Differentiation of progenitors in the liver

Differentiation of progenitors in the liver: a matter of local choice

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The liver is a complex organ that requires multiple rounds of cell fate decision for development and homeostasis throughout the lifetime. During the earliest phases of organogenesis, the liver acquires a separate lineage from the pancreas and the intestine, and subsequently, the liver bud must appropriately differentiate to form metabolic hepatocytes and cholangiocytes for proper hepatic physiology. In addition, throughout life, the liver is bombarded with chemical and pathological insults, which require the activation and correct differentiation of adult progenitor cells. This Review seeks to provide an overview of the complex signaling relationships that allow these tightly regulated processes to occur.

Introduction

Progenitor cells have highly regulated mechanisms governing proliferation and differentiation to achieve accurate tissue repair. In several organ systems, the hierarchy of stem cells, transit amplifying-progenitor cells, and differentiated mature cells is well described, including the signaling pathways that define these stages (1–4), though much remains to be characterized for this hierarchy in liver. The adult liver has an impressive regenerative capability, and classical experiments in rats following partial hepatectomy demonstrated that the liver can regrow to its original size within ten days. This capability is utilized in clinical scenarios in which partial hepatectomy is used to resect liver tumors, and in living donor transplantation in which a portion of the liver is taken from a donor and transplanted into a recipient, with both remnants regrowing into a functional liver mass. This regeneration occurs through division of the mature epithelial cells (hepatocytes and cholangiocytes) within the liver, which leave their normal mitotically quiescent state, termed G_0, and enter cell cycle and mitosis.

During chronic liver injury this normally efficient renewal from mature epithelial cells becomes impaired. In many liver diseases, such as chronic viral hepatitis (5) and non-alcoholic fatty liver disease (6), hepatocytes become senescent and unable to efficiently regenerate the parenchyma. In this scenario, hepatic progenitor cells (HPCs) become activated and are sufficient to regenerate the biliary and hepatocellular epithelium (7, 8). The biology of HPCs is less studied in comparison to analogous progenitor cells in other adult tissues, and markers that delineate the stem versus transit amplifying populations are not clearly defined (9–11).

While the putative adult liver stem cell is often thought to reside within the canals of Hering, located at the terminal branches of the biliary tree, multiple lines of recent work suggest that a label-retaining stem cell might exist throughout the biliary network (12, 13) and even in the common bile duct (14, 15). One model posits that these putative stem cells may be differentially activated in diverse disease patterns and may have different differentiation potencies depending on their location, though this idea has not yet been formally addressed. What is known is that this putative stem cell gives rise to a transit-amplifying population of HPCs. Mechanisms of HPCs have been increasingly studied, and a broad class of factors that can activate the proliferation of HPCs has been identified (16, 17). To restore function to the damaged liver, these bipotential HPCs must differentiate into hepatocytes, the key metabolic cells of the liver, and cholangiocytes, which line the biliary tree and transport bile into the intestine. The mechanism by which HPCs differentiate into these two key epithelial types is poorly defined, but recent advances in transgenic mouse technology and the ability to manipulate HPCs in vitro and in vivo has shown that the fate of HPCs depends not only upon which signaling cascades are activated within the HPCs, but also the disease context in which HPCs evolve.

Expansion of HPCs is a multifactorial process

HPCs are facultative and not activated in healthy liver. The induction of HPC proliferation is critical to generate sufficient numbers of transit-amplifying cells, which can subsequently differentiate into the mature hepatic epithelia. Multiple signaling pathways have been identified as mitogenic for HPCs (for a comprehensive review, see ref. 18). TGF-β is a potent mitogen associated with the inflammatory response that is able to act differentially on TGF-βR2 expressed by hepatocytes and HPCs through variable glycosylation of the TGF-β receptor (19). Hepatocyte proliferation is restricted by TGF-β, although HPCs with glycosylated TGF-βR are not repressed by TGF-β and can proliferate in an inflammatory environment. TNF-like weak inhibitor of apoptosis (TWEAK) acts though its receptor, Fn14, activating NF-κB signaling and potently inducing HPC proliferation. Knockout of either the TWEAK ligand or the Fn14 receptor results in a severely abrogated HPC response; moreover, exogenous injection of TWEAK is sufficient to induce HPCs in an undamaged mouse liver (16, 17).

In vitro assessment of the stemness and differentiation capacity of HPCs

Due to limitations in the available lineage-tracking system in the adult liver, much of our understanding of HPC differentiation has largely been defined in vitro. HPCs are isolated, and their ability to form clones (clonogenicity) and to differentiate into multiple lineages (multipotentiality) can be assayed.

Wang et al. sought to identify a bipotential population of cells with self-renewing capacity that were transplantable and could contribute to hepatic parenchyma in vivo (20). c-kit–positive

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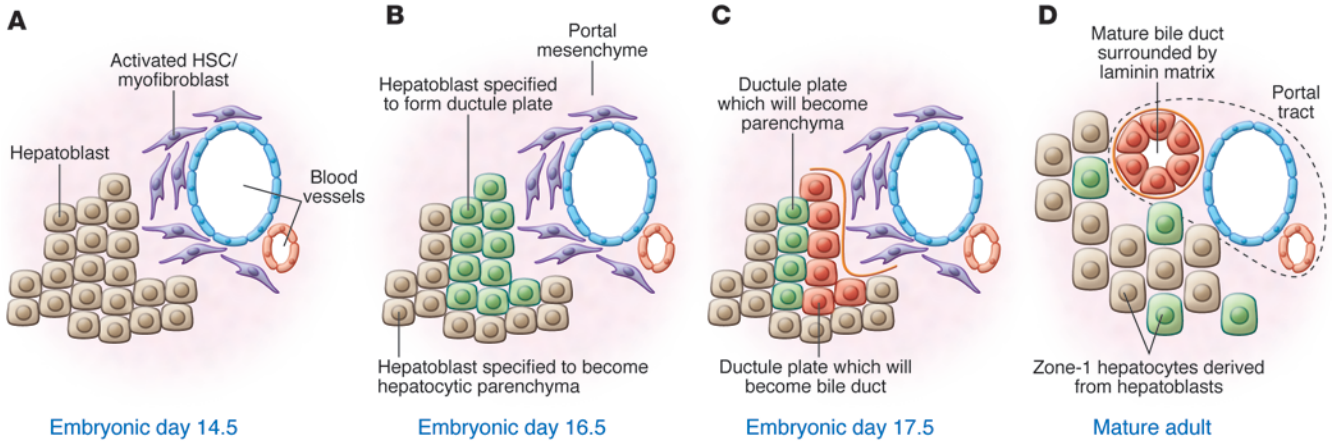

Figure 1
Liver development involves multiple phases of specification and differentiation. (**A**) During liver development, the liver bud is specified from the remainder of the endoderm by Wnt expression from the cardiac mesoderm. HSC, hepatic stellate cell. (**B**) Once specified, the endoderm matures into fetal hepatoblasts that are capable of bilineage differentiation into hepatocytes and cholangiocytes. (**C**) Spatial heterogeneity across the developing liver results in the specification of the ductal plate though activation of the Notch signaling pathway, in close proximity to what will ultimately become the portal tract. (**D**) Once specified, the ductal plate is patterned, and the side adjacent to the portal mesenchyme closely associates with laminin and expresses high levels of Sox9, whereas the side adjacent to the developing parenchyma expresses TGF-β–mediated C/EBP and contributes to zone-1 hepatocytes.

progenitor cells were isolated from wild-type donors and transplanted into fumaryl acetoacetate hydrolase–null (*Fah*$^{-/-}$) recipient mice, in which hepatocytes lack a tyrosine metabolic pathway (20). The c-kit–positive cells differentiated into genetically normal hepatocytes, which proliferated to rescue the *Fah*$^{-/-}$ phenotype (20). In later studies this group generated a suite of antibodies against HPCs, such as MIC1-1C3 and OC-2, which were used to identify a population of putative HPCs (21). However, a number of the antibodies generated in these studies also cross-react with bile ducts in the healthy adult mouse, and as such these markers may not isolate a pure HPC population. This problem was addressed by culturing sorted cell populations in vitro at clonal density using selection based on the expression pattern CD45^{-}CD11b^{-}CD31^{-}MIC1-1C3^{+}CD133^{+}CD26; these cells were bipotent, with the capacity to differentiate into two distinct cell types, hepatocytes and cholangiocytes (22). Furthermore, additional data suggested that a liver-regenerating cell resides within the epithelial cell adhesion molecule–positive (EpCAM-positive) population within the uninjured adult liver, and that these cells are capable of forming epithelial colonies with bilineage potential (23). However, the identity of these cells within the EpCAM-positive pool remains enigmatic.

Lineage tracing in the adult liver

The contribution of progenitors to tissue turnover and regeneration is difficult to address in adult organs, which are already structured and contain terminally differentiated cells. To this end, several stem cell lineage–tracing tools have been developed to assess the location of HPCs and the mechanisms governing their contribution to regeneration. The basic principle of lineage-tracing animals is that the putative stem or progenitor cell is indelibly marked; as such, any progeny arising from this cell will also carry the same distinct mark. The most common method of lineage tracing is to use an inducible, lineage-specific Cre-recombinase crossed with a mouse that harbors a flox-stop-flox reporter (such as GFP or LacZ). Following induction, Cre recombinase excises the stop codon and the stem cell is subsequently labeled irreversibly with either GFP or LacZ. Therefore, its progeny can be tracked during tissue homeostasis and regeneration (24).

The initial liver studies from Linda Greenbaum and colleagues identified Foxl1 as a marker of adult HPCs (25). This was an important advance because previous attempts to track cells in the adult liver had been limited primarily to markers of adult HPCs, including the cytokeratins Krt7 and Krt19 and other liver-restricted markers (e.g., α-fetoprotein) expressed on fetal hepatoblasts, and, as such, made adult analysis of these tissues impossible (26, 27). The use of *Foxl1Cre* flox-stop-flox *LacZ* reporter mice demonstrated that HPCs could contribute to mature hepatocytes when livers were damaged and progenitor cells were activated, but the number of positive hepatocytes was rare (0.5% Foxl1 and hepatic nuclear factor 4α [HNF4α] dual-positive hepatocytes) and likely too low for mechanistic studies.

The use of *inducible* Cre provides an attractive alternative to constitutive reporter systems. In this context the Cre recombinase is fused to an estrogen response element, which upon administration of 4-hydroxytamoxifen results in the translocation of the fusion protein to the nucleus, where the enzyme can then induce recombination (28). Administration of an inducing agent means labeling of HPCs can be strictly timed, and the cellular progeny that result from this recombination can be tracked over time. Utilizing this inducible Cre technology under the control of the Sox9 transcriptional control elements, Furuyama et al. described how Sox9 is expressed in HPCs and biliary epithelium in the regenerating liver (29). Using both an inducible *Sox9-Cre/LacZ* mouse and also a *Sox9-GFP* reporter mouse in which GFP is expressed in Sox9-expressing cells, the authors found Sox9^{+} cells in close proximity to the biliary tree in normal liver. Interestingly, when healthy animals were left for up to 12 months, the parenchyma of these animals was replaced by cells of a Sox9 origin, the putative HPCs (29). This result was remarkable and challenged the dogma about normal homeostatic mechanisms in the liver — rather than the mature

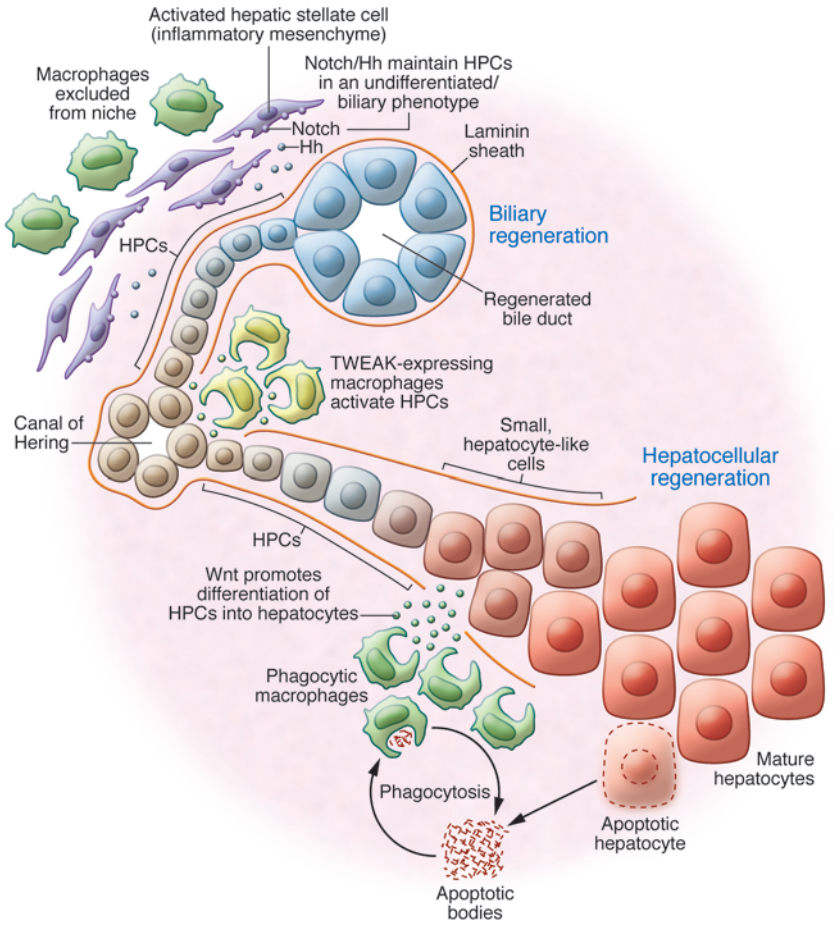

Figure 2
HPCs are involved in a complex microenvironment and are subject to multiple signals. Regeneration in the liver can be broadly characterized into hepatocellular or biliary regeneration. In the former context, progenitor cells irradiate from the portal tracts, in which they are sheathed in laminin, which facilitates their expansion. Upon exit from the laminin niche, these cells are subject to differentiation cues, such as Wnt and HGF, which activate the pro-hepatocyte transcriptional cascade in HPCs. In biliary regeneration, HPCs emerge in a similar fashion, but they remain in the laminin ECM, in which fibroblasts are able to influence their maturation though activation of the Notch signaling pathway. This pathway influences the activation of the HNF6/HNF1β transcriptional network to correctly specify cholangiocytes.

parenchyma being responsible for maintaining itself, turnover of the healthy liver was progenitor mediated. Furthermore, when *Sox9-Cre/LacZ* mice were injured there was a startling expansion of Sox9-positive progeny that quickly restored normal epithelial function (29). However, in 2011 a second paper investigating the embryonic origin of HPCs shed some light on these exceptional Sox9 results (30).

Work from Frédéric Lemaigre's group clearly demonstrated that the Sox9-positive bile duct and progenitor cell are deposited from the ductal plate during hepatic ontogeny; in a thorough study the authors demonstrated that the embryonic ductal plate gives rise not only to biliary epithelium, as previously expected, but also to HPCs and to a subpopulation of hepatocytes in periportal zone 1 of the adult liver (30). Moreover, upon tamoxifen administration to adult Sox9 reporter mice, the zone 1 hepatocytes upregulated Sox9. This control suggests that the Sox9-positive cells identified by Furuyama et al. (29) may in fact be periportal hepatocytes that upregulate Sox9 and activate the transgene in response to tamoxifen administration.

The work from Uemoto and Furuyama (29), however, is not without considerable merit because this paper clearly demonstrates a streaming gradient of cells that arise at the portal tract and then divide and potentially migrate through the zones of the liver until they reach the central vein. This confirms the so-called "streaming liver hypothesis" originally described by Zajicek and colleagues (31, 32).

The culmination of these lineage-tracing strategies has resulted in an important recently published work by the Leclercq group (7). Using osteopontin-1 as a marker, the authors demonstrate that HPCs express osteopontin (a glycoprotein that marks HPCs), emerge from bile duct, and are capable of directly differentiating into hepatocytes (7). Importantly, HPCs regenerated hepatocytes following chronic hepatocyte injury (2.4% of hepatocytes were positive for GFP after two weeks of a choline-deficient, ethionine-supplemented [CDE] diet), but not following biliary injury and regeneration (3,5-diethoxycarbonyl-1,4-dihydrocollidine diet, with which 0% YFP-positive hepatocytes were seen), demonstrating that the HPC microenvironment is critical for HPC expansion and differentiation, as we have previously suggested (33). Interestingly, the HPCs radiated from their niche to the site of injury but contributed little to hepatocyte regeneration during the injury on the CDE diet. However, during recovery with a normal diet, HPCs differentiated into mature, replication-competent hepatocytes that could then maintain parenchymal regeneration themselves. This insight has relevance to the study of human liver disease, which may have intermittent relapsing-remitting disease activity.

There has been debate in the liver field as to whether hepatic stellate cells are capable of undergoing mesenchymal-epithelial transition (MET), thereby contributing to parenchymal regeneration. Work from Yang and colleagues utilized a constitutive GFAP-Cre line, which labeled the biliary tree and hepatic stellate cells (34). This study demonstrated for the first time that GFAP-expressing

cells could be traced into hepatocytes, and by analyzing the kinetics of disease recovery, Yang et al. concluded that the parenchyma can be regenerated from non-epithelial, GFAP$^+$ cells (34). However, the use of a constitutive GFAP-Cre expression system does not fully exclude the possibility that GFP-labeled hepatocytes are derived from GFAP-expressing bile ducts. This observation, however, offers a tantalizing suggestion that stellate cells may have a progenitor role in the adult liver; this has been investigated by other groups in the liver (35, 36) and other tissues (37). Of course stellate cells are also subject to regional signaling in the liver, and multiple developmental regulators, including Necdin-Wnt (38–40) and Hedgehog, have been implicated in the differentiation of hepatic stellate cells into fibroblasts, the latter of which control cellular metabolism to regulate fate (41). Intriguingly, differentiation of these fibroblasts is in part mediated through epigenetic regulation of PPARγ (42–44), although there is little evidence to indicate which signals might facilitate MET.

Do these murine studies shed any light on the human condition? Alison and colleagues have harnessed the inability of the cell to repair mutations in mitochondrial DNA to ask whether human livers have such progenitors (8, 45). By identifying cells in which the mitochondria have a *COX* mutation, this group described patches of epithelium in the liver in which the mitochondria were mutated, and when random, discrete regions of the patch were analyzed the mitochondrial mutation was consistent across the sample. This result suggests that the *COX* mutation carried in these mitochondria arose from a common ancestor of cells throughout the patch. Of course, this does not necessarily point to a progenitor cell, but if both hepatocytes and cholangiocytes could be identified with the same mitochondrial mutation, then this would be compelling evidence that in the adult human liver both key epithelial types can originate from a common precursor.

Inspiration from development: liver patterning during ontogeny

During development, embryonic progenitors (hepatoblasts) must make lineage decisions similar to adult HPCs and form the biliary tree and hepatocytes. Once the liver bud has formed from the primitive endoderm, epithelial lineage separation occurs; a population of hepatoblasts adjacent to the portal mesenchyme is specified and forms the ductal plate (46). This process requires the differentiation of hepatoblasts into the bile ducts and repression of the ductal phenotype in the developing hepatocytes (47–49), summarized in Figure 1. The delineation of bile duct from the rest of the parenchyma occurs early in development and is initiated by asymmetry of the ductal plate where, in E-cadherin–positive cells, HNF4α and Sox9 are asymmetrically expressed on the parenchymal and the portal faces, respectively. This asymmetry of the ductal plate is enhanced through deposition of laminin on the portal side of the presumptive bile duct, whereas the repression of biliary phenotype in the parenchymal side of the ductal plate is driven through TGF-β signaling, putatively through the TGF-βR2 (47). This regulatory asymmetry is critical, though not for the propagation of a biliary phenotype. As in *Sox9* knockout livers, there is little change in the expression of HNF1β, HNF6, and hematopoietically expressed homeobox (HEX), but rather there is a failure of hepatocyte fate repression. Without Sox9 repression of C/EBPα, there is a failure to resolve the ductal plate during embryogenesis.

A critical parameter for lineage specification of the adult biliary system is the Notch signaling pathway (50). During development, this signaling cascade is critically implicated in the formation of cholangiocytes and also in the maturation and terminal patterning of the biliary tree in a multitude of species (51–53). In the normally developing liver, Notch2 is activated in the presumptive cholangiocytes by jagged 1, which is expressed by the portal mesenchyme (54). In this context, an HNF6/HNF1β signaling cascade is activated as a direct result of Notch intracellular domain interactions with the promotor of HNF6 (55, 56). Loss of Notch signaling in biliary development in mice, through genetic ablation of jagged 1 or haplosufficiency of Notch2, results in a reduction in biliary development and failure to pattern the biliary tree (57–60). The human congenital disease Alagille syndrome, which is caused by mutations in Notch pathway components, is characterized by a biliary paucity with failure to correctly resolve the ductal plate during development; this phenotype can also be observed in adult mice that harbor a fringe mutant in which Notch is inappropriately activated (61). Huppert and colleagues demonstrated that the Notch1 intracellular domain must act in a dose-dependent fashion, showing that overexpression of the Notch1 intracellular region during development results in a hyper-arborized biliary network, presumably at the cost of mature parenchyma (62).

Whereas the role for Notch in development is highly stereotyped, and its effect is common between species, the role for Wnt/β-catenin signaling in the developing liver is confounded and complex. Wnt plays critical roles not only in expansion of the liver bud (63–65), but also in formation of the definitive hepatoblasts (66), biliary proliferation (67), and hepatocyte maturation (68, 69). As such, elucidating the temporal and spatial action of the canonical Wnt pathway is complex.

Initial transgenic studies that looked at activating the Wnt/β-catenin signaling pathway through embryonic loss of adenomatosis polyposis coli (APC), a critical regulator of β-catenin stability, demonstrated that this signaling cascade was required for the expansion of fetal hepatoblasts (66). Furthermore, the presence of constitutively activated β-catenin resulted in hypotrophy of the developing liver with perturbed hepatoblast differentiation occurring at embryonic day E11.5 and resulted in late lethality in utero at E16.5–E18.5 (66). These data, however, are in contrast to data from the Monga group (70). Tan et al. demonstrated that embryonic deletion of the gene encoding β-catenin under the control of the *FoxA3-Cre* transgene (expressed by E9.5) results in a failure of liver development through reduced hepatocyte differentiation, increased hepatoblast apoptosis, and reduced hepatoblast proliferation (70). The phenotype in the embryos is only realized at E12, a timing that is similar to APC mutant embryos. These data together indicate not only that Wnt signaling is critical for fetal hepatogenesis, but also that in normal development, modulation of this pathway must be exquisitely timed for correct liver development. Interestingly, in the postnatal liver, activation of the canonical Wnt signaling pathway is required for the expansion of hepatocytes and is responsible for expansion of the liver. Selective postnatal loss of β-catenin results in a reduced organ size, and as such suggests a conserved functional outcome of these signaling pathways during development and maturation (71). Intriguingly, targets of β-catenin such as lymphoid enhancer binding factor 1 and Myc are directly responsible (in association with liver-enriched transcription factors) for patterning the developing liver and are critical for correct metabolic zonation (68).

Mechanisms of differentiation during adult regeneration

Whereas the mechanisms of lineage specification during hepatic ontogeny are increasingly understood, the cues by which HPCs in the adult liver are specified into different lineages are not well described in situ. However a handful of studies have looked at the mechanism by which HPCs differentiate in vivo. The HGF receptor c-Met is required for the maturation of HPCs into hepatocytes, and until recently this was all that was known about the potential mechanisms by which HPCs become hepatocytes (72). More recently, lineage-tracing evidence has emerged demonstrating that inhibition of laminin signaling with iloprost, a synthetic analog of prostacyclin PGI_2, in HPCs during regeneration results in the precocious differentiation of HPCs into hepatocytes (7), reiterating previous descriptive work examining laminin in the HPC response (73, 74).

We have identified a re-establishment of the embryonic state in regeneration; Notch and Wnt are required for HPC differentiation, and their interaction is necessary for appropriate delineation of hepatocellular versus biliary fate (33). During activation of HPCs in biliary diseases of both mouse and human, HPCs express the receptors Notch1 or Notch2, respectively; these are activated though interaction with jagged 1. In the mouse, the periportal fibroblasts that surround the regenerating bile ducts express this ligand and appear to recapitulate in some regards the portal mesenchyme seen in development (33, 75).

This Notch-high state appears to delineate the default pathway for the development of HPCs into cholangiocytes, and critically this pathway is inhibited during regeneration of hepatocytes through the ubiquitin ligase Numb. In HPCs undergoing hepatocyte differentiation, *Numb* transcription is activated by β-catenin and as such represents a critical node at which the Notch and Wnt pathways can interact to enable lineage specification. In recent years, macrophages have been described as a source of Wnt, often in cancer, though some regeneration studies have also described macrophage-derived Wnt (76, 77). Ablation of macrophages during hepatocyte regeneration removed the stimulus for HPCs to become hepatocytes; instead, they differentiated into cholangiocytes and formed biliary structures. These results have also been confirmed in independent studies in which macrophages have been depleted in the liver (78). Notably, phagocytosis of the hepatocyte debris promoted profound Wnt upregulation in macrophages, providing a critical link between hepatocyte death and HPC fate that enables co-ordinated and appropriate tissue renewal. Furthermore, this model provides a mechanism linking local tissue injury and inflammation patterns of specific diseases with the extra- and intracellular signaling controls that govern HPC fate.

Clinically, in the context of severe liver injury accompanied by a failure of hepatocyte regeneration and consequent functional decrease in parenchymal mass, a marked ductal reaction occurs, which likely represents a regenerative response that is "too biliary" (79, 80). In such situations, promoting hepatocytic differentiation of progenitors may provide a legitimate future target for drugs and small molecules. Indeed, by genetically expressing stabilized Wnt in mouse liver progenitors, we have found that HPCs can be coaxed in vivo into a hepatocyte fate rather than a biliary fate (33).

Parallels in cancer

Recent intriguing work in mice suggests that the overactivation of the Notch signaling pathway (through ectopic expression of the Notch1 intracellular domain in hepatocytes in vivo) can transdifferentiate these mature cells into cancerous cholangiocytes (81, 82). This phenomenon obviously parallels the mechanisms of biliary development discussed earlier, in that repression of the hepatocyte phenotype and promotion of the biliary phenotype can specify cells toward a cholangiocyte phenotype. In these mouse models of cancer, Notch deregulation clearly results in the propagation of a naive phenotype that is carcinogenic. Similarly, Wnt deregulation has been implicated in the development of hepatocellular carcinoma (83). These cancers occur most commonly in the context of chronic liver injury and regeneration, and emerging evidence suggests that parallels in the signaling pathways linking development, regeneration, and cancer may enable new treatment modalities to promote healthy liver regeneration and inhibit the formation of liver cancer.

Conclusions and perspectives

Clearly the activation and evolution of HPCs during disease is one of multiple pathogenic phases analogous to disease onset in other adult tissues. In this model a resident (and yet to be identified) hepatic stem cell is activated, giving rise to a transit-amplifying pool known as HPCs. These cells then undergo rapid division and expansion, likely driven by inflammatory cues, principally TWEAK. These HPCs evolve in a laminin-rich microenvironment, which is necessary to facilitate their expansion and maintain their naivete. In this steady state, once the niche is established, HPCs are subject to multiple classical signaling cascades that are context (disease) dependent. If the HPCs are exposed to Notch signaling whilst in the niche, they form cholangiocytes and maintain a laminin rich basement membrane around themselves. When HPCs exit the laminin sheath, these cells are exposed to multiple ligands from their local environment, such as macrophage derived Wnt or HGF, which acts through c-Met expressed by HPCs and initiates the activation of a pro-hepatocyte signaling cascade and results in differentiation of HPCs into hepatocytes (Figure 2).

Regeneration in the liver is undoubtedly an important clinical target. The ability to influence HPCs to proliferate and to regulate their differentiation may ultimately lead to exciting new therapies for chronic liver diseases. Until recently, HPC research has moved slowly compared with research for other organ systems such as gut, blood, and skin. Difficulties identifying HPCs in vivo, limited tools available to identify their targets, and difficulties culturing highly pure populations capable of modeling the in vivo situation have been at least partly overcome, making the present a prime time for progress in this field.

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