCs) – commonly referred to as "oval cells" – within the liver parenchyma (Farber, 1956; Popper et al., 1957). ADCs have a ductal morphology, but their arrangement into an intricate anastomosing configuration that extends into the hepatic lobule gives them a histologic appearance that is distinct from that of normal biliary epithelial cells (BECs) (Desmet, 1985). ADCs are thought to arise from BECs within the Canals of Hering, structures that reside at the interface of the intrahepatic bile ducts and hepatocyte-lined canaliculi (Factor et al., 1994; Preisegger et al., 1999).

Based on *in vitro* studies, ultrastructural analysis and cell transplantation assays, ADCs have been proposed to function as bipotent FSCs, giving rise to both hepatocytes and BECs, during toxin-mediated liver injury, although this issue is controversial (Espanol-Suner et al., 2012; Fausto and Campbell, 2003; Friedman and Kaestner, 2011; Furuyama et al., 2011; Huch et al., 2013; Malato et al., 2011;

Zaret and Grompe, 2008). Furthermore, adult hepatocytes exhibit significant plasticity *in vivo* (Michalopoulos et al., 2005; Yanger et al., 2013), a phenomenon that may give the appearance of stem cell-mediated differentiation. In order to obtain evidence for liver stem cell activity *in vivo*, I specifically labelled distinctive cell populations in the liver – BECs and hepatocytes, and transit amplifying cells (the results of the latter done in collaboration will be discussed briefly) – using both direct genetic and unbiased nucleoside analogue-based lineage labelling tools under multiple ADC-inducing injury conditions. Our results suggest that hepatocytes, not stem cells, serve as the major source for renewal and regeneration in the liver.

2.2 Results

2.2.1 ADC-inducing injuries

The liver was injured in several ways to induce ADCs including chow containing DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine) (Wang et al., 2003a), CDE diet (choline-deficient ethionine supplemented) (Carpentier et al., 2011), CCl_4 administration (carbon tetrachloride) (Malato et al., 2011), and ANIT (alpha-naphthyl-isothiocyanate) (Faa et al., 1998), all of which act as hepatotoxins that has been used extensively for studies of ADC/oval cell biology (henceforth, the term ADCs will be used to refer to cells with an "oval cell" or "progenitor" phenotype). As expected, I observed the emergence of numerous ADCs within 2 weeks of DDC, ANIT and CCl_4 treatment and within 4 days of CDE treatment. ADCs first arose in the portal regions and expressed both "classical" ADC markers (e.g. A6 and KRT19) as well as a number of newly-generated ADC markers (Figure 2.1; (Dorrell et al., 2008; Wang et al., 2003a)). Based on pilot experiments, I adopted a regimen whereby animals received a normal diet for 3-5 weeks following DDC treatment and 2 weeks after CDE and ANIT diet, a protocol that resulted in a return to near-normal liver histology. All hepatotoxins were associated with substantial hepatocyte death as assessed by cleaved caspase 3 staining and proliferation with BrdU incorporation (Figure 2.2). Thus, all treatment regiments were associated with a robust ADC response during which a substantial fraction of hepatocytes underwent turnover.

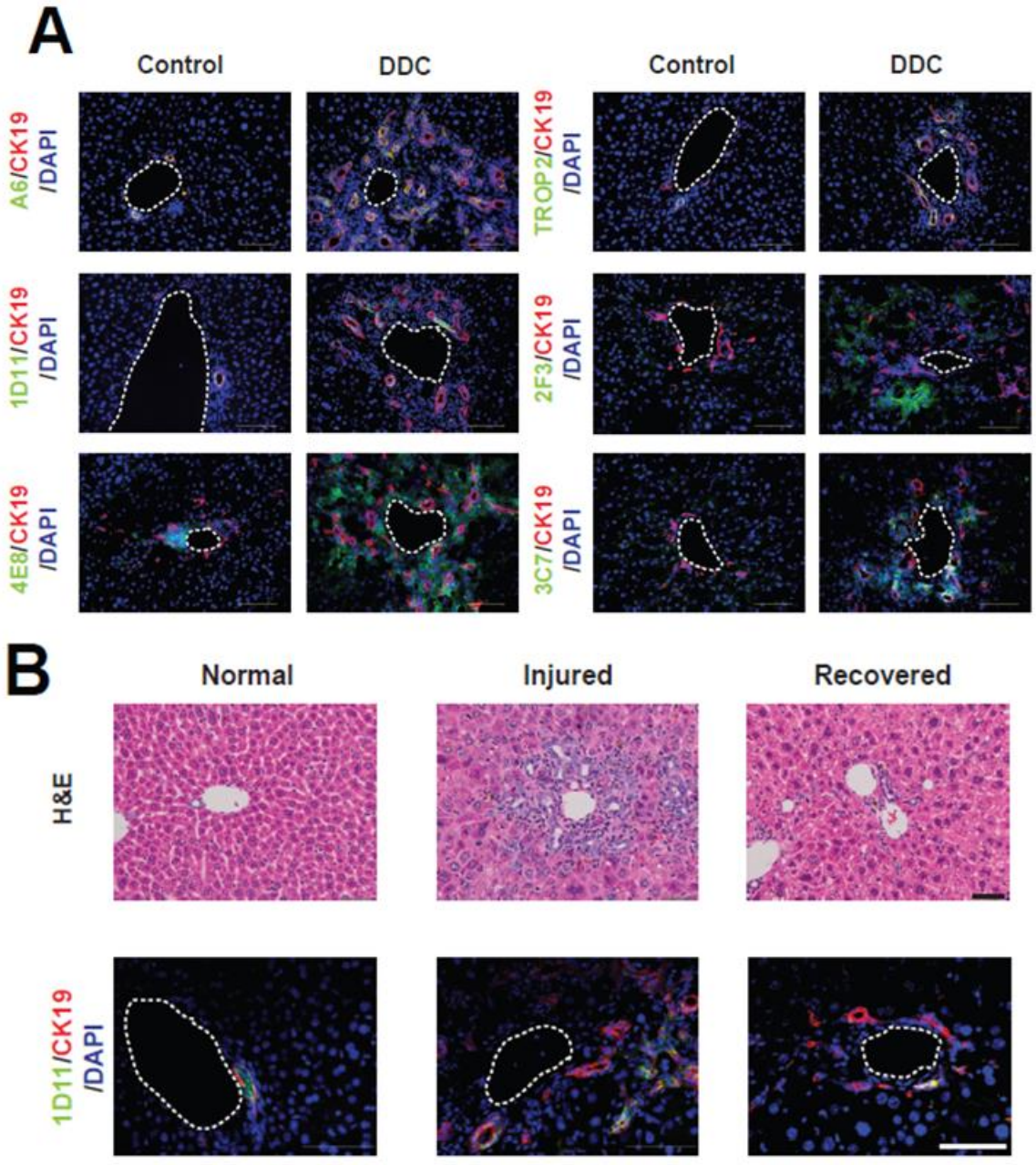

Figure 2.1 Example of liver injury resulting in an atypical ductal cell response and cessation after recovery

Figure 2.1 (A) DDC treatment is associated with an expansion of liver cells expressing a variety of oval cell/ADC markers compared to control livers. Several markers of "non-parenchymal" cells (2F3 and 3C7) were expressed only following DDC treatment, whereas all epithelial-specific markers (A6, 1D11, Trop2, and KRT19) were expressed in both normal ducts and oval cells/ADCs. (B) The injury-recovery protocol (2 weeks of DDC followed by 5 weeks of normal diet) is associated with an expansion and regression of the $KRT19^{+}1D11^{+}$ population. Scale: white bar=50μm; black bar=100μm; yellow bar=50μm.

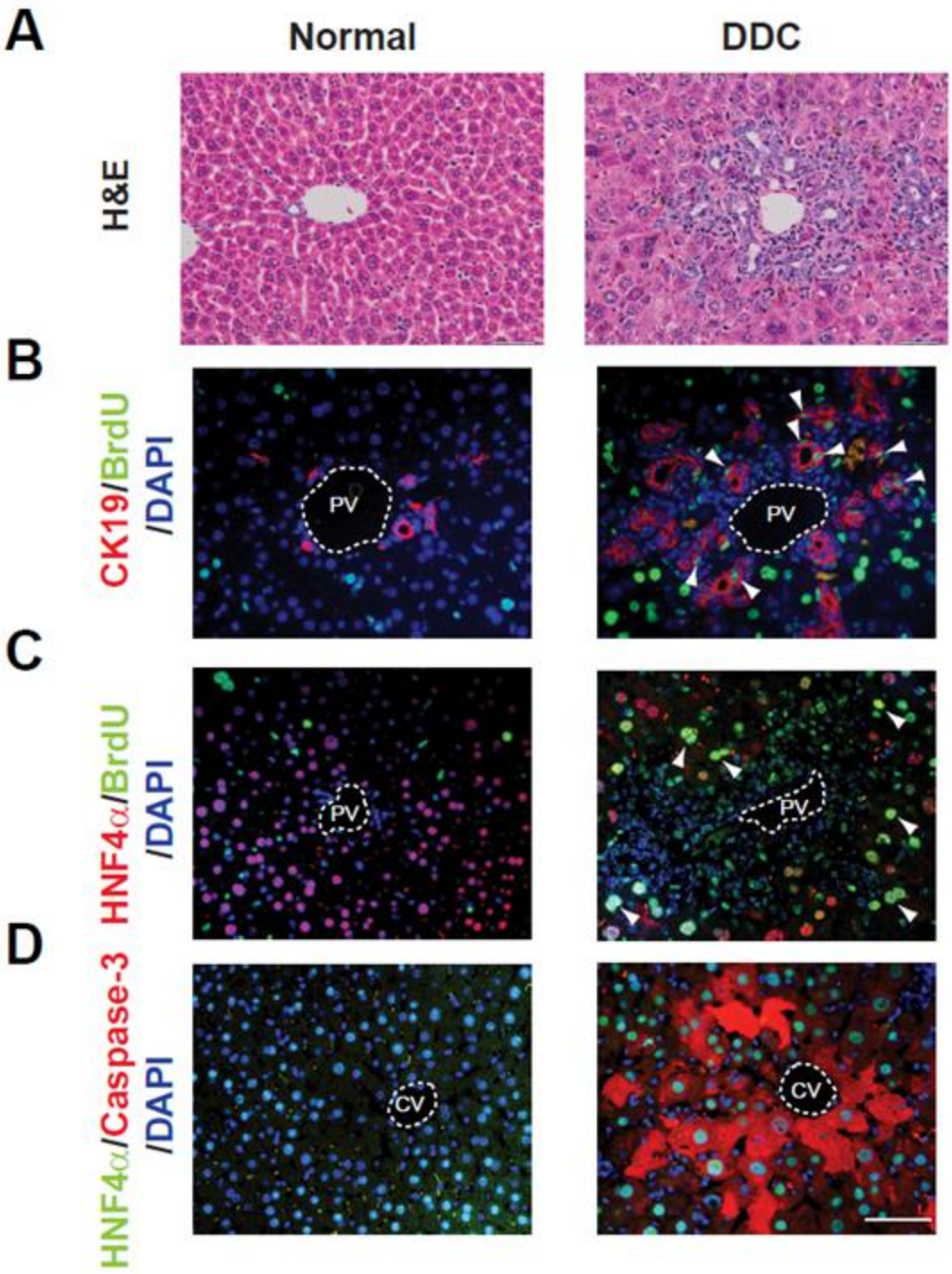

Figure 2.2 Example of toxin injury associated with significant cellular turnover

Figure 2.2 (A) Administration of a DDC diet leads to an ADC response, characterized by the expansion of small BEC-like cells from the peri-portal region into the lobule. Animals given BrdU in the drinking water for two weeks with either regular chow (Normal) or in combination with a DDC-containing diet (DDC) exhibit marked proliferation of both BECs, marked by CK19 **(B**, arrowheads**)**, and hepatocytes, marked by HNF4α **(C**, arrowheads**)**. **(D)** DDC treatment also leads to a significant increase in hepatocyte cell death, as detected by staining for cleaved caspase-3. Scale bar=50μm.

2.2.2 Lineage tracing biliary epithelial cells *in vivo*

It has been proposed that BECs residing within the Canals of Hering serve as precursors of liver progenitor cells (Figure 2.3 (Factor et al., 1994; Wang et al., 2003a)). Therefore, I crossed inducible cytokeratin-19 promoter (*Krt19*) *CreER* knock-in mice (Means et al., 2008) to *Rosa26*^{YFP} reporter mice (Srinivas et al., 2001) to label cells from the BEC lineage – prior to injury – to look for evidence that these cells could give rise to hepatocytes (Figure 2.3). Bigenic *Krt19-CreER/R26*^{YFP} mice animals were given tamoxifen (TM), resulting in pulse labelling of BECs with an efficiency of 36.2±8.7% (Figure 2.4 A, top, n=4), including cells within the Canals of Hering (Figure 2.4 A, side panels). Importantly, labelling was completely restricted to BECs, as previously reported (Figure 2.4 A; (Scholten et al., 2010)).

I then tested whether labelled BECs give rise to hepatocytes under injury or homeostatic conditions. 6-8 week old bigenic animals were given TM and after a washout period were subjected to either an injury-recovery protocol with various ADC-inducing injury models including DDC, CDE diet, CCl_4 administration, and ANIT or left uninjured. Under all injury-recovery circumstances, YFP^+ cells co-stained for biliary markers but not hepatocyte markers, indicating that labelled BECs did not give rise to hepatocytes (Figure 2.4 B and data not shown; DDC:1191 cells counted, n=3; CDE: 1157 counted, n=3). I next determined whether BECs might generate hepatocytes over longer periods of time, as has been previously

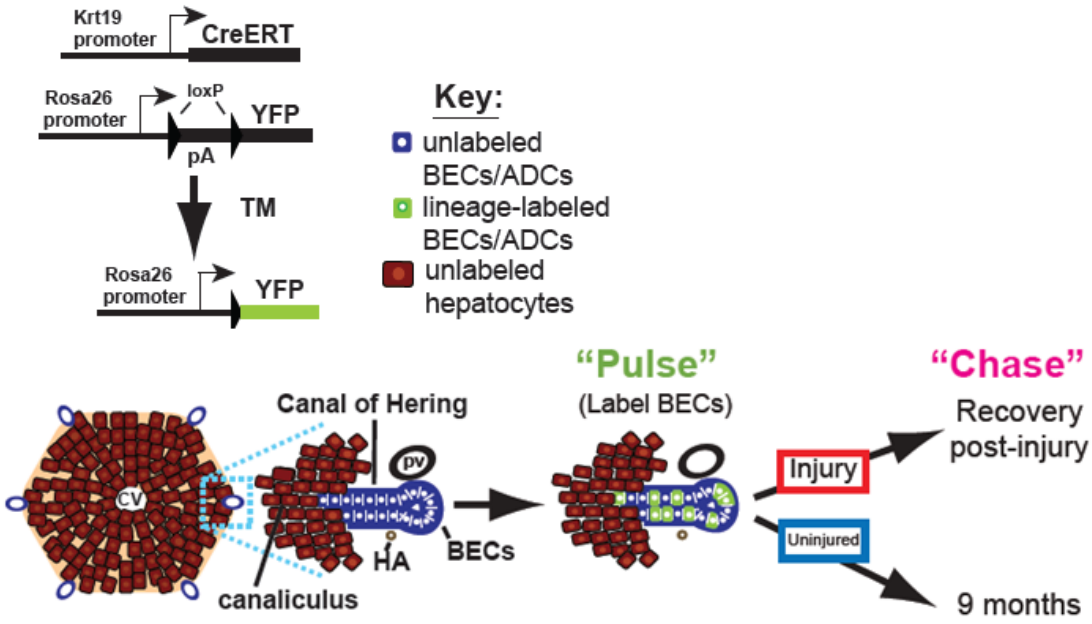

Figure 2.3 Mechanisms for lineage tracing biliary epithelial cells

Figure 2.3 Schematic view of BEC labelling using *Krt19-CreER*; *R26*^{YFP}mice. Cells are marked in a mosaic fashion upon TM injection ("Pulse"), and the ability of labelled BECs to give rise to hepatocytes is assessed following injury and recovery /under homeostatic conditions ("Chase").

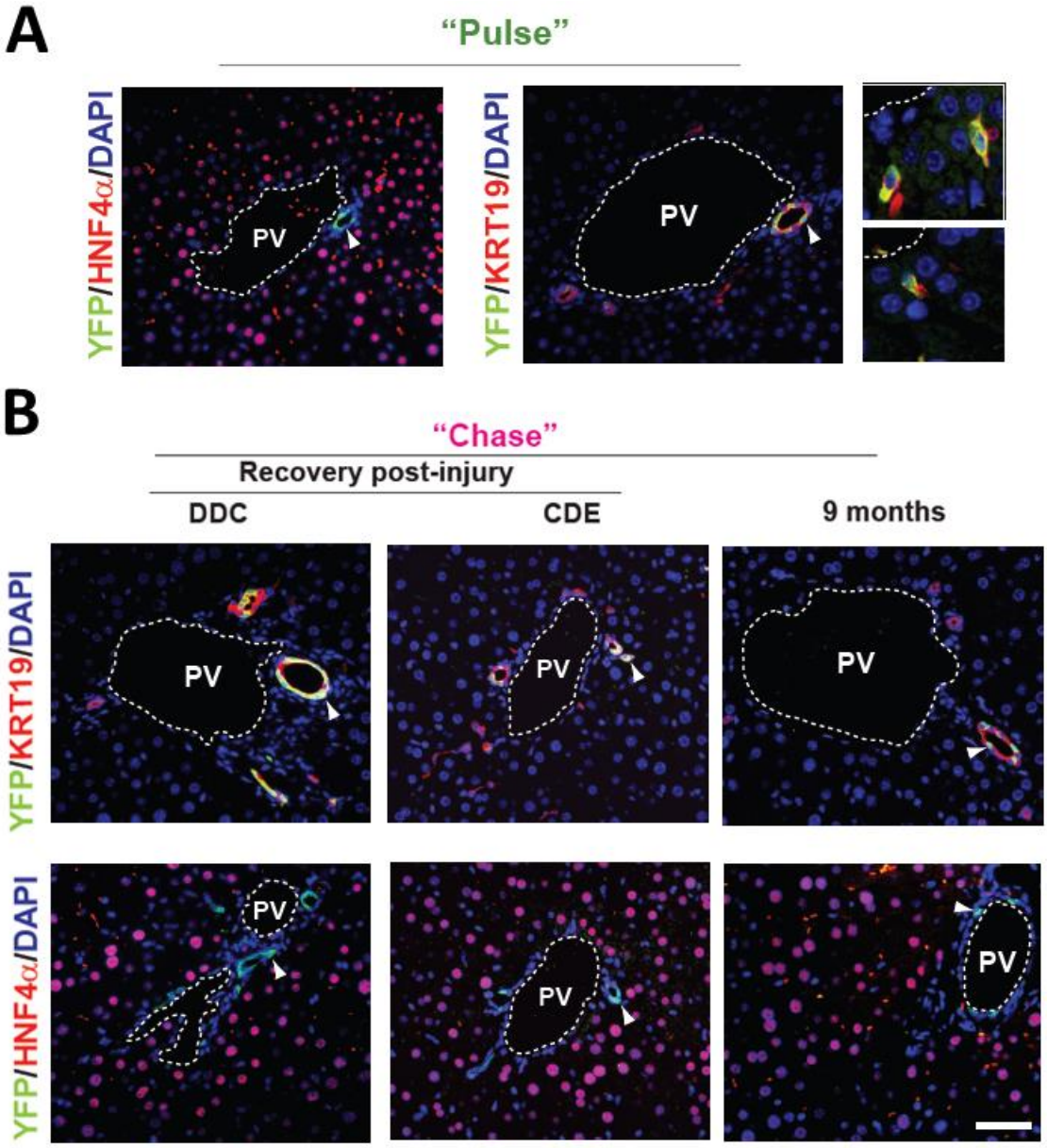

Figure 2.4 BECs lack detectable progenitor cell activity in vivo

Figure 2.4 (A) YFP-labelling at "pulse." Labelling occurs exclusively in BECs - encompassed BECs lining ducts (arrowhead) as well as BECs within the Canal of Hering (side panels). (B) Following injury-recovery, a similar pattern and degree of labelling is observed after the chase; no YFP label is observed in hepatocytes ("Chase" – DDC and CDE). Unperturbed livers studied after 9 months do not show any hepatocytes bearing the YFP label ("Chase" = 9 months). The images shown are representative of multiple experiments (Pulse: n=7; DDC: n=8; CDE: n=3; 9 months chase group: n=5). CV, central vein; PV, portal vein; HA, hepatic artery; Scale bar=50μm.

reported for Sox9^{+} BECs (Furuyama et al., 2011). Following a 9 month labelling "chase," all YFP^{+} cells expressed biliary markers (1154 counted; n=4) but not hepatocyte markers or morphology (Figure 2.4 B, 4773 counted). Hence, KRT19-expressing BECs do not appear to give rise to hepatocytes following injury or during normal liver turnover.

2.2.3 Lineage tracing atypical ductal cells *in vivo*

It is possible that KRT19^{+}ADCs arise from a unique cell population that is not labelled in the quiescent state. To this end, I administered TM to *Krt19-CreER/R26^{YFP}* animals during the second-half of DDC injury, thus labelling newly-formed ADCs (Figure 2.5 A). Injury alone (in the absence of TM) did not induce recombination of the reporter allele, an important control to verify the stringency of the inducible promoter (Figure 2.5 B). TM administration during DDC treatment resulted in YFP labelling of 22.7±4.6% of K19^{+} cells, including A6^{+} ADCs ("Pulse"; n=3; Figure 2.5 C). Labelling encompassed cells within large mature-appearing ductal structures as well as isolated ductal cells that penetrated the lobule. Labelling was highly specific for ADCs, as all labelled cells (1010 counted, n=6) exhibited a biliary morphology and stained positive for ADC/biliary markers, but lacked a hepatocyte morphology and were negative for HNF4α (Figure 2.5 C).

Following a 3-week recovery ("Chase"), YFP^{+} cells were readily detected in the livers of *Krt19-CreER/R26^{YFP}* mice (Figure 2.5 D). Consistent with our results from BEC labelling prior to injury, YFP expression was never observed in cells

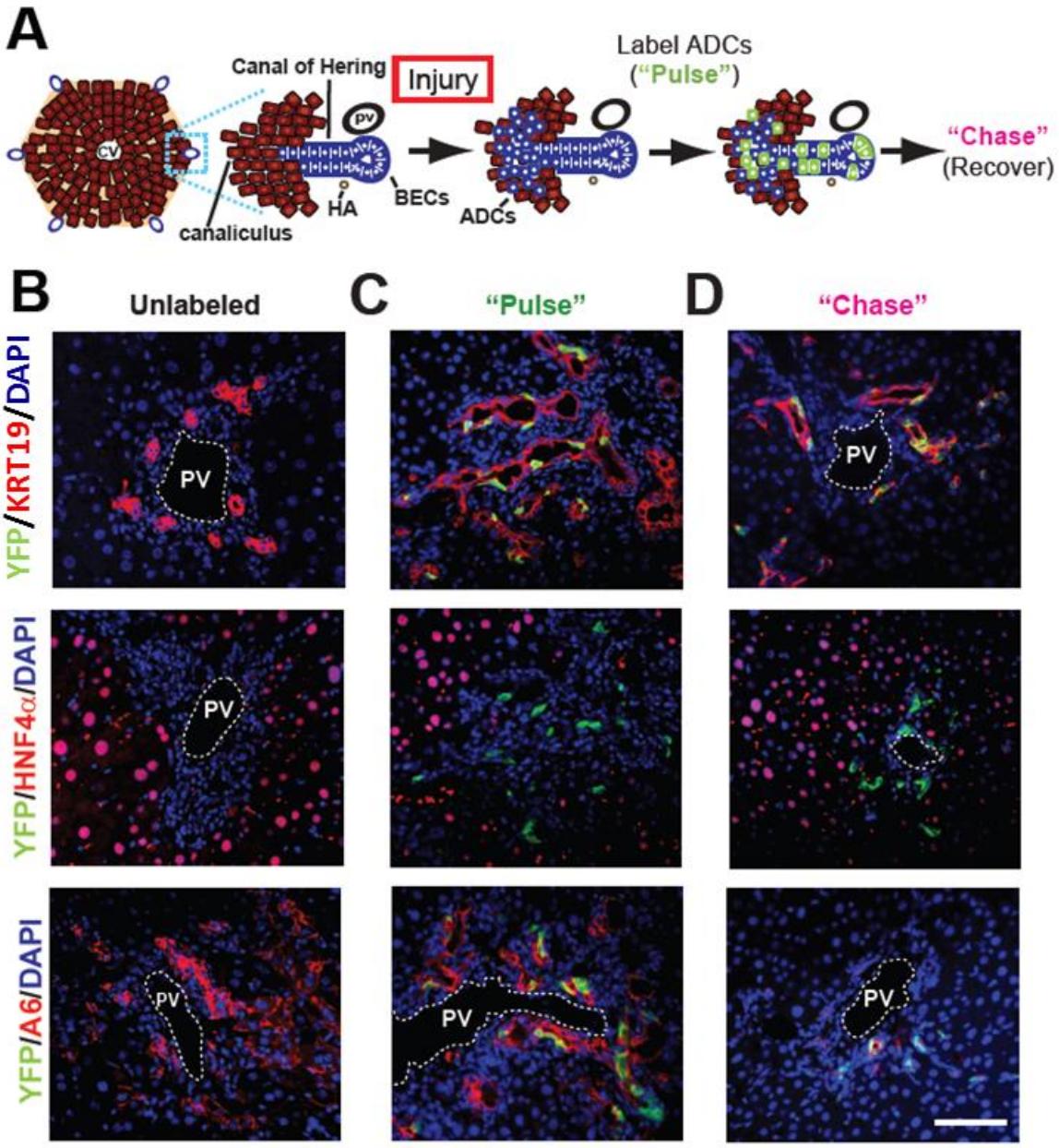

Figure 2.5 ADCs do not give rise to hepatocytes

Figure 2.5 (A) Schematic view of lineage tracing of ADCs. An idealized hexagonally-shaped lobule is shown on the left. A blow-up of one portal tract illustrates the interface between biliary epithelial cells (BECs; blue) and hepatocytes (crimson). Treatment of animals with the toxin DDC ("Injury") leads to the emergence of ADCs in the lobule (blue cells mingled with hepatocytes). Lineage labelling ("Pulse") results in the heritable marking of ADCs (green) but not hepatocytes, whose progeny can be followed after recovery (2 weeks DDC followed by 5 weeks normal chow; "Chase"). (B) DDC-treated *Krt19-CreER*; $R26^{YFP}$ mice do not exhibit YFP expression in the absence of TM. (C) The *Krt19-CreER* transgene permits specific labelling of $KRT19^{+}$ and $A6^{+}$ ADCs after DDC treatment. No hepatocytes were labelled during the pulse. (D) Following recovery ("Chase"), no label-bearing hepatocytes were observed. White bar=50μm

with a hepatocyte morphology or HNF4α expression (2474 counted; n=5). YFP^+ cells in the recovery group resembled normal BECs, exhibiting a biliary morphology and staining with biliary markers (Figure 2.5 D). As some stem/progenitor cell populations undergo replication rates that differ from that of surrounding cells (Blanpain et al., 2007), I ensured there was equal YFP labelling of proliferating and non-proliferating ADCs using Ki-67 (Figure 2.6). These results indicate that ADCs labelled during injury do not give rise to hepatocytes. Taken together, the results reveal that neither ADCs nor BECs (their presumptive cell of origin), give rise to hepatocytes under homeostatic conditions or after toxin-mediated injury.

2.2.4 Determining hepatocyte neogenesis *in vivo*

These experiments do not rule out the possibility that hepatocytes arise from progenitor cells that were not marked by the *Krt19-CreER* labelling approach (including "marker negative" cells). To test this possibility, I labelled differentiated hepatocytes and determined the contribution of "non-hepatocytes" to recovery following injury (Figure 2.7 A). The rationale for the approach follows from the expectation that unlabelled stem cells contributing to liver regeneration would give rise to unlabelled progeny, resulting in decreased hepatocyte-labelling index (Figure 2.7 B (i)). Alternatively, if new hepatocytes are derived solely from existing hepatocytes, then the hepatocyte labelling index would remain unchanged (Figure 2.7 B (ii)). This method has been used as a general means of determining the degree

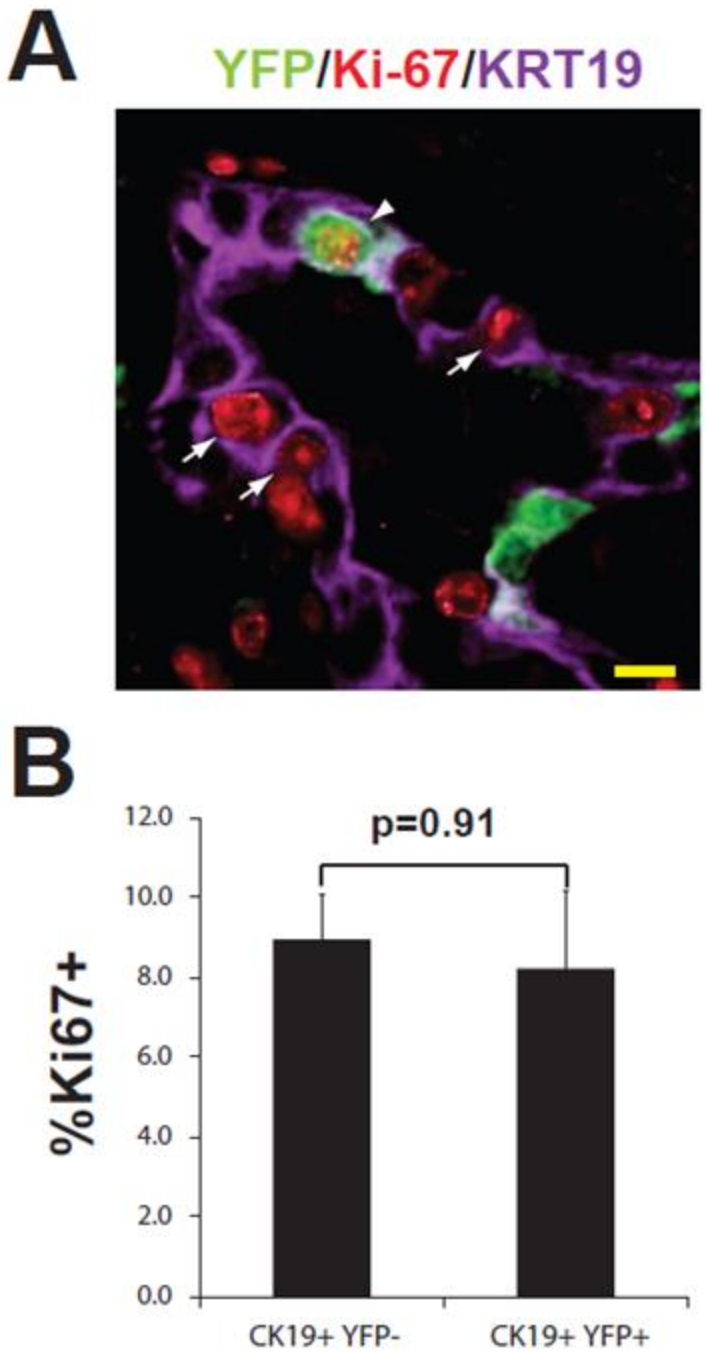

Figure 2.6 TM administration to Krt19-CreER/R26YFP mice results in equal labelling of proliferating and non-proliferating ADCs

Figure 2.6 I sought to ensure that TM administration to *Krt19-CreER*/*R26*^{YFP} mice resulted in equal labelling of proliferating and non-proliferating ADCs. Hence, I used Ki-67 to compare the fraction of proliferating cells in (A) both YFP^{-} (arrows) and YFP^{+} (arrowheads) ADCs with (B) with equal frequency of Ki-67^{+} cells comprising approximately 8% in both populations (mean ±SD). Yellow bar=10 μm

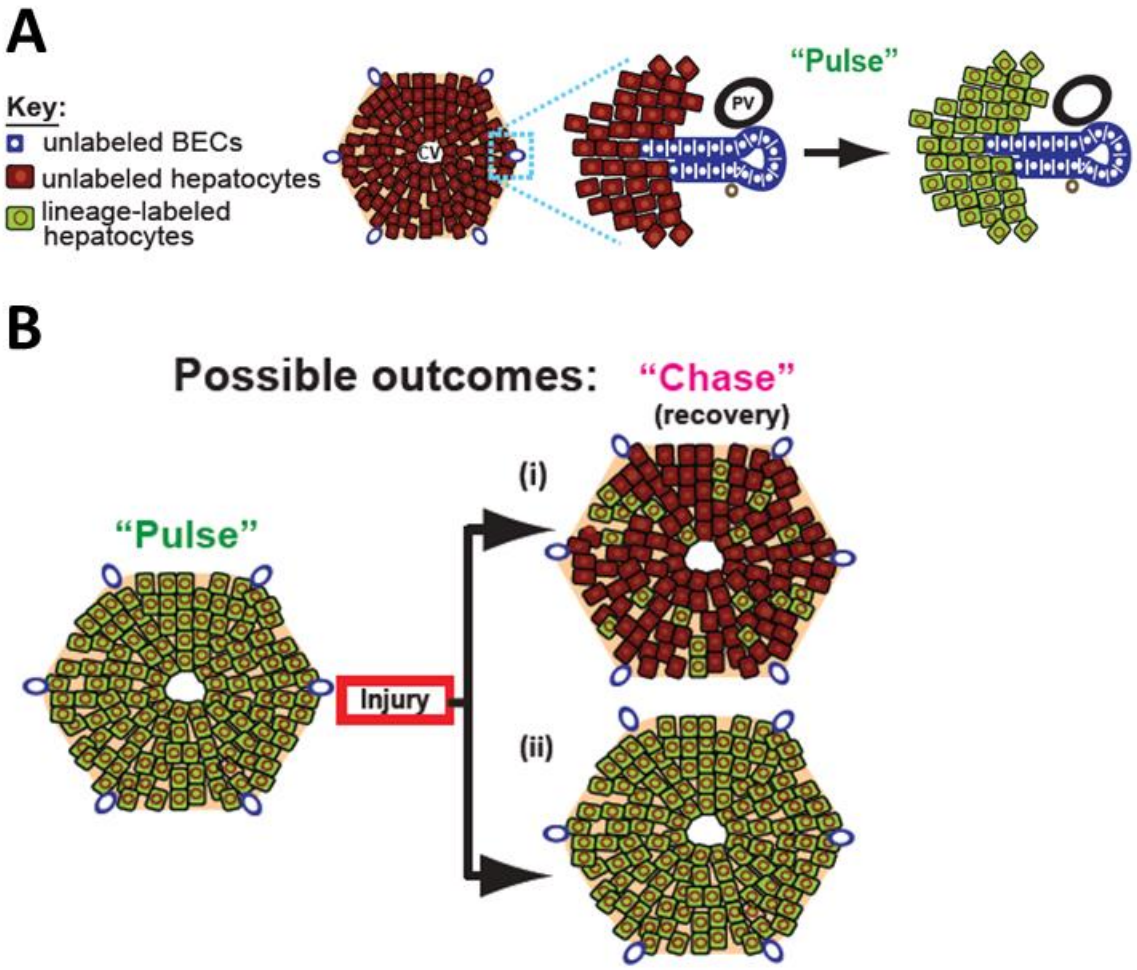

Figure 2.7 A pulse-chase system for determining the origin of regenerating hepatocytes

Figure 2.7 (A) Schematic view of lineage tracing hepatocytes. Lineage labelling ("Pulse") results in the heritable marking of hepatocytes (green) but not BECs. (B) Predictions from different models of liver regeneration. After injury and recovery ("Chase"), stem cell-based repair would result in a decrease in the hepatocyte labelling index (i), while hepatocyte-mediated recovery would result in no change (ii).

to which putative stem/progenitor cells contribute to tissue regeneration when markers of such cells are lacking (Dor et al., 2004).

Hepatocyte labelling specificity

To label hepatocytes – defined here as postnatal cells expressing HNF4α but not expressing BEC markers – we utilized a recombinant adeno-associated virus serotype 2/8 expressing Cre recombinase driven by the hepatocyte-specific promoter (thyroid hormone-binding globulin, AAV8-TBG-Cre) (Figure 2.8 A). This transduction was highly specific and effective, as all YFP^+ labelled cells expressed HNF4α, a hepatocyte marker (>99%, 3948 YFP^+ cells examined, N = 4 mice), while none expressed KRT19, a BEC marker (2056 $KRT19^+$ cells examined, N=6 mice) (Figure 2.8 A; (Gao et al., 2006; Malato et al., 2011; Wang et al., 2010; Yanger et al., 2013; Zincarelli et al., 2008)). This labelling pattern was true for liver analysed 9 months later (Figure 2.8 B). As an additional controlled, $R26^{YFP}$ mice were administered with AAV8-Cre virus under the control of the ubiquitous cytomegalovirus (CMV) promoter (AAV8-CMV-Cre). Efficient marking of hepatocytes ($HNF4\alpha^+$ cells) with no marking of BECs ($KRT19^+$ cells) was observed under both conditions demonstrating that viral tropism (and not the TBG promoter) accounts for hepatocyte-specific labelling (Figure 2.8 C-D). Additionally, all YFP^+ labelled cells were found to be positive for HNF4α with no labelling of non-hepatocytes (Figure 2.8 E).

I further confirmed AAV-specificity by fluorescence-activated cell sorting (FACS). A BEC marker, EpCAM, was used to isolate BEC cells as has been

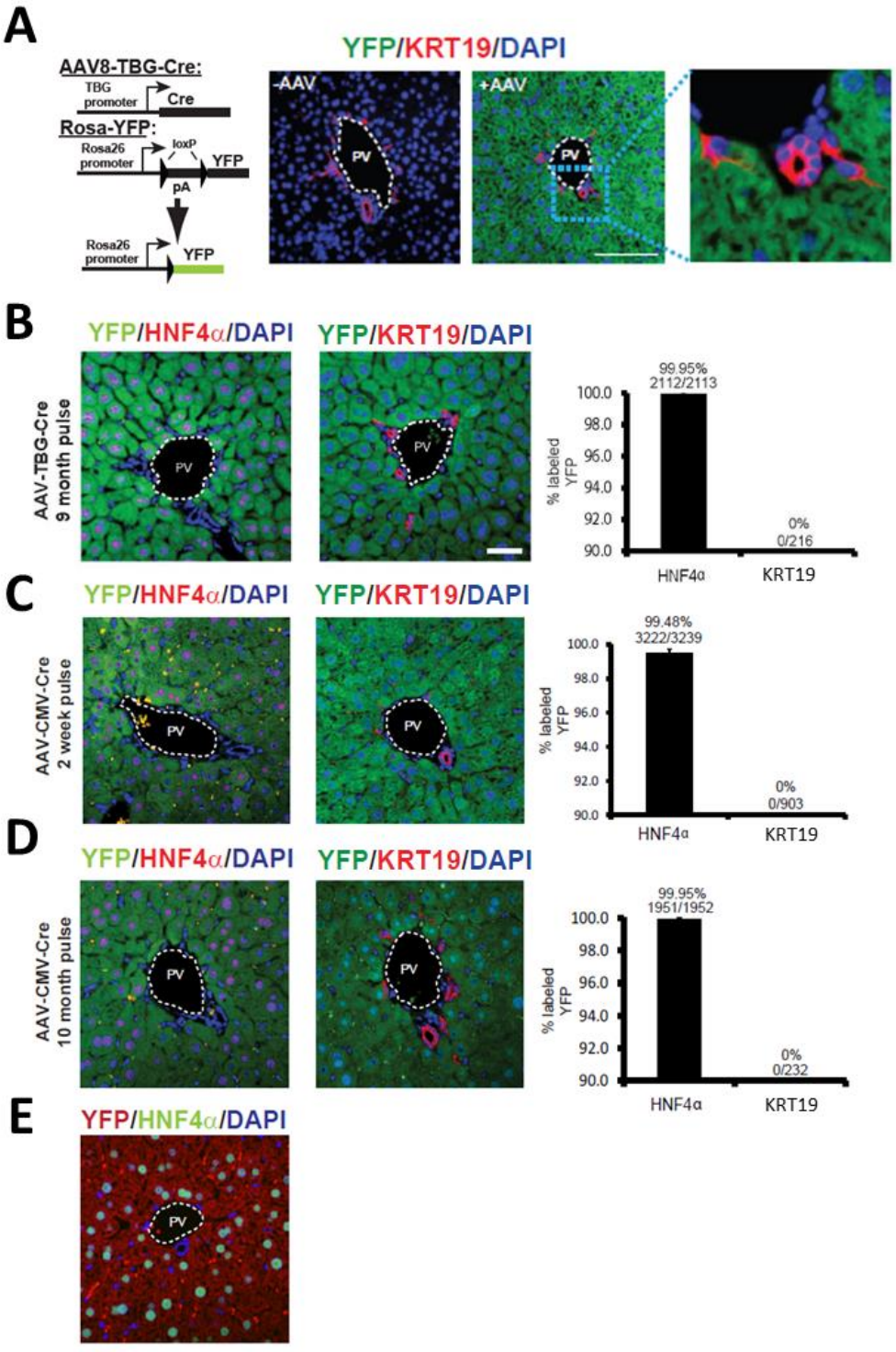

Figure 2.8 The AAV2/8 serotype specifically transduces hepatocytes

Figure 2.8 (A) Hepatocyte labelling was achieved by administrating AAV2/8 viruses containing Cre recombinase to *R26*^{YFP} mice under the control of the hepatocyte-specific thyroid binding globulin (TBG) promoter (AAV8-TBG-Cre) to determine the specificity, sensitivity, and tropism of viral infection. Immunofluorescent images show specific and efficient labelling of hepatocytes. Labelling of BECs was never observed following AAV (right panel, n= 6). (B) AAV8-TBG-Cre also results in persistent specific labelling of hepatocytes 9 months after infection; again there is no labelling of CK19^{+} BECs with this virus. (C, D) AAV2/8 viruses containing Cre recombinase under the control of the ubiquitous cytomegalovirus (CMV) promoter was used to further assess viral tropism. The images depict YFP^{+} lineage-labelled cells either 2 weeks **(**C**)** or 10-months **(**D**)** after injection of AAV8-CMV-Cre virus into R26^{YFP} animals. **(**E**)** Additionally, all YFP^{+} cells resulting from AAV8-TBG-Cre recombination (3948 cells counted) were HNF4α^{+}. Scale bar=25μm.

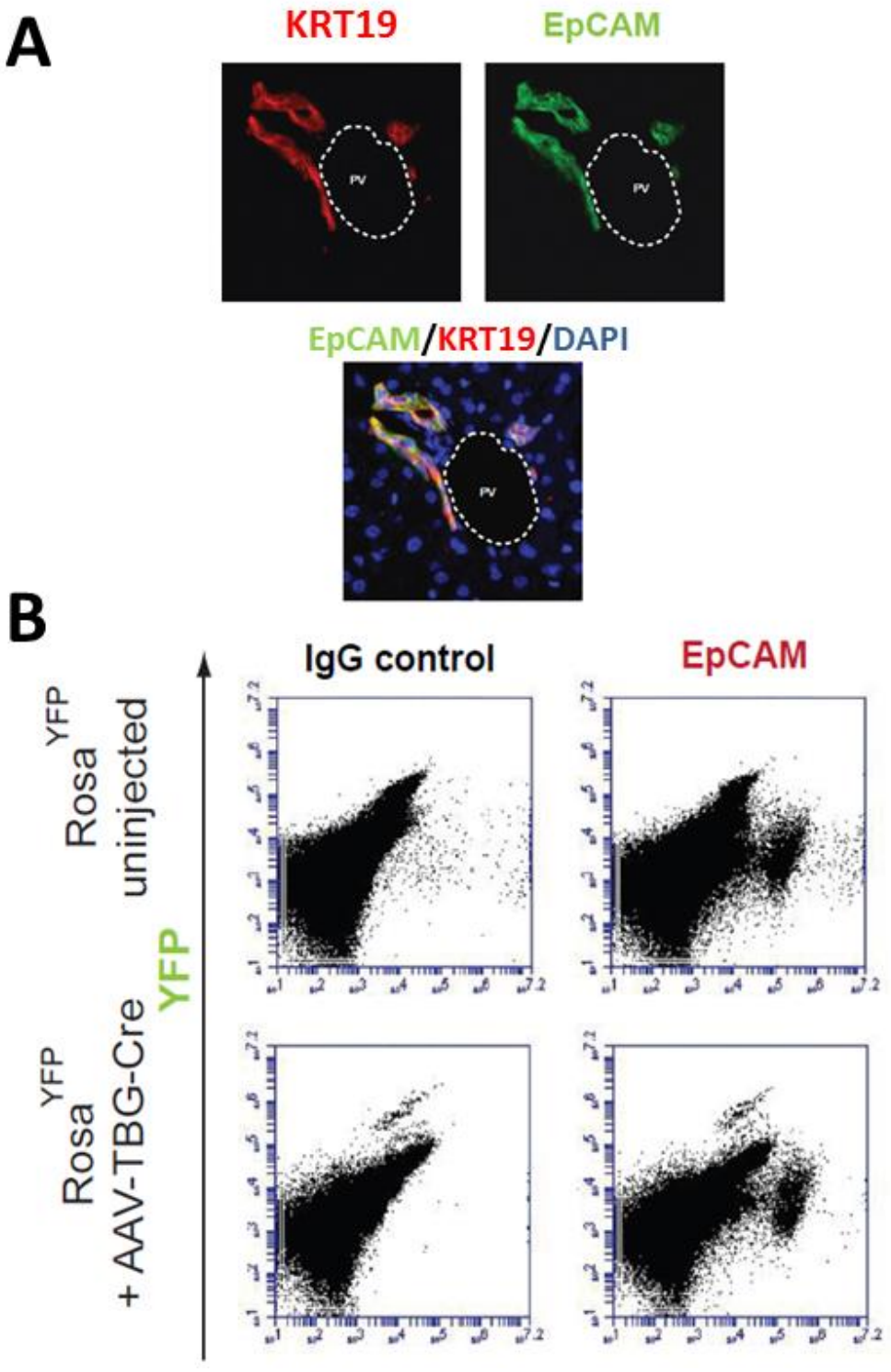

Figure 2.9 Testing AAV2/8 serotype by fluorescence-activated cell sorting (FACS)

Figure 2.9 (A) BEC marker, EpCAM, overlaps and is co-expressed in $CK19^{+}$ cells. **(**B**)** Uninjected (control) and injected (YFP^{+}) $R26^{YFP}$ animal livers were perfused and stained for EpCAM. Double positive cells for YFP and EpCAM were not detected in normal animals by FACS.

previously demonstrated (Okabe et al., 2009) (Figure 2.9). EpCAM overlaps and marks KRT19^{+} BECs (Figure 2.9 A). AAV-TBG-Cre injected *R26*^{YFP} mice were subjected to liver perfusion and further assessed by FACS to find no overlap between YFP^{+} cells and EpCAM^{+} cells, thus confirming BECs are not transduced.

Hepatocyte pulse-chase outcome

I then subjected lineage-labelled AAV8-TBG-Cre; *R26*^{YFP} mice ("Pulse") to the injury-recovery protocols used previously ("Chase"). Under these conditions, the percentage of labelled hepatocytes remained unchanged (Figure 2.10 B, C). Specifically, the labelling index for the pulse group (99.36±0.96%, 11,359 counted) did not decrease following recovery after DDC (99.31±1.00%, 5830 counted), CDE (99.83±0.17%, 1838 counted), CCl_4 (99.75±0.38%, 1677 counted) or ANIT (100%, 1944 counted). As a control, we performed 2/3 partial hepatectomy which also showed no change in the YFP labelling index (99.71±0.36%, 695 counted). Thus, by this sensitive labelling technique, I failed to find evidence that hepatocytes arise from non-hepatocytes after recovery from multiple models of ADC-inducing injuries.

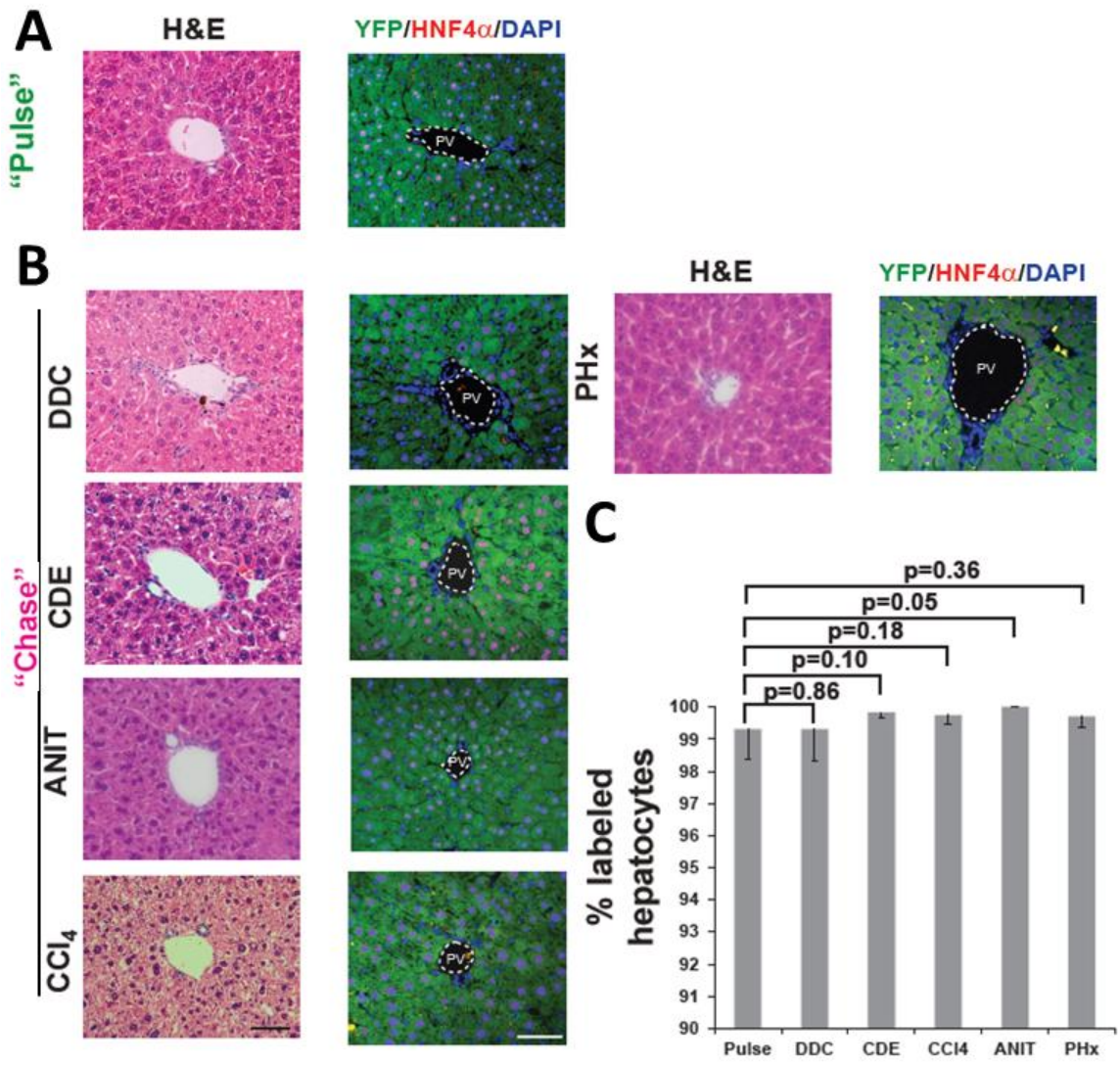

Figure 2.10 Hepatocyte pulse-chase outcomes

Figure 2.10 (A) Liver histology (haematoxylin and eosin, left panel) and immunofluorescence (right panel) of uninjured "pulse" liver. (B) Liver histology returns to normal following injuries with DDC, CDE, CCl_4, ANIT and PH with no appreciable change in the frequency of hepatocyte labelling. Scale bar=50μm. (C) Quantification of hepatocyte labelling following AAV injection ("Pulse") and recovery from DDC, CDE, CCl_4, ANIT and PH injuries. Labelling index was quantified from 6, 6, 4, 4, 2 and 2 animals for each of the conditions, respectively (mean ± SD). Absolute numbers of cells counted for pulse and chase are provided in the text.

2.3 Summary

In this study, I have used two distinct lineage tracing approaches to test the hypothesis that regeneration from toxin-induced liver damage is mediated by FSCs. First, I genetically labelled KRT19-expressing BECs and ADCs – the tissue compartment in which liver stem cells are believed to reside – and found no evidence that these cells give rise to hepatocytes in the four injury models tested. Second, we labelled hepatocytes with high efficiency and specificity, and found that there was no decrease in the labelling index with the same types of injury, a result that is consistent with the notion that new hepatocytes are derived from pre-existing hepatocytes.

In addition, in collaboration with Dr. David Knigin in Dr. Eli Pikarsky's laboratory at the Hebrew University, Israel, we utilized an unbiased approach using nucleoside analogue labelling to trace the fate of highly proliferative liver cells. Stem cells contribute to tissue homeostasis and regeneration by generating rapidly-dividing progeny – referred to as transient amplifying (TA) cells – which expand prior to final differentiation (Blanpain et al., 2007). ADCs with their high proliferative index are characterized as liver TA cells (Alison et al., 2004; Hu et al., 2007; Jensen et al., 2004). Teta et al. have previously reported that TA cells (Teta et al., 2007) can be labelled by incorporation of two thymidine analogues (i.e. dual-labelling) when administered in succession. Adopting these methods for the liver, we found no evidence that rapidly-dividing non-hepatocytes differentiated into hepatocytes in the course of CDE-mediated injury. Taken together, these data

suggest that ADCs and other non-hepatocyte populations do not contribute significantly to hepatocyte neogenesis during liver regeneration, complementing the results from our genetic approaches.

Our results do not eliminate the possibility that under more demanding circumstances ADCs could exhibit bipotency. Likewise, we cannot rule out the possibility that our lineage labelling techniques have labelled a small subset of hepatocyte-like cells that remain multipotent. However, the finding that all cells labelled with AAV8-TBG-Cre (out of nearly four thousand examined) exhibit a mature hepatocyte phenotype argues against this possibility. Moreover, IdU/CldU labelling experiments failed to reveal such a rapidly dividing (and expanding) subset of hepatocytes. Thus, our data are most consistent with a scenario in which ADCs play a very minor, if any, role undetectable using our tools with new adult hepatocytes coming from pre-existing hepatocytes, not only following partial hepatectomy but in the setting of toxin injuries as well.

Finally, these conclusions have not fully addressed the data from electron microscopy studies which reveal that following injury with hepatotoxins, cells with ultrastructural features of both ductular cells and hepatocytes, a "transitional" cell phenotype, emerge within the liver lobule (Factor et al., 1994). Due to their median size compared to other ADCs and hepatocytes, and variable nucleocytoplasmic rations, mitochondrial content, and glycogen rosettes, these observations allude to ADCs being a heterogeneous population of cells. The cell of origin for these intermediate-looking cells

exhibiting features “between” hepatocytes and BECs will be addressed in the next chapter.

3 ROBUST CELLULAR REPROGRAMMING DURING LIVER REGENERATION[2]

[2] This chapter, with modifications, has been published: Yanger, K., Zong, Y., Maggs, L.R., Shapira, S.N., Maddipati, R., Aiello, N.M., Thung, S.N., Wells, R.G, Greenbaum, L.E., Stanger, B.Z. *Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes Dev*. 2013 Apr 1;27(7):719-24

3.1 Introduction

In adult tissues, rates of cell birth and death are tightly controlled to maintain tissue mass. During amphibian regeneration, de-differentiation and/or transdifferentiation is a major source of new cells (Nacu and Tanaka, 2011). By contrast, mammalian regeneration is driven by the replication of existing cells or differentiation from stem cells. Transdifferentiation, or cellular reprogramming, has been observed in mammals following the introduction of defined factors *in vivo* (Xie et al., 2004; Zhou et al., 2008), but spontaneous inter-conversion of differentiated cells seems to occur only in the setting of strong selective pressure (Thorel et al., 2010). "Metaplasia" – a condition in which the replacement of one tissue type with another predisposes to cancer – may involve cellular reprogramming, but the connection between metaplasia at the tissue level and transdifferentiation at the cellular level remains unresolved (Slack, 2009).

The mammalian liver is exceptional among regenerative organs in that the mode of injury is thought to dictate the cellular mechanism of recovery. Following partial removal of the liver (partial hepatectomy; PHx), liver mass is restored by replication and/or growth of existing cells (Miyaoka et al., 2012), whereas following toxin-mediated injury, animals and humans exhibit an accumulation of atypical ductal cells (ADCs) within the liver, often referred to as "oval cells" (Farber, 1956; Popper et al., 1957). In this study, we sought to determine whether cellular plasticity underlies the regenerative response of the liver.

3.2 Results

3.2.1 Notch mediated reprogramming

In new-born mice, immature hepatocytes remain responsive to Notch signals and undergo a fate switch, becoming biliary epithelial cells (BECs) upon ectopic activation of the pathway (Zong et al., 2009). To determine whether this plasticity and competence was true in the adult animal, we induced Notch signalling in adult hepatocytes. We bred $R26^{YFP}$ mice to $R26^{NICD}$ mice, which harbour a Cre-inducible constitutively active form of Notch1 (Murtaugh et al., 2003; Zong et al., 2009). Next, we simultaneously labelled cells and induced Notch signalling using the same AAV8-TBG-Cre virus. As predicted, infection of bigenic $R26^{NICD/YFP}$ mice with AAV8-TBG-Cre resulted in the activation of Notch signalling (assessed by Hes1 expression) in more than 95% of hepatocytes. Over the course of Notch signalling, lineage-labelled cells started to express KRT19 and by 6 weeks, 23% of all KRT19 cells were YFP^{+}. Hence, adult hepatocytes retain this plasticity and ectopic activation of Notch signalling is sufficient to reprogram a subset of hepatocytes into BEC-like cells (Yanger et al., 2013).

3.2.2 Injury mediated reprogramming

Injury induced "intermediate cells"

Toxic liver injuries result in an atypical ductal cell (ADC) or "oval cell" response. ADCs have a biliary phenotype, but their arrangement into an intricate anastomosing configuration that extends into the hepatic lobule gives them a histological appearance that is distinct from normal bile ducts (Desmet, 1985). We

noted that the reprogrammed BEC-like cells resulting from Notch activation resembled ADCs, prompting us to hypothesize that some ADCs might be hepatocyte-derived. To test this possibility, I administered AAV8-TBG-Cre to $R26^{YFP}$ mice and, following a washout period, treated animals with the hepatotoxin DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine). As previously shown, DDC treatment was associated with a robust ADC response during which a substantial fraction of hepatocytes and BECs underwent turnover. Within 1-2 weeks of DDC exposure, cells that co-stained with biliary and hepatocyte markers were readily detectable. These bi-phenotypic "intermediate" cells were most abundant in peri-portal regions and many were binucleated (for example, 41% of $A6^{+}HNF4\alpha^{+}$ cells had two nuclei; Figure 3.1 A). Staining for the YFP lineage marker demonstrated that these intermediate cells were of hepatocyte origin, appearing within 2 weeks (Figure 3.1 B)

Conversion of hepatocytes into mature BECs

After 2-3 weeks of DDC treatment, YFP-labelled hepatocytes started expressing the mature BEC marker, KRT19. YFP^{+} $KRT19^{lo}$ cells (weak KRT19 expression) appeared first at this time point (Figure 3.2A). After 6 weeks of DDC treatment, a time-point when ADCs were most plentiful, I observed that many YFP^{+} cells had assumed a biliary morphology and co-stained with OPN, A6, Sox9, or KRT19 (Figure 3.3 A). At this time-point, YFP^{+} cells with strong KRT19 staining ($KRT19^{hi}$) comprised 4.4% of the total $KRT19^{+}$ population (Figure 3.2 B). The

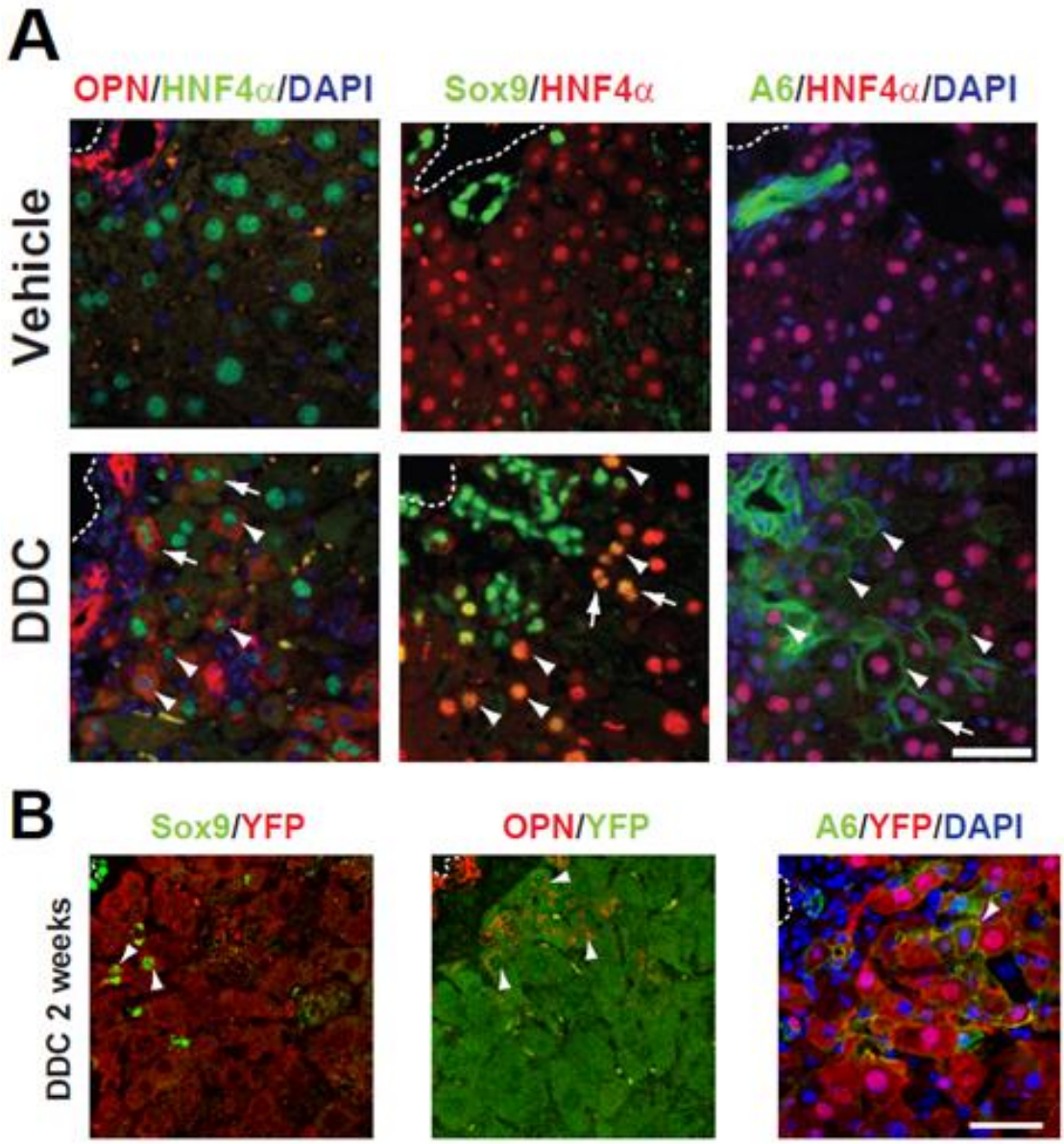

Figure 3.1 Appearance of "intermediate" cells expressing both biliary and hepatocyte makers upon injury

Figure 3.1 (A) Mice treated with DDC for 1 week exhibit co-staining with a hepatocyte marker (HNF4α and one of three biliary markers (OPN, Sox9, A6). Double positive cells have a hepatocyte morphology (arrowheads) and many are binucleated (arrows). The images are representative of multiple experiments. (B) "Intermediate" cells emerge early following treatment with DDC (2 weeks). Most YFP^+ cells with co-staining for the biliary markers Sox9, OPN, or A6 (arrowheads) retain a hepatocyte morphology at this time-point. Scale bars=25μm.

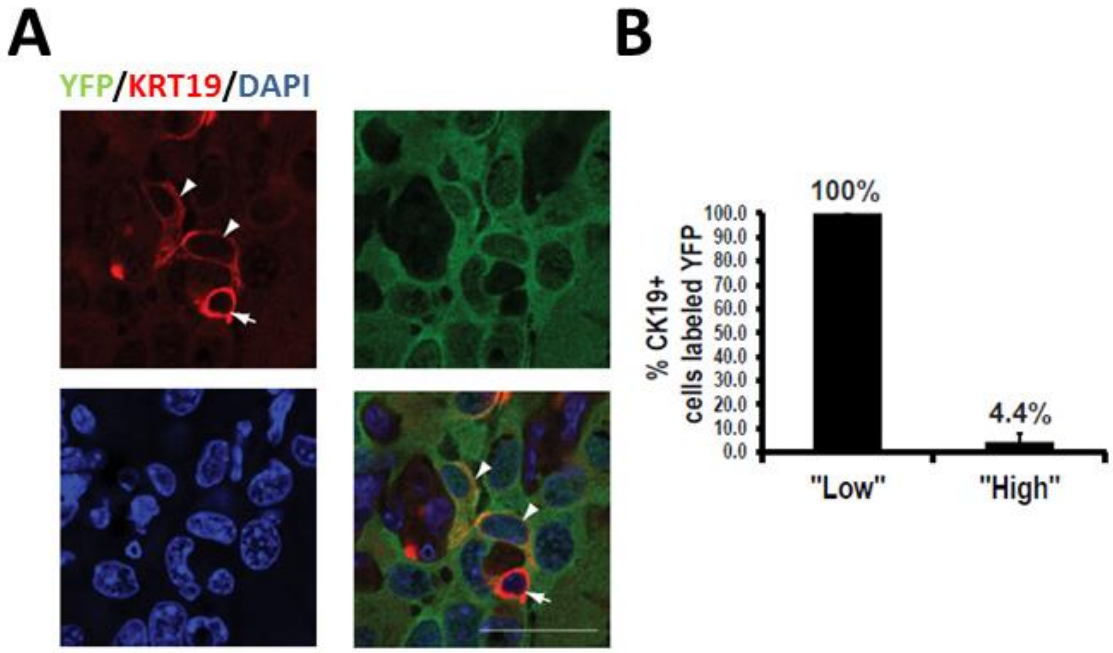

Figure 3.2 Low expression of KRT19 in hepatocytes during injury

Figure 3.2 (A**)** Hepatocyte-derived $CK19^{+}$ cells following injury (6 weeks of DDC) can be divided into $CK19^{lo}$ cells and $CK19^{hi}$ cells based on the intensity of staining (arrowheads denote $CK19^{lo}$ and arrow denotes $CK19^{hi}$). **(**B**)** All $CK19^{lo}$ cells are YFP^{+} (100%), while a fraction of $CK19^{hi}$ cells are also YFP^{+} (4.4%) (N=4 animals). $CK19^{lo}$ cells are never observed in control livers and appear early in the injury process (after 2-3 weeks of DDC), whereas $YFP^{+}CK19^{hi}$ cells (such as those in Figure 3.3), emerge later. Scale bar=25μm.

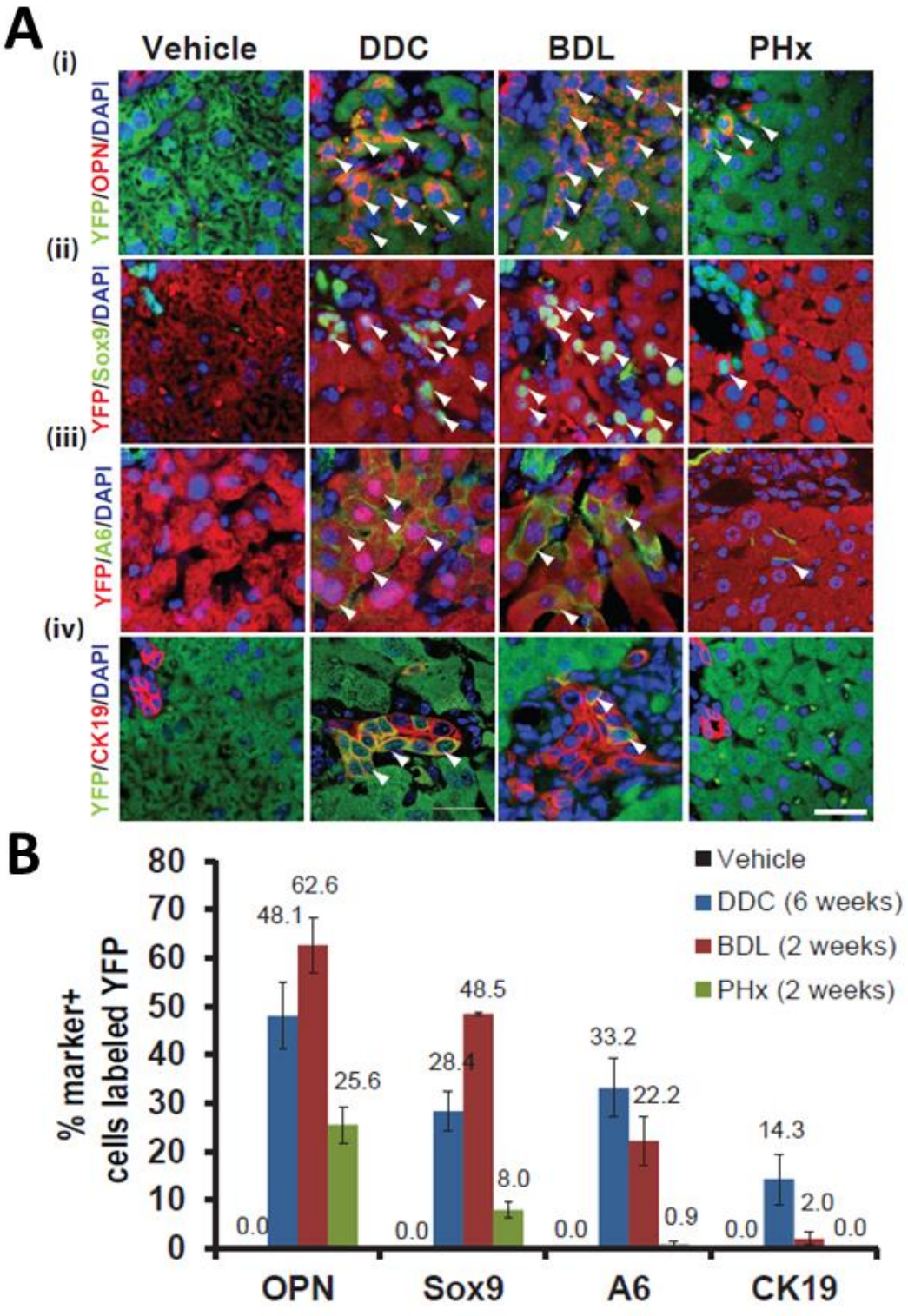

Figure 3.3 Direct evidence for hepatocyte-to-ADC reprogramming in vivo

Figure 3.3 (A) Co-staining of the biliary markers (i) OPN, (ii) Sox9, (iii) A6 and (iv) CK19 with the YFP lineage label is observed following DDC treatment (6 weeks), BDL (2 weeks), or PHx (2 weeks); co-stained cells are denoted with arrowheads. Note that many of the co-stained cells are smaller than neighbouring hepatocytes. (B) Biliary markers are induced in a stepwise fashion following injury. The percentage of marker^{+} cells (i.e. cells that stained positive for OPN, Sox9, A6, or CK19) which co-express the YFP lineage label is shown. Both CK19^{hi} and CK19^{lo} cells were included in the analysis. Double positive cells were not detected in vehicle-treated livers. Each bar represents the mean (+/- SE) of at least 3 mice; a minimum of 950 marker^{+} cells were examined for each data point. Scale bars=25μm.

frequency of co-staining with YFP depended on the biliary marker used, ranging from 48.1% of OPN^+ cells to 14.3% of $KRT19^+$ cells (Figure 3.3 B).

To determine whether this phenotypic conversion is a generalized feature of liver damage, I performed other types of injury in animals with lineage-labelled hepatocytes. As with DDC treatment, I observed changes in the morphology of YFP^+ cells and co-staining of YFP and biliary markers in the setting of ductular reactions caused by other stimuli – including bile duct ligation (BDL) and other injuries (Figure 3.3 A-B, Figure 3.4, respectively) – although the frequency of co-staining varied with the type of injury). Following PHx, by contrast, YFP^+ cells that had adopted a biliary morphology or stained weakly for KRT19 were never observed (N=5), although staining for OPN, Sox9, and (rarely) A6 was seen in some hepatocytes (Figure 3.3 A,B). These results suggest that toxin-based injuries and BDL – insults that provoke an ADC response – are associated with stepwise hepatocyte-to-BEC reprogramming while PHx – an injury that predominantly involves cell replication and hypertrophy – does not lead to cellular conversion.

Cellular characterization of reprogrammed BECs

The appearance of bi-phenotypic cells in a variety of rodent injury models prompted us to analyse human liver specimens for evidence of hepatocyte-to-BEC reprogramming in the setting of injury. As described for other liver diseases with biliary involvement (Limaye et al., 2008a), I found abundant cells which co-expressed the hepatocyte markers HNF4α and HepPar1 and the BEC marker Sox9 in liver sections from patients with several types of liver disease, whereas such cells

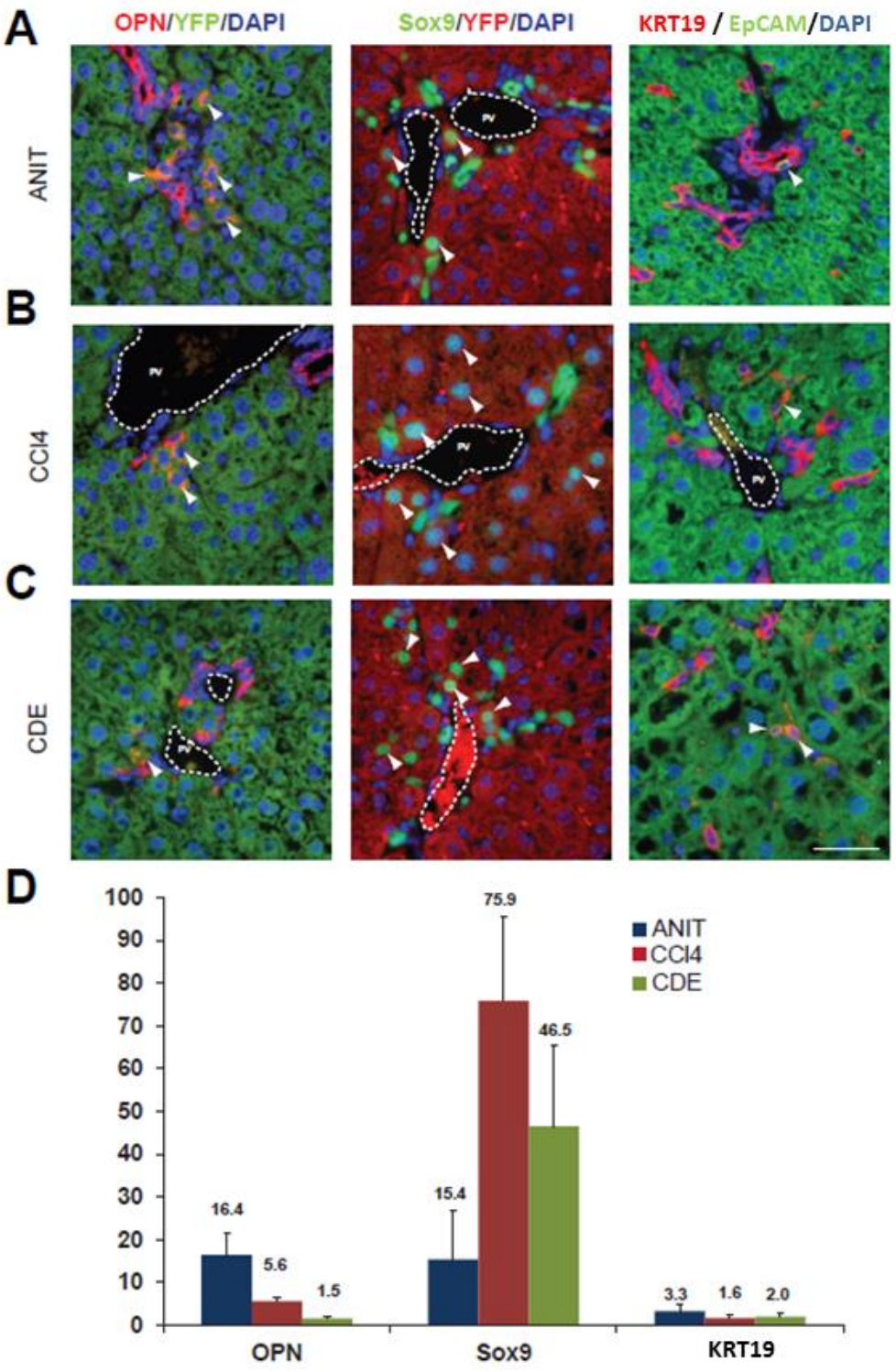

Figure 3.4 Hepatocytes express biliary cell markers in multiple liver toxin injuries

Figure 3.4 Co-staining of various BEC markers, OPN, Sox9 and CK19, appear in YFP^+ hepatocytes in various toxin injuries such as ANIT diet (A), CCl_4 administration (B) and CDE diet (C); co-stained cells denoted by arrowheads. (D) The percentage of $marker^+$ cells in YFP^+ hepatocytes. Each bar represents the mean of (+/- SE) of at least 3 mice. Scale bar=25 μm.

were never observed in control human liver specimens (Figure 3.5). These results suggest that the cellular plasticity observed in the rodent injury models may also operate during human liver injury.

To determine the extent to which hepatocyte-derived BECs acquire features of normal BECs, I performed a more detailed morphological and molecular analysis of DDC-treated livers. Within 3 weeks of DDC treatment, YFP^+ cells underwent dramatic morphological changes, including the acquisition of a distinctive apical-basal polarity (detected by staining for PKCζ and Par6), a reduction in cell size, and coalescence into neo-lumens (Figure 3.6 A). YFP^+ cells in DDC-treated livers also exhibited the development of primary cilia, a biliary-specific organelle marked by acetylated-tubulin (Ac-tub; Figure 3.6 B).

Next, I sought independent evidence for a hepatocyte origin of ADCs using ploidy characterization. More than 30% of normal murine hepatocytes are binucleated, whereas BECs are mono-nucleated (Gupta, 2000). We reasoned, therefore, that some hepatocyte-derived ADCs might be binucleated. Consistent with this notion, binucleated ADCs were found in DDC-treated livers, whereas none were detected in control livers (Figure 3.6 C-D; 0.6% vs. 0.0%). All binucleated ADCs expressed the YFP lineage maker, suggesting that polyploidy was restricted to hepatocyte-derived BECs (Figure 3.6 C).

Molecular characterization of reprogrammed BECs

Additionally, I obtained molecular evidence that the hepatocyte-derived BECs closely resemble normal BECs. Hepatocyte-derived YFP^+ BECs were

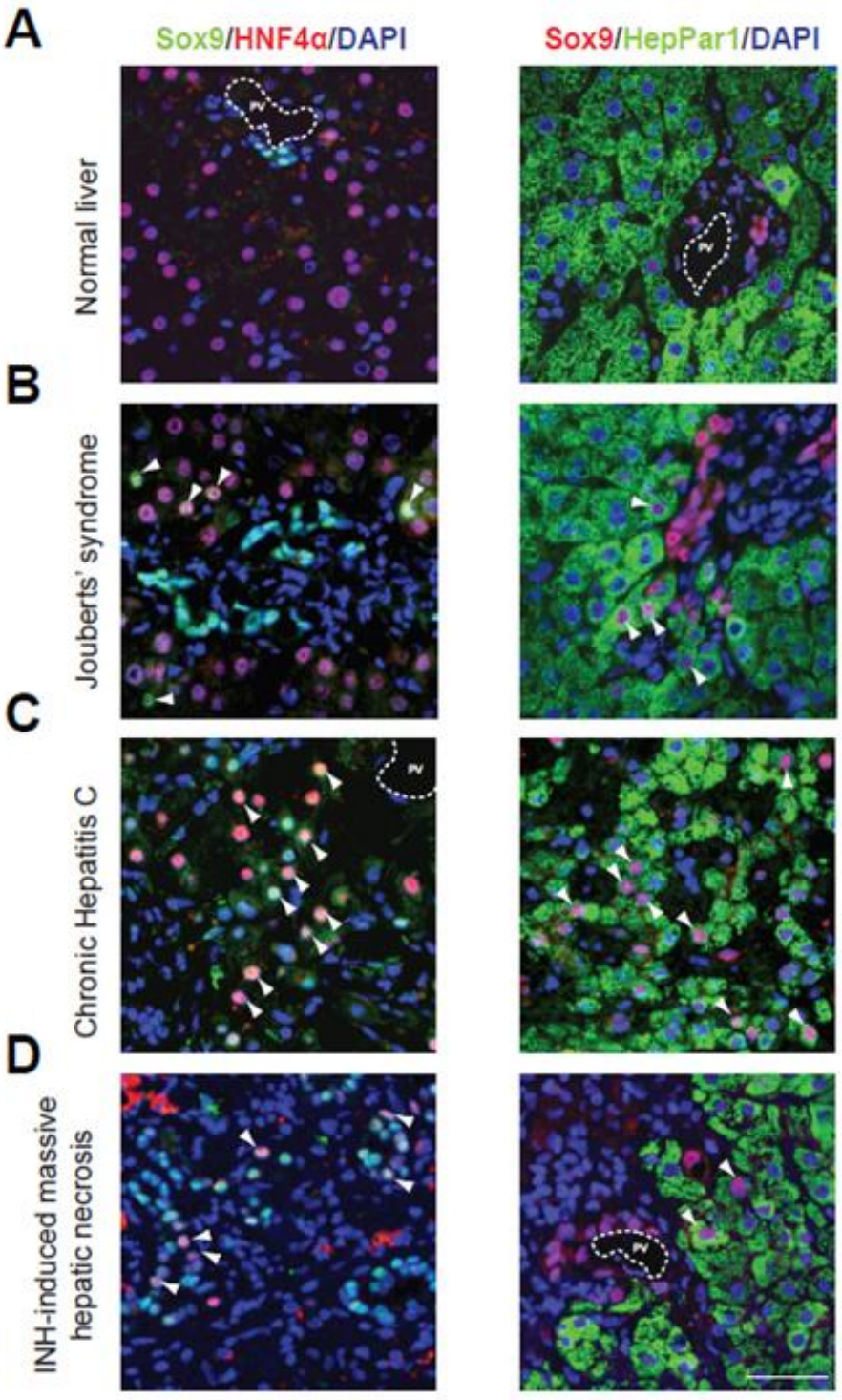

Figure 3.5 Human liver diseases exhibit "intermediate" cell phenotype

Figure 3.5 Sox9 segregates BECs from hepatocytes marked with HNF4α or HepPar1 in normal human liver (A). Co-expression of Sox9 in hepatocytes is found in liver diseases such as (B Joubert's Syndrome, (C) chronic Hepatitis C and (D) INH-induced massive hepatic necrosis. Scale bar=25 μm.

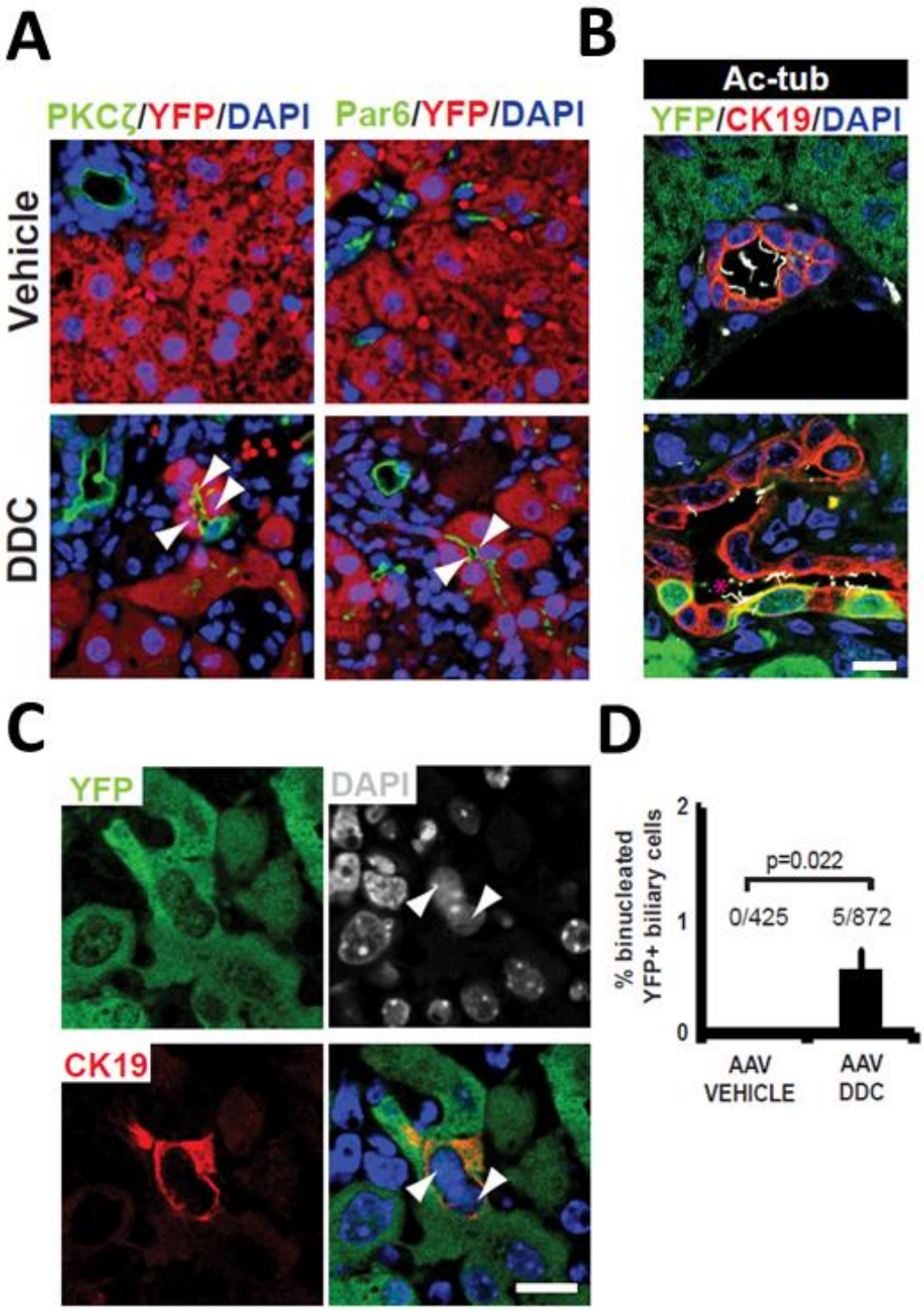

Figure 3.6 Hepatocytes undergo morphological changes during reprogramming

Figure 3.6 (A) YFP^+ hepatocytes change their morphology after DDC injury, organizing into duct-like structures with neo-lumens marked by the apical polarity markers PKCζ and Par6 (arrowheads). (B) Reprogrammed cells have cilia, marked by acetylated tubulin (6 weeks, Ac-tub; pink asterisk). (C) Reprogramming leads to binucleated ADCs. Sections of liver from AAV8-TBG-Cre-labelled animals given DDC were co-stained with DAPI (grey/blue), YFP (green) and CK19 (red) and examined by confocal microscopy. Binucleated ADCs were present in the YFP^+ population. (D) Quantification of binucleated BECs in labelled mice given normal chow (AAV vehicle) or DDC (AAV DDC); the number of cells counted (N=3 mice in each group) is shown. Scale bars=10μm.

captured using FACS and assessed by qPCR for multiple BEC markers. These reprogrammed cells exhibited transcript levels for these genes comparable to those of *bona fide* BECs (Figure 3.7 A-C) indicating that they had acquired many of the transcriptional hallmarks of normal biliary cells. Notably, hepatocyte-derived BECs and YFP^+ hepatocytes (including those at early stages of biliary reprogramming) did not express the hepatoblast marker α-fetoprotein (AFP), suggesting that the conversion process does not go through a dedifferentiation step (Figure 3.7 D). Collectively, these results indicate that following injury *in vivo*, hepatocytes can turn into cells that closely resemble normal BECs at the morphological, structural, and molecular level.

3.2.1 Notch signalling is required for reprogramming

Finally, we examined the molecular mechanism underlying the hepatocyte-to-BEC conversion. As Notch overexpression itself induced hepatocyte reprogramming, we employed mice with a liver-specific deletion of RBP-Jκ – an essential component of the canonical Notch pathway – to determine whether Notch signalling is also required for reprogramming under injury conditions. We and others have shown that deletion of RBP-Jκ in the embryonic liver using the AFP^{Cre} strain results in bile duct paucity due to Notch's role in biliary specification (Sparks et al., 2010; Zong et al., 2009). We found that treatment of AFP^{Cre}; $RBP^{L/L}$ mice (referred to as "AFP-RBP" mice) with DDC resulted in a marked reduction in the ADC response compared to controls, a result that provides genetic evidence that Notch signalling is required for the generation of ADCs (Yanger et al., 2013).

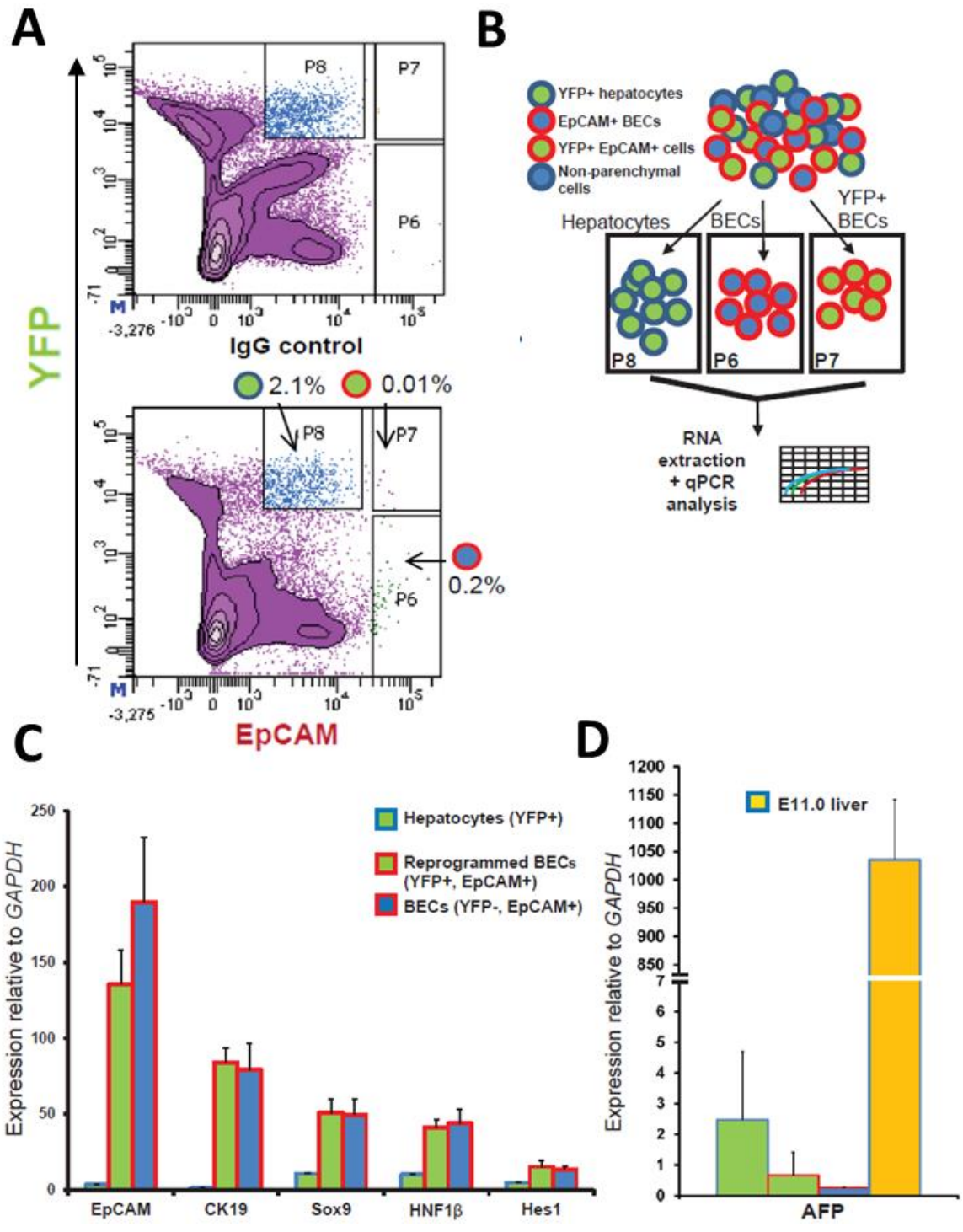

Figure 3.7 Molecular analyses of hepatocyte-derived/reprogrammed BECs

Figure 3.7 (A) AAV8-TBG-Cre injected $R26^{YFP}$ mice underwent DDC injury and livers were sorted and collected for 3 different populations: YFP^+ hepatocytes (P8), $EpCAM^+$ YFP^+ double positive reprogrammed BECs (P7) and $EpCAM^+$ BECs (P6). (B) Schematic of cellular population isolated for downstream analysis such as qPCR. (C) qPCR analysis of BEC markers gene expression levels comparing populations isolated from (B). Expression levels relative to housekeeping gene, GAPDH. (D) Expression level for the hepatoblast marker, AFP, was negligible in all populations including reprogrammed BECs. Positive control for AFP expression was whole livers from E11.0 embryos.

However, since AFP^{Cre} mediates deletion in embryonic hepatoblasts, AFP-RBP mice lack RBP-Jκ in both hepatocytes and BECs. Hence, the defective ADC response could be the result of RBP-Jα loss in either, or both, of these cellular compartments.

To determine whether Notch is involved in hepatocyte-to-ADC reprogramming, I assessed the degree of reprogramming in the context of Notch signalling impairment via RBP-Jκ knockdown. In order to do so, I administered AAV8-TBG-Cre to $RBP^{L/\Delta}$ mice harbouring one "floxed" allele and one null allele of RBP-Jα ("L/Δ") and for controls administered either 1) AAV8-TBG-GFP virus lacking Cre to control $RBP^{L/L}$ mice ("L/L') or 2) AAV8-TBG-Cre to $RBP^{L/WT}$ mice harbouring one wild-type allele of RBP-Jα ("L/WT"; Figure 3.8 A). Following a week-long washout period, animals in both groups were fed DDC for 3 weeks. As expected, control animals (L/L, L/WT) exhibited abundant bi-phenotypic cells, (Figure 3.8 B, D; left panels). By contrast, RBP-Jκ KO mice (L/Δ) exhibited dramatic decreases in bi-phenotypic cells (Figure 3.8 B, C; right panels) and hepatocyte-derived $Sox9^{+}$ and OPN^{+} cells (Figure 3.8 D, E; right panels). This result indicates that RBP-Jκ is required for biliary reprogramming.

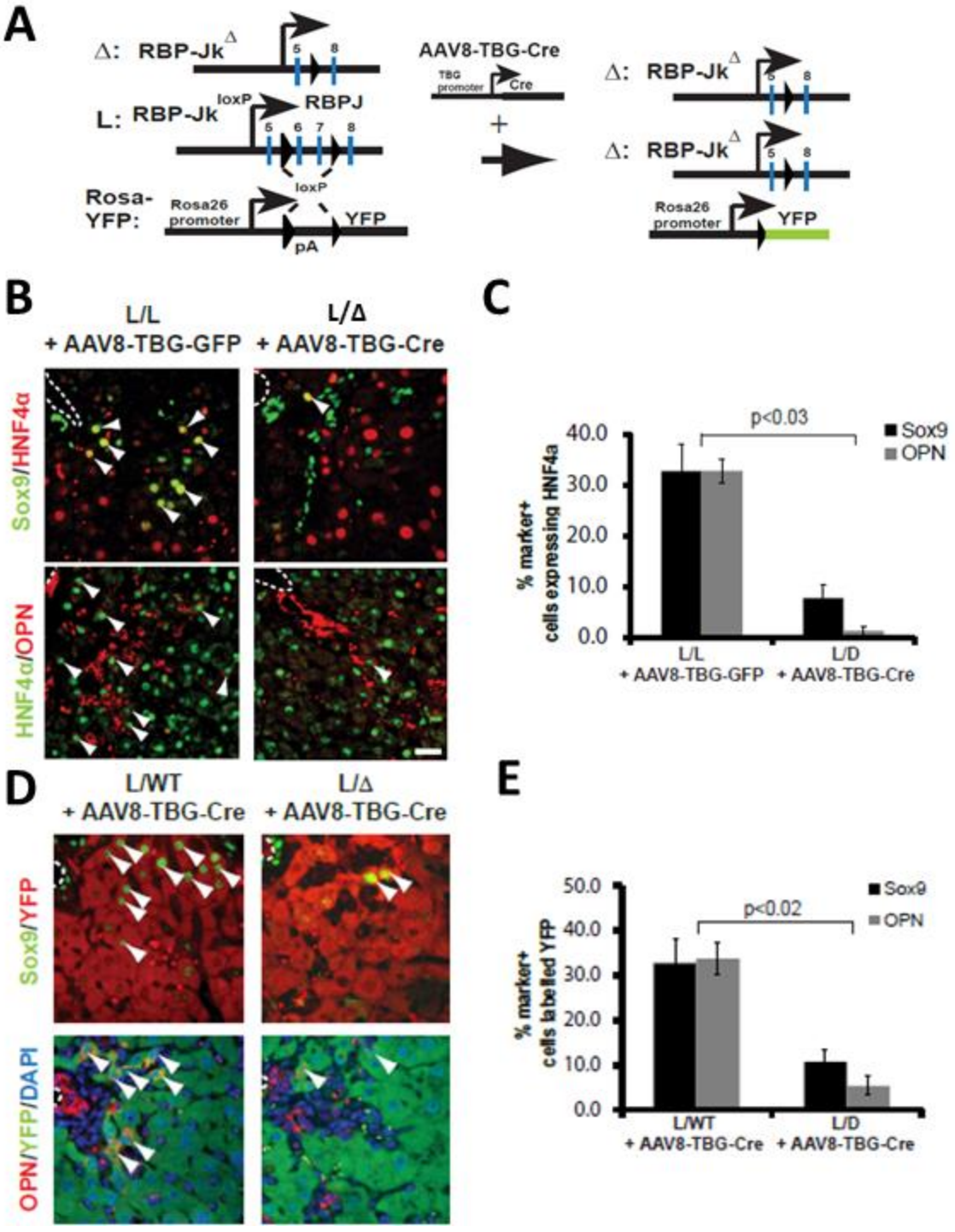

Figure 3.8 Hepatocyte reprogramming requires Notch signalling

Figure 3.8 (A) Schematic of simultaneous deletion of RBPJκ and lineage tracing of hepatocytes. (B-E) Mice with hepatocyte-specific deletion of RBP-Jκ have a significant reduction in the number of "intermediate" cells. (B) RBP-Jκ deletion results in a reduction in the percentage of HNF4α "intermediate" cells co-localizing with $Sox9^+$ or OPN^+ cells after DDC treatment (3 weeks). (C) Quantitation of (B), depicting the percentage of $Sox9^+$ or OPN^+ cells which co-stain with HNF4α. (D) Concomitant hepatocyte labelling and RBP-Jκ deletion results in a reduction in the percentage of hepatocyte-derived $Sox9^+$ or OPN^+ cells after DDC treatment (3 weeks). (E) Quantification of (D), depicting the percentage of $Sox9^+$ or OPN^+ cells which carry the YFP lineage marker (N=4 mice each group). Scale bars=25μm.

3.3 Summary

In summary, I have shown that hepatocytes initiate an *in vivo* differentiation program following injury that results in functioning as FSCs in their conversion into biliary cells. This process occurs in a stepwise fashion that involves the induction of biliary markers, a decrease in cell size, formation of a polarized epithelial layer, and the formation of new and potentially functional organelles. Thus, we also address the long-standing observations from electron microscopy studies revealing cells with both hepatocyte and biliary features in toxin-injured livers (Factor et al., 1994), with hepatocytes being the cell of origin for a subset of the ADC population discussed in Chapter 2. These results thus provide a dramatic example of how cellular reprogramming – a process that has been mainly associated with lower organisms – can contribute to mammalian regeneration. This plasticity is mimicked by overexpressing a single transcription factor, Notch, a signalling pathway utilized during biliary specification in liver development. Truncation of the pathway attenuates this reprogramming process and thus Notch signalling is required. Our findings also suggest that hepatocyte pliability may serve as a cellular source for the production of BECs in diseases that are characterized by loss or dysfunction of these cells, such as cholestasis or Alagille Syndrome.

4 DISCUSSION

The longstanding notion that ADCs are FSCs has been based largely on static observations, *in vitro* studies and cellular transplantation assays (Dorrell et al., 2011; Huch et al., 2013; Wang et al., 2003a). For example, Wang et al. (2003a) employed cellular fractionation to isolate distinct populations of non-parenchymal cells from DDC-treated chimeric mice, and then transplanted genetically marked cells from the ADC-containing fraction into conditioned recipients to test their potential when a selective pressure was applied. Since the transplanted cells were able to repopulate hepatocytes killed during the pre-transplant conditioning protocol, the authors concluded that ADCs give rise to hepatocytes. However, as the starting population was heterogeneous, this study could not rule out the possibility that the hepatocytes – with their nearly unlimited capacity for self-renewal – mediated the recovery. Moreover, the transplantation assay is a test of cell *potential* under selective pressure rather than cell fate in the absence of experimental perturbation. Hence, while repopulation studies suggest that fractions of cells that contain ADCs *can* give rise to hepatocytes under strong selective pressure, the fate of ADCs during normal liver regeneration is not addressed by this technique.

Similarly, *in vitro* studies which demonstrated that cells with a biliary/ADC phenotype can differentiate into hepatocyte-like cells have limitations (Dorrell et al., 2011; Okabe et al., 2009; Shin et al., 2011). In particular, such assays rely on the assumption that hepatocytes and BECs represent stable end-products of differentiation that are not easily inter-convertible, an assumption that might not be valid for cell types that retain a high degree of plasticity. In other words, if the

barrier to transdifferentiation between two cell types is low, such *in vitro* assays cannot serve as evidence for stem cell activity. Previous studies have demonstrated significant plasticity between hepatocytes and BECs *in vitro* (Grisham, 1980; Limaye et al., 2008b; Nishikawa et al., 2005; Tsao et al., 1984; Yaswen et al., 1984) and my own work and work from others has demonstrated significant plasticity *in vivo* (Michalopoulos et al., 2005; Yanger et al., 2013). Thus while cell transplantation and *in vitro* culture systems can provide insight into cell potential under these experimental conditions, lineage tracing permits a more accurate indication of cell fate.

Several groups have used lineage tracing to characterize the origin and fate of ADCs. Initial work employing [^{3}H]-thymidine incorporation gave rise to discordant conclusions, with experimental evidence both favouring (Evarts et al., 1987; Evarts et al., 1989) and refuting (Grisham and Porta, 1964; Rubin, 1964; Tatematsu et al., 1984) an ADC-to-hepatocyte differentiation lineage relationship. In addition, more recent lineage tracing studies have also led to disparate results (Friedman and Kaestner, 2011). As discussed earlier, results from the Foxl1-Cre mice (Sackett et al., 2009) cannot stringently assess the bipotency of oval cells. As the promoter is not an inducible one, hepatocytes and biliary cells can both potentially activate *Foxl* independently and thus giving a false ontological conclusion of a progenitor-to-hepatocyte relationship. More recently, several Cre-based labelling- inducible drivers resulted in the inheritance of label in hepatocytes, although the contribution to the hepatocyte pool was low. For instance, Cre-based

labelling- employing osteopontin (*Opn*) gave rise to between 0.78%-2.45% of labelled cells co-staining with hepatocyte markers (Espanol-Suner et al., 2012). Similarly, in a study utilizing the *Lgr5*-inducible driver reports, the initial percentage of labelled hepatocytes was not reported but the authors claimed these cells arose from a non-hepatocyte *Lgr5*$^{+}$ population that emerged and got labelled upon TM administration in the context of injury. These cells were further grown as organoid cultures and used for transplantation which resulted in a total of 5 out of 15 *FAH-/-* mice being successfully engrafted, of which approximately 0.1-1% of hepatocytes were labelled (Huch et al., 2013). The authors of the study noted this result was in contrast to a more robust engraftment and 100% rescue displayed by hepatocyte transplantation in their previous studies (Azuma et al., 2007; Huch et al., 2013).

More dramatic evidence for liver progenitors was provided by Furuyama et al. (2011), who reported that a significant percentage (up to 90%) of the liver was eventually marked following pulse-labelling with a *Sox9-CreER* strain, a result which on face value is strong evidence for physiologically-active stem cells. By contrast, other studies utilizing a similar but separately constructed *Sox9-CreER* strain did not find evidence for such liver progenitors (Carpentier et al., 2011). Additionally, using a similar hepatocyte labelling method with adeno-associated virus – no decrease in labelling was observed following most forms of injury and a scant 1.3% reduction in labelling following CCl_4 injury (an effect whose statistical

significance from baseline was not reported), indicating that stem cells play a minor, if any, role in liver homeostasis and regeneration (Malato et al., 2011).

The ability to reach strong conclusions about lineage is deeply dependent upon the specificity of the tracing tools employed. For example, the *Opn*-, *Lgr5*-, and *Sox9* studies all rely upon the assumption that Cre-mediated recombination never occurs in hepatocytes; if such a lack of specificity were to exist, even to a small extent, one would be left with the impression that hepatocytes are derived from BECs or ADCs, when in fact no such progenitor-progeny relationship existed. An example of this is the *RIP-CreER* strain that has been used for numerous β-cell studies in the pancreas, which was shown to induce recombination independent of TM administration (Liu et al., 2010). Several other studies have highlighted the importance of using appropriate controls and careful handling with TM used for the inducible strains and studies (Anastassiadis et al., 2010; Danielian et al., 1998; Reinert et al., 2012). Thus, numerous controls have to be done to ensure "tightness" of inducible strains to show there is no "leakiness" in the absence of TM in normal and perturbed animals. We speculate that this technical point accounts for the discrepancy between our results and those of others who have reported liver progenitor cell activity *in vivo*. Indeed, when I tested the specificity of the same *Sox9-CreER* strain (Soeda et al., 2010) used by Furuyama and colleagues with a $R26^{YFP}$ reporter, I found that this strain confers substantial hepatocyte labelling (Figure 4.1). This result is consistent with the observation that *Sox9* is induced in hepatocytes by TM administration alone (Carpentier et al., 2011) and under

pathological conditions (Yanger et al., 2013). Hence, "ectopic" *Sox9* expression in hepatocytes, resulting in their labelling, may have led to the conclusion that they were derived from biliary cells. A similar phenomenon may have contributed to the low-level of hepatocytes marked with *Opn-CreER* (Espanol-Suner et al., 2012) and *Lgr5-CreER* (Huch et al., 2013), as hepatocytes have a propensity for expressing

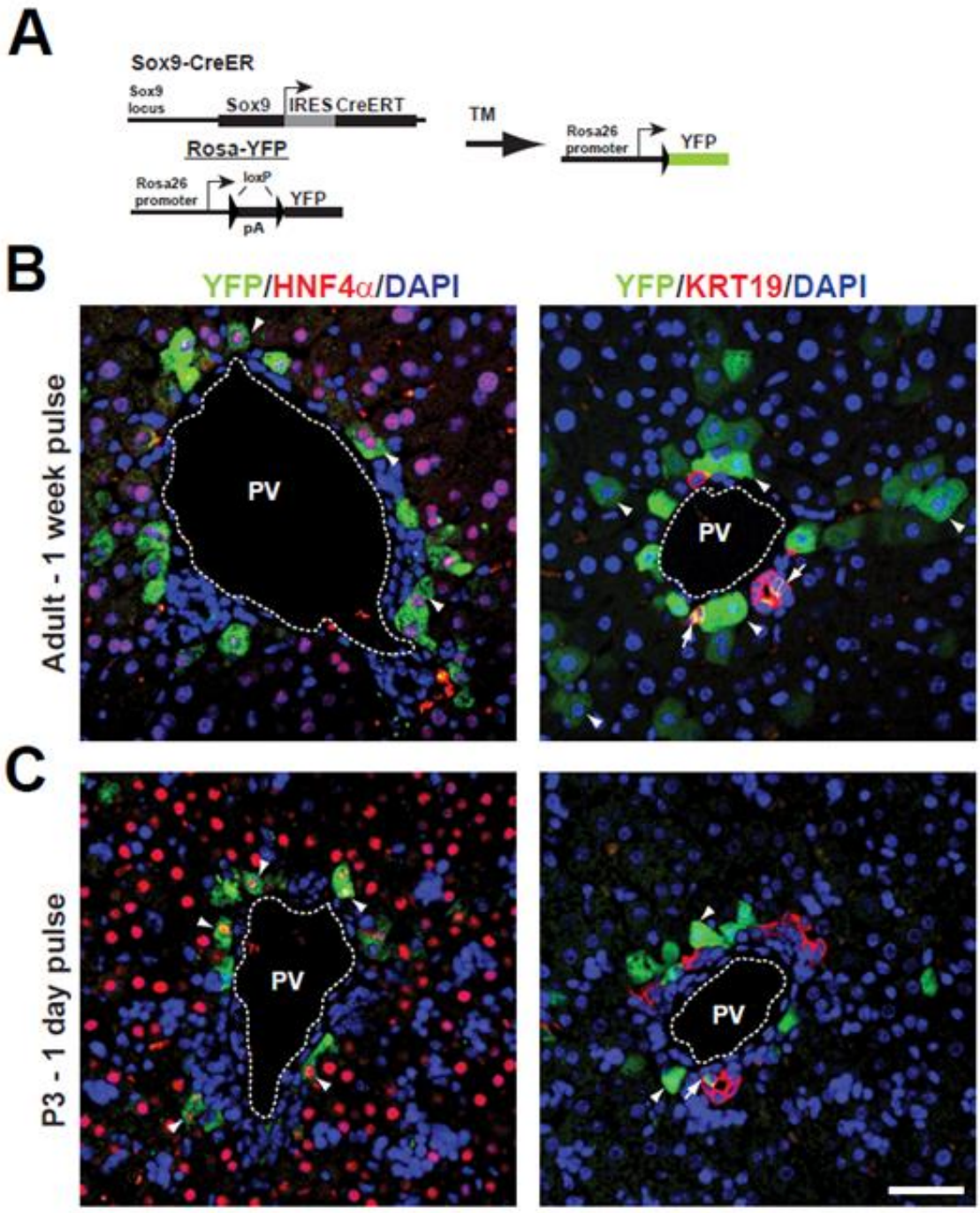

Figure 4.1 Sox9-CreER labels BECs and hepatocytes

Figure 4.1 (A) Schematic of *Sox9-CreER*animal crossed to $R26^{YFP}$ reporter mice to label cells upon TM administration. (B) 6-8 week adults given 40 mg of TM in a 1 week pulse result in labelling of hepatocytes (arrowheads) and inefficient labelling of $KRT19^{+}$ BECs (arrows). (C) A 1 day pulse in post-natal animals administered TM via the mother results in unspecific labelling of hepatocytes (arrowheads) while inefficiently labelling BECs (arrows). Scale bar=50μm.

certain biliary markers including OPN, upon stress (Limaye et al., 2008a; Yanger et al., 2013). As a "terminal" biliary marker, KRT19 appears to be an exception to this specificity problem; unlike *Sox9* and *Opn*, hepatocytes do not express KRT19 upon injury and become KRT19^{+} only after prolonged toxin exposure as part of a hepatocyte-to-BEC reprogramming process (Figures 3.1-3). Hence, *Krt19-CreER* mice are likely to represent a more specific – and hence more reliable – tool for assessing the contribution of BECs and ADCs to liver repopulation.

Our results are in accordance with older studies that utilized [^{3}H]-thymidine incorporation, immunostaining, and electron microscopy to examine the origins of ADCs *in vivo* (Factor et al., 1994). In those experiments, newly emergent ADCs had ultrastructural features of hepatocytes and were commonly found in close apposition to hepatocytes as they expanded into the lobule. In light of our lineage tracing results that show hepatocytes functioning as FSCs, I propose that these observations reflect the *retention*, rather than the *acquisition*, of hepatocyte features during reprogramming, an idea that first emerged more than fifty years ago when ADCs were initially described as "ductular hepatocytes" (Leduc, 1959).

The notion that a differentiated cell can be—under certain circumstances—recruited to function as a stem cell is related to the larger concept of cellular plasticity. In many organisms, terminally differentiated cells are able to undergo a process of dedifferentiation to reconstitute the stem cell state. In the *Drosophila* male germline, for example, cells that have undergone the first step in spermatogonial differentiation (to become "transient amplifying" cells) can reverse direction and take on a stem cell

identity (Brawley and Matunis, 2004). In mammalian studies, the landmark discovery that fibroblasts can be converted to pluripotent cells, known as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006) , utilizes the ectopic expression or activity of defined factors *in vitro*. In 2004, Wagers and Weissman established a set of stringent criteria by which to assess *bona fide* reprogramming events *in vivo*, a list that includes demonstrating proper integration of the trans-differentiated cells within the tissues. My studies regarding the conversion of hepatocytes to BECs fulfil this, and do so in a robust manner unique to the liver and not shown in other organs.

Using a lineage tracing approach, I show that hepatocytes are FSCs and can indeed undergo a robust process of biliary reprogramming, a process dependent on Notch signalling. We have shown that following the expression of a single transcription factor (NICD) or multiple types of hepatic injury, hepatocytes gradually lose their identity and acquire a biliary phenotype *in vivo*. As part of this Notch-dependent process, our studies suggest that BEC reprogramming occurs as a cascade, transitioning through an intermediate state, characterized by the co-expression of hepatocyte- and BEC-specific transcription factors, and adopting morphologic features of BECs (Figure 4.2). Given the role that Notch plays as a major fate determinant during normal biliary differentiation (Si-Tayeb et al., 2010; Zong et al., 2009), we postulate that adult hepatocyte-to-BEC reprogramming is a recapitulation of this developmental process. However, the question regarding whether a 'dedifferentiation' process is involved is not very clear as I could not detect any levels of α-fetoprotein, *AFP*, a marker of hepatoblasts - progenitor cells during liver development, in the reprogrammed cells.

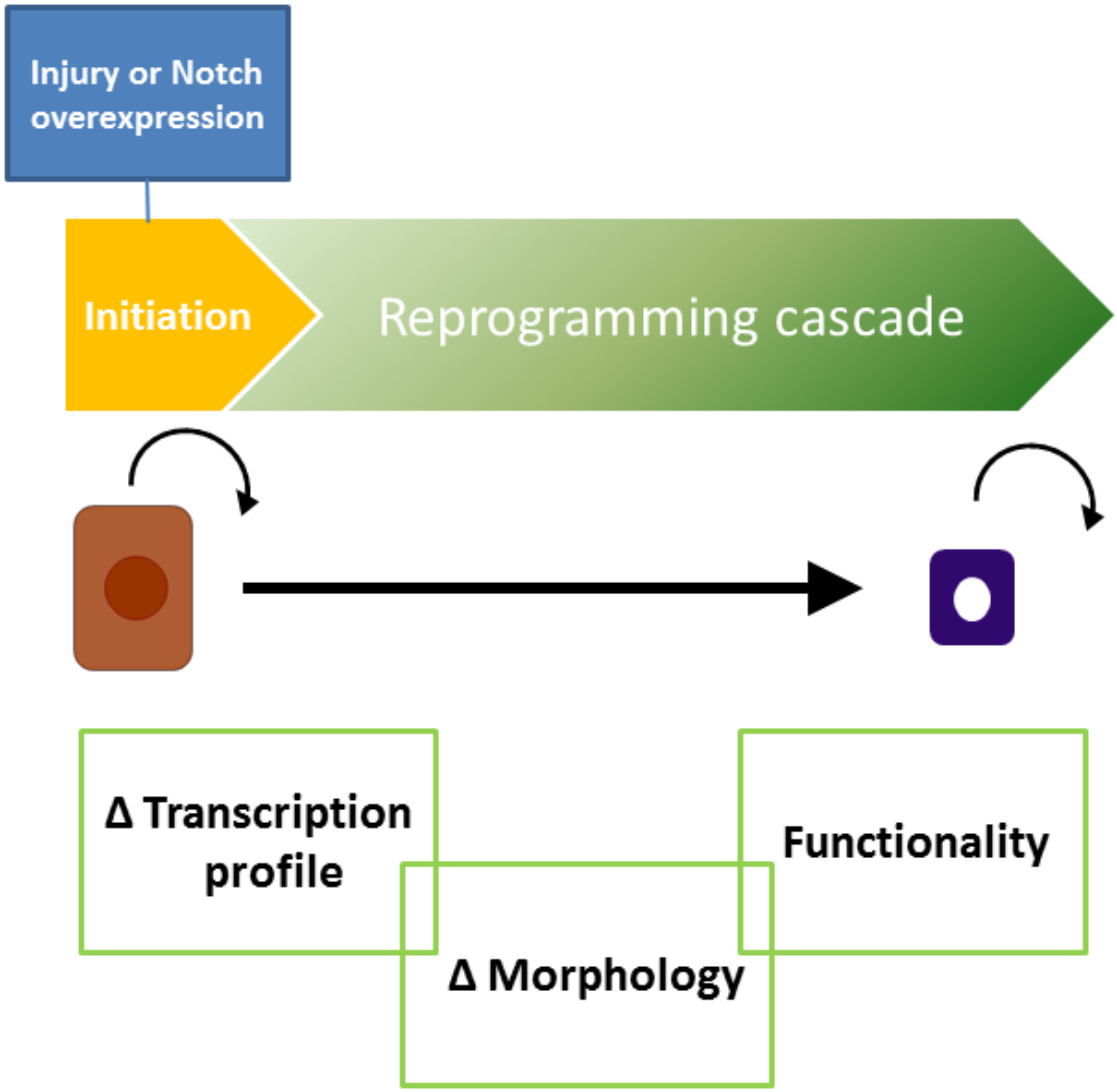

Figure 4.2 Model for hepatocyte-to-ADC reprogramming

Figure 4.2 Toxin-based injuries or Notch-signalling initiates a hepatocyte-to-ADC reprogramming cascade. This cascade includes transcriptional changes, alterations in cellular morphology and eventually function.

Several features of liver cell reprogramming are reminiscent of the process of iPSC reprogramming. The route from hepatocyte to BEC is a gradual one involving a "reprogramming cascade," with distinct and sequential changes in morphology and gene expression patterns. For example, hepatocytes acquire primitive biliary features (e.g. decrease in cell size and re-alignment of cell polarity) and express primitive biliary markers (e.g. OPN and Sox9) soon after injury. Similarly, iPSC generation being a multistep process involves early phases of reprogramming with cells decreasing in size, obtaining epithelial cell characteristics and upregulating various proliferative genes (Plath and Lowry, 2011). It is only later that reprogrammed hepatocytes adopt more mature cellular and molecular features of BECs, such as the ability to form tubes, cilia, and express the definitive biliary marker, KRT19. Likewise, the late phase of iPSC reprogramming is characterized by the generation of ESC-like colonies and by the loss of repressive chromatin marks leading to the expression of pluripotent genes such as endogenous Oct4 (Plath and Lowry, 2011).

Despite expression of all transcription factors, iPSC reprogramming is heterogeneous (Carey et al., 2009), as exhibited in our hepatocyte reprogramming study. During injury, hepatocytes located closest to the portal veins, known as "Zone 1," undergo biliary reprogramming with a much higher efficiency than "Zone 3" hepatocytes, which are centrally located within the liver lobule and do not reprogram fully. Similarly, despite activation of the Notch pathway in more than 95% of hepatocytes (including all zones), only Zone 1 and Zone 2 hepatocytes undergo biliary conversion, though not with 100% efficiency. This result suggests

that heterogeneity in reprogramming is a result of either a lack of competence of Zone 3 hepatocytes to be converted due, for example, to a difference in epigenetic state or a requirement for a second signal that is absent from Zone 3. For instance, Notch signalling is not the sole pathway involved in biliary specification during development (Si-Tayeb et al., 2010). Thus, full maturation of hepatocyte reprogramming may require, in addition to Notch activation, a "second-hit" from other signalling pathways such as TGF-β, akin to the requirement of Nanog for the final stages of iPSC reprogramming (Silva et al., 2009). Thus, these extracellular signals may be more permissive in the diverse extracellular milieu zone 1 hepatocytes are in with the biliary cells and fibroblasts around the portal veins, which are void in the area zone 3 hepatocytes are in. While proliferation is required for iPSC generation, (Takahashi and Yamanaka, 2006), other forms of reprogramming such as acinar-to-β cell reprogramming (Zhou et al., 2008) and heterokaryon-generated iPSCs do not (Bhutani et al., 2010). In our system, injuries that provoke biliary reprogramming are associated with a high degree of cellular turnover and occur over a long period of time (2-4 weeks); hence, more refined methods will be needed to probe this cellular conversion process.

Many clinical implications arise from our findings. The prevalence of the phenomenon suggests that cellular reprogramming is a generalized component of the liver's normal response to injury, particularly those injuries cause by multiple toxins. I and others have shown that indeed, various human diseases display characteristics of this reprogramming with the abundance of "intermediate"/bi-

phenotypic cells (Figure 3.5) (Limaye et al., 2008a). Cancer studies have also revealed that "extreme" or prolonged reprogramming could be detrimental. For instance, Notch signalling in the liver (our findings in Appendix 1), in the hepatocytes (Fan et al., 2012; Sekiya and Suzuki, 2012) and prolonged toxin injury (Wang et al., 2012) can lead to hepatocellular carcinoma, cholangiocarcinomas or both, respectively. Thus, this transition and switch between hepatocyte reprogramming being a reparative process to potentially becoming disease initiating is an interesting question to probe further.

Our findings also suggest hepatocyte pliability being a potential cellular source for the production of BECs in diseases such as cholestasis or Alagille Syndrome that are characterized by loss or dysfunction of these cells. Alagille Syndrome is a paediatric, genetic autosomal diseases affecting 1/70,000 new-borns (Piccoli and Spinner, 2001). It is characterized by jaundiced and cirrhotic livers due to the build-up of bile caused by the lack of BECs for efficient transportation out of the liver, though the severity is heterogeneous (Alagille et al., 1987; Piccoli and Spinner, 2001). Interestingly, human patients display mutations within the Notch pathway, with a significant portion having *JAG1* mutations while a smaller portion have *NOTCH2* mutations (Li et al., 1997; McDaniell et al., 2006; Oda et al., 1997). Results of mouse mutation models of Jag1 (Xue et al., 1999) and Notch2 (McCright et al., 2001) were initially dismissed as not mirroring the BEC paucity seen in human samples, except when both mutations were combined (McCright et al., 2002). I have found, contrary to these earlier studies, both mutations promote

defects in biliary formation in early post-natal livers, with the double mutant animal being the most severe (P2, P16 (Figure 4.3)). Notably, at later time points, the double mutant animals displayed "intermediate-looking" cells, with hepatocyte-looking cells starting to adopt a BEC-phenotype by expressing OPN but not KRT19 (P16, (Figure 4.4)). This suggests that hepatocyte reprogramming may potentially be a compensatory mechanism Alagille livers use to make up for the BEC paucity to survive into adulthood. Understanding the mechanism underlying this process may therefore have therapeutic relevance.

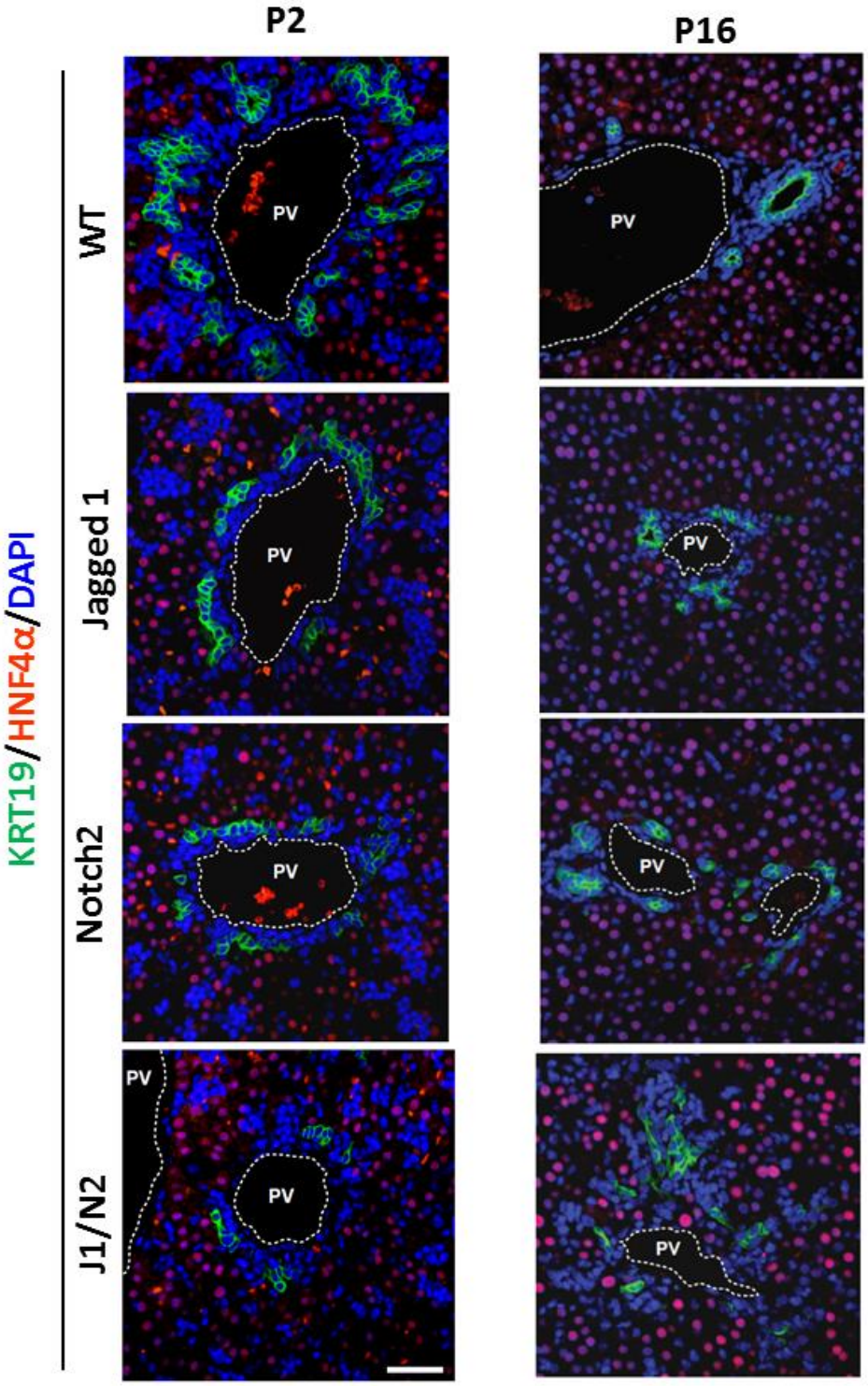

Figure 4.3 Biliary development in Alagille mice

Figure 4.3 Single (Jagged1, Notch2) and compound mutants (J1/N2) exhibit BEC paucity compared to wildtype litter mates in early post-natal livers. Scale bar=25 μm.

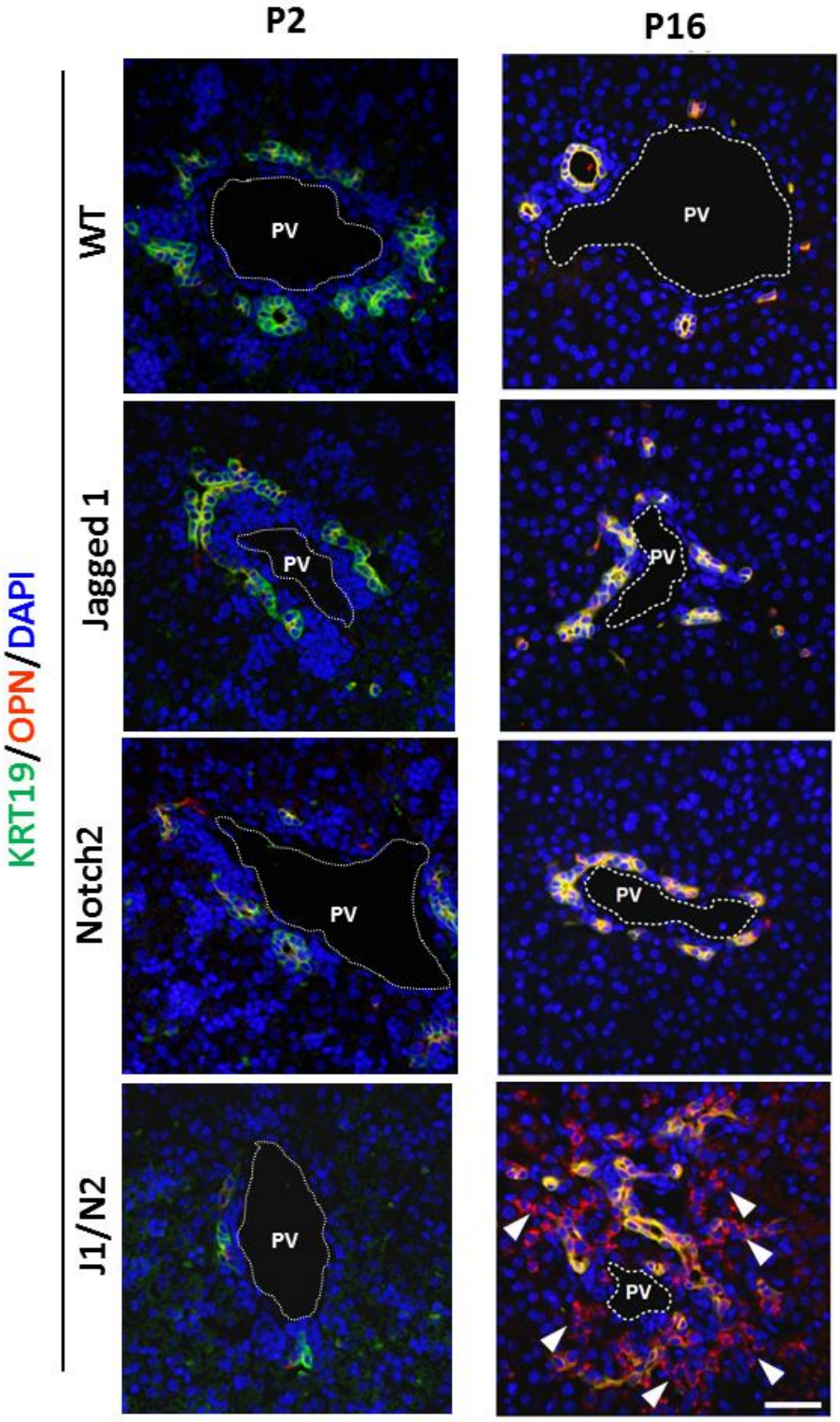

Figure 4.4 Intermediate cells in double mutant Alagille livers

Figure 4.4 BEC markers OPN and KRT19 overlap and mark BEC in wildtype and Jagged1 and Notch2 mutant animals. Double mutant (J1/N2) liver samples have more OPN single positive cells than OPN/KRT19 double positive cells (arrowheads). Scale bar=25 μm.

5 APPENDIX 1: ROLE OF NOTCH SIGNALLING IN HEPATOCELLULAR CARCINOMA[3]

[3] This appendix, with modifications, has been published: Villanueva, A., Alsinet, C., Yanger, K., Hoshida, Y., Zong, Y., Toffanin, S., Rodriguez-Carunchio, L., Solé, M., Thung, S., Stanger, B.Z., Llovet, J.M. *Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice*. *Gastroenterology*. 2012 Dec;143(6):1660-1669

5.1 Introduction

This work was done in collaboration with Dr. Augusto Villanueva and Dr. Clara Alsinet (laboratory of Dr. Josep Llovet; University of Barcelona, Spain), resulting in a joint first-authorship among the three of us. We utilized bioinformatics from both human patients and mice along with *in vivo* mouse models and cell culture experiments to study the role of Notch signalling in hepatocellular carcinoma (HCC).

Hepatocellular carcinoma (HCC)

Primary liver cancer is the third leading cause of cancer death worldwide (Jemal et al., 2011), and its incidence in the United States has tripled between 1975 and 2005 (Altekruse et al., 2009).The most frequent subtype is HCC, which has a complex pathogenesis related to its diverse etiologic factors including cirrhosis because of viral hepatitis (B and C) and/or alcohol abuse. Overall, less than 30% of newly diagnosed HCC patients are eligible for curative therapies such as resection, liver transplantation, or local ablation (Llovet et al., 2003). Patients diagnosed at advanced stages have a bleak prognosis, although the recent identification of sorafenib as an effective molecular therapy has extended their survival to a median of approximately 1 year (Llovet et al., 2008). Although this therapy is not curative, it has changed the landscape of translational research in the field, underscoring the importance of dissecting the molecular drivers of HCC.

The Notch pathway is an evolutionarily conserved signalling module that participates in embryonic cell fate decisions and regulates stem/progenitor cell states (Zaret, 2008).In the liver, Notch acts in a temporal- and dose-dependent manner to

coordinate biliary fate and morphogenesis (Zong et al., 2009). A causative role for Notch signalling is well established in T-cell acute lymphoblastic leukaemia, where mutations or chromosomal aberrations affecting the *NOTCH1* gene are found with high frequency (Ranganathan et al., 2011). In addition, activation of the Notch pathway has been described in several other solid tumours (Santagata et al., 2004; Westhoff et al., 2009). Data regarding Notch involvement in HCC are limited and ambiguous in terms of antitumoural effects following its inhibition (Giovannini et al., 2009; Qi et al., 2003; Wang et al., 2009). Of note, none of the previously reported studies on Notch and HCC included genetic engineered models for pathway deregulation. We previously developed a series of gain-of-function and loss-of-function reagents to characterize the role of Notch during liver development (Zong et al., 2009). Herein, we report that long-term exposure to constitutive Notch signalling in the liver induces HCC in mice with high penetrance and that Notch pathway activation occurs in roughly one-third of human HCCs.

5.2 Results

5.2.1 Liver-Specific Activation of Notch Pathway Promotes Oncogenesis

We sought to evaluate long-term effects of Notch signalling in vivo by using bigenic AFP-NICD mice, in which Cre-mediated recombination in embryonic hepatoblasts results in the expression of a constitutively active form of Notch1 in > 95% of hepatoblasts and cholangiocytes postnatally (Zong et al., 2009) (Figure 5.1 A). At 6 months of age, AFP-NICD mice developed liver tumours at an estimated frequency of 33% (1/3) and 50% (2/4) at 9 months. Beyond 12 months, all AFP-NICD mice showed macroscopic liver tumours (12/12, 5.1 B), whereas none of the control monogenic mice ($R26^{NICD}$) had tumours. Bigenic animals had significant worse survival than monogenics (Figure 5.1 C).

H&E staining of tumours revealed HCC with varying degrees of differentiation in the majority of analysed tumours (7/10, Figure 5.1 D). Accompanying these HCCs, we also identified areas formed by proliferative nonmalignant hepatocytes that tended to group together and replace normally shaped hepatocytes, resembling dysplastic foci found in HCC patients (Figure 5.1 D, "Dysplasia"). All livers displayed various degrees of ductular proliferation, highlighted by CK19 staining (Figure 5.1 D), a feature present in livers of bigenic mice at birth (Zong et al., 2009). In the remaining tumours (3/10), $CK19^{+}$ cells infiltrated the malignant nodules and provoked a significant distortion of liver architecture (Figure 5.1 D, "Atypical pattern"). In addition to hepatocytes (normal, dysplastic, or malignant) and $CK19^{+}$ cells, there was an additional cell population formed by high-proliferative small cells, with reduced cytoplasm and oval nuclei. These cells

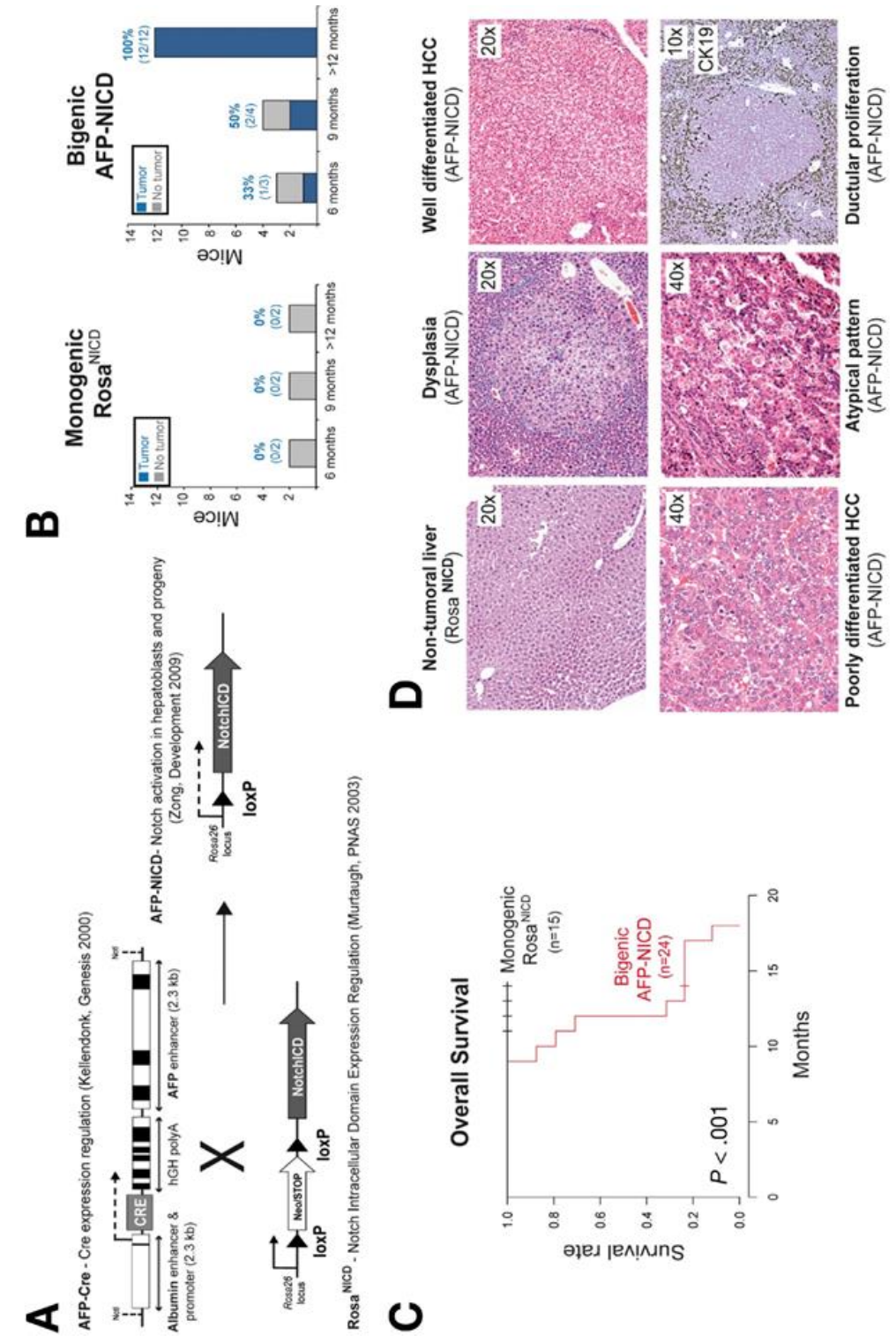

Figure 5.1 Activated Notch induces liver oncogenesis in vivo

Figure 5.1 (A) Schematic representation of the generation of bigenic mice over-expressing a constitutively active form of Notch specifically in the liver (*AFP-NICD*). (B) Tumour incidence in AFP-NICD bigenic and control NICD monogenic mice. (C) Kaplan–Meier curves of mice survival. (D) Representative H&E images of control liver, a dysplastic nodule, HCC with different degrees of differentiation, HCC with atypical pattern (i.e., infiltration of duct-like cells with marked architectural distortion), and intense ductular proliferation (as observed in CK19 staining image).

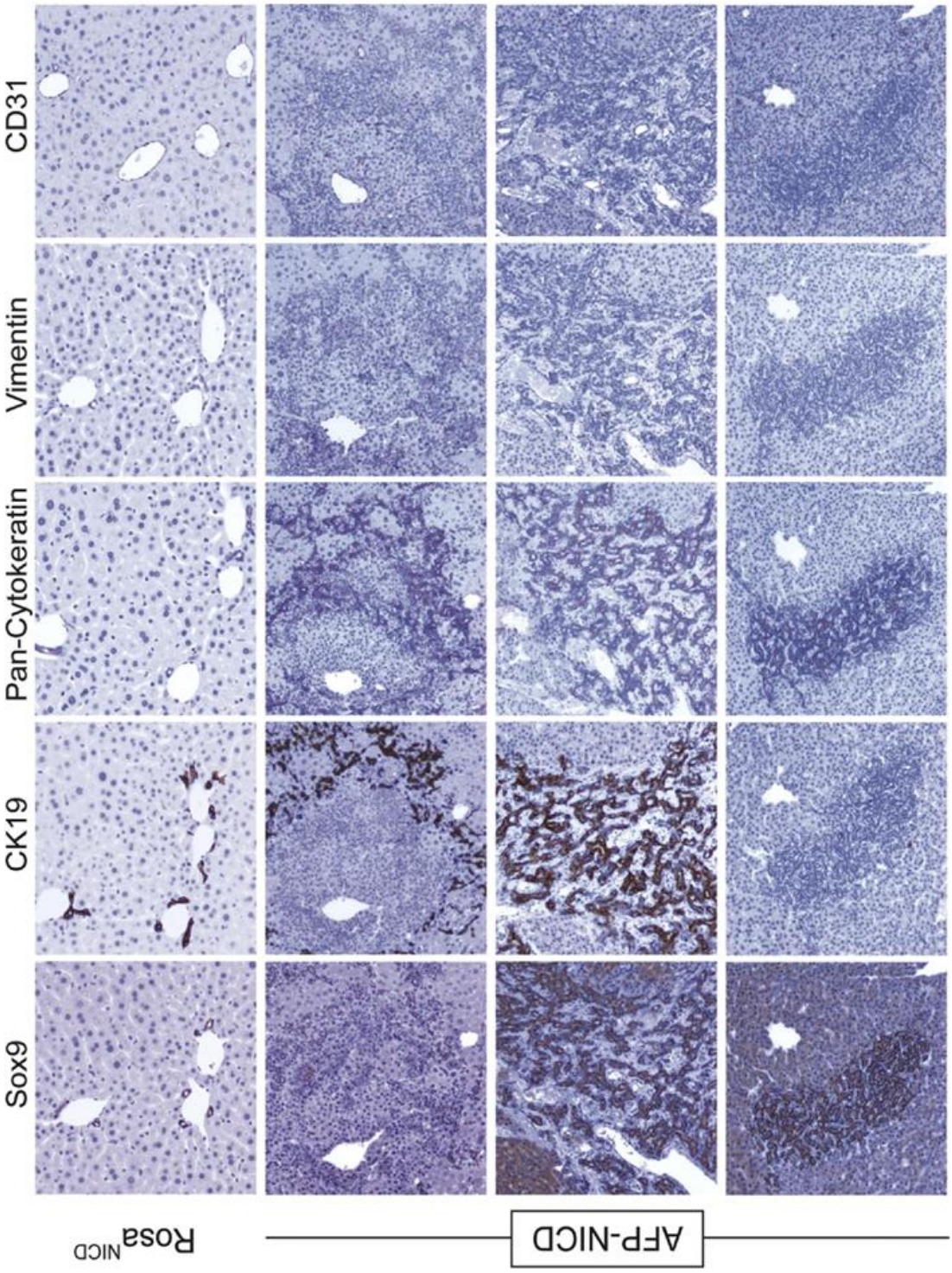

Figure 5.2 Phenotypic characterization of Notch-induced proliferation of oval-shaped cells

Figure 5.2 Representative images of immunohistochemistry of Sox9, CK19, pan-Cytokeratin, Vimentin, and CD31 in murine tissues. *Top panel* shows nontumoural liver from monogenic mice ($R26^{NICD}$) with positive staining for Sox9, CK19, and pan-Cytokeratin in cholangiocytes, and CD31 in endothelial cells. Vimentin is negative in parenchymal cells. *Bottom panels* show staining for the same markers in 3 different tumours from Notch-activated bigenic animals (AFP-NICD) with intense proliferation of atypical small oval-shaped cells. These cells are typically Sox9 positive, CK19 negative, and pan-Cytokeratin scattered positive.

showed histologic features of so-called progenitor cells, exhibiting scattered staining for pan-Cytokeratin and progressive loss of CK19 staining (Figure 5.2). These cells lacked expression of either CD31 or vimentin, excluding a mesenchymal phenotype. They were located either forming nests within dysplastic/tumour nodules or dispersed among normal hepatocytes. Overall, these data indicate that long-term activation of Notch in the mouse liver recapitulates different stages of human hepatocarcinogenesis and results in full-blown HCC, including histologic features associated with progenitor cell expansion.

5.2.2 Notch-Induced Tumors Exhibit Insulin-Like Growth Factor 2 Promoter Reactivation

To examine the possible molecular alterations governing Notch-induced tumourgenesis, we performed genomic profiling of 5 tumours derived from AFP-NICD mice using whole-genome transcriptome arrays and compared them with 4 monogenic livers. Analysis of differentially expressed genes (Villanueva et al., 2012) revealed significant (q value < .05) up-regulation of Notch target genes such as *Spp1* (37.4-fold), *Sox9* (4.4-fold), *Nrarp* (2.98-fold), *Hes1* (2.9-fold), and *Hey2* (2.8-fold) in tumours. In addition, genes involved in cell cycle regulation were significantly up-regulated, including *Dnase1*, *Cdk1*, *Ccna2*, *Ccnd1*, *Ccnb2* (all > 2.5-fold up-regulated). Tumours showed significant down-regulation of members of the cytochrome P450 superfamily, which are involved in hepatic detoxification and homeostasis (e.g., *Cyp2a22, Cyp2b13*, and *Cyp2b9*, all > 15-fold down-regulated). As expected, gene set enrichment analysis identified gene sets defining oncogenic phenotypes in tumours, whereas control livers were markedly enriched in gene sets associated with metabolic biosynthesis (Villanueva

et al., 2012). Analysis of gene ontology terms found concordant results (Villanueva et al., 2012).

The mitogen insulin-like growth factor 2 (*Igf2*) was markedly induced in AFP-NICD tumours (16.3-fold up-regulated). *H19*, an imprinted gene for a long noncoding RNA that is commonly co-regulated with *Igf2*, was also over-expressed (39.5-fold). Typically, fetal liver express *Igf2* transcribed from 3 different promoters that can be detected using exon-specific PCR (Figure 5.3 A). Because previous reports have documented *IGF2* overexpression and reactivation of fetal *IGF2* promoters in human HCC (Nardone et al., 1996; Tovar et al., 2010),we tested whether this was also occurring in Notch-induced tumours in mice. *Igf2* promoters P1–P3 were strongly induced in tumours (4/5) when compared with control liver, mirroring the *Igf2* messenger RNA (mRNA) array levels (Figure 5.3 B).

We also sought to identify candidate oncogenic partners during Notch-induced transformation. Hence, we profiled livers from AFP-NICD mice (n = 6) at birth (postnatal days 0–2)—prior to the development of HCC or dysplasia—and compared them with monogenic livers (n = 5). As predicted, Notch target genes were significantly up-regulated in bigenic new-born livers (Villanueva et al., 2012). Consistent with these results, the Notch pathway appeared among top canonical pathways enriched upon ingenuity pathway analysis (Villanueva et al., 2012). In contrast to the tumours, the imprinted genes *Igf2* and *H19* were not significantly deregulated in new-born AFP-NICD mice. This finding suggests that *Igf2* may cooperate with activated Notch during malignant progression.

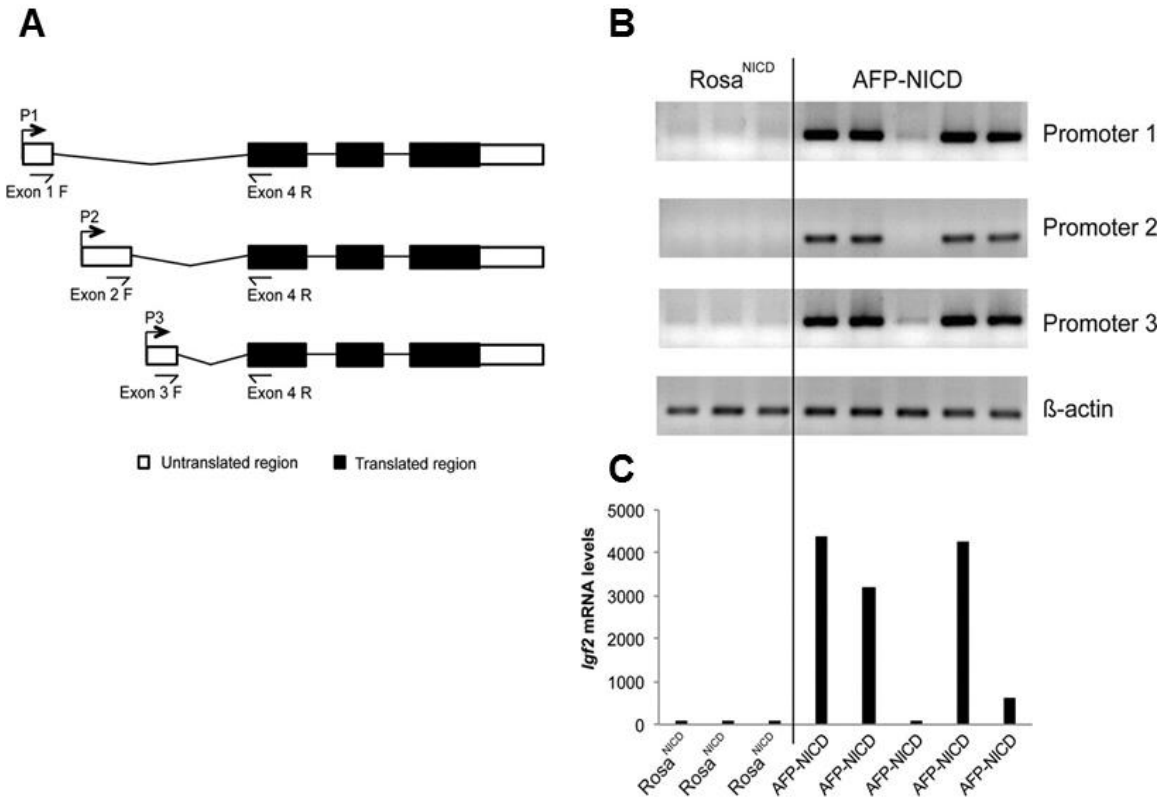

Figure 5.3 Insulin-like growth factor 2 (IGF2) promoter reactivation in Notch-induced tumours

Figure 5.3 (A) Schematic representation of murine *Igf2* mRNAs structure according to differential promoter usage events. (B) Exon specific PCR identifies *Igf2* P1, P2, and P3 promoter reactivation in Notch-induced tumours in bigenic mice (AFP-NICD), whereas none of the control livers evaluated ($R26^{NICD}$ mice) showed promoter reactivation. (C) Promoter reactivation correlates with *Igf2*microarray mRNA levels. The tumour without promoter reactivation did not show high mRNA levels of *Igf2*.

5.2.3 Activation of the Notch Pathway Occurs Frequently in Human HCC

We then used comparative functional genomics to determine whether Notch pathway activation occurs frequently in human HCC samples. For that purpose, we generated a 384-gene signature using differentially expressed genes between Notch-induced tumours in AFP-NICD mice and control liver from monogenics (Villanueva et al., 2012). The Notch tumour signature was first tested in our cohort of human samples recapitulating hepatitis C virus (HCV)-associated hepatocarcinogenesis (normal liver [n = 10], cirrhosis [n = 13], dysplastic nodules [n = 18], and full-blown HCC [n = 91] (Chiang et al., 2008; Wurmbach et al., 2007)) using NTP (Hoshida, 2010). Interestingly, a subset of cancerous tissues held a significant prediction for the signature but none of noncancerous tissue, which suggested that the signature was mostly capturing Notch activation in transformed tissues. When NTP was performed in HCCs, the Notch signature predicted activation of Notch in 29 out of 91 (31.8%) human HCC (Figure 5.4). To rule out whether the signature was capturing unspecific genomic signals of malignant transformation unrelated to Notch activation, we defined a separate signature—consisting of 276 genes—using liver tissue from new-born bigenic animals (before tumour onset). When applied to human HCC, even though fewer samples were predicted with this Notch new-born signature (18%, 17/91), they were still significantly enriched in the Notch tumour signature ($P < .001$; Figure 5.5). Interestingly, both signatures were enriched in a robust HCC subclass recently identified (proliferation class (Chiang et al., 2008),$P < .001$; Figure 5.5). Thus, the 386-gene Notch tumour signature identifies a subgroup of

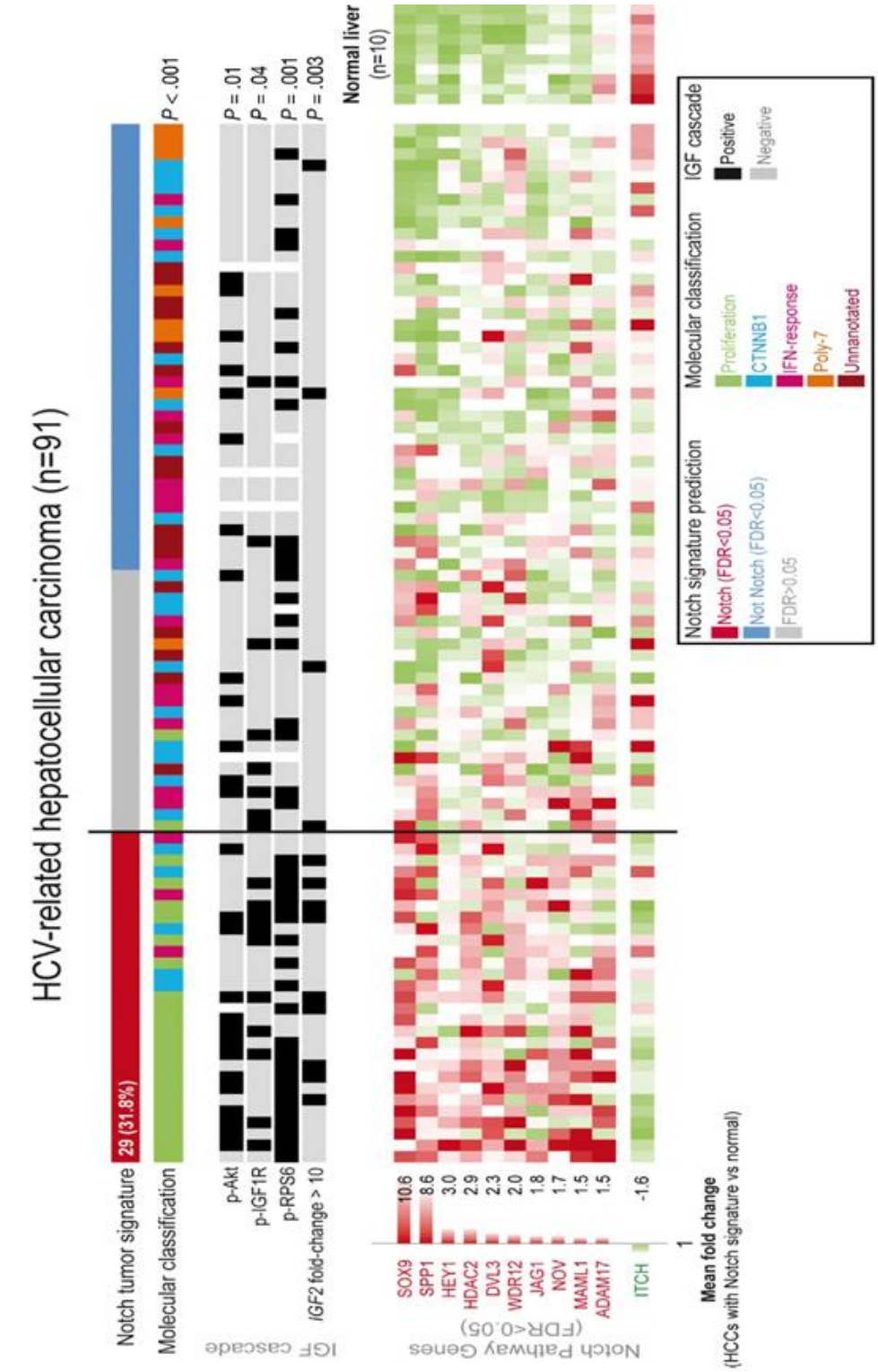

Figure 5.4 Notch activation and de-regulation in human HCC

Figure 5.4 Prediction of the Notch signature (*red* in the first row) in HCCs of the HCV-related data-set (n = 91). Each *square* represents data of each individual sample. One third of the samples (31.8%) had activation of Notch based on a significant prediction for the presence of the signature (false discovery rate [FDR] < 0.05, nearest template prediction method). Chiang et al. showed tumours with the Notch signature were significantly enriched in the proliferation class of our molecular classification of HCC (Proliferation = *green*). In addition, HCCs with activated Notch were significantly enriched in different markers of IGF pathway activation including phosphorylated (p)-Akt, p-IGF1R, p-RPS6 (immunohistochemistry), as well as high expression levels of *IGF2 according to data from Tovar et al.* The *bottom panel* shows Notch Pathway Gene List (see Materials and Methods section for details) genes found significantly deregulated between Notch-activated HCC and those HCC without the signature as well as with normal liver (FDR < 0.05, *red* and *blue bars* in the first row, respectively). Deregulation magnitude is graded based on a *red* (over-expression) and *green* gradient (down-regulation) normalized to the median expression value in normal liver.

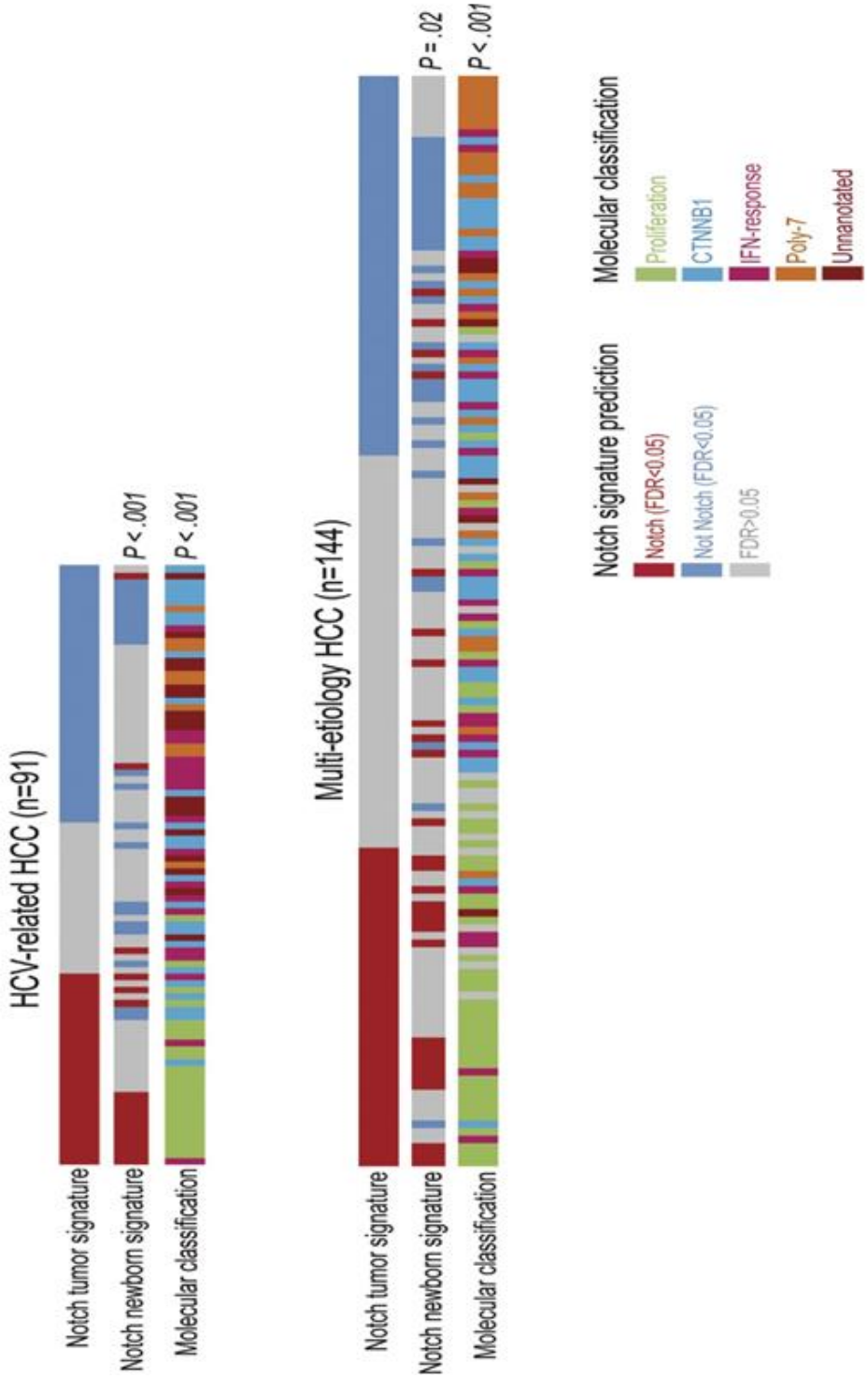

Figure 5.5 Prediction overlap between Notch tumour and new-born signatures in human HCC

Figure 5.5 Comparative analysis of predictions from the Notch tumour (384 genes) and new-born (276 genes) signatures. There is a significant association between them and also with the proliferation class of the molecular classification of HCC[4] (Fisher exact test).

nearly one-third of human HCCs associated with HCV.

Having captured a subset of Notch-associated human HCCs based on the signature generated in murine tumours, we next determined the status of potential downstream effectors in these samples. To do so, we first crafted a Notch gene list (n = 96) by merging previously reported lists of Notch pathway genes (Higgins et al., 2007; Kanehisa and Goto, 2000; Villanueva et al., 2012). When evaluating their differential expression between Notch-activated HCC (n = 29) and normal liver (n = 10, Figure 5.4), we found a significant up-regulation of several Notch activators such as ligands (e.g., *JAG1*), α-secretases (e.g., *ADAM17*), effectors (e.g., *MAML1*, *NOV*) and target genes (eg,*SOX9*, *SPP1*, *HEY1*). Pathway inhibitor *ITCH* was significantly down-regulated. In accordance with data from murine tumours, Notch-activated human HCCs were significantly enriched for high levels of *IGF2* expression (Fisher exact test, *P* = .003; Figure 5.4), an alteration we previously found to be associated with fetal promoter reactivation (Tovar et al., 2010). In addition, these tumours exhibited activation of the IGF, AKT, and MTOR pathways as determined by phospho(p)-IGF-1R, p-Akt, and p-RPS6 IHC staining (Figure 5.4). To address possible mechanisms underlying Notch activation in humans, we integrated gene expression data with DNA copy number changes in the same cohort for Notch pathway genes. We failed to find high-level amplifications affecting any putative Notch gene, but there were DNA gains affecting the *HEY1* locus (chromosome 8).*HEY1* is a Notch target gene significantly up-regulated in HCCs with the Notch signature compared with normal tissue and tumours without the signature, mostly in those samples with DNA gains (Figure 5.4 and Figure 5.6).

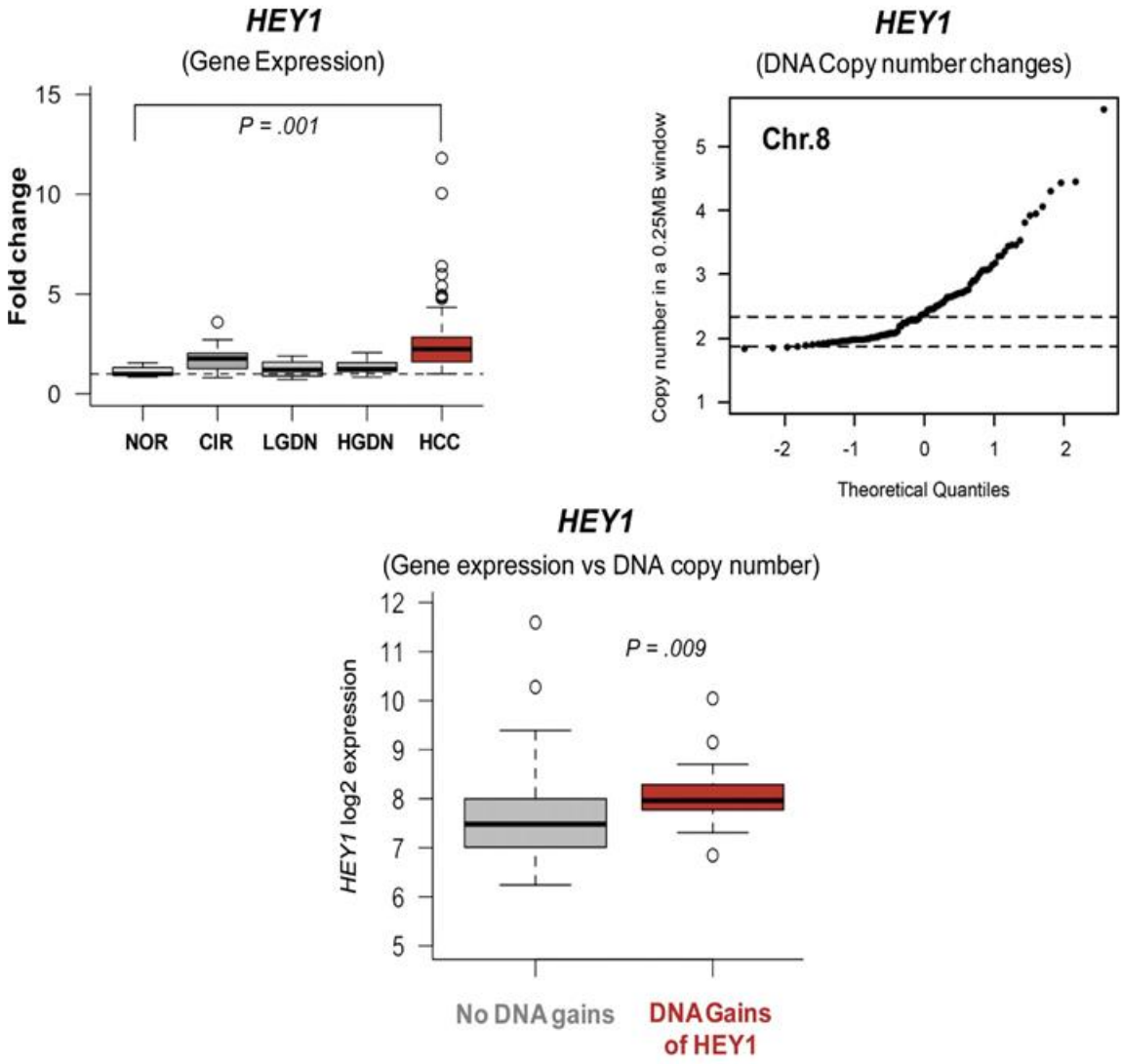

Figure 5.6 HEY1 alterations in human HCC

Figure 5.6 (*Top left*) *HEY1* expression in normal (*NOR*, n = 10), cirrhotic (*CIR*, n = 13), low-grade dysplastic nodule (*LGDN*, n = 10), high-grade dysplastic nodule (*HGDN*, n = 8), and hepatitis C virus (HCV)-associated HCC (*HCC*, n = 91). (*Top right*) Copy number alterations in HCV-associated HCCs (n = 104) in *HEY1* locus; *dashed lines* correspond to maximum and minimum copy number values in paired cirrhotic tissue. (*Bottom*) Correlation between *HEY1* gene expression data and *HEY1* locus copy number alterations.

We further tested the performance of the signature on an additional set of 144 HCCs from different etiologies including also hepatitis B infection and alcohol-induced HCC (Villanueva et al., 2011). Consistent with the results obtained using the HCV set, 29.2% (42/144) of these tumours exhibited the Notch signature. To assess the validity of this signature in biologic samples, we performed IHC for the Notch target SOX9 in a subset of HCC with (n = 27) and without (n = 24) the Notch signature. Nuclear staining of SOX9 (Figure 5.7 A) was more prevalent in HCCs with the Notch signature than without (19/27 vs 6/24, respectively, $P = .001$), and mRNA levels of SOX9 were associated with both SOX9 nuclear staining and the Notch signature prediction ($P < .001$, Figure 5.7 B).

SOX9 has been shown to maintain pancreatic progenitors, and it has been proposed as a marker of progenitor cells with bipotentiality in the adult liver (Furuyama et al., 2011; Seymour et al., 2007). An emerging role of SOX9 in cancer promotion and metastasis seeding has also been suggested (Guo et al., 2012). *SOX9* was highly up-regulated in Notch-activated HCC (10.6-fold compared with normal liver, Figure 5.4). Additionally, we observed increased *SOX9* levels in an aggressive microRNA-based molecular subclass of HCC (C3 subclass (Toffanin et al., 2011)) and identified miR-30 as a candidate regulator of *SOX9* (Figure 5.8).

Previous studies have identified activating *NOTCH1* mutations in solid tumours (Westhoff et al., 2009). Hence, we sought to estimate their prevalence in our data set and searched for the presence of mutations at hotspot exons 26, 27, and 34 of the *NOTCH1* gene using direct sequencing in 50 human HCCs from our HCV-related

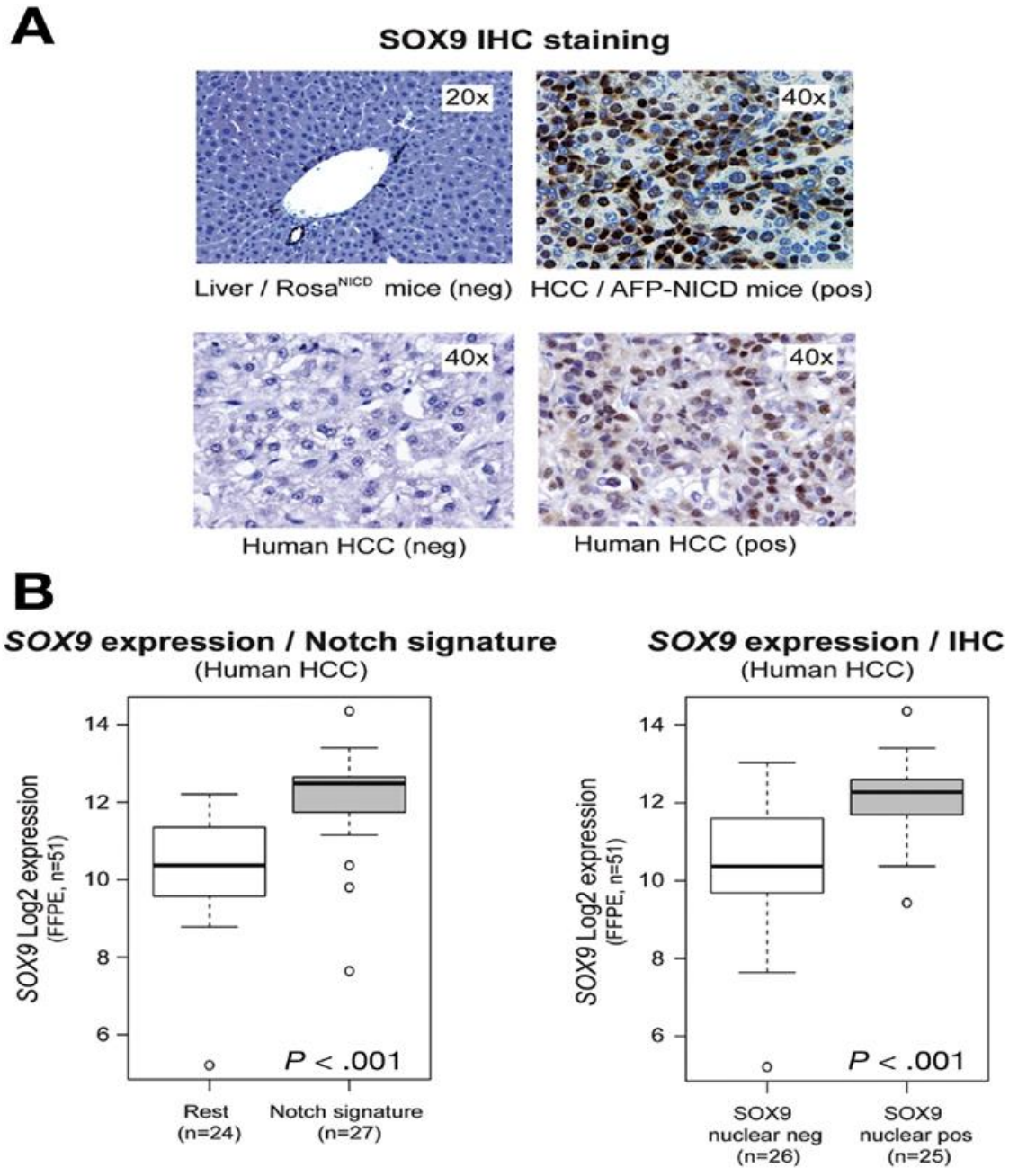

Figure 5.7 Deregulation of the Notch target gene SOX9 in HCC

Figure 5.7 (A) Representative images of nuclear SOX9 staining. *Top panels* show SOX9 staining in mice: stained bile duct cells in livers from control monogenic mice (*upper left*) and stained hepatocytes and cholangiocytes in murine tumours (*upper right*). *Bottom panels* show human HCC samples with negative (*bottom left*) and positive (*bottom right*) nuclear staining. (B) Boxplots of *SOX9* messenger RNA levels from HCC samples (n = 51) with and without the Notch signature (*left*) or SOX9 nuclear staining (*right*).

data set. Two samples showed nonsynonymous mutations (4%), indicating that activating mutations in hotspots of the*NOTCH1* gene do not account for pathway deregulation in most HCC cases.

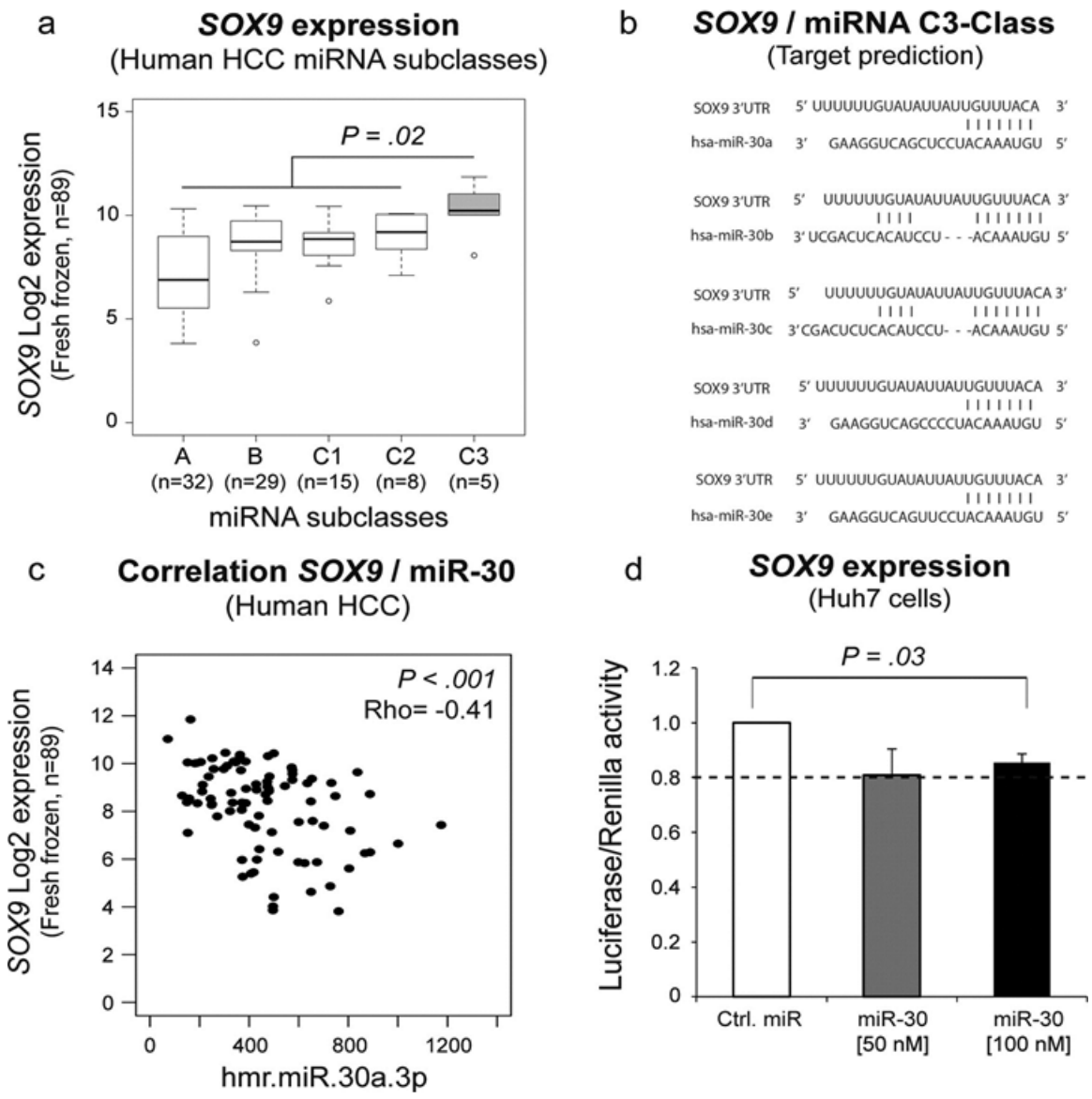

Figure 5.8 SOX9 regulation by miR-30 in HCC

Figure 5.8 (A) Expression of *SOX9* in HCC (n = 89, hepatitis C virus-related data set) according to our previously described microRNA subclasses by Wurmbach et al., which overlaps with our mRNA-based classification. (B) In silico prediction shows sequence complementary between miR-30 members and *SOX9* 3′ untranslated region (UTR) (TargetScan software). (C) *Dot plot* representing the inverse correlation between *SOX9* mRNA levels and miR-30a.3p in HCCs (n = 89). (D) Decrease in luciferase activity from a *SOX9*-3′UTR-containing dual firefly/Renilla luciferase reporter following transfection of miR-30a in Huh7 cells.

5.2.4 The Notch Signature Coclusters Within the Proliferation Class and Predicts Response to Selective Notch Inhibition

To understand better the predictive performance of the Notch 384-gene signature in human HCC, we further evaluated 5 publicly available HCC data sets, including a total of 407 samples using the NTP method (Villanueva et al., 2012). We also integrated data of Notch predictions with our previously described molecular classification of HCC (Chiang et al., 2008). As a whole, between 20% and 37% of samples in each HCC data set showed the presence of the Notch signature (Figure 5.9 A). Of note, when we focused on those data sets with higher number of significant predictions based on a false discovery rate < 0.05, the prediction rate for Notch ranged between 32% and 37%, very close to that of our own cohorts (~30%). In most data sets, there was a significant correlation between the Notch signature and our previously defined proliferation class (Chiang et al., 2008), a class characterized by genomic signals related to cell cycle progression and proliferation. Thereafter, we tested the similarity between the Notch signature and signatures previously reported to confer aggressive clinical behaviour in HCC using Cramer's V coefficient (Villanueva et al., 2011). The Notch signature coclustered with a group including the proliferation class (Chiang et al., 2008), a $CK19^{+}$ progenitor-derived HCC signature generated in rats (Andersen et al., 2010), a cholangiocarcinoma-like gene expression trait described in HCC (Woo et al., 2010), and a prognostic transforming growth factor-β signature generated in mouse hepatocytes (Coulouarn et al., 2008) (Figure 5.9 B). The Notch signature was not correlated with

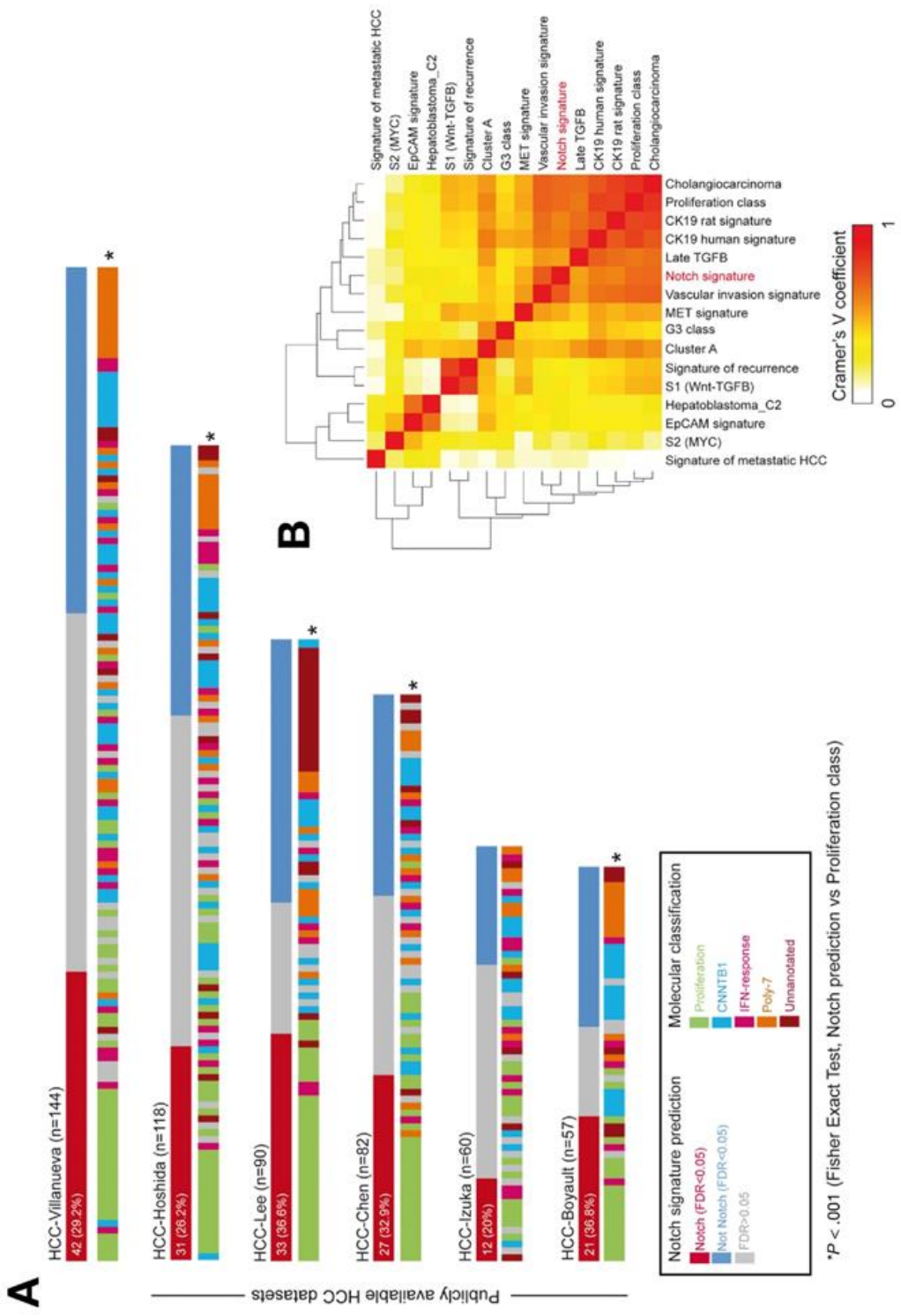

Figure 5.9 Signature-based activation of Notch across HCC data sets

Figure 5.9 (A) Performance of the Notch signature across HCC data sets. *Top rows* in each data-set represent Notch predictions, and *bottom rows* show our molecular classification of HCC (Chiang et al., 2008) (Proliferation = *green*) for each sample. In all except 1 data set (*HCC-Izuka*), tumours with Notch activation are enriched in patients of the Proliferation class. (*B*) Heatmap of Cramer's V coefficient showing correlation between the Notch signature and other previously reported signatures that predict clinical aggressive behaviour (from Villanueva et al., 2001 and Hoshida et al., 2009). Briefly, Cramer's V statistic values range from 0 to 1, being 0.36–0.49 substantially correlated, > 0.5 strongly correlated, and 1 identical.

clinical outcome in these patients, which points toward its limited role as a prognostic biomarker in HCC (Hoshida et al., 2009).

To evaluate further the Notch 384-gene signature and determine whether it predicts sensitivity to Notch pathway inhibitors, we applied this signature to a large panel of cancer cell lines (n = 318) whose expression data are available online (caBIG). We found that the signature captured Notch activation mainly in cells derived from solid Tumours, as opposed to hematologic malignancies, and liver cancer cell lines showed varied profiles (Figure 5.10 A). Next, we focused on the Notch signature prediction in a panel of 21 liver cancer cell lines (CCLE (Barretina et al., 2012), Figure 5.10 B) and selected 3 lines with the Notch signature (SNU423, SNU475, and SNU449) and 2 without (HepG2 and Huh7) for further analysis. When incubated with 2 μmol/L Compound E, a GSI that inhibits ligand-dependent Notch signalling, SNU423 and SNU475 showed significant decrease in cell viability, whereas HepG2 and Huh7 remained irresponsive (P = .002, Figure 5.10 C). To obtain a more specific inhibition of the pathway, we transfected SNU449, SNU475, and HepG2 cells with a construct containing a dominant negative form of the transcriptional coactivator of Notch, MAML1 (DN-MAML-GFP). SNU449 and SNU475 (Notch signature positive) showed decreased proliferation upon DN-MAML-GFP infection in comparison with GFP-only cells (P < .03, Figure 4.10 D). In contrast, HepG2 (Notch signature negative) showed an increase in proliferation (P = .036, Figure 5.10 D).

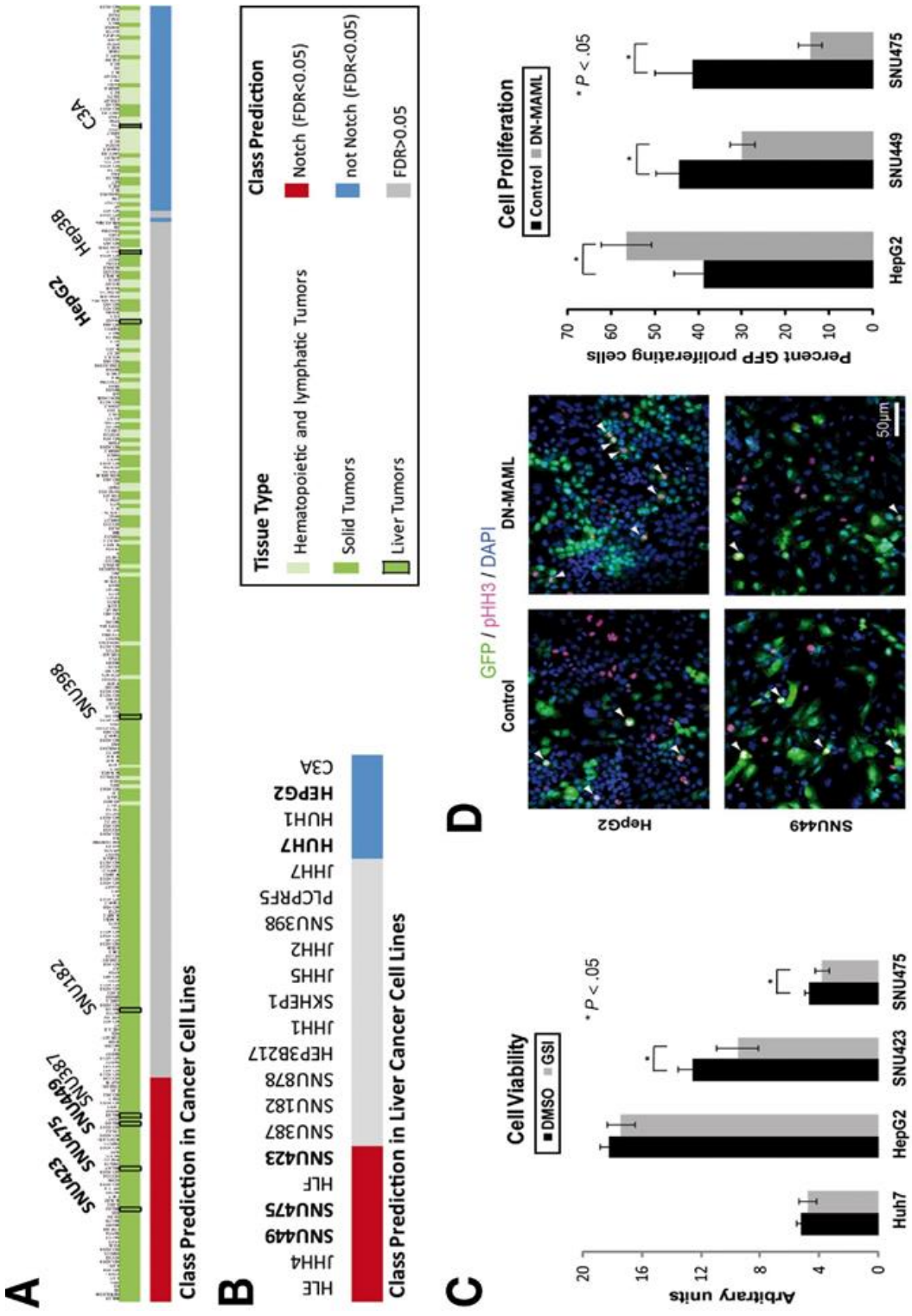

Figure 5.10 Notch signature predicts response to Notch inhibition in vitro

Figure 5.10 (A) Prediction of the Notch signature in 318 cancer cell lines from the Cancer Biomedical Informatics Grid, caBIG National Cancer Institute (Bethesda, MD) (*dark green*: solid tumours' cell lines; *light green*: hematologic malignancies' cell lines). (*B*) Notch signature prediction in 21 liver cancer cell lines (Broad-Novartis Cancer Cell Line Encyclopedia, Barretina et al.). Cell lines used in further analysis are highlighted in *bold*. (*C*) Cell viability upon 5-day incubation with a γ-secretase inhibitor. Decrease in viability was restricted to cells harbouring the Notch signature; standard deviation is shown. (*D*) Representative images of phospho-histone H3 (*pHH3*) staining in DN-MAML-GFP and GFP-only (*control*) transfected cells; *white arrowheads* highlight transfected proliferating cells (GFP^+ and $pHH3^+$). Quantification of cell proliferation was evaluated by enumerating $pHH3^+$ GFP^+ cells as a percentage of total GFP^+ cells in liver cancer cell lines 48 hours post infection; standard deviation is shown.

5.3 Discussion

In this study, we have undertaken a comprehensive and integrative approach to explore the role of Notch signalling in liver cancer pathogenesis. We have found that Notch signalling promotes liver carcinogenesis in a genetically engineered mouse model and that this pathway is activated in one third of human HCCs. Specifically, our studies demonstrate that (1) liver-specific Notch activation in mice recapitulates features of human hepatocarcinogenesis, including dysplasia and HCC; (2) several genes involved in the Notch signalling cascade are deregulated in HCC, particularly *SOX9*; (3) *Notch1* is a bona fide oncogene in experimental liver cancer; (4) Notch and IGF signalling are frequently coactivated in experimental and human HCCs, and *Igf2* induction occurs via reactivation of silenced *Igf2* promoters; (5) a 384-gene signature obtained from Notch-induced tumours is able to recognize pathway activation in approximately 30% of human HCCs from different etiologies; (6) inhibiting Notch signalling in liver cancer cells lines that harboured the Notch signature, but not those that lacked it, resulted in growth inhibition. Taken together, these data suggest that activation of Notch signalling is pivotal in liver oncogenesis.

The role of the Notch cascade in solid tumours is controversial (Ranganathan et al., 2011), and, despite strong data indicating that Notch activation facilitates tumour progression in the liver (Lim et al., 2011; Liu et al., 2012), some reports suggest the opposite (Qi et al., 2003; Viatour et al., 2011; Wang et al., 2009), a discrepancy that may be due to the high context dependency of the Notch cascade (Ranganathan et al., 2011). Ours is the first study to use a genetically engineered mouse model to test the

tumorigenicity impact of liver-specific Notch activation in vivo. In our model, HCCs arose with a latency of greater than 6 months, suggesting that additional hits may be needed in addition to Notch deregulation to achieve cell transformation. In this regard, we found strong up-regulation of *Igf2* in Notch-derived tumours, raising the possibility that *Igf2* acts as an oncogenic partner of Notch in this setting. Reactivation of *Igf2* fetal promoters and their tight correlation with mRNA levels lends credence to such a cooperative pathogenic role because both promoter reactivation and *IGF2* up-regulation are well-recognized features of human HCC (Nardone et al., 1996; Tovar et al., 2010). Whether there is a functional cross talk between both cascades in HCC needs further evidence. Previous reports suggested a cooperative oncogenic role between Notch and other pathways such as RAS (Weijzen et al., 2002).

We undertook a comparative functional genomics (Lee et al., 2004) approach to connect molecular pathogenic features of human cancer to the mouse model by integrating high-density genomic data. This approach has previously been used in HCC (Lee et al., 2004), which resulted in the identification of best-fit mouse models to model human HCC. In line with this concept, we aimed to translate detection of aberrant Notch activation in rodent HCC to humans by identifying common transcriptome deregulation patterns in murine Notch-activated tumours and human HCC. To this end, we generated a 384-gene signature of Notch-activated HCC from mice and determined its predictive performance in 642 human HCC samples. Overall, 30%–35% of HCCs were confidently predicted to harbour the signature, regardless of etiology and disease stage. Strikingly, tumours with the signature were significantly enriched in the “Proliferation class” of our

previously described molecular classification (Chiang et al., 2008) and showed deregulation of several Notch pathway genes such as *HEY1*, which has been recently proposed as an oncogene in HCC (Jia et al., 2011). Recent data suggest that this class is heterogeneous, capturing different genomic signals related to transforming growth factor-β (Hoshida et al., 2009), MET (Kaposi-Novak et al., 2006), IGF (Chiang et al., 2008; Tovar et al., 2010), and progenitor-derived HCC (Lee et al., 2006).

Detailed pathologic examination of Notch-induced HCC identified 3 major cell populations: (1) "proliferative" duct-like cells, which were positive for CK19; (2) hepatocyte-like cells, which were negative for CK19, including normal, dysplastic, and malignant hepatocytes; and (3) atypical small oval-shaped cells, which formed nests within tumours that were CK19 negative but positive for scattered pan-Cytokeratin staining. All Notch-induced tumours showed various degrees of nuclear staining for the Notch target gene *Sox9*, and *SOX9*overexpression was frequently observed in human HCCs, particularly those tumours bearing the Notch signature. A recent study (Furuyama et al., 2011) suggests that Sox9 marks a pluripotent population within the liver, and, thus, it is possible that Notch acts during hepatocarcinogenesis by expanding a pre-existing progenitor-like cell or conferring progenitor-like properties to differentiated cells during hepatocarcinogenesis.

The success of targeted therapies that block oncogene addiction loops, such as vemurafenib (Chapman et al., 2011) in mutant BRAF melanomas or crizotinib in lung cancer patients with ALK rearrangements (Kwak et al., 2010), underscores the importance of identifying molecular drivers of cancer. At present, HCC lacks any known

oncogene addiction loop amenable for selective targeting. Recent failures in phase 3 trials with systemic agents (e.g., sunitinib negative in first line and brivanib negative in first and second line) highlight the need for new therapeutic targets and a change in trial design to routinely include enrichment strategies based on molecular markers of response, and the Notch signature fulfils both criteria. Similarly to other molecular biomarkers of response in oncology (e.g., ALK rearrangements in lung cancer), lack of prognosis performance of the Notch signature does not diminish its potential role as a therapeutic biomarker. We provide evidence of differential response to selective Notch inhibition depending on the presence or absence of a Notch signature, both with a pharmacologic inhibitor (GSI) and a specific molecular inhibitor (dominant negative MAML) of Notch signalling. This finding raises the possibility that the estimated 30% of HCCs exhibiting Notch pathway activation could be responsive to Notch inhibition. Prospective trials, ideally including populations enriched with biomarkers of Notch activation, will be necessary to address this issue. Of note, interrogation of a cohort of 318 cancer cell lines for the presence of the 384-gene signature predicted Notch activation only in cells derived from solid tumours. The presence of differential genomic traits between Notch-induced solid tumours and hematologic malignancies might be expected with such a context-dependent pleiotropic pathway.

In summary, this study provides evidence that the Notch pathway is involved in the pathogenesis of HCC, with 30%–35% of tumours exhibiting pathway activation according to a novel gene signature across different data-sets. Overall, our findings

indicate that Notch may be an appealing target for new drug development initiatives in HCC.

6 MATERIALS AND METHODS

Mouse Studies

Mice were maintained in a pathogen-free environment. AFP^{Cre}, $R26^{YFP}$, RBP-Jκ, and $R26^{NICD}$ strains have previously been described (Han et al., 2002; Kellendonk et al., 2000; Murtaugh et al., 2003; Srinivas et al., 2001). To generate an ADC response, animals were given 0.1% wt/wt DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine; Sigma-Aldrich) in PMI mouse diet #5015 (Harlan Teklad) for 1-6 weeks as described (Wang et al., 2003a). Choline deficient + ethionine diet (CDE) was administered for 3 weeks with choline-deficient pellets (MP Biomedicals) ad-libitum and fresh drinking water with 0.15% ethionine (Sigma-Aldrich) prepared every 2-3 days as described (Carpentier et al., 2011). α-naphthylisothiocyanate pellets (ANIT) (Dyets) were administered for 2 weeks (Faa et al., 1998). For recovery studies, animals were switched back to normal show for 2-5 weeks before assessing the livers. CCl_4 intoxication was conducted as described (Malato et al., 2011) with injections twice weekly at a dose of 0.5ul/g of body weight and diluted in corn oil (Sigma-Aldrich). Partial hepatectomy (PHx) and bile duct ligation (BDL) were performed as described (Chu AS, 2011; Greenbaum et al., 1995).All studies were conducted in accordance with the policies of the National Institutes of Health and the University of Pennsylvania Institutional Animal Care and Use Committee guidelines. *In* vivo BrdU labelling was performed by adding BrdU (Sigma, Lot#040M1344) to the drinking water at a concentration of 0.5 mg/ml coinciding with 1-2 weeks of DDC treatment.

Immunostaining and quantification

Antibody staining was performed as previously described (Zong et al., 2009) and listed in Table 1. The percentage of marker-positive cells and binucleated biliary cells was determined by taking representative images and directly counting cell number. Samples were obtained from at least three animals; cell enumerations for each experiment are listed in the text or Figure legends. Human explant liver tissues were obtained from tissue banks at the Children's Hospital of Philadelphia or Mount Sinai School of Medicine, collected with IRB approval.

Antibody	Species	Source	Catalogue #	Dilution
Cytokeratin19	Rabbit	D. Melton	NA	1:1000
GFP	Chicken	Abcam	ab13970	1:500
GFP	Goat	Abcam	ab6673	1:500
A6	Rat	V. Factor	NA	1:100
Hnf4α	Goat	Santa Cruz	SC-6556	1:250
Hnf4α	Rabbit	Santa Cruz	SC-8987	1:250, TSA
Ki-67	Mouse	BD	561165	1:100
1D11	Rat	Novus Biologicals	NBP1-18963	1:100
4E8	Rat	Novus Biologicals	NBP1-18971	1:100
2F3	Rat	Novus Biologicals	NBP1-18964	1:100
3C7	Rat	Novus Biologicals	NBP1-18970	1:100
TROP2	Mouse	R&D	AF1122	1:100
Ki-67	Rabbit	Thermo Scientific	MA1-90584	1:100
Hes1	Rabbit	In-house	NA	1:1000, TSA
Hnf1β	Goat	Santa Cruz	SC-7411	1:250, TSA
A6	Rat	V. Factor	NA	1:100
Par6	Rabbit	Santa Cruz	sc67393	1:250, TSA
PKCζ	Rabbit	Santa Cruz	Sc-216	1:250, TSA
Osteopontin	Goat	R&D	AF808	1:500
Sox9	Rabbit	Millipore	ab5535	1:1000, TSA
Ac-tubulin	Mouse	Sigma	T6793	1:200
EpCAM	Rat	BD	552370	1:500

Table 1. List of Antibodies

Quantitative PCR

Total RNA was extracted from FACS sorted cells using the RNeasy Mini Kit (Qiagen) and 30-50 ng was used to synthesize cDNA using the iScript cDNA Synthesis Kit (BioRad, 170-8890). Quantitative PCR was performed with SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), 312 nM of Primer Mix (IDT) and cDNA template diluted 10-fold. Fold enrichment was determined using the difference of C_t method with values normalized to GAPDH. Primer sequences are listed in Table.

Gene	Forward	Reverse
GAPDH	ATGTTCCAGTATGACTCCACTCACG	GAAGACACCAGTAGACTCCACACA
CK19	GACCTAGCCAAGATCCTGAGT	TCAGCTCCTCAATCCGAGCA
EpCAM	GCGGCTCAGAGAGACTGT	CCAAGCATTTAGACGCCAGTTT
Sox9	ACTCTGGGCAAGCTCTGGAG	CGAAGGGTCTCTTCTCGCTCT
HNF1β	CCCAGCAATCTCAGAACCTC	AGGCTGCTAGCCACACTGTT
Hes1	AAAGCCTATCATGGAGAAGAGGCG	GGAATGCCGGGAGCTATCTTTCTT
AFP	CCTCCCAGTGCGTGACGGAGAA	CACTTCCTCCTCGGTGGCTTCC

Table 2. List of Primers

Viral infections

Replication incompetent AAV viruses were obtained from the Penn Vector Core. AAV8-TBG-Cre carries Cre recombinase under the regulatory control of the hepatocyte-specific thyroid binding globulin (TBG) promoter, while AAV8-CMV-Cre carries Cre recombinase under the regulatory control of the ubiquitous CMV promoter. *In vivo* cell labelling with AAV8-TBG-Cre and AAV8-CMV-Cre was achieved by giving mice of the appropriate genetic background either 2.5 X 10^{11} viral particles (regular dose) diluted in

sterile 1X PBS. For activating Notch signalling in differentiated hepatocytes, AAV-TBG-Cre was administered to mice via retro-orbital injection at a dose of $1x10^{11}$ GC/animal (or $1x10^{10}$ GC/animal diluted in PBS for low dose experiments).

Tamoxifen administration

KRT19-CreER transgenes was achieved by giving mice 40 mg of tamoxifen (TM) over 5 doses. For labelling ADCs, *KRT19-CreER*; $R26^{YFP}$ mice were given 3-5 doses of 8 mg/dose TM during the second week of DDC treatment. All studies were conducted in accordance with the policies of the National Institutes of Health and the University of Pennsylvania Institutional Animal Care and Use Committee guidelines.

Liver cell preparation

Livers of normal and DDC-treated mice were perfused initially with 1X HBBS solution followed by perfusion media (1X HBSS, 0.1 M $CaCl_2$) containing 5mg/ml Liberase (Fisher Scientific). The digested liver and cells were further agitated by pipetting and collected through a 70-µm cell strainer for FACS analysis/collection.

Flow cytometry staining

Cells were counted and blocked, followed by staining with EpCAM and IgG isotype control antibodies. After washing, cells were passed through a 40-µm cell strainer and analysed/collected by FACS Aria (Becton Dickinson) using DAPI to exclude dead cells.

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