

## Identification and differentiation of hepatic stem cells during liver development

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## 1. ABSTRACT

Stem cells responsible for maintenance and repair of tissues are found in a number of organs. The liver's remarkable capacity to regenerate after hepatectomy or chemical-induced injury does not involve proliferation of stem cells. However, recent studies suggest that liver stem cells exist in both embryonic and adult livers. Using fluorescence-activated cell sorting and a culture system in which primitive hepatic progenitor cells form colonies, a novel class of cells with the marker profile c-Met<sup>+</sup>CD49<sup>+/low</sup>c-Kit<sup>+</sup>CD45<sup>-</sup>TER119<sup>-</sup> was found in the developing liver. This class apparently represents the population of cells that form colonies containing distinct hepatocytes and cholangiocytes. When cells in this class are transplanted into the spleen or liver of mice subjected to liver injury, the cells migrate and differentiate into liver parenchymal cells and cholangiocytes that are morphologically and functionally indistinguishable from their native counterparts. During mid-gestation, hematopoietic cells migrate into the liver from a region bounded by aorta, gonad, and mesonephros and produce oncostatin M (OSM). In combination with glucocorticoid hormones, OSM induces maturation of liver stem and progenitor cells, including those of the c-Met<sup>+</sup>CD49<sup>+/low</sup>c-Kit<sup>+</sup>CD45<sup>-</sup>TER119<sup>-</sup> class. The ability to manipulate the proliferation and differentiation of liver stem cells *in vitro* will greatly aid in analyzing mechanisms of liver development and offers promise in stem cell therapy of liver diseases.

## 2. INTRODUCTION

While in adulthood liver is the central organ for intermediary metabolism, fetal liver has few metabolic functions associated with adult liver (1-3). Instead, fetal liver functions as the major hematopoietic organ during

mid- to late gestation (4, 5). Hepatocytes, the primary cells of the liver, have many functions such as synthesis and degradation of amino acids, lipids, and cholesterol; gluconeogenesis and glycogen storage; and detoxifying countless xenobiotic chemicals. In addition, hepatocytes also have an endocrine function, secreting large quantities of protein into blood plasma, including apolipoproteins, coagulation factors, and serum albumin. In contrast, fetal hepatocytes express several cytokines that support growth of hematopoietic cells expanding in the mid-gestation liver. That hepatocytes dramatically change during liver development is evident.

Stem cells are generally defined as clonogenic cells exhibiting the characteristic properties of self-renewal or maintenance (do not proliferate rapidly and are slow-cycling), multipotency (producing progeny in two lineages or more), and long-term tissue repopulation after transplantation (6). During tissue development, stem cells give rise to non-self-renewing progenitors with restricted differentiation potential and, while maintaining a subpopulation of primitive stem cells, to functionally mature cells. After birth, in response to homeostatic or regenerative signals, such cells are believed to play a central role in the maintenance, repair, and reconstitution of tissues to which their progeny contribute (7). In contrast, fully differentiated, organ- or tissue-specific cells in adult mammalian tissues are generally considered to have little or no proliferative potential. Interestingly, recent studies found that self-renewing and multipotent stem cells exist in the nervous system, a tissue that had never been considered capable of regenerating (8, 9). To use stem cells in therapy, it is essential to understand the mechanisms regulating their proliferation and differentiation potential *in vivo*. The hematopoietic system is one of the most well-studied

organs involving stem cells. Purification of hematopoietic stem cells with flow cytometric cell sorting has enabled examination of their full potential for regeneration of the hematopoietic system. A single CD34<sup>+</sup>/cKit<sup>+</sup>/Sca-1<sup>+</sup>/lineage<sup>-</sup> hematopoietic cell derived from bone marrow can reconstitute bone marrow of irradiated mice (10). Several specific lineage cell surface markers have been proven very useful for analyses of each step of differentiation from stem cells to totally differentiated blood cells (11, 12). In order to advance stem cell biology in other organs, prospective identification and clonal expansion of stem cells is very important.

Adult liver has a unique capacity to regulate cell growth (13, 14). Although under normal conditions hepatocytes rarely divide, their proliferative capacity and the ability of the liver to adapt to various metabolic functions are not in question. Several growth factors and cytokines are important for the proliferation of mature hepatocytes during liver regeneration *in vivo*. Mature hepatocytes can even undergo serial transplantation under very selected circumstances (15). These data are not equivalent with the observation of telomere shortening during high cellular turnover, as in chronic liver diseases; it thus remains unknown how the liver is regenerated and what cells are responsible for such regeneration (16). Under normal conditions, undifferentiated liver progenitors do not take part in acute liver regeneration. However, hepatocyte progenitors are still required in some chronic injury responses, especially when the ability of differentiated hepatocytes to divide is impaired. In this review, we describe recent findings in embryonic liver development and the fetal and adult liver stem cell system. Fetal liver stem cells can be isolated and induced to mature as adult hepatocytes *in vitro*. In addition, hepatic stem cells may exist in the adult livers and participate in liver regeneration under severe conditions in which mature hepatocytes cannot proliferate.

### 3. GROWTH OF HEPATIC ENDODERM INTO A LIVER BUD

An important question in gut organogenesis is how individual tissues are specified at different domains along the anteroposterior axis of the endoderm. Studies in model organisms have shown that endodermal domains are usually patterned by interactions with contiguous mesodermal tissue. The cardiac mesoderm, which adjoins the prospective hepatic endoderm, provides a signal that is important for inducing liver specification in the endoderm (17, 18). This process begins on embryonic day 8.5 (E8.5) in the mouse with proliferation of undifferentiated endodermal cells of the ventral foregut and their migration into the septum transversum. The ventral foregut endoderm gains the competence to develop into various tissues as a result of the expression of transcription factors. During the course of tissue specification, several soluble factors initiate the liver gene expression program in the proximal endoderm (reviewed by Zaret) (19). Using an embryonic tissue explant system, fibroblast growth factor (FGF) signalling from the cardiac mesoderm was shown to be necessary and sufficient to induce hepatic gene

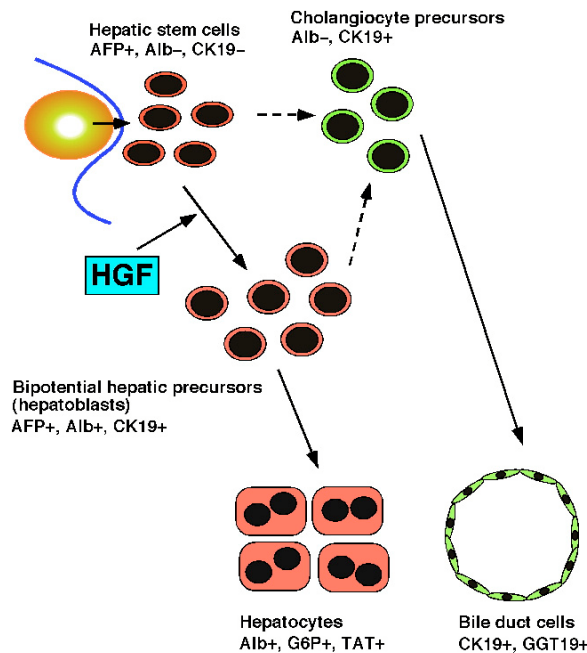
expression in the endoderm (20). Septum transversum mesenchyme cells surround the developing cardiac region near the ventral foregut endoderm. Inclusion of these cells in embryonic tissue explant cultures led to production of significant levels of bone morphogenetic proteins (BMPs) that are also important for early hepatogenesis (21). Deletion of Bmp4 also perturbs the development of various ventral structures in the embryo (22). Interestingly, ventral endoderm tissue culture was found to start expressing pancreatic genes without FGF and BMP signals (23). These results indicated that FGF signals from the cardiac mesoderm, in conjunction with BMP produced by septum transversum mesenchyme, determine the fate of the ventral endoderm as a precursor of hepatic cells.

After the commitment of endoderm to the liver bud, the liver bud starts to expand and to express the functions of a hematopoietic organ. Several mouse mutants are known in which the growth of fetal liver at this stage is affected. Earlier studies revealed that c-jun and SEK1 signals are important for maintenance of cell survival of fetal hepatocytes (24, 25). Defects in genes encoding these proteins caused significant apoptosis during the mid-gestation fetal liver. *Hex* is a homeobox-containing gene expressed in the anterior endoderm cells at E7 and subsequently in the ventral-lateral foregut (26). *Hex* is also expressed in the liver bud. *Hex*-null embryos grow to the E11.5 stage and undergo normal turning and gut tube closure, but they lack a liver, a thyroid, and parts of the forebrain (27, 28). *Hex* accordingly was suggested to be strictly required for either specification or growth of the liver bud. Bort et al. have recently shown that *Hex* controls growth, rather than specification, of the liver bud. Hepatocyte-specific genes are first detected at the E8.5 stage in the ventral endoderm (29). Expression of these genes can also be detected in the E8.5 *Hex*-null ventral endoderm, suggesting that *Hex* is not necessary for specification of the liver bud. In contrast, cell growth in *Hex*-null hepatic endoderm in the E8.5 and E9.5 stages was down-regulated as revealed by bromodeoxyuridine incorporation, suggesting that a defect in liver bud development of *Hex*-null mice is due to a deficiency in proliferation of hepatic endoderm.

### 4. IDENTIFICATION AND PROPAGATION OF LIVER STEM CELLS

In the developing liver, both hepatocytes and bile ductal cells have been reported to differentiate from a common cell, the hepatoblast (30). Transplantation experiments using fetal rat liver cells indicated that these cells could reconstitute both bile-duct and hepatocyte structures. This suggests that the developing liver has bi-potent hepatic stem or progenitor cells. However, the existence and phenotype of stem cells in the developing liver remain unproven and undefined. To identify hepatic stem cells, *in vitro* and *in vivo* clonal analyses were used for the isolation and characterization of candidate cells (31, 32). Using monoclonal antibodies and fluorescence-activated cell sorting (FACS), liver stem cells were prospectively isolated and their capacity for self-renewal and bi-potent differentiation was arrayed out. In addition to

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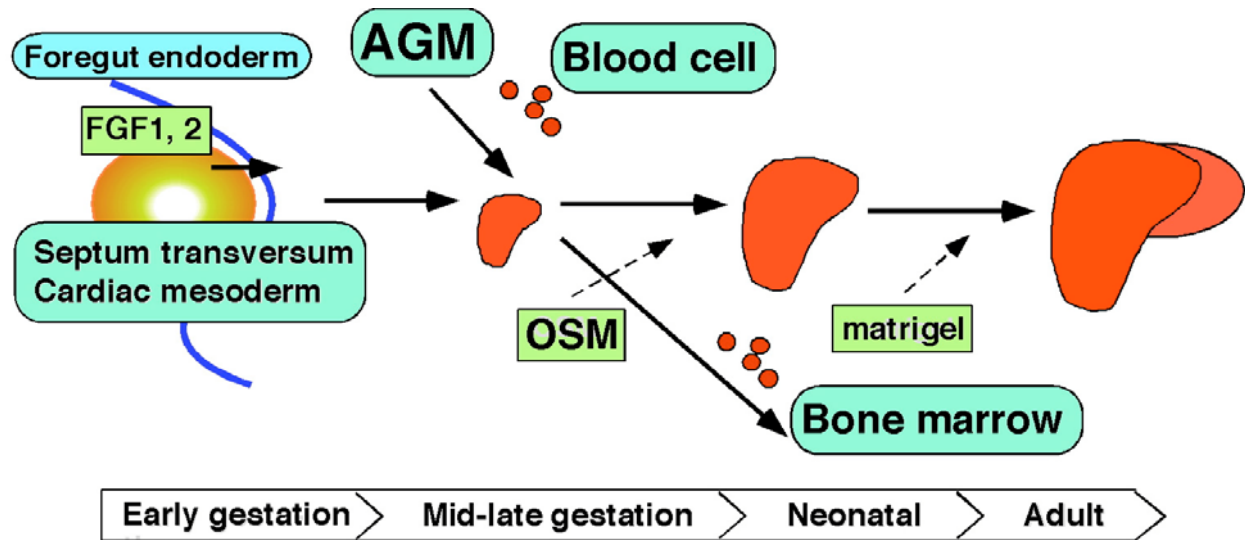
**Figure 1.** Schematic representation of possible mechanisms of the hepatic stem cell system. Hepatocyte growth factor (HGF) induced early transition of albumin-negative stem cells into albumin-positive hepatic progenitor cells having bipotential differentiation (hepatocytes and cholangiocytes).

characterizing the capacity of stem cells, *in vitro* colony assays for hepatic stem cells were established using special culture conditions in which single cells can proliferate to form clusters of up to several hundred cells (31). Sorted cells were cultured on collagen I-, collagen IV-, or laminin-coated dishes and formed clusters of different sizes by days 5 to 21 after plating. Liver cells were dissociated from E13.5 mouse embryos and sorted for the absence of surface antigens characteristic of hematopoietic cells (CD45, leukocyte common antigen, or TER119, a molecule resembling glycophorin and exclusively expressed on immature erythroid cells). Cells strongly reactive to antibodies directed against integrin beta1 and alpha6 (CD29<sup>+</sup> and CD49f<sup>+</sup>) and selected for the absence of c-kit represented 3.3% of the isolate. In the presence of HGF, approximately 0.2% of cells in this subgroup were able to form H-CFU-C (hepatic colony-forming units in culture), which is defined as colonies containing more than 100 cells after 5 days culture. After prolonged culturing, H-CFU-C colonies differentiated into both cell types, hepatocytes and cholangiocytes, based on the expression of marker genes such as albumin (for hepatocytes) and CK19 (for cholangiocytes).

Next, in order to further enrich the yield of H-CFU-C and to permit clonal analysis of this cell class, FACS sorting of H-CFU-C was improved using antibodies against c-met (HGF receptor) to identify a positive cell surface marker and sorting for demonstrable but weak expression of integrin alpha6 (33). Sorting for c-Met<sup>+</sup>CD49f<sup>+</sup>/low c-Kit<sup>+</sup>CD45<sup>-</sup>TER119<sup>-</sup> cells achieved a 560-fold enrichment in H-CFU-C compared with total fetal liver

cells. Cells identified by clone sorting with FACS were cultured in individual wells of laminin-coated 96-well plates. These analyses clearly showed that single hepatic stem cells could proliferate and give rise to both hepatocytes and cholangiocytes. While only a fraction of the Met<sup>+</sup>CD49f<sup>+</sup>/low c-Kit<sup>+</sup>CD45<sup>-</sup>TER119<sup>-</sup> cell subpopulation formed H-CFU-C colonies, serial clone-sorting of the progeny of individual H-CFU-C revealed that 60% of them produced their own cell types in culture from a single daughter cell, with few morphological and functional differences among them. Ultrastructural studies of the secondary colonies showed that they could differentiate into hepatocytes, forming bile canaliculi-like structures with luminal spaces occupied by microvilli, and into cholangiocytes, forming well-organized bile duct-like structures with luminal membranes covered with short microvilli. These results revealed that Met<sup>+</sup>CD49f<sup>+</sup>/low c-Kit<sup>+</sup>CD45<sup>-</sup>TER119<sup>-</sup> cells are hepatic stem cells capable of self-renewal and with bi-directional differentiation potential. This self-renewal status persisted for more than six months in culture, with spontaneous production of hepatocytes and cholangiocytes. In addition, when transplanted into the spleen of mice subjected to severe hepatic damage by carbon tetrachloride treatment ("regenerative induction"), these cells migrated to the recipient liver and differentiated into liver parenchymal cells *in vivo*. Next, H-CFU-C cell clones were transplanted into liver, pancreas, and intestine. The cells differentiated into hepatocytes or cholangiocytes in recipient livers after regenerative induction. Interestingly, when these cells were injected into the pancreas, they integrated into and formed pancreatic ducts and acinar cells. These cells also could differentiate into intestinal epithelium and reconstituted intestinal villi and crypts when injected into duodenal wall (34). Met<sup>+</sup>CD49f<sup>+</sup>/low c-Kit<sup>+</sup>CD45<sup>-</sup>TER119<sup>-</sup> cells were also separated into albumin-negative (ALB<sup>-</sup>) and positive (ALB<sup>+</sup>) cells using FACS, following gene transfer of the albumin enhancer/promoter-EGFP construct into stem cell cultures. When cultured with HGF, the ALB<sup>-</sup> cells were converted into ALB<sup>+</sup> cells; both ALB<sup>-</sup> and ALB<sup>+</sup> cells could proliferate in culture, with differentiation into hepatocytes and cholangiocytes (Figure 1). These results suggested that the expression of albumin begins at a very early stage of stem cell differentiation, and that lineage specification into either hepatocytes or cholangiocytes cannot be determined by albumin expression. Indeed, albumin-negative hepatic stem cells are differentiated by soluble factors such as HGF into albumin-positive hepatic progenitor cells during fetal liver development (34).

In contrast to the existence of fetal stem cells, the existence of stem cells in the adult liver is a very controversial issue. Identification of unique markers for adult liver stem cells and establishment of these cells in culture have proven difficult. Several studies suggested that some stem-like cells increase in number during liver regeneration under conditions in which hepatocyte proliferation is blocked. Rats treated with 2-acetylaminofluorene, which causes extensive DNA damage in hepatocytes, followed by two-thirds partial hepatectomy, could not increase the proliferation of epithelial cells in the periportal region (35). These cells, which have oval nuclei,



**Figure 2.** A possible model for mid-fetal liver development regulated by hepatic maturation factors. Hematopoietic cells migrate from the aorta-gonad-mesonephros (AGM) region, proliferate in the liver, and produce oncostatin M (OSM).

were designated “oval cells”. They express both hepatocytic markers (AFP and albumin) and bile-duct markers (CK7 and CK19) (36, 37). Oval cells can differentiate into both hepatocytes and biliary epithelial cells under certain conditions, suggesting that these cells may be adult hepatic progenitor or stem cells. In a mouse model, treatment with a 3- or 4-week 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet causes hepatocyte apoptosis and massive bile-ductular cell proliferation with differentiation of some of the oval-like bile duct cells into hepatocytes (38). Using transplantation into fumarylacetoacetate hydrolase (FAH) deficient mice, the DDC-induced oval cells were able to differentiate into fully functional hepatocytes and to repopulate the FAH-deficient livers, suggesting that these oval cells progenitor or stem cells (39). Oval cells are known to express several hematopoietic cell surface markers. For example, rat oval cells have been shown to express cell surface markers such as Thy-1, CD34, and c-kit (40-42). In addition, mouse DDC-induced oval cells expressed the antigen recognized by A6, a monoclonal antibody that is specific for biliary cells and proliferating oval cells (43). Immunohistochemistry revealed that the cells expressing Sca-1/CD34/CD45 were indeed oval cells that were also expressing A6 as well as AFP (43). A recent report also supports a hematopoietic stem cell origin of hepatocyte-like cells after liver injury (44). These studies showed that both rat and mouse oval cells share several cell surface markers with hematopoietic cells and that these markers may be useful to identify adult hepatic stem cells. Some studies, however, do not support a hematopoietic origin of hepatocytes (45). Since oval cells are a morphologically defined cell type, they may be a heterogeneous cell population. Oval cells that express hematopoietic markers may represent cells of monocytic origin that scavenge apoptotic hepatocytes and cholangiocytes. Identification of oval cell specific cell surface markers and prospective isolation of oval cells will clarify these issues.

## 5. DEVELOPMENT OF LIVER PROGENITOR CELLS IS REGULATED BY SOLUBLE FACTORS AND EXTRACELLULAR MATRICES

Embryonic liver formation consists of multiple stages and is regulated by hormonal factors as well as intercellular and matrix-cell interactions. As summarized above, the liver primordium proliferates and invades the mesenchyme of the septum transversum to give rise to the hepatic cords and buds. Fetal hepatocytes then proceed through a series of maturation steps that are accompanied by a decrease in hematopoietic activity and an increase in the expression of several genes associated with liver maturation, such as glucose-6-phosphatase (G6Pase) and tyrosine aminotransferase (TAT) (46, 47). The final stage of differentiation takes place after birth, and the fully mature liver expresses adult liver-specific enzymes that include tryptophan oxygenase (TO) and serine dehydratase (48-50). In addition, commencing with the late-fetal and perinatal stage, hepatocytes start to express various types of cytochrome P450s (CYPs) that play key roles in the detoxification of drugs (51).

Maturation of hepatocytes is affected by extracellular signals, both soluble factors and signals originating in extracellular matrices (Figure 2). In the mid-to late fetal liver, several soluble factors exist that affect hepatic progenitor cells. For example, dexamethasone, a synthetic glucocorticoid, is known to suppress AFP production and DNA synthesis, while it up-regulates albumin production (52-54). Transforming growth factor-beta is a potent inhibitor of hepatocyte proliferation and was shown to augment albumin production in prenatal hepatocytes (55). As previously described, an *in vitro* culture system of fetal hepatic cells derived from E14 fetal livers was established in order to investigate the molecular basis of fetal hepatic development (56). Using this system, molecules that stimulate hepatic development *in vitro* were

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sought and oncostatin M (OSM), an interleukin 6 family cytokine, was shown to promote hepatic maturation. Fetal hepatocytes induced to differentiate by OSM express metabolic enzymes, accumulate glycogen and lipids, and remove ammonia (56, 57). Interestingly, OSM is expressed in CD45<sup>+</sup> hematopoietic cells in the mid-fetal livers, whereas the OSM receptor is mainly detected in hepatic cells. It was suggested that OSM produced from hematopoietic cells is a paracrine regulator that plays a pivotal role during fetal liver development.

Cell-cell contact is also involved in hepatic maturation since high cell density promotes many metabolic functions of hepatocytes in culture (57). However, stimulation by cytokines and cell-cell interactions could not induce genes expressed in mature liver, such as those encoding TO and CYP (for which expression begins after birth), suggesting that terminal differentiation of fetal hepatocytes requires another factor. Extracellular matrices play an important role in the regulation of hepatic functions in cultures of adult hepatocytes (58-60). During the late fetal and perinatal stages, hematopoiesis shifts from the liver to the bone marrow, and the liver starts acquiring the functions of mature liver. With relocation, the numbers of non-parenchymal cells are increased and the typical liver architecture is formed. Stellate cells, one of the non-parenchymal type cells, are known to produce a large amount of matrix (61). Thus, the effect of extracellular matrices on fetal liver development was analysed (62). The addition of Matrigel (including collagen type IV and laminin) significantly induced expression of *To* and *Cyp* in E14-derived fetal hepatic culture. Since the integrin family is known to include receptors for several extracellular matrices, including collagen and laminin, a role for integrin beta1 in hepatic maturation was examined. Indeed, antibody to integrin beta1 partly inhibited hepatic maturation in this culture system. In addition, another group described that integrin alpha3beta1 is important for the attachment of hepatocytes to matrices and also for expression of albumin mRNA as analyzed by using a mouse cell line derived from hepatocytes (63). These results suggested that extracellular matrices-integrin interactions are important for both maintenance of liver functions and for the progression of fetal liver development.

In addition to OSM, several soluble factors are expressed in the liver during embryonic development. In the adult liver, tumor necrosis factor-alpha (TNFalpha) is mainly expressed during liver injury or inflammation. TNFalpha also is involved in embryonic liver development: Knockout mice lacking expression of several proteins involved in NF-kappaB signal transduction die as embryos due to enhanced hepatic apoptosis and this lethality can be rescued by inactivation of the TNFalpha receptor, TNFR1 (64, 65). TNFalpha is mainly expressed in the prenatal and postnatal liver but not in adult liver. TNFR1 mRNA expression continues in the adult liver (66). Using fetal hepatocytes in primary culture, this factor suppressed fetal hepatic maturation induced by OSM and matrigel and down-regulated the expression of mature-liver genes such

as TAT, G6Pase, apolipoprotein (Apo) AI and ApoAIV. In contrast, expression of M-CSF and MCP-1, hematopoietic cytokines and chemokines, was suppressed by hepatic maturation factors (OSM and Matrigel) (67), whereas TNFalpha induced expression of these genes. In addition, the expression of cyclin A2, repressed by OSM and matrigel, was induced by TNFalpha. These results indicated that hepatic maturation factors and TNFalpha have opposite roles in fetal liver development. Interestingly, stimulation with OSM and matrigel significantly reduced the relative proliferation of fetal hepatocytes. The addition of TNFalpha recovered the proliferation of fetal hepatocytes inhibited by OSM and Matrigel, suggesting that TNFalpha regulates hepatic maturation through the control of cell proliferation. It is noteworthy that OSM is expressed in mid- and late-fetal livers and that matrigel is important for postnatal liver development. Thus, both the hepatic maturation inducer (OSM / Matrigel) and the putative hepatic maturation suppressor (TNFalpha) coexist in the developing perinatal liver. A balance between these factors may control the extent of maturation of fetal hepatocytes (66).

## 6. TRANSCRIPTION FACTORS REGULATING FETAL LIVER DEVELOPMENT

Cell differentiation is a result of changes in gene expression that are mainly regulated at the level of transcription. Regulation of specific transcription factor expression in response to extracellular signals such as hormones, cytokines, and extracellular matrices is important for cell differentiation. Several transcription factors that control embryonic cell differentiation are often required to maintain and to regulate gene expression in adult cells. Previous studies have suggested that hepatocyte nuclear factors (HNFs) 1alpha, 1beta, 3alpha, 3beta, 3gamma, 4alpha and 6 as well as members of the CCAAT/enhancer-binding protein (C/EBP) family (alpha, beta and delta) are involved in both liver development and mature liver functions (68). HNF4alpha, a member of the nuclear receptor family, was identified as a factor within hepatoma cell extracts that bound to the promoter of the transthyretin and ApoCIII genes (69). Targeted disruption of *Hnf4alpha* in mice results in an embryonic-lethal phenotype caused by failure of complete gastrulation due to visceral endoderm dysfunction (70). This early-lethal phenotype could be rescued by the complementation of HNF4alpha-null embryos with a tetraploid embryo-derived visceral endoderm (71). This study showed that HNF4alpha was required for expression of several genes with liver-enriched expression, such as HNF1alpha and pregnane X receptor (PXR), and is a key transcription factor for xenobiotic responses in early-fetal livers. In addition, deficiency in HNF4alpha in late-fetal livers correlates with disruption of hepatocytic glycogen accumulation and cell-cell interaction of hepatic epithelia (71). Deficiency in HNF4alpha induced the downregulation of expression of several cell adhesion and cell junction molecules such as E-cadherin, ZO-1 and CEACAM (72). Interestingly, HNF4alpha directly regulated the proximal promoter of PXR in fetal hepatocytes, suggesting that HNF4alpha is important for

drug metabolism in the fetal liver. However, adult-hepatocytes specific disruption of HNF4alpha could not cause downregulation of expression of PXR. That transcription factors regulating xenobiotic responses change during liver development can be inferred (73-75).

C/EBPalpha is also an important transcription factor in liver organogenesis. Mice having a defect in C/EBPalpha did not store hepatic glycogen and died from hypoglycemia within 8 hours after birth (76). In these mutant mice, glycogen synthase mRNA was decreased and the transcriptional induction of the genes for two gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and G6Pase, was delayed. In contrast, liver-specific C/EBPalpha -null mice were produced using the albumin-Cre transgenic mouse (77, 78). Unlike whole body C/EBPalpha-null mice, mice lacking hepatic C/EBPalpha expression did not exhibit hypoglycemia, nor did they show reduced hepatic glycogen as adults. Expression of liver glycogen synthase, phosphoenolpyruvate carboxykinase, and G6Pase remained at normal levels. However, these mice exhibited impaired glucose tolerance due in part to reduced expression of hepatic glucokinase, and hyperammonemia from reduced expression of hepatic carbamoyl phosphate synthase-I. These mice also had reduced serum cholesterol and steatosis; these changes were increased along aging (77). We further showed that HGF induces expression of C/EBPalpha in isolated ALB<sup>+</sup> hepatic progenitor cells isolated from E13.5 fetal livers. Blocking C/EBPalpha activity by means of a dominant-negative C/EBP mutant inhibits the transition of ALB<sup>+</sup> hepatic progenitor cells to ALB<sup>+</sup> precursor cells (34). These results suggest that C/EBPalpha is important for differentiation of hepatic stem and progenitor cells but not for maintenance of adult liver function.

In contrast to HNF4alpha and C/EBPalpha, HNF6 is a key factor for hepatic stem cell differentiation into intrahepatic cholangiocytes. HNF6 is expressed in the epithelial cells of the developing intrahepatic bile ducts. The inactivation of HNF6 causes defects of the gallbladder and the extrahepatic bile ducts; prenatal intrahepatic bile duct differentiation is premature and excessive as well in HNF6-null mice (79). Interestingly, blocking C/EBPalpha in hepatic stem cells reduced expression of HNF6, while moderately inducing the biliary differentiation markers CK19 and gamma-glutamyltranspeptidase (34). These findings suggest that HNF6 is required for attenuation of commitment of early bile-duct progenitor cells and helps regulate stem cell bipotency.

Cell isolation technology has enabled prospective isolation of various somatic stem cells, including hepatic stem cells. Our system can isolate and identify fetal hepatic stem cells using *in vitro* and *in vivo* clonal analyses. *Ex vivo* expansion of fetal liver-derived hepatic stem cells, unlike hematopoietic stem cells, appears relatively easy. In the next step, if such stem cells can be found in fetal and adult human livers, they will be even more useful for stem cell therapy in several liver diseases, permitting virus-mediated gene transfer and serving as

theoretically unlimited sources of cells for transplantation or artificial liver structures.

## 7. ACKNOWLEDGMENT

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