

Review Article

Liver Development, Regeneration, and Carcinogenesis

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The identification of putative liver stem cells has brought closer the previously separate fields of liver development, regeneration, and carcinogenesis. Significant overlaps in the regulation of these processes are now being described. For example, studies in embryonic liver development have already provided the basis for directed differentiation of human embryonic stem cells and induced pluripotent stem cells into hepatocyte-like cells. As a result, the understanding of the cell biology of proliferation and differentiation in the liver has been improved. This knowledge can be used to improve the function of hepatocyte-like cells for drug testing, bioartificial livers, and transplantation. In parallel, the mechanisms regulating cancer cell biology are now clearer, providing fertile soil for novel therapeutic approaches. Recognition of the relationships between development, regeneration, and carcinogenesis, and the increasing evidence for the role of stem cells in all of these areas, has sparked fresh enthusiasm in understanding the underlying molecular mechanisms and has led to new targeted therapies for liver cirrhosis and primary liver cancers.

1. Introduction

Worldwide, liver cancer is the fifth most common malignancy [1] and the third most common cause of cancer death [2]. Five hundred million individuals are infected with Hepatitis B or C and a proportion will progress to liver failure and cancer. As a result, the imperative to understand the mechanisms of liver disease and to improve treatments for liver disease has resulted in a dramatic expansion in liver research. Accordingly, this has driven forward the technical developments essential to the isolation, maintenance and propagation of highly purified cell subtypes which will underpin the therapies of the future. Better understanding of the physiology of liver development and the responses to liver injury has revealed emerging themes common to ontogeny, regeneration, and carcinogenesis. Regulatory pathways observed in one may form the basis for therapeutic intervention in another. This review describes recent insights into liver development, and the relationships with liver stem

cells, hepatocyte proliferation, differentiation, and cancer. Potential therapeutic options are now emerging from our developing understanding of these pathways.

2. Liver Development

During embryonic development, totipotent stem cells of the blastocyst inner cell mass differentiate into multipotent tissue-specific progenitor cells. Fate-mapping experiments have shown that the liver arises from the lateral domains of endoderm in the ventral foregut [3, 4] and from a small group of cells tracking down the ventral midline [4]. During foregut closure, the medial and lateral domains fuse together and the endoderm cells are specified to a hepatic fate under the influence of inductive signals and genetic regulatory factors that are highly conserved among vertebrates. Studies in chick, frog, mouse, and zebrafish models indicate that coordinated signalling of fibroblast growth factors (FGF) from the cardiac mesoderm and

bone morphogenetic proteins (BMP) from the septum transversum mesenchyme is critical in hepatic induction [5–9].

Following hepatic specification of the foregut endoderm, the cellular responses to inductive signals elicit new gene expression programmes required for cell differentiation. Wnt signalling, initially repressed by Wnt inhibitors to maintain foregut identity and allow hepatic induction [10, 11], becomes necessary to promote liver bud emergence and differentiation [10, 12, 13]. The newly specified hepatic cells, at this stage referred to as hepatoblasts, change to a columnar shape and invade the septum transversum mesenchyme to form the liver bud [14]. This transition involves coordinated interkinetic nuclear migration and proliferation, loss of intercellular adhesion, hepatoblast migration, and tissue-specific differentiation [15]. A number of studies in mutant mice have shown that liver bud formation is tightly controlled by a network of transcription factors, including haematopoetically expressed homeobox factor (Hex) [14, 16, 17], GATA-6 [18], hepatocyte nuclear factor (HNF)-6 [19], Onecut (OC)-2 [19], T-box transcription factor 3 (Tbx3) [20], and prospero-related homeobox 1 (Prox-1) [21]. Once hepatoblasts bud into the local mesenchyme, they continue to proliferate under the influence of a variety of cytokines and growth factors secreted by mesenchymal cells in the septum transversum, such as FGF, epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factor (TGF)- β , tumour necrosis factor (TNF)- α , and interleukin-6 (IL-6) [22–25]. Stimulatory signals to hepatoblasts from neighbouring endothelial cells are of particular importance as the presence of endothelial cells, independent of the blood supply, is critical for normal liver organogenesis throughout development [26, 27]. The specific molecular signals from endothelial cells are being studied; a recent study in the developing chick showed that Wnt9a secreted by the hepatic sinusoids not only stimulates hepatoblast proliferation, but it also controls global liver morphology [28, 29].

Hepatoblasts in the liver bud express serum protein genes specific to hepatocytes such as albumin (*alb*), transthyretin (*ttr*), and α -fetoprotein (*afp*) [30, 31]. These cells are bipotential and soon after mesenchyme invasion differentiate into hepatocytes (α -fetoprotein⁺/albumin⁺) and cholangiocytes (cytokeratin (CK)-19⁺) [5, 32]. The proper balance in the numbers of hepatocytes and cholangiocytes from hepatoblasts is achieved by integrated signalling and transcriptional networks. The Jagged-Notch pathway controls differentiation of hepatoblasts towards a biliary epithelial phenotype [33, 34], while HGF antagonises biliary differentiation and in conjunction with oncostatin M (OSM) promotes hepatocyte differentiation [35]. Following lineage segregation, the percentage of bipotent cells is markedly reduced and most cells are unipotent and irreversibly committed to either the hepatocytic or cholangiocytic lineage. Committed cells exhibit progressive change in morphology and physiologic functions, and this maturation process extends until several weeks after birth as demonstrated by a number of gene array analyses in rodent liver development [36–38]. An overview of embryonic liver development is presented in Figure 1.

3. Stem/Progenitor Cells in Human Foetal Liver

Studies in human liver development are relatively few in number as they rely heavily on ex vivo liver specimens. These studies are invaluable as not only do they provide direct observations and knowledge of the regulatory factors involved in human liver organogenesis but their findings could also lead to successful isolation and in vitro propagation of foetal liver progenitor cells suitable for clinical use. Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) hold great promise for a potentially abundant source of hepatocytes; however, directing their differentiation into specific, fully functional adult cell lineages remains a significant challenge. The use of foetal human liver progenitor cells abrogates the issue of forced differentiation, as foetal progenitors have undergone sufficient morphological and physiological differentiation so that they are committed to a hepatic fate, and yet they retain their “stemness” by maintaining their bipotentiality, proliferative capacity, and transplantability.

The phenotype of foetal human liver progenitor cells remains controversial. A range of cell markers based on rodent studies, such as Liv2 [39, 40], E-cadherin [41], and delta like kinase-1 (Dlk-1) [42], have only been characterised in human livers by immunodetection methods in vitro [43, 44]. To date, the only convincing evidence to show that liver progenitors can be isolated from human foetal livers comes from immunoselection for epithelial cell adhesion molecule (EpCAM)-positive cells [45]. In situ studies reveal that EpCAM⁺ foetal liver progenitors are located in the ductal plate. Once isolated, these cells are capable of self-renewal and clonogenic expansion, as well as differentiation into both hepatocytic and biliary lineages in defined culture conditions [45]. Moreover, purified EpCAM⁺ foetal liver progenitors when transplanted are able to engraft the livers of immunodeficient adult mice yielding mature human liver tissue [45].

Another potential stem cell population, side population (SP) cells, has been found to contribute to haematopoietic and epithelial lineages in the early gestational phase of human liver development [46]. SP cells have been isolated using fluorescence-activated cell sorting based on their ability to efflux DNA-labelling Hoechst dye [47], a phenotype determined by expression of ATP-binding cassette (ABC) transporters encoded by the multidrug resistance (MDR)-1 gene [46]. Their location in situ however remains uncertain, not least because of the widespread distribution of ABC transporters in the liver [48]; clearly the vast majority of cells in the liver expressing ABC proteins are not stem cells [49].

4. Translating Liver Development to Disease Models

4.1. Liver Regeneration. The normal adult liver has considerable inherent regenerative capacity. Following acute liver injury, such as partial hepatectomy, the tissue mass is restored

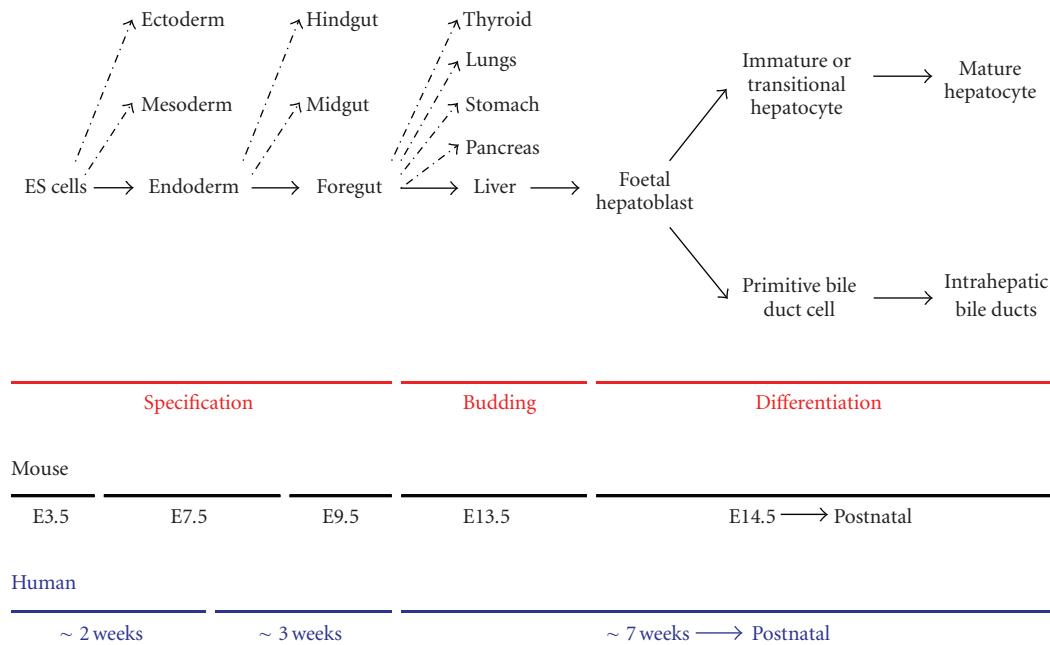


FIGURE 1: The lineage of the developing liver in vivo. Pluripotent embryonic stem (ES) cells from the blastocyst inner cell mass give rise to three principal germ layers: ectoderm, mesoderm, and endoderm. The anterior region of the endoderm will form the foregut. Following hepatic specification of foregut endoderm, hepatic cells (now called hepatoblasts) will bud into the septum transversum and continue to proliferate and differentiate. Maturation into hepatocytes and bile epithelial cells continue until several weeks after birth. The red bars highlight the key stages of liver development. The black bars in the middle are mouse embryos at different stages of development, and the blue bars at the bottom indicate the equivalent stages in human development.

by mitotic division of mature hepatocytes [50]. This division of mature hepatocytes provides an efficient means by which the normal liver can regain liver mass. The molecular signals underpinning this form of regeneration are now well understood (reviewed in [51]). However, this regenerative capacity is overwhelmed during massive or chronic injury and facultative liver progenitors (in rodents called oval cells) are activated. The two reparative processes are quite distinct yet not entirely mutually exclusive, as liver progenitors and hepatocyte replication can be observed simultaneously in some injury models [52, 53]. Nonetheless, gene ontology analysis shows that restoration after hepatectomy and liver development differ significantly with regards to transcription factors and chromatin structure modification [54].

On the contrary, although the exact mechanisms controlling progenitor activation in chronic liver injury remain elusive, collective data suggest that in progenitor-mediated regeneration of the adult liver, the molecular signals may follow a pattern suggestive of a recapitulation of foetal development. Whilst undoubtedly simplistic, this helps provide a framework to understand the adult response, though unequivocally this will not be a complete parallel. First, EpCAM⁺ cells purified from normal and injured adult human livers possess similar biological characteristics to those from foetal livers and function as bipotential progenitor cells [45, 55]. Second, in situ antigenic profiling shows that EpCAM⁺ progenitor cells are located at the canals of Hering, adult remnants of the ductal plates [55]. The progenitor nature of cells at the canals of Hering is further

supported by a recent lineage tracing experiment [56]. Third, the same cytokines and growth factors involved in foetal development are also implicated in adult regeneration (reviewed in [57]). In rodent models of chronic liver injury, HGF [58–60] and EGF [58, 61] upregulate proliferation and expansion of oval cells while TGF- β [62] and OSM [63] have the opposite effect. Fourth, upregulation of the Wnt/ β -catenin pathway in rodent models of both acute chemical [64] and chronic [65] liver injuries suggest symmetry between foetal development and adult regeneration at the transcription level. Taken together, adult liver regeneration parallels foetal development and involves progenitor cells that can be identified by anatomic, antigenic, and biochemical profiles.

It is evident that knowledge of the developmental biology of the liver provides clues to the molecular mechanisms in liver regeneration. This is critical in the search for a cure for chronic liver disease as specific pathways may be selectively targeted via pharmacological means to manipulate the progenitor cell compartment in adult liver. This has already been proven to be feasible with imatinib mesylate, a tyrosine kinase inhibitor, which has been shown to inhibit the liver progenitor cell response, liver fibrosis, and liver cancer formation in a mouse model of chronic liver injury [66]. Alternatively, this method of progenitor manipulation may be used to enhance liver regeneration in situ without the complications of cell engraftment and immunological rejection associated with transplantation. Further, efforts in programming human ESCs and iPSCs

to generate hepatocytes *de novo* (reviewed in [67, 68]) are founded on understanding how hepatocytes normally develop and differentiate in the embryo and how hepatocytes arise during regeneration in adults, in response to tissue damage and disease. The precise conditions that exist within the embryo which promote the differentiation of hepatocytes from pluripotent stem cells can be mimicked *in vitro*, for example, by using extracellular factors and recruiting accessory cell types to yield highly functional derivatives for drug screening, human bioartificial liver construction and, potentially, transplantation therapy.

4.2. Liver Stem Cells and Cancer. In the UK, the incidence of primary liver cancer has tripled in the last 30 years, and the associated mortality has increased by 40% over the last decade. Most worryingly, provisional data for 2008 indicate that the number of deaths from primary liver cancer is accelerating, with a 9% increase over the previous 12 months [69]. Given these statistics, there is an urgent need to understand the mechanisms of carcinogenesis in the liver and thereby aid the development of new forms of cancer therapy.

The cellular origin of HCC has long been debated, but whether HCC originates from mature hepatocytes, stem/progenitor cells, or both remain unclear. The fact that many liver tumours arise during cirrhosis when hepatocyte senescence triggers the activation of liver progenitors causes further confusion [70]. In the liver, there may be at least three distinct cell lineages susceptible to neoplastic transformation: hepatocytes, intrahepatic stem cells, or small hepatocytes [71, 72]. Most well-differentiated HCCs in the early stages are detected as small nodules with normal levels of AFP. Subsequently, they increase in size and become moderately or poorly differentiated cancerous tissues producing AFP [73]. This suggests that HCC might arise due to dedifferentiation of mature hepatocytes that have retained their ability to divide [74]. In addition, it is now accepted that the arrested differentiation of tissue-based stem cells or their immediate progenitors, the concept of blocked ontogeny, is linked to hepatocarcinogenesis [75–77]. It has been suggested that intrahepatic stem cells can give rise to HCC and cholangiocarcinoma (CC) [78], as activation of oval cells has been demonstrated in rodent models of HCC and CC [79, 80]. Further, the role of intrahepatic stem cells in carcinogenesis is supported by a histological subtype of liver malignancies that displays features of both HCC and CC (HC-CC) combined with the presence of numerous liver progenitor cells [81, 82]. The development of HC-CC in mice implanted with *p53*-null oval cells suggests that dysregulated propagation of liver progenitors is an important early step in hepatocarcinogenesis [83].

There are several lines of evidence to suggest that hepatocarcinogenesis in part recapitulates foetal liver development, as both cell types have the capacity to self-renew, produce heterogeneous progeny, and divide limitlessly. First, hepatoblastomas presenting in their less differentiated phenotype in the livers of human infants represent an early stage in the cellular lineage pathway observed in the development of highly

differentiated HCCs seen in adults, thereby supporting the progenitor cell differentiation arrest model [72]. Second, SP cells, which have hepatocytic and cholangiocytic potential in foetal livers, have been isolated in a number of HCC cell lines and are capable of tumour formation with a starting population of as few as 10^3 cells in serial xenotransplantation experiments [84]. Third, foetal liver progenitors and HCC cell lines share a number of oncofetal markers. A recent study has shown that Huh1, Huh7, and Hep3B cell lines all express EpCAM to varying degrees, with up to 99.2% of Hep3B cells being EpCAM⁺ [85]. Purified EpCAM⁺ cells from HCC cell lines and human clinical specimens possess progenitor features and, when injected into immunodeficient adult mice, exhibit tumorigenic and invasive capacity [85, 86]. Moreover, individuals with HCC whose gene expression profiles match that of foetal hepatoblasts have a poorer prognosis compared to those with adult genomic profiles [82, 86]. Fourth, signalling mechanisms central to embryonic liver development have been implicated in hepatocarcinogenesis. Activation of Wnt/ β -catenin signalling is observed in OV6⁺ HCC cells in rats [87] and EpCAM⁺ HCC cells in humans [88] and is linked to their excessive self-renewal capability and tumorigenicity. Aberrant TGF- β and IL-6 signalling has also been found to promote the growth of stem/progenitor cells in their undifferentiated state and contributes to the modulation of HCC [89].

In recognition of the symmetry between liver development and carcinogenesis and the possible role of liver cancer stem cells, new approaches to the treatment of primary liver cancer have been proposed. The inhibition of specific molecular pathways is one promising strategy. A recent study has shown that Hep3B cell growth can be inhibited by RNA interference-based blockage of EpCAM, a direct transcriptional target of Wnt/ β -catenin signaling [85]. Encouragingly, EpCAM-specific antibodies, though not licensed for treating HCC, are currently in phase II clinical trials for the treatment of EpCAM⁺ colorectal carcinoma [90]. An alternative strategy is differentiation therapy, whereby liver cancer stem cells are induced to differentiate and in so doing lose their self-renewal capacity and tumorigenic potential. In transgenic mice in which the *c-Myc* oncogene is inactivated, tumorigenesis is reversed with HCC cells losing their neoplastic properties and differentiating into hepatocytes and biliary cells [91]. It is also conceivable that the fate of liver progenitors may be manipulated by targeting the stem cell niche, as the specified microenvironment in which stem cells reside often dictates self-renewal and differentiation [92]. One potentially effective target is endothelial cells, as not only are they a critical component of the liver progenitor cell niche but they are responsible for neovascularisation which is a crucial prerequisite for hepatocarcinogenesis [93, 94]. A number of anti-angiogenic agents, namely, bevacizumab, erlotinib, and sorafenib, have already entered clinical trials for HCC and shown efficacy in some instances [95, 96]. Although these agents hold much promise, their effect on the improvement in overall survival is still marginal [95, 96]. There is no doubt that much more research on the microenvironment supportive for HCC progression is urgently needed.

5. Conclusion

There are common threads which span liver development, regeneration and carcinogenesis, notably the cellular functional phenotype responsible for each process and the molecular machinery dictating appropriate cell fate. The identification of stem cell candidates in the developing liver, and cancer stem cells in liver tumours, has revealed physiological themes relating to surface markers and the regulation of proliferation and differentiation. Further progress towards the clinical application of stem cells and ES-derived liver cells is critically dependent on detailed understanding of all of these mechanisms. Only when the generation of nontumorigenic cells for liver therapy and the interactions of these cells with host tissues are understood, can the promise of liver stem cell therapy be fully realised in human liver disease.

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