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REVIEW

Stages based molecular mechanisms for generating cholangiocytes from liver stem/progenitor cells

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Abstract

Except for the most organized mature hepatocytes, liver stem/progenitor cells (LSPCs) can differentiate into many other types of cells in the liver including cholangiocytes. In addition, LSPCs are demonstrated to be able to give birth to other kinds of extra-hepatic cell types such as insulin-producing cells. Even more, under some bad conditions, these LSPCs could generate liver cancer stem like cells (LCSCs) through malignant transformation. In this review, we mainly concentrate on the molecular mechanisms for controlling cell fates of LSPCs, especially differentiation of cholangiocytes, insulin-producing cells and LCSCs. First of all, to certificate the cell fates of LSPCs, the following three features need to be taken into account to perform accurate phenotyping: (1) morphological properties; (2) specific markers; and (3) functional assessment including *in vivo* transplantation. Secondly, to promote LSPCs differentiation, systematical attention should be paid to inductive materials (such as growth factors and chemical stimulators), progressive materials including intracellular and extracellular signaling pathways, and implementary materials (such as liver enriched transcriptive factors). Accordingly, some recommendations were proposed to standardize, optimize, and enrich the effective production of cholangiocyte-like cells out of LSPCs. At the end, the potential regulating mechanisms for generation of cholangiocytes by LSPCs were carefully analyzed. The differentiation of LSPCs is a gradually progressing process, which consists of three main steps: initiation, progression and accomplishment. It's the unbalanced distribution of affecting materials in each step decides the cell fates of LSPCs.

Key words: Liver stem/progenitor cells; Cholangiocytes; Biliary differentiation; Unbalanced distribution of materials; Cell therapy

Core tip: After liver stem/progenitor cells (LSPCs) are isolated by different groups from both fetal and adult livers, it is urgent to decide the cell fates of LSPCs. Especially, it is found that the core issue for LSPCs application lies in their accurate differentiation. Because there are lots of literatures concentrating on self-renewal and hepatic differentiation of LSPCs, in this review, we mainly summarize the molecular mechanisms for controlling other cell fates of LSPCs, especially differentiation into cholangiocytes. For biliary differentiation, we propose that it is a gradually progressing process consisting of three main steps: initiation, progression and accomplishment.

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INTRODUCTION

Liver stem/progenitor cells (LSPCs) possess high pro-

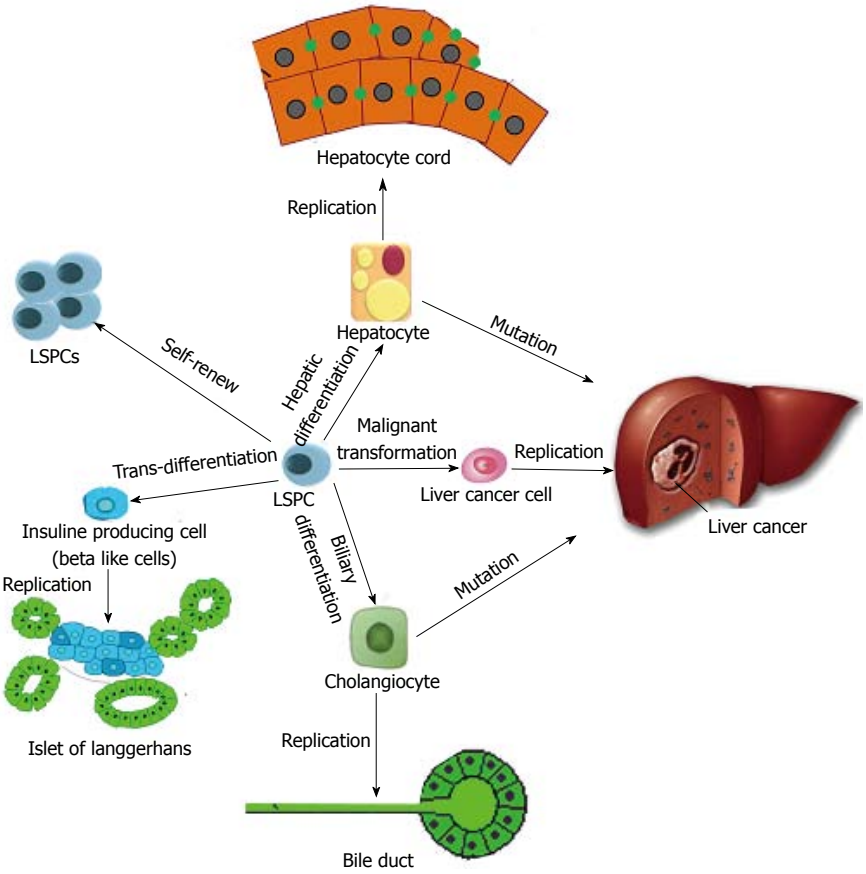

Figure 1 The different cell fates of liver stem/progenitor cells under distinct situations. Under special stem microenviroment, LSPCs would probably self renew to keep stem properties. In the contrary, under differentiating stimuli, LSPCs could give birth to two kinds of fundermental mature cells in the liver, hepatocytes and cholangiocytes. This is very important for liver development and liver regeneration. Except for the traditional differentiation directions, LSPCs have the capacity to trans-differentiate into insulin producing cells, which is promising for treating diabetes. While in some bad situations, LSPCs may carry out malignant transformation to become liver cancer cells, even liver cancer stem cells, as a result, these cancer cells replicate themselves to cause liver cancer. This is a different way from mutation of hepatocytes for liver carcinogenesis. LSPC: Liver stem/progenitor cell.

liferative capacity and low immunogenicity and are robust in the face of cryopreservation or ischemic injury: properties that could enhance their engraftment within a recipient liver. Because of this, these LSPCs are very promising for the treatment of end-stage liver disease[1]. A series of animal models transplanted with LSPCs have been established, and several clinical trials have been reported. In one animal model, transplanted rat embryonic day (ED) 14 fetal liver stem/progenitor cells (FLSPCs) differentiated into the two mature epithelial cell phenotypes in the liver, *i.e.,* hepatocytes and cholangiocytes, and long-term, *in vivo* functional reconstitution of the liver tissue was achieved (Figure 1)[2,3]. The important recent progress is the use of human FLSPCs engrafted into naturally derived scaffolds to create a liver-like tissue *in vitro*[4]. However promising LSPCs are for cell therapy or tissue engineering, the fundamental purpose lies in generating mature, functional cells[5,6].

LSPCs constitute approximately 0.5%-2.5% of liver parenchyma at all donor ages. The self-renewal capacity of LSPCs is demonstrated by their phenotypic stability after expansion for > 150 population doublings in a serum-free, defined medium, with a doubling time of approximately 36 h[7]. In fetal liver, LSPCs are commonly called hepatoblasts[2,3]. In some studies, some groups have used other terms than hepatoblasts to represent the cell populations with stem properties in fetal liver, such as embryonic hepatic stem cells or fetal liver stem-like epithelia. Thus, it would be more appropriate to denote these cells as the "FLSPCs", and we will adopt this description in this review. In adult liver, LSPCs are generally referred to as oval cells (OCs), with scant, lightly basophilic cytoplasm and pale blue-staining nuclei[8]. The appearance of OCs has been reported in rat livers treated with hepatotoxins, such as 2-acetylaminofluorene, combined with partial hepatectomy (PHx) and D-galactosamine[9,10]. However, in addition to OCs, small hepatocytes (SHs) are also well known in adult liver, and they are better suited to the appellation "progenitor" cells. As it is not an easy task to distinguish stem cells from progenitors because of the difficulty of proving the unlimited self-renewal activity of stem cells in many situations, we use the term "adult liver stem/progenitor cells (ALSPCs)" to describe such cells, including both OCs and SHs in this review article. In the field of liver biology, the definitions of "LSPCs" include the following: (1) cells responsible for normal tissue turnover; (2) cells that regenerate liver after PHx; (3) cells responsible for progenitor-dependent regeneration; (4) transplantable liver repopulating cells; and (5) cells that adopt hepatocyte and bile duct phenotypes *in vitro*.

Currently, researchers are working hard to characterize, localize and isolate LSPCs, though this has been difficult because of the lack of specific markers[11]. To avoid the restriction of lacking specific markers, Liu *et al*[12,13] have tried other strategies for isolating LSPCs. Based on the concept that stem cells have specific physical and morphological properties, Liu *et al*[12] isolated FLSPCs by a percoll continuous gradient centrifugation-centered

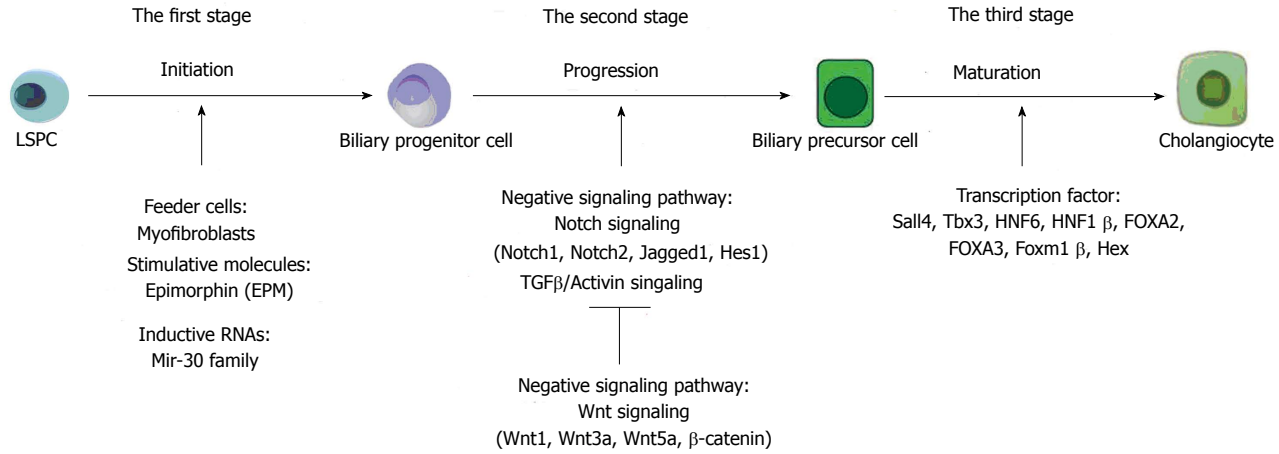

Figure 2 The molecular mechanisms in each step of biliary differentiation of liver stem/progenitor cells. The biliary differentiation of LSPCs can be divided into three main stages comprising of initiation, progression and maturation. Some important jacent or feeder cells and specific molecules are proposed to be responsible for initiating the first stage of biliary differentiation. When the biliary differentiation goes on, several key signaling pathways including Notch and TGFβ have essential impacts on guarantee of the second stage. After some transcription factors activated, the third stage of biliary differentiation could be accomplished and LSPCs could be matured into cholangiocytes. LSPCs: Liver stem/progenitor cells; TGFβ: Transforming growth factor β.

three-step method. Because stem cells have specific functional characteristics, such as excluding biological vital dyes such as Hoechst 33342, Liu *et al*[13] obtained ALSPCs by side population (SP) enrichment. Although many groups have isolated LSPCs using various strategies, LSPCs for disease application remain far off. The core issue is how to manipulate LSPC differentiation, which is essential for both cell therapy and liver regeneration[14,15]. Thus, to guarantee the efficiency and security of LSPC-based therapy for liver diseases, it is important to clarify the strategies and related mechanisms for proper differentiation of LSPCs.

In recent years, many studies have shed light on the tangle of regulatory mechanisms that govern the complex process of LSPC differentiation, but an overall understanding remains a challenge. Here, we review the current understanding of the exact mechanisms related to the differentiation of LSPCs, especially toward cholangiocytic differentiation. We divided the process of LSPC differentiation into three stages (Figure 2). The first stage is the onset of differentiation, when LSPCs are induced to mature into certain cell types. In this stage, the lineage-specific cytokines/growth factors (GFs), their (relative) doses and order of application are crucial for directing the lineage specification of the LSPCs[16]. The second stage is the acceleration of differentiation, when LSPCs are quickly progressing through the process. Many developmental regulatory signaling pathways, including the Wnt, Notch, bone morphogenetic protein and fibroblast growth factor pathways, may play a role in directing the cell fates of LSPCs[17]. The third stage is to guarantee the accomplishment of differentiation. In this stage, transcription factors are vital to make cells express lineage-specific markers[5].

IDENTIFICATION OF CHOLANGIOCYTES GENERATED FROM LSPCs

LSPCs can differentiate into a wide range of cell types, including hepatocytes, cholangiocytes, pancreatic cells and intestinal epithelial cells (Figure 1)[18]. However, in this review, we focus on cholangiocytic specification from LSPCs. To ascertain the cell fates of LSPCs, the following three features inherent to LSPC transitions must be taken into account for accurate phenotyping: (1) the differentiation of LSPCs toward a specific lineage often involves uncontrolled processes, resulting in a heterogeneous cell population; (2) the differentiation into mature cells is a steady process; and (3) the ultimate proof of functional cell behavior is *in vivo* transplantation of *ex vivo* generated LSPC-based mature cells into immunodeficient animal models with liver injury[19,20].

As LSPCs differentiate into cholangiocytes, the cells grow in size to > 12 μm and display a keystone morphology with cholangiocyte-type epithelial polarity. These cells are concentrically layered to form a cyclic structure or arranged in lines to form ductal plates. Under the electron microscope, these cells acquired the classic cholangiocyte features of small numbers of organelles and many primary cilia on their surface.

Aside from morphological identification, the analytical work is limited to the elucidation of (1) cholangiocytic RNA transcripts *via* (quantitative) reverse transcriptase polymerase chain reaction and (2) cholangiocytic proteins by immunofluorescence. During the process of LSPC differentiation into cholangiocytes, cells transition from the expression of early biliary markers (such as Sox9, which is a representative transcriptional factor expressed in biliary precursor cells), to the expression of mid-stage

biliary markers (such as the cytokine CK19 and E-cadherin), and then mature biliary markers (such as CK7)[21]. In addition, gamma-glutamyl transpeptidase (GGT), a major enzyme of glutathione homeostasis, is often used as a biliary marker to follow the differentiation of LSPCs[22]. Furthermore, multidrug resistance-associated protein 3[23] and secretin receptors[24] are also found to be expressed in cholangiocytes.

Although the induced differentiation of cholangiocytes has been performed, the functional examination of LSPC-derived cholangiocytes is very scarce. Thus, the *in vivo* identification of induced cholangiocytes is essential, and to some extent it can be considered the "gold standard" of certifying the cell fates of LSPCs[25]. LSPC-derived cholangiocytes *in vivo* should be able to replace injured cholangiocytes or lost bile duct cells.

STRATEGIES FOR CHOLANGIOCYTIC DIFFERENTIATION OF LSPCs

The components of the stem-cell microenvironment regulating differentiation include distinct cell-cell interactions and paracrine signals, which comprise both soluble and extracellular matrix factors, as well as the three-dimensional architecture, which shapes and dictates the delivery of these cues. It is reported that mature stellate cells and/or myofibroblasts resulted in differentiation of LSPCs into cholangiocytes[26]. These feeder cells control the cell fates of LSPCs through either paracrine signaling pathways or cell-cell interaction[27-30]. Thus, if the paracrine signals produced by the feeders are replaced with similar components, the same induced differentiation of LSPCs could be achieved. There are feeder-free conditions that yield equivalent results, consisting of the embedding of LSPCs into hydrogels containing type I collagen (60%) and Matrigel (40%) with modified Kubota's medium for cholangiocytes. It is also demonstrated that the murine FLSPC cell line, hepatoblast cell line-3, can be induced to differentiate toward cholangiocyte by plating in Matrigel[31]. Furthermore, Matrigel-coated films are also widely used for manipulating LSPCs. Although PLL-terminal t-(poly-l-lysine/poly-l-glutamic acid) (PLL/PLGA) films are less favorable for stem cell cultures than PLGA-terminal t-(PLL/PLGA) films, the cell fates of LSPCs are correlated with the film thickness on both types of film, with differentiation favored on the thinner films[32].

Recent evidence has shown that expression of miRNAs can regulate the divergent differentiation pathways of stem cells[33]. Therefore, Liu *et al*[13] reasoned that miRNAs could be responsible for regulating cell fate decisions in LSPCs by regulating the cells' responses to ubiquitous GFs. It was found that the miR-23b cluster, including miR-23b, miR-27b, and miR-24-1 and miR-10a, miR-26a and miR-30a, was highly expressed in LSPCs[34]. MiR-23b cluster repressed bile duct gene expression in LSPCs while promoting their growth; low levels of the miR-23b miRNAs were needed in cholangiocytic differentiation and bile duct formation[34].

MOLECULAR MECHANISMS OF CHOLANGIOCYTIC DIFFERENTIATION FROM LSPCs

The process of intrahepatic bile duct (IHBD) formation from LSPCs involves cholangiocyte differentiation (lineage specification) and morphogenesis of ductal structures[21]. Understanding how LSPCs can generate differentiated bile ducts is crucial for studies on epithelial morphogenesis and for development of cell therapies for hepatobiliary diseases. Many groups[35-37] have demonstrated that, during *in vivo* liver development and *in vitro* differentiation, LSPCs located around the portal vein first develop as biliary precursor cells and then generate cholangiocytes. Nevertheless, the molecular mechanisms behind these events have yet to be fully elucidated. It is shown that Wnt and Notch signaling are active in the adult human liver to drive proliferation and differentiation of LSPCs into the hepatocyte or cholangiocyte lineages[38]. The Notch pathway is triggered by expression of the Notch ligand Jagged1 by myofibroblasts, thereby promoting biliary differentiation of LSPCs, and the enhancement of Wnt3a expression in macrophages after uptake of hepatocyte debris and paracrine activation of Wnt signaling in neighboring LSPCs specifies hepatocytic differentiation[39,40]. The opposing roles of Wnt and Notch signals in cholangiocyte fate determination in the LSPCs are described below. The molecules responsible for differentiation of LSPCs into cholangiocytes are also discussed in this section (Figure 2).

INITIATION OF CHOLANGIOCYTIC DIFFERENTIATION

When the LSPCs are cultured in Matrigel, they are likely to differentiate into cholangiocytes. Recently, the key stimulator has been found. Epimorphin/syntaxin 2 (EPM) is a highly conserved and very abundant protein involved in epithelial morphogenesis in various epithelial organs[41], and in the liver, it is exclusively expressed on the surface of hepatic stellate cells and myofibroblasts[42]. Biliary differentiation markers elevated by EPM include Yp, Cx43, aquaporin-1, CK19 and GGT[41]. Moreover, the signaling pathway of EPM was analyzed by focal adhesion kinase (FAK), extracellular regulated kinase 1/2 (ERK1/2) and RhoA. Most importantly, RhoA was found to be necessary for EPM-induced activation of FAK and ERK1/2 and bile duct formation. In addition, EPM regulated GGT Ⅳ and GGT Ⅴ expression differentially, and this was possibly mediated by C/EBPβ. Taken together, these data demonstrated that EPM regulates biliary differentiation of LSPCs through effects on RhoA and C/EBPβ, implicating a dual aspect of this morphoregulator in bile duct epithelial morphogenesis. In another study, it was reported that EPM selectively induced bile duct formation through upregulation of CK19 expression and suppression of hepatocyte nuclear factor

(HNF) 3α and HNF6[43]. These results demonstrate a new biophysical action of EPM in bile duct formation, during which the determination of LSPCs play a crucial role. MiRNAs could also initiate biliary differentiation of LSPCs. In the previous section, we described the requirement for miR-23b miRNAs in growing hepatocytes to repress bile duct genes and repress tumor growth factor (TGF) β signaling. There has also been another report providing evidence that miR-30 family miRNAs were required for complete bile duct formation to repress hepatocyte genes[44].

PROGRESSION OF CHOLANGIOCYTIC DIFFERENTIATION

Bile ducts are formed only around the portal side, suggesting that region-specific signals induce cholangiocytes from LSPCs. Two signaling pathways, TGFβ/Activin[21,45] and Notch[46,47], are specifically activated in LSPCs near the portal vein. Although differentiation of LSPCs to cholangiocytes by TGFβ and Notch signaling occurs in mid-gestation, surprisingly, LSPCs can be induced to differentiate into cholangiocytes and form ectopic duct structures in the parenchyma upon Notch activation after birth[48]. That is, the Notch pathway plays an essential role in the morphogenesis of bile duct structures[49]. Indeed, conditional knockout of Recombination signal binding protein Jκ, an essential downstream signal component of the Notch receptor, results in a reduced number of cholangiocytes at ED 16.5, confirming a role for this signaling pathway in cholangiocyte cell fate specification[48]. In general, Notch signaling is likely to play the most important role in controlling biliary differentiation of LSPCs.

A study using an *in vitro* culture of FLSPCs has shown that activation of the Notch signaling pathway promotes LSPC differentiation into the cholangiocyte lineage by coordinating a network of LETFs including HNF1α/β, HNF4α and C/EBPα[46]. Among multiple Notch signaling components, Notch1, Notch2 and Jagged1, Hes1 are widely accepted as essential for promoting bile duct differentiation[49-51], while Notch3 and Jagged2 play key roles in hepatic differentiation[52]. Lacking Hes1, a target of the Notch signaling, ductal plate formation occurs normally, but the subsequent remodeling and tubular structure formation is completely blocked[53]. In humans, mutations in Jagged1, a ligand for the Notch receptors, are associated with Alagille syndrome, an autosomal dominant disorder characterized by multiple developmental defects including neonatal cholestasis caused by a paucity of IHBD[54-56]. In addition, another form of Alagille syndrome has been found to be caused by mutations in the *Notch2* gene[57].

TGFβ is necessary for the formation of bile ducts[58]. The inhibition of TGFβ signaling allows LSPCs to undergo normal hepatocyte differentiation[45]. Wnt signaling is also involved in regulating biliary epithelial cell fate. The addition of Wnt3a in *ex vivo* culture experiments supports biliary epithelial cell differentiation of FLSPCs[59]. However, as to Wnt5a, a non-canonical Wnt ligand, *in vitro* differentiation assays showed that Wnt5a-mediated signaling in FLSPCs suppresses biliary differentiation through the activation of phosphorylated Calcium/calmodulin-dependent protein kinase Ⅱ[60]. Similarly, in the absence of Wnt1 signaling, LSPCs failed to differentiate into hepatocytes and underwent atypical ductular hyperplasia, exhibiting epithelial metaplasia and mucin production[61,62]. Furthermore, the inhibition of β-catenin, a core component of canonical Wnt signaling, prevents LSPCs from expressing biliary markers[63].

In brief, Notch signaling promotes LSPCs differentiation into the biliary epithelial lineage and concurrently inhibits hepatic differentiation by reducing the expression of hepatic genes. In contrast, Wnt signaling is more likely to aid in promoting hepatic differentiation and repressing biliary differentiation. The unbalanced activation of Wnt and Notch signaling pathways influences the cell fates of LSPCs.

ACCOMPLISHMENT OF CHOLANGIOCYTIC DIFFERENTIATION

With regard to the molecular mechanisms involved in cholangiocytic differentiation, several transcription factors have been implicated, including Sal-like 4 (Sall4), T-box transcription factor 3 (Tbx3), the Onecut transcription factor HNF6 and HNF1β, HES1, FOXA2, FOXA3, forkhead Box (Fox) m1β (Foxm1β), and Hex[36,37,64-68]. Sall4 is expressed in LSPCs but not in mature liver cells. The expression level of Sall4 gradually falls during liver development. Sall4 has been shown to play a role in regulating the lineage commitment of LSPCs by inhibiting their differentiation into hepatocytes while driving differentiation toward cholangiocytes[36]. When bile duct-like structures were induced by collagen gel-embedded culture conditions, overexpression of Sall4 markedly augmented the size and number of $CK19^+$ branching structures. These results suggest that Sall4 plays a crucial role in controlling the lineage commitment of LSPCs not only by inhibiting their differentiation into hepatocytes but also by driving their differentiation toward cholangiocytes[36]. Tbx3 also contributes to the hepato-biliary lineage decision[37,69]. Tbx3 functions to maintain expression of the hepatocyte transcription factors HNF4α and C/EBPα while suppressing expression of the cholangiocyte transcription factors HNF6 and HNF1β[69]. In addition, as a direct and critical target of HNF6, HNF1β shows a decisive effect in bile duct development[65].

DIFFERENTIATION OF LSPCs INTO INSULIN-POSITIVE CELLS

Although organ-specific stem cells possess plasticity that permits differentiation along new lineages, production of endocrine pancreas and insulin-secreting beta cells from stem cells has not been fully demonstrated. The liver and pancreas share a common developmental origin, and a

bipotential precursor cell population for these organs has been identified within the embryonic endoderm[70]. Consistent with these facts, many studies have demonstrated that LSPCs can be converted to insulin-producing cells by stable expression of pancreatic duodenal homeobox 1 (Pdx1) or its super-active form (Pdx1-VP16) or to functional pancreatic beta-cell-like cells, and/or islet-like cell clusters containing other pancreatic lineages under certain other conditions[71-74]. The most common condition under which LSPCs are induced to differentiate into insulin-producing cells is a high-glucose environment[75]. In addition, there are studies indicating an efficient chemical protocol for differentiating LSPCs into functional insulin-producing cells using small molecules, and they represent a promising LSPC-based treatment for diabetes mellitus. When ALSPCs were incubated with a combination of 5 mmol/L sodium butyrate and 1 nmol/L betacellulin, most of the cells were converted into morphologically beta cell-like cells. An immunoreactive pancreatic polypeptide, somatostatin, and insulin were detected in sodium butyrate and betacellulin-treated ALSPCs[72]. Based on induction by a combination of 5-aza-2'-deoxycytidine, trichostatin A, retinoic acid and a mix of insulin, transferrin and selenite, LSPCs could also trans-differentiate into beta-like cells[76]. Furthermore, transduction of pancreatic transcription factors, such as Pdx1, Neurogenin3, NeuroD and MafA, can induce the formation of ectopic islet-like cells and the production of insulin in ALSPCs[77,78]. Stepwise differentiation from LSPCs into functional insulin-secreting cells will identify key steps in beta-cell development and may yet prove useful for transplantation therapy for diabetic patients[79].

MALIGNANT TRANSFORMATION OF LSPCs INTO LIVER CANCER STEM LIKE CELLS

Stem cells have potential for therapy of liver diseases, but they may also be involved in the formation of cancer[80]. At present, it is widely accepted that cancer arises from the malignant transformation of stem cells[81,82] because these are the only cells that persist sufficiently long to acquire the required number of genetic changes. Specifically, LSPCs are hypothesized to be the precursors for a subset of liver cancer[83,84]. Presently, accumulating evidence supports the above notion as follows[85]: (1) similar signaling pathways may regulate self-renewal in LSPCs and liver cancer cells; and (2) liver cancer contains rare cells with stem cell-like properties, which may derive from malignant transformation of LSPCs. Herein, we propose that liver cancer stem like cells (LCSCs) might arise from LSPCs it would facilitate our understanding of stem-cell origin of liver cancer. It has been demonstrated that deletion of p53 from LSPCs is sufficient to induce tumor formation[86]. Recently, through loss expression of Tg737, You *et al*[87] successfully induced FLSPCs to malignantly transform into LCSCs. These LCSCs from LSPCs could generate liver cancer after transplantation into immunoinsufficient mice. In addition to gene manipulation, dysregulated miRNAs may also initiate malignant transformation. To find the possible target miRNAs, Liu *et al*[13] compared the miRNA profiles between LSPCs and LCSCs. As a result, Liu *et al*[13] found 78 miRNAs were dysregulated, including miR-200a (the most down-regulated miRNA in LCSCs) and miR-181 (the most greatly upregulated miRNA in LCSCs)[13]. After inhibition of miR-200a in LSPCs, Liu *et al*[13] found that cells displayed malignant properties such as unlimited proliferation and strong metastasis. A novel regulatory link between miR-181 and LCSCs was proven by a study from another group[88]. They found that miR-181 could induce LSPCs' malignant transformation by directly targeting hepatic transcriptional regulators of differentiation (for example, caudal type homeobox transcription factor 2 and GATA binding protein 6) and an inhibitor of Wnt/beta-catenin signaling (nemo-like kinase).

CONCLUSIONS AND FUTURE DIRECTIONS

Despite uncertainty surrounding the mechanism underlying the role of LSPCs in liver regeneration[89], there is great hope for the use of these cells in liver-based therapies[90]. First, LSPCs can be used for the treatment of inherited end-stage liver disease. Second, they can also serve as a source of cells for cell transplantation in acquired liver diseases such as acute failure due to toxic or viral injury. Third, because LSPCs can be expanded *in vitro* to a desired extent, they can be used to populate liver assist devices or artificial livers based on bioengineered matrices. Lastly, they can be used as targets for gene therapies in primary liver diseases or diseases where extra-hepatic manifestations arise from abnormal gene expression or defective protein production in the liver. Considering the strong proliferative potential and amenability for *in vitro* manipulation, LSPCs may be attractive candidates for liver disease treatment. In addition, LSPCs may be useful for cell therapy to treat diabetic patients, given their potential to be effectively reprogrammed toward pancreatic lineages[91]. Furthermore, the development of such protocols would reduce the likelihood of malignant transformation upon transplantation.

Although LSPCs are promising for the future use in many fields, the accurate control of cell fates of LSPCs is far from accomplishment. Thus, it is necessary to clear the mechanisms for LSPCs differentiation and build standardisation of the production of functional cholangiocytes from LSPCs. Here, we want to list several directions that may help to guide future research of LSPCs differentiation: (1) The knowledge of biliary development and liver regeneration can best provide detailed information for *in vitro* cholangiocytic differention of LSPCs. It is a good choice to thoroughly investigate the molecular basis of biliary development during the period from fetal liver to adult liver; (2) LSPCs react differently to stimulative

materials at different stages. The dosage, timing, and combinations of materials should thus be fine-tuned according to the differentiated stage LSPCs located. Hence, it is important to figure out what state LSPCs are presented; (3) The molecular mechanism in each step of cholangiocytic differentiation from LSPCs is essential for cell-based therapies. Both positive and negative factors responsible for the initiation, progression and maturation of cholangiocytic differentiation should be specially considered; and (4) Although we divide the process of cholangiocytic differentiation into three stages, it is actually a continuous evolving process. That is to say, it should be kept in mind that many key factors may not only take effect in some stage of cholangiocytic differentiation from LSPCs.

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