Stem Cell Populations Giving Rise to Liver, Biliary Tree, and Pancreas

Mark E. Furth, Yunfang Wang, Vincenzo Cardinale, Guido Carpino, Giacomo Lanzoni, Cai-Bin Cui, Eliane Wauthier, David Gerber, Tsunekazu Oikawa, Juan Dominguez-Bendala, Luca Inverardi, Domenico Alvaro, Eugenio Gaudio, and Lola M. Reid

Abbreviations

The stem cell or progenitor cell populations are indicated by an acronym which is preceded by a small letter indicating the species: m = murine; r = rat; h = human.

AFP	Alpha-fetoprotein
CD133	Prominin 1
CFTR	Cystic fibrosis transmembrane conductance regulator
CK	Cytokeratin
C-PEP	C-peptide
CS-PG	Chondroitin sulfate proteoglycan
CXCR4	CXC-chemokine receptor 4
CYP450	Cytochrome p450
DS-PG	Dermatan sulfate proteoglycan
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule (CD326)
ES cells	Embryonic stem cells
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXA2	Forkhead box A2
GAG	Glycosaminoglycan
GCG	Glucagon
GFAP	Glial fibrillary acidic protein
HA	Hyaluronan
hBTSC	Human biliary tree stem cell

M.E. Furth, Ph.D.
Wake Forest Innovations, Wake Forest Baptist Medical Center, 391 Technology way, Winston Salem, NC 27101, USA
e-mail: mfurth@wakehealth.edu

Y. Wang, M.D., Ph.D.
The Stem Cell and Regenerative Medicine Lab, Beijing Institute of Transfusion Medicine, Beijing 100850, China
e-mail: wangyf1972@gmail.com

V. Cardinale, M.D. • D. Alvaro, M.D., Ph.D.
Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, 37, Viale dell'Università, 00185 Rome, Italy
e-mail: vincenzo.cardinale@uniroma1.it; domenico.alvaro@uniroma1.it

G. Carpino, M.D., Ph.D.
Department of Health Sciences, University of Rome "Foro Italico", Piazza Lauro de Bosis 15, 00194 Rome, Italy
e-mail: guido.carpino@uniroma1.it

G. Lanzoni, Ph.D. • J. Dominguez-Bendala, B.Sc., M.Sc., Ph.D.
L. Inverardi, M.D.
Diabetes Research Institute, University of Miami Miller School of Medicine, 1450 Northwest 10th Avenue, Miami, FL 33136, USA
e-mail: glanzoni@med.miami.edu; jdominguez2@med.miami.edu; linverar@med.miami.edu

C.-B. Cui, M.D., Ph.D.
Department of Surgery, University of North Carolina School of Medicine, 101 Mason Farm Road, Glaxo Building, Room 154, Chapel Hill, NC 27599, USA
e-mail: cuicb@yahoo.com

E. Wauthier • L.M. Reid, Ph.D. (✉)
Department of Cell Biology and Physiology and Program in Molecular Biology and Biotechnology, University of North Carolina School of Medicine, 101 Mason Farm Road, Glaxo Building, Room 32–36, Chapel Hill, NC 27599, USA
e-mail: elainew@med.unc.edu; Lola.M.Reid@gmail.com

D. Gerber, M.D.
Department of Surgery, University of North Carolina School of Medicine, CB#7211, 4025 Burnett-Womack Building, Chapel Hill, NC, 27599, USA
e-mail: david_gerber@med.unc.edu

T. Oikawa, M.D., Ph.D.
Department of Cell Biology and Physiology, University of North Carolina School of Medicine, 101 Mason Farm Road, Glaxo Research Building, Room 34, Chapel Hill, NC 27599, USA
e-mail: oitsune@gmail.com

E. Gaudio, M.D., Ph.D.
Department of Anatomical, Histological, Forensic Medicine and Orthopedics Sciences, Sapienza University of Rome, Via Borelli 50, 00161 Rome, Italy
e-mail: eugenio.gaudio@uniroma1.it

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HDM	Serum-free, hormonally defined medium
HGF	Hepatocyte growth factor
hHB	Human hepatoblast
hHpSC	Human hepatic stem cell
HNF	Hepatocyte nuclear factor
HP-PG	Heparin proteoglycan
HS-PG	Heparan sulfate proteoglycan
ICAM-1	Intercellular adhesion molecule-1
INS	Insulin
iPS	Induced pluripotent stem
KM	Kubota's Medium
LGR5	Leucine-rich repeat-containing G protein coupled receptor 5
MIXL1	Mix paired-like homeobox gene (expressed in primitive streak in embryos)
MUC6	Mucin 6, oligomeric mucus/gel-forming
NCAM	Neural cell adhesion molecule
NGN3	Neurogenin 3
PBG	Peribiliary gland
PCNA	Proliferating cell nuclear antigen
PDG	Pancreatic duct gland
PDX1	Pancreatic and duodenal homeobox 1
PROX1	Prospero homeobox protein 1
SALL4	Sal-like protein 4
SEM	Scanning electron microscopy
SMAD	Homolog of the *Drosophila* protein, mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein, SMA
SOX	Sry-related HMG box
TEM	Transmission electron microscopy
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial cell growth factor

Introduction

Liver, biliary tree, and pancreas are midgut endodermal organs central to handling glycogen and lipid metabolism, detoxification of xenobiotics, processing of nutrients for optimal utilization, regulation of energy needs, and synthesis of diverse factors ranging from coagulation proteins to carrier proteins (*e.g.*, AFP, albumin, transferrin). The integrity of the body depends heavily on liver, biliary tree, and pancreatic functions, and failure in any of them, especially the liver, results in rapid death. In recent years it has become apparent that these tissues comprise maturational lineages of cells that are in epithelial-mesenchymal cell partnerships. Each lineage tree begins with an epithelial stem cell (*e.g.*, hepatic stem cell) partnered with a mesenchymal stem cell (MSC) (*e.g.*, an angioblast). These give rise to cellular descendants that mature coordinately. The maturational process generates epithelial and mesenchymal cells that change stepwise with respect to their morphology, ploidy, growth potential, biomarkers, gene expression, and other phenotypic traits. More detailed presentation of the literature on the phenotypic traits of the biliary tree [1], pancreas [2], and of the liver [3] have been given in prior publications (Fig. 1). Moreover, the properties of maturational lineages in the biliary tree are not fully known as few studies have been completed. Here we note only a few examples of changes in the intrahepatic lineages to demonstrate the phenotypic gradients in phenotypic traits that can occur (Table 1).

The net sum of the activities of cells at the sequential maturational lineage stages yields the composite tissue. In this review we provide an overview of stem cell populations giving rise to liver, biliary tree, and pancreas. Several recently published reviews present further details [1, 3–5]. For the sake of brevity, we will not discuss studies involving the lineage restriction of embryonic stem (ES) cells or induced pluripotent stem (iPS) cells to a hepatic or pancreatic fate. This topic is covered elsewhere in the book (Chap. 10). In addition, we have focused this review almost entirely on studies of human tissues. Other chapters in the book address closely related endodermal stem and precursor cells for the stomach (Chap. 19), and others provide further information on stem cells on liver or pancreas (Chaps. 20, 22, 30, and 34) (Fig. 2).

Embryonic Development

During early development definitive endoderm derives from stem cells through the effects of a number of pluripotent transcription factors, including goosecoid, MIXL1, SMAD2/3, SOX7, and SOX17 [6]. Endoderm subsequently segregates into foregut (lung, thyroid), midgut (pancreas, biliary tree, and liver), and both foregut and hindgut (intestine), also through the effects of specific mixes of transcription factors. Those dictating the midgut organs include SOX9, SOX17, FOXA1/FOXA2, Onecut2/OC-2, and others [7–10] (Fig. 3). The liver, biliary tree, and pancreas derive from midgut endoderm established at the gastrulation stage of early embryonic development [11]. Among the other organs of endodermal origin, endogenous adult stem cells have been identified in most, including the small and large intestines [12], the stomach [13], and the lungs [14, 15]. The pancreas is distinct in that lineage tracing experiments indicate that there are only very rare stem cells in the postnatal organ [16–18]. Subsequently, we found evidence that pancreatic stem cells are not located within the organ itself but rather in the biliary tree, particularly the hepato-pancreatic common duct. These stem cells give rise to committed progenitors located in pancreatic duct glands (PDGs) [2].

The formation of the liver and pancreas occurs with outgrowths on either side of the duodenum that extend and ramify into a branching biliary tree structure that, at its end, engages the cardiac mesenchyme to form liver [19].

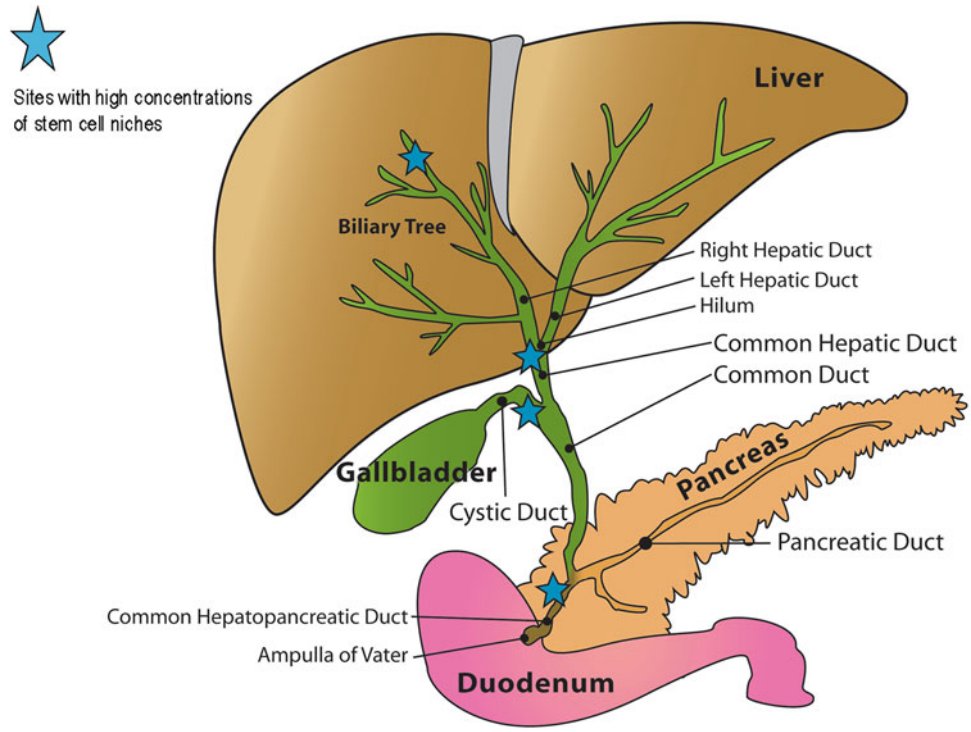

Fig. 1 Schematic of liver, pancreas, and biliary tree. Figure reproduced from Cardinale et al. (2012)

Table 1 Intrahepatic lineage-dependent phenotypic traits in human livers

Maturational lineage stage	Early (stages 1–4; zone 1)	Intermediate (stages 5–6; zone 2)	Late (stages 7–10; zone 3)
Cell size	7–9 μm—stem cells 10–12 μm—hepatoblasts 12–15 μm—committed progenitors 17–18 μm—adult cells	~20–25 μm	~25–35 μm
Ploidy	Diploid	Diploid, and some tetraploid depends on age	Tetraploid or higher
Proliferation	Hyperplastic growth (DNA synthesis with cytokinesis)	Hyperplastic growth and some hypertropic growth (depends on the extent of cytokinesis)	Hypertrophic growth (DNA synthesis with negligible cytokinesis)
Representative genes expressed	*Stem cells*: NCAM, EpCAM, CD44H (no AFP and little to no albumin), CS-PGs[a, d] *Hepatoblasts*: ICAM-1[a], EpCAM, AFP[a], CD44H, constitutive albumin[b], P450A7[a], HS-PGs[a, d] *Hepatocytes*: glycogen synthesis enzymes[a], CX 28[a], HS-PGs[d], partially regulatable albumin[b]	Transferrin[c], TAT[a], fully regulatable albumin[b]	P4503A4[a], glutathione-S-transferase, HP-PGs[d] factors associated with apoptosis[a]

AFP alpha-fetoprotein, *CD44* receptor for hyaluronans, *CS-PG* chondroitin sulfate proteoglycan, *CX* connexins (gap junction proteins), *Cyp450* cytochrome P450s, *HS-PG* heparan sulfate proteoglycan, *ICAM-1* intercellular adhesion molecule-1, *NCAM* neural cell adhesion molecule, *TAT* tyrosine aminotransferase

[a]Levels of expression are due to lineage-dependent activation of transcription

[b]Acquisition of relevant regulatory elements in transcription

[c]Translational mechanism(s)

[d]Posttranscriptional modifications (*e.g.*, in Golgi)

One of the branches connects the gallbladder to the biliary tree via the cystic duct. The branch closest to the duodenum forms the ventral pancreas. On the other side the ducts extend and connect to the dorsal pancreas. The formation of the intestine incorporates a twisting motion that swings the ventral pancreas anlage to the other side where it subsequently merges with the dorsal pancreas anlage to form the complete organ. The liver cannot swing to the

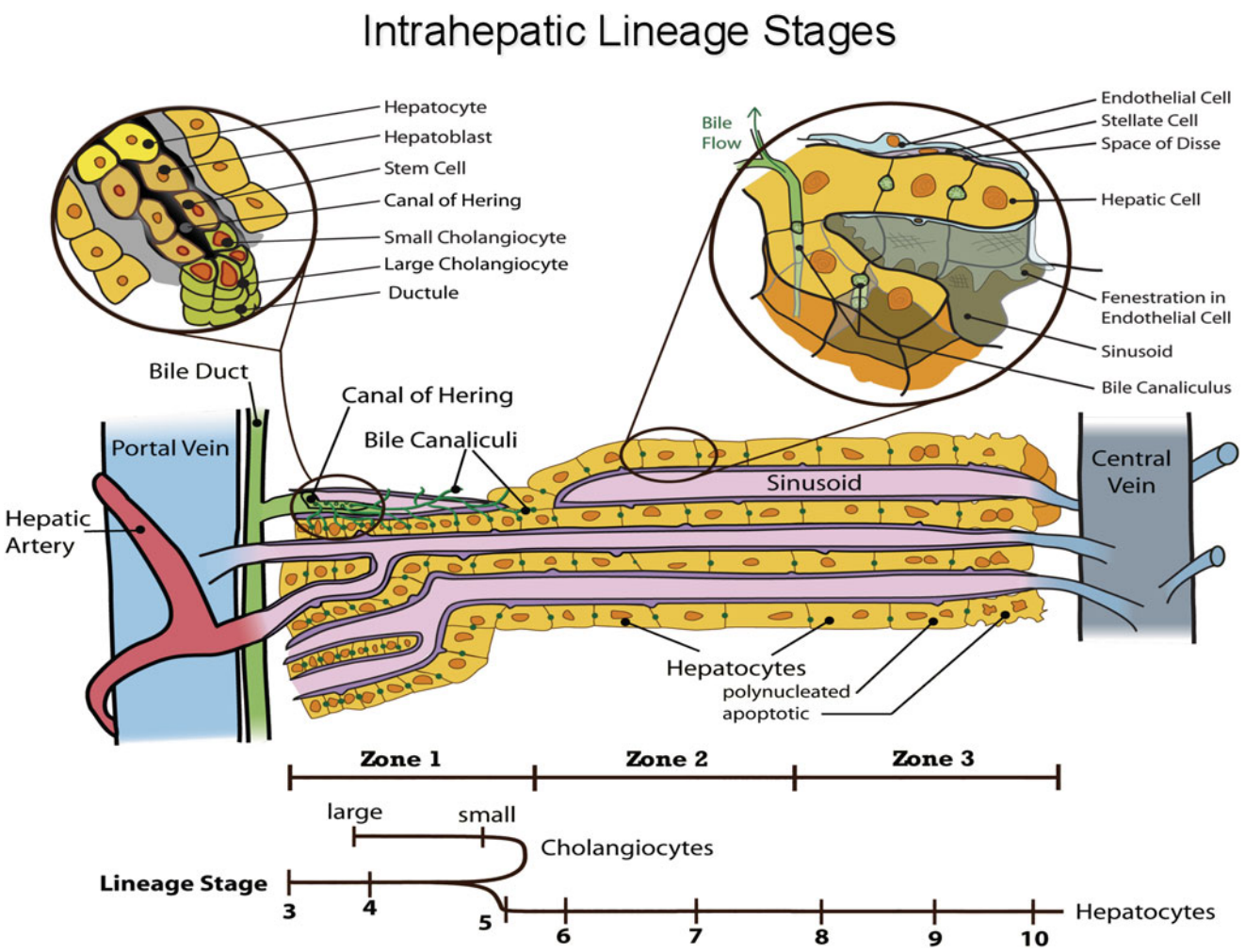

Fig. 2 Schematic of intrahepatic parenchymal lineages in the human liver. Figure is reproduced from Turner et al. (2011)

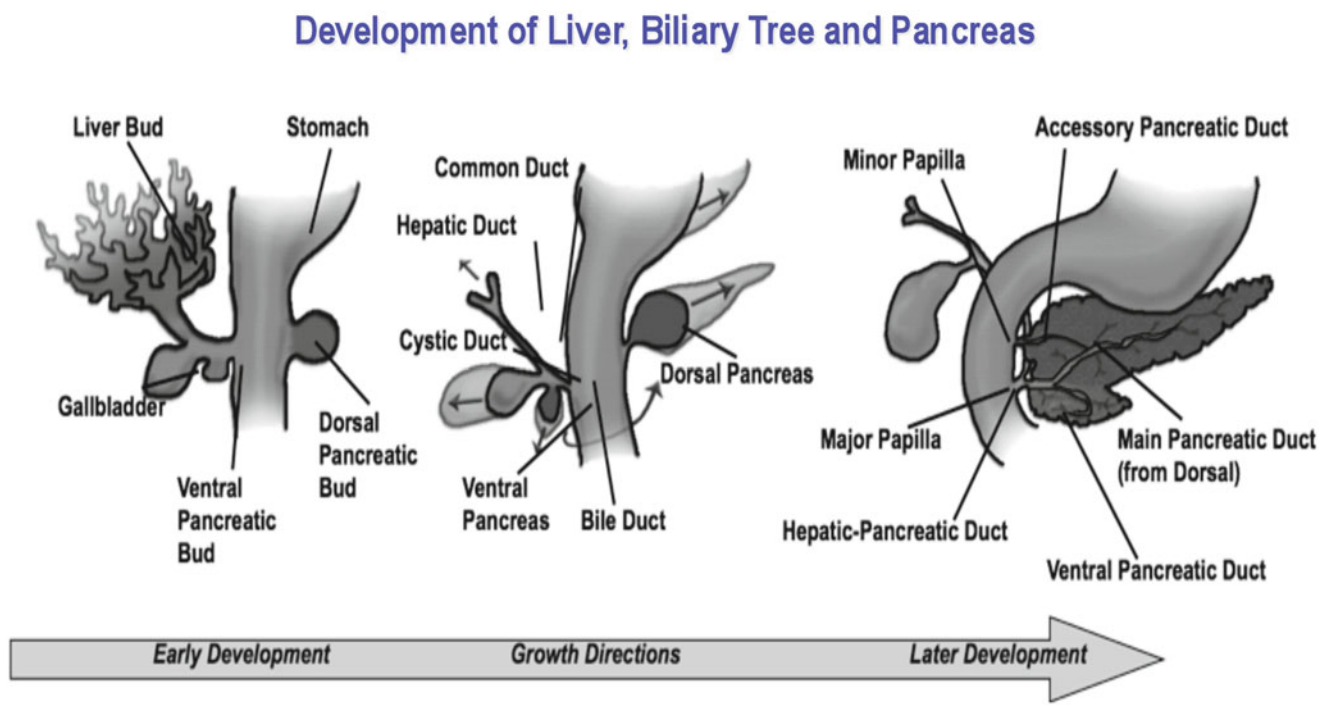

Fig. 3 Development of liver, pancreas, and biliary tree. Reproduced from Cardinale et al. (2012)

opposite side, given its size and its connections into the mesenchyme, connections which are associated with rapid vascularization of the forming tissue. This results in the liver and the ventral pancreas sharing the hepato-pancreatic common duct that connects them to the duodenum, while the dorsal pancreas has a separate connection to the duodenum.

Stem Cell and Progenitor Cell Niches

Stem and progenitor cells reside in discrete locations called niches, each with a unique environment [20] (see also Chaps. 1 and 4) (Fig. 4). The niches for the midgut organs include *peribiliary glands* (PBGs) in the extrahepatic and intrahepatic biliary tree; [2, 21, 22] *the ductal plates* in fetal and neonatal livers; the *canals of Hering*, which are derived from the ductal plates, in pediatric and adult livers; [23–25] and the PDGs [2, 26–28]. These niches form a network that is continuous throughout the biliary tree, with those in the biliary tree anatomically connecting directly to the canals of Hering within the liver and to the PDGs, the reservoirs of committed progenitors, within the pancreas. Many of the cellular components of the niches are known. Stem cell niches comprise epithelial stem cells and their mesenchymal cell partners, angioblasts. Transit amplifying niches or committed progenitor cell niches comprise epithelial transit amplifying cells and committed progenitors and their mesenchymal cell partners, precursors to endothelia, stellate cells, or stromal cells. Paracrine signaling between the epithelial and the mesenchymal cells is essential for viability, proliferative potential, and specilized cell functions. It can be mimicked *in vitro* by use of feeder cells of the relevant mesenchymal type, or by defined mixes of matrix components and soluble signals. To date, the matrix and soluble signals in the stem cell and progenitor cell niches have been only partially defined [29–33]. The known components are listed in Table 2.

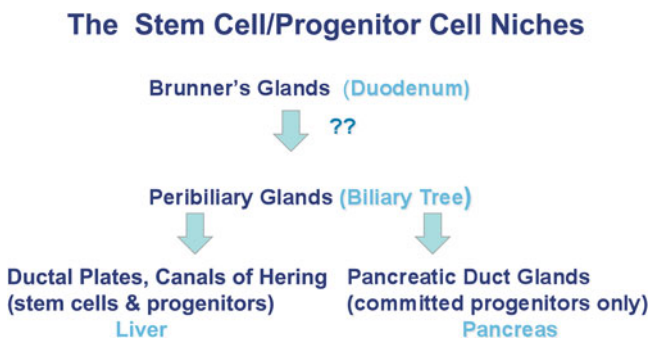

Fig. 4 Network of stem and progenitor cell niches in liver, biliary tree, and pancreas

There are hints, but no proof, that the network of niches begins with the Brunner's glands. These submucosal glands in the duodenum are located between the major papilla, the entranceway to the hepato-pancreatic duct, and the minor papilla, the port connecting the duodenum to the dorsal pancreatic duct. Brunner's glands are not found elsewhere within the intestinal tract. Indeed, they are used to define the transition from the duodenum to the beginning of the small intestine. Future studies should determine their possible relevance to the stem cell and progenitor cell niche network in the biliary tree, liver, and pancreas.

The PBGs occur throughout the biliary tree as *intramural glands*, found within the bile duct walls, and *extramural glands* that are tethered by extensions to the bile ducts [38]. PBGs occur in highest frequencies at the branching points of the biliary tree, with the greatest numbers found in the hepato-pancreatic common duct and the large intrahepatic bile ducts [1] (Fig. 5). Beyond pioneering studies of Nakanuma and associates [38–40], almost nothing is known of the roles of the extramural PBGs.

Each PBG contains a ring of cells at its perimeter and is replete with mucous (PAS-positive material) in its center. The cells in the ring are phenotypically quite homogeneous at some sites (*e.g.*, hepato-pancreatic common duct, large intrahepatic bile ducts) but heterogeneous at other sites (*e.g.*, cystic duct, hilum, common duct). The variations identified thus far implicate maturational lineages for which there are two axes: [22, 41]

- *A radial axis* starting with high numbers of primitive stem cells (characterized by elevated expression of pluripotency genes and other stem cell markers) located in PBGs near the fibromuscular layer in the interior of the bile ducts and ending with mature cells at the lumens of the bile ducts (Figs. 6 and 7)
- *A proximal-to-distal axis* starting with high numbers of primitive stem cells near the duodenum and progressing along the length of the bile ducts to mature cells near the liver or pancreas.

Thus, the radial axis in the biliary tree near the liver results in mature hepatic parenchymal cells. That near the pancreas results in mature cells of pancreatic fate. Radialaxes between liver and pancreas yield cells with mature bile duct markers. The PBGs connect directly into the canals of Hering, the intrahepatic stem cell niches, and at the level of the hepato-pancreatic common duct, the PDGs. The network provides a biological framework for ongoing organogenesis of liver, biliary tree, and pancreas throughout life.

These phenomena parallel the well-described intestinal lineage system. The radial axis of maturation in the intestine progresses from stem cells in the crypts to fully differentiated cells at the tops of the villi. The proximal-to-distal axis follows the length of the intestine and results in distinct

Table 2 Cellular and microenvironment components in precursor cell niches [25, 33–37] (see also Chap. 4)

A. Stem cell niches			
Cells types	Biliary tree and hepatic stem cells	Mesenchymal cell partners: angioblasts	
Markers	NCAM, CD133, LGR5, SOX9, SOX17, PDX1, pluripotency genes that include Nanog, OCT4, SOX2, KLF4, SALL4	CD117, CD133, VEGF-receptor, Von Willebrand factor	
Extracellular matrix components	Type III collagen, hyaluronans, minimally sulfated chondroitin sulfate proteoglycans, a form of laminin that binds to alpha6/beta4 integrin (laminin-5?)		
Soluble factors	Leukemia inhibitory factor (LIF), interleukin 11 (IL-11), and others still being identified		
B. Transit amplifying cell and progenitor cell niches			
Cell types	Hepatoblasts, hepatocytic and biliary committed progenitors, pancreatic committed progenitors	Mesenchymal cell partners: Stellate cell precursors	Endothelial cell precursors
Markers	EpCAM, ICAM-1, SOX9, no expression of pluripotency genes —AFP, albumin, CYP450A7 in hepatoblasts —Glycogen, albumin, CX28 in hepatocytic committed progenitors —Secretin receptor, CFTR in biliary committed progenitors —NGN3, MUC6, amylase, low levels of insulin, and other islet hormones in pancreatic committed progenitors	CD146, alpha-smooth muscle actin (ASMA), low levels of desmin, ICAM-1, but no GFAP and minimal levels of vitamin A	CD133, CD31, VEGF-receptor, Von Willebrand factor
Extracellular matrix components	Hyaluronans, type IV collagen, form(s) of laminin binding to alpha/beta1 integrin, sulfated forms of CS-PGs, and minimally sulfated form of HS-PGs		
Soluble factors	HGF, EGF, bFGF, IL-11, IL-6, and others		

AFP alpha-fetoprotein, *CFTR* cystic fibrosis transmembrane conductance regulator, *CS-PG* chondroitin sulfate proteoglycan, *EGF* epidermal growth factor, *FGF* fibroblast growth factor, *GFAP* glial fibrillary acidic protein, *HGF* hepatocyte growth factor, *HS-PG* heparan sulfate proteoglycan, *ICAM-1* intercellular adhesion molecule-1, *IL* interleukin, *MUC6* Mucin 6, oligomeric mucus/gel-forming, *NGN3* neurogenin 3, *VEGF* vascular endothelial cell growth factor

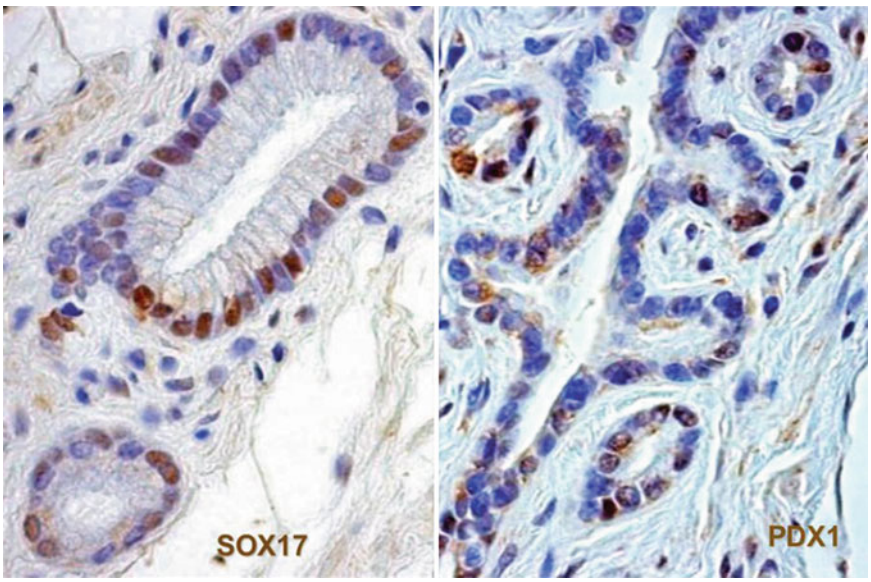

Fig. 5 PBGs in adult human biliary tree tissue stained for SOX17 or PDX1. Figure modified from Cardinale et al. (2011)

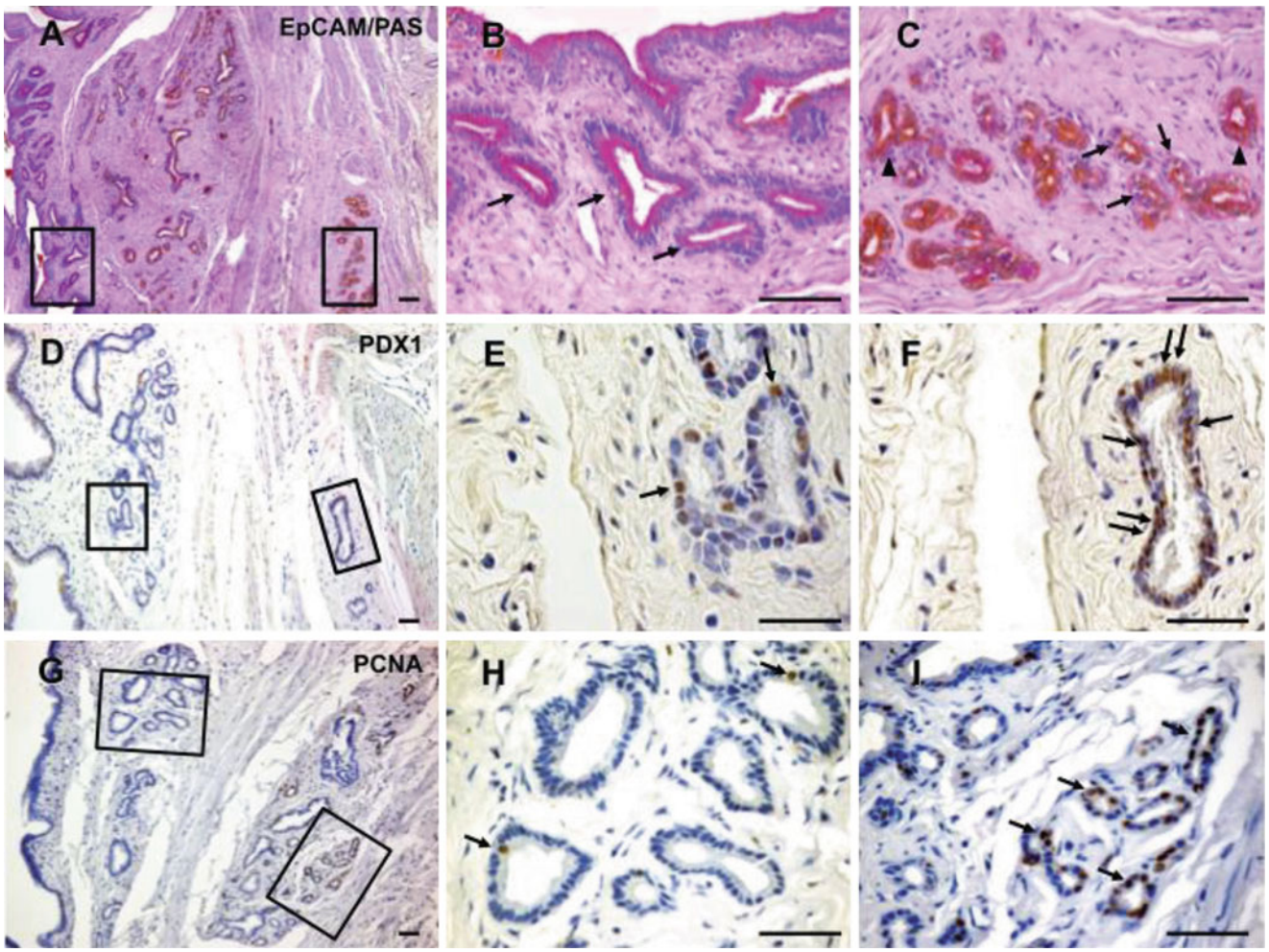

Fig. 6 Radial axis maturational (*blue*) lineage within bile ducts. Gradient in expression of stem cell markers and of cell proliferation in PBGs (*red*). (**a**–**c**) Immunohistochemistry for EpCAMs counterstained with PAS. Glandular elements just beneath the surface epithelium (see magnification in (**b**)) are mostly EpCAMs-negative and PAS-positive (goblet cells); by contrast, acini deeply located near the fibromuscular layer (see magnification in (**c**)) are composed of cells that are or [EpCAM+, PAS+] cells (*arrowhead*). a mix of [EpCAM−, LGR5+] and [EpCAM+, LGR5+] and PAS or negative (*arrows*). (**d**–**f**) Immunohistochemistry for PDX1. PDX1+ cells (*brown*) are mostly situated deeply within duct walls (see magnification in (**f**): *arrows*). PBGs near the surface epithelium are occasionally PDX1+ (*arrow* in (**e**)). (**g**–**i**) Immunohistochemistry for PCNA. Proliferating cells are mostly present in glandular elements located near the fibromuscular layer (*arrows* in (**i**)). Few cells are positive in more superficial acini. Notably, surface epithelial cells are (*brown*) mostly negative for PCNA. Scale Bar = 50 μm. Figure reproduced from Carpino et al. (2012)

mature cells depending on whether they are located in the esophagus, stomach, duodenum, small or large intestine.

The phenotypic changes in cells in the PBGs along the identifiable maturational lineages indicate the existence of multiple subpopulations of stem cells. Populations in PBGs at the start locations (near the fibromuscular layers in the interior of the bile ducts) and those near the duodenum have the highest numbers of stem cells that co-express endodermal transcription factors essential for liver and pancreas formation (*e.g.*, SOX9, SOX17, PDX1). These cells also express genes associated with pluripotency (Nanog, OCT4, SOX2, KLF4, SALL4) and other early lineage stage markers (NCAM, LGR5, CD133) or indicators of proliferation (*e.g.*, Ki67). Furthermore, they do not express detectably markers of mature cells (*e.g.*, insulin, albumin) [22, 41]. The PBGs between those with the most primitive stem cell traits and those with mature markers are characterized by cells with an intermediate phenotype: expression of epithelial cell adhesion molecule (EpCAM); some but not all of the endodermal transcription factors (*e.g.*, PDX1 or SOX17, but not both); less or negligible amounts of the pluripotency genes; fewer, if any, of the other stem cell traits (*e.g.*, LGR5 or CD133); and low but detectable expression of one or more mature cell markers (*e.g.*, albumin or insulin). The extent of expression

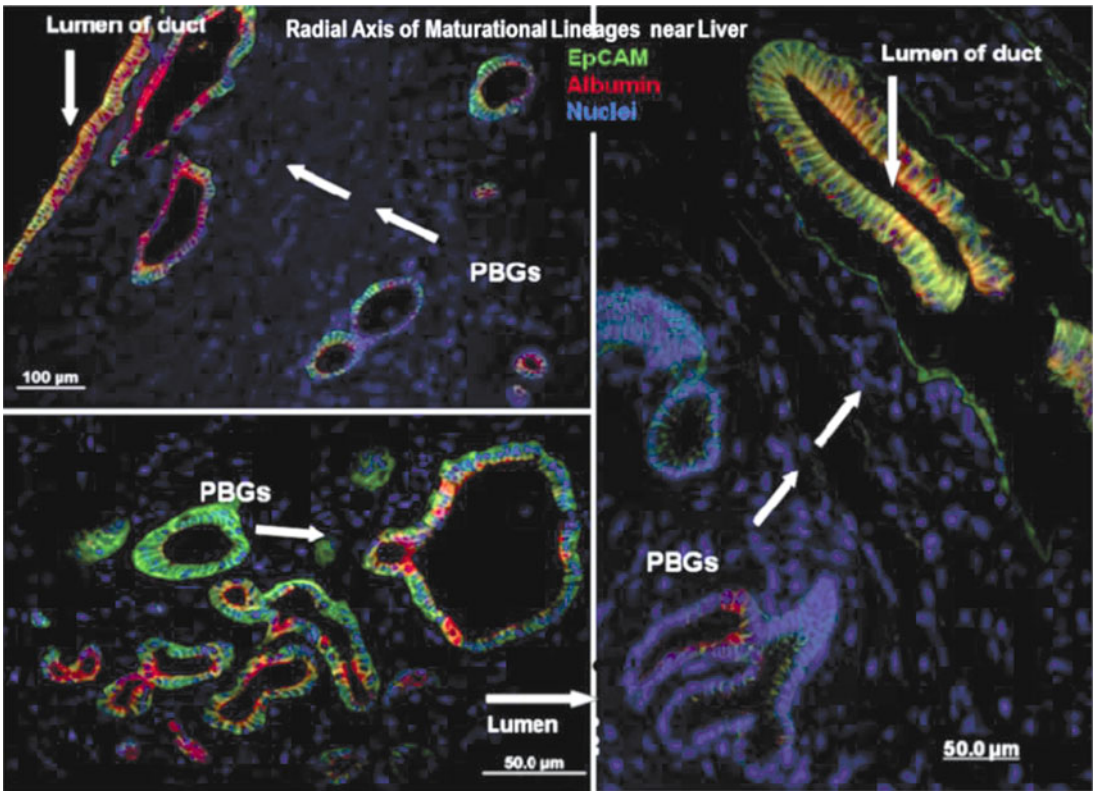

Fig. 7 Radial axis maturational lineage near liver. The markers change from those found in the PBGs near the fibromuscular layer to those found in cells at the lumens of the bile ducts. Here are images tracking two markers, albumin and EpCAM, from the PBGs to the luminal surface of the ducts. Note that some of the cells within the PBGs express EpCAM or albumin, while some do not. With progression towards the surface, there are PBGs in which all of the cells express EpCAM but may be devoid of albumin expression. Finally, the cells at the lumen of the ducts express both EpCAM and albumin. We hypothesize that these findings are evidence for a maturational lineage progressing from the PBGs deep within the walls of the bile duct to the cells at the luminal surface of the ducts and that EpCAM is an intermediate marker and albumin a more mature marker for the cells that are maturing towards a liver fate. This occurs in the portion of the biliary tree closest to the liver. Figure reproduced from a figure in the online supplement of Cardinale et al. (2011)

of the mature lineage markers increases with proximity to the bile duct lumens and to the liver or the pancreas (Figs. 6, 7, and 8).

The *in situ* studies summarized above have been complemented by *in vitro* studies of the biliary tree stem cells, hepatic stem cells, hepatoblasts, and committed progenitors of liver or pancreas. More details on those in the liver are given later in this review. Here, we will summarize observations on biliary tree stem cells (Fig. 9).

These cells can be dispersed into a cell suspension and grown on culture plastic or on certain extracellular matrix components and in *Kubota's Medium*, a serum-free formulation tailored for culture selection and expansion of endodermal stem cells and progenitors. The same medium has proven useful also for angioblasts and their descendants [1, 29, 31, 33, 42] (Fig. 10). Kubota's Medium comprises any rich basal medium with low calcium (~0.3 mM), no copper, selenium (~10^{-10} M), zinc (~10^{-12} M), insulin (~5 µg/mL), transferrin/fe (~5 µg/mL), high-density lipoprotein (~10 µg/mL), and a defined mixture of purified free fatty acids bound to highly purified albumin. Notably, the medium contains no cytokines or growth factors. Mature cells do not survive in Kubota's Medium, only the stem cells and progenitors from both epithelial and mesenchymal cell lineages. Thus, it co-selects for endodermal stem cells and progenitors and their mesenchymal stem/progenitor cell partners, angioblasts and their descendants, precursors of stellate cells or endothelia [29, 33].

We observed two major types of biliary tree stem cell colonies in cultures. Type 1 colonies have cells that undulate ("dancing cells"), are very motile, and initially do not express EpCAM (CD326) but acquire it at the edges (the perimeters) of the colonies, corresponding to slight cellular differentiation.

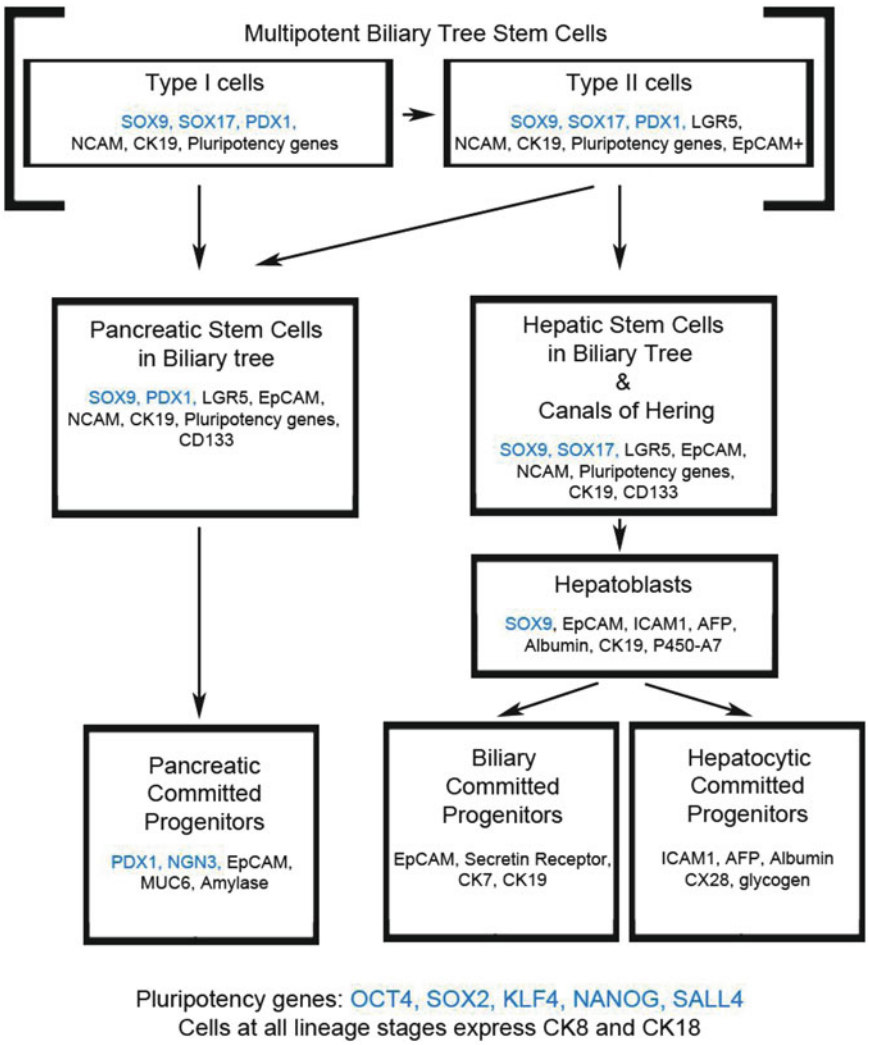

Fig. 8 Major subpopulations of stem cells and progenitors in liver, biliary tree, and pancreas. It is unknown at this time whether the pancreatic stem cells, located within the biliary tree, derive from type I or type II biliary tree stem cells or both. Although the two populations of biliary tree stem cells and pancreatic stem cells are present in highest numbers in the hepato-pancreatic common duct, they are found also in large numbers in the PBGs of the large intrahepatic bile ducts (figure prepared with information from Carpino et al. 2012; Wang et al. 2012)

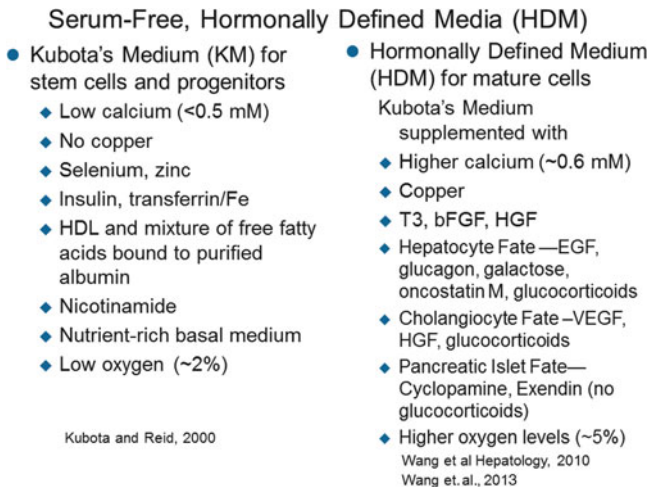

Fig. 9 Serum-free, hormonally defined media (HDM) used for expansion or for differentiation of stem/progenitors. They include Kubota's Medium used for stem/progenitors and HDM tailored to a mature cell type

The type 1 colonies are precursors to type 2 colonies. The latter show uniform expression of EpCAM from the outset and display a carpet-like appearance with cells of uniform morphology. The expansion potential of the cells in culture in Kubota's Medium is considerable: 2–3 cells can grow to colonies of more than 500,000 cells in ~8 weeks [1]. The cells retain a stable stem cell phenotype (i.e., self-renew) throughout months of culture and may be subcultured ("passaging"). Initially cells show a typical division time of about 1–2 days, but within a week, they slow to a division every 2–3 days. At 8 weeks the colonies contain cells in the centers that are morphologically uniform, are small (7–9 μm), and express high levels of stem cell markers. Cells at the edges of the large colonies are slightly larger (~10–12 μm) and have weak expression of EpCAM and expression of markers intermediate in the differentiation pathways, indicating potential loss of stemness and transition to more mature progenitors.

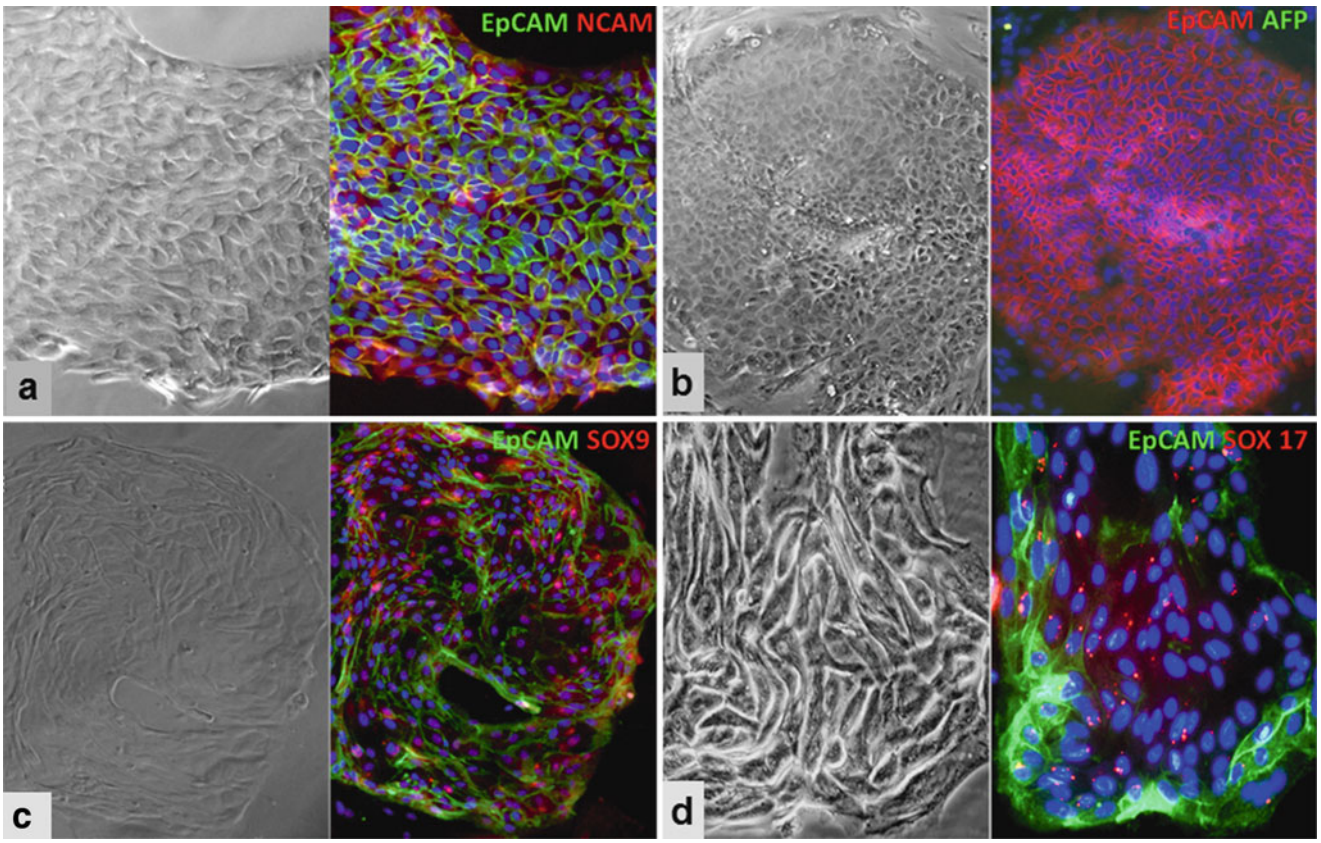

Fig. 10 There are two types of biliary tree stem cell colonies. Type II colonies (**a**, **b**) contain cells those that express EpCAM on every cell from the outset. Type I colonies (**c**, **d**) are those that are EpCAM negative initially but acquire expression of EpCAM at the perimeter of the colonies. Reproduced from Cardinale et al. (2011)

Using three-dimensional (3D) hydrogels and appropriate signaling molecules, the biliary tree stem cells can be induced to differentiate to hepatocytes, cholangiocytes, or pancreatic neo-islets [1]. We have not done studies yet to learn if they can give rise to acinar cells. The differentiation is achieved by embedding the stem cells in specific mixes of extracellular matrix components (hyaluronans and type I collagen for bile ducts, hyaluronans and type IV collagen and laminin for hepatocytes or islets) and providing a serum-free, hormonally defined medium (HDM) tailored for a specific mature cell type. The HDM are prepared by supplementing Kubota's Medium with copper (10^{-12} M), higher calcium (0.6 mM), and bFGF (10 ng/mL) and then adding a unique set of hormones and growth factors for hepatocytes (*HDM-H*, glucagon, galactose, T3, oncostatin M, hepatocyte growth factor (HGF), epidermal growth factor (EGF), glucocorticoids), cholangiocytes (*HDM-C*, HGF, EGF, VEGF, glucocorticoids), or pancreatic islets (*HDM-P*, B27, ascorbic acid, cyclopamine, retinoic acid, HGF, and, after 4 days, replacement of bFGF with Exendin-4). Further optimization of these conditions is underway.

The gene expression profiles of cells in the 3D hydrogels complemented the morphological observations. For example, cells cultured under conditions for hepatocytes produced albumin, transferrin, and P450s. Cells in conditions for cholangiocytes expressed anion exchanger 2 (AE2), cystic fibrosis transmembrane conductance regulator (CFTR), gamma glutamyl transpeptidase (GGT), and secretin receptor. Cells in conditions for pancreatic islets expressed transcription factor PDX1 and the hormones glucagon, somatostatin, and insulin. Specific staining for human C-peptide confirmed *de novo* synthesis of proinsulin, and its secretion was regulated in response to the level of glucose. *In vivo* studies provided further evidence for the multipotency of the human biliary

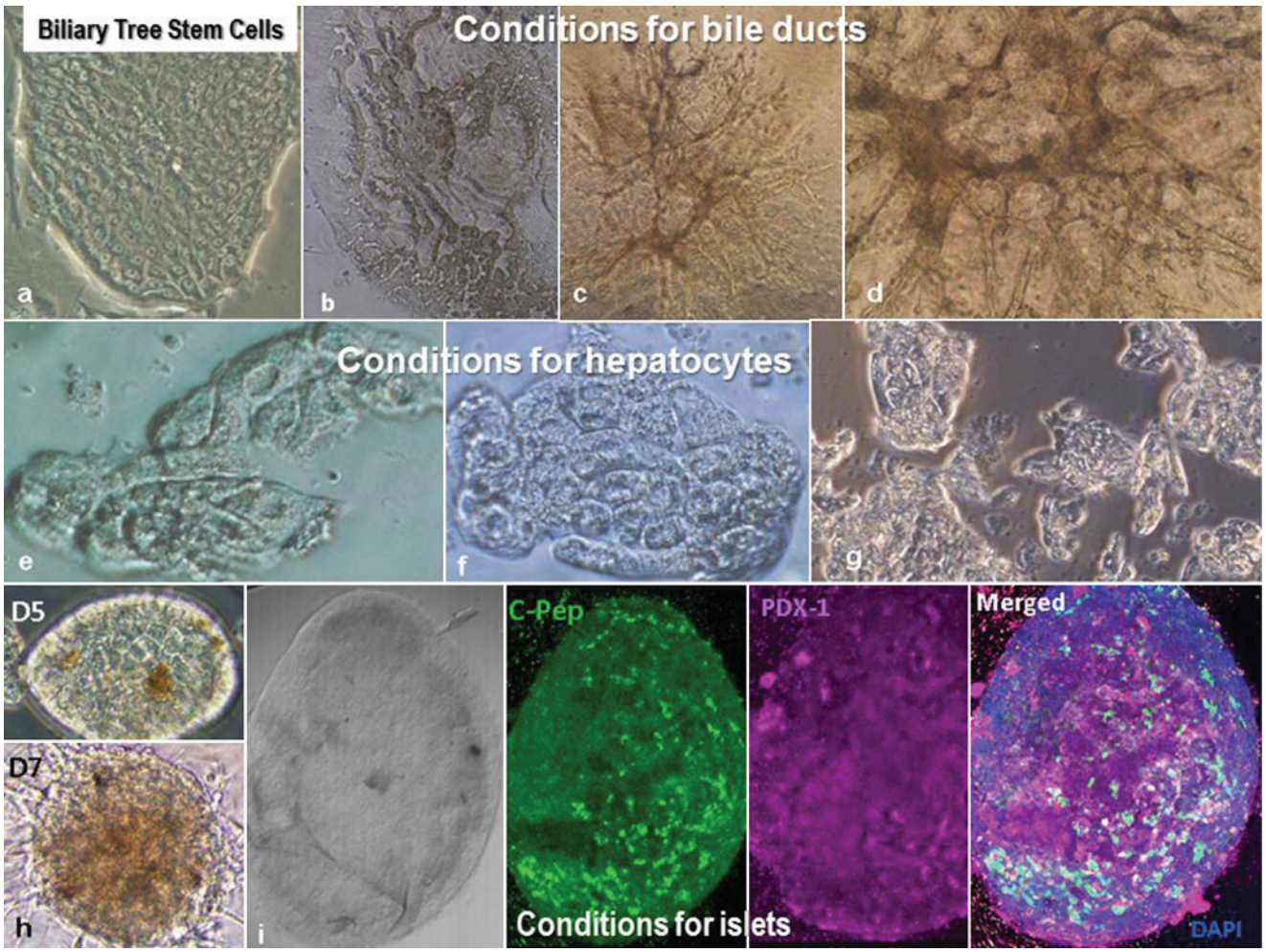

Fig. 11 Self-replication of biliary tree stem cells in Kubota's Medium (**a**). Lineage restriction of the biliary tree stem cells to bile ducts (**b**–**d**) vs. hepatocytes (**e**–**g**) vs. pancreatic islets (**h**–**l**). In (**h**) are shown two images of a neo-islet stained for zinc, a co-factor in insulin synthesis. In (**i**) is a phase contrast image of the hydrogel that was then stained for C-Peptide (**j**), PDX1 (**k**) and a merged image of (**j**, **k**). Figure is modified from a figure in Cardinale et al. (2011)

stem cells for hepatic, biliary tree, and pancreatic fates. Direct injection of the stem cells into the livers of immune-deficient mice generated mature human hepatocytes and cholangiocytes (Fig. 11).

To confirm endocrine pancreatic differentiation, pre-induced neo-islet structures were implanted into mouse fat pads, and the animals were treated with a toxin (streptozotocin) at a dose sufficient to destroy their own pancreatic beta cells, but not human beta cells. Those mice transplanted with the human neo-islets showed significant resistance to hyperglycemia compared to controls that did not receive cell therapy. The presence of functional beta-like cells derived from the biliary tree stem cells produced serum levels of human C-peptide, which was regulated appropriately in response to a glucose challenge [1] (Fig. 12). Further studies have confirmed and expanded upon these initial findings, leading us to conclude that the hepato-pancreatic common duct is the major reservoir of stem cells giving rise to committed progenitors found in PDGs and thence to pancreatic islets throughout life [41]. Ongoing studies are testing whether the maturational lineage involves a migration of cells or, as in the intestine, a type of "conveyer belt" leading to mature cells. See Table 3 for markers occurring at varying stages along the proximal-to-distal axes.

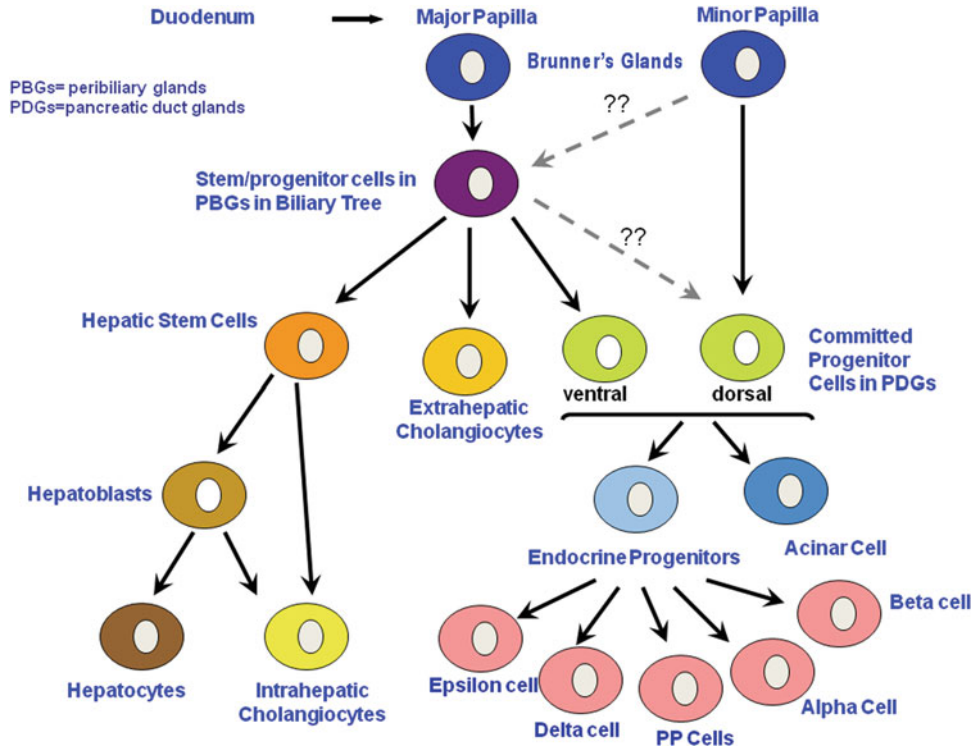

Fig. 12 Schematic of probable lineage pathways for stem cells and progenitors in the biliary tree. Figure is from a review by [1]

Table 3 Comparison of markers of stem/progenitor cells in liver, biliary tree, and pancreas (reproduced from a table in the online supplement of [2]

Proximal-to-distal axis of the maturational lineages						
	Liver		⟷			Pancreas
Cells	Hepatoblasts—adjacent to Canals of Hering [25, 29]	Hepatic stem cells in Canals of Hering [3]	Biliary tree stem cell subpopulations in peribiliary glands (PBGs) [2, 21, 22, 64]			Pancreatic committed progenitors in pancreatic duct glands (PDGs) [2]
Endoderm markers	SOX9	SOX9, SOX17	SOX9, SOX17	[a]SOX 9, SOX17, PDX1	SOX9, PDX1	SOX9, PDX1
Epithelial markers			CK8 and 18, CK19, E-cadherin			
Cell adhesion molecules	αβ1 integrin, ICAM-1 EpCAM	α6β4 integrin [33], NCAM, EpCAM	NCAM, EpCAM	NCAM	NCAM, EpCAM	Integrins [100] EpCAM [101]
			Integrins not yet studied			
Pluripotency genes[b]	None	moderate	Strong OCT4A, SOX2, NANOG, KLF4			None [102, 110]
Other stem cell markers	Weak CXCR4, CD133, SALL4	Strong CXCR4 CD133, LGR5, SALL4 CD117 [31][c]	CXCR4, CD133, SALL4, LGR5			CXCR4 [106], CD133 [104, 107] CD24 [105]
Hedgehog proteins	Weak Indian and Sonic [32]	Strong Indian and Sonic [32]	Indian; Sonic hedgehog expressed			Weak Sonic [102, 104, 111]
Matrix proteins	Laminin, type IV collagen [33]	Laminin-5, type III collagen [33, 62]	Not tested			Fetal islets have collagen IV, V, VI, nidogen, elastin; fetal acinar cells have primarily fibrillar collagens, fibronectin
GAG/PGs	HA, CD44, syndecans, and CS-PGs [33, 66, 67]	HA, CD44, minimally sulfated CS-PGs [33, 103]	HA, CD44; others not yet tested			HA, CD44, fetal islets have syndecans (HS-PG-1 and 3), glypicans; fetal acinar cells have primarily CS-PGs (Wang and Reid, unpublished studies)

(continued)

Table 3 (continued)

Liver traits	Albumin++, AFP+++, P450A7, Glycogen [31]	Albumin+/−, AFP− [31]	None of the mature traits	None
Pancreatic traits	None	None	ISL1, PROX 1, NeuroD, PAX4	NGN3, MAFA, MUC6, Nkx6.1/ NKx6.2 (Nkx6) and Ptf1a [108, 112], GLUT2 [109]
Multidrug resistance genes	MDRI-negative ABCG2-moderate [64]	MDRI-1 moderate, MVR-1 Strong ABCG2 Strong [64] ABCG2 [64]		None

PBGs peribiliary glands, *PDGs* pancreatic duct glands, *HA* hyaluronans, *HS-PGs* heparan sulfate proteoglycans, *CS-PG*$_S$ chondroitin sulfate proteoglycans, *Syndecans* HS-PGs that have transmembrane core proteins, *Glypicans* HS-PGs linked to plasma membrane by PI linkages, *MDRI* multidrug resistance genes

[a]These biliary tree stem cells are the most primitive and found near the fibromuscular layer within the bile ducts; they give rise in the radial axis maturational lineage to EpCAM+ cells

[b]*Pluripotency genes* = OCT4, NANOG, KLF4, SOX2

[c]*CD117* is found in canals of Hering and present on angioblasts that are tightly bound to the hepatic stem cells; it is hypothesized to be found in the PBGs in association with the various stem cell subpopulations. *Hepatoblasts*, transit amplifying cells, give rise to *hepatocytic and biliary committed progenitors* that do *not* express SOX17, pluripotency genes, LGR5, or other markers of stem cells. See also Fig. 8

Hepatic Stem Cells

Those familiar with the myth of Prometheus will recall that the liver possesses a remarkable capacity for regeneration [43]. Yet liver diseases, potentially leading to organ failure due to hepatitis viruses, alcohol consumption, diet and metabolic disorders, and other causes, constitute a major medical burden [44–46] (Fig. 13).

Cell-based therapies and tissue engineering represent possible approaches to address these needs [3, 47–50]. Sourcing of cells for such applications is a significant challenge. In some countries it is possible to obtain fetal tissues. In others neonatal or adult tissues can be used. Given the newly discovered source of stem cell population in the biliary tree, this tissue represents a major potential source of the stem cells for cell therapy and tissue engineering for both liver and pancreas.

Here we will focus specifically on stem/progenitor cells of the liver and biliary tree as they pertain to formation of liver (Fig. 14). The role(s) of stem cells in the normal maintenance of the liver and in regeneration from various insults remains a subject of active research and debate [23, 24, 43, 49, 51–56]. This section of the review focuses more on hepatic stem cells (hHpSCs) and hepatoblasts (hHBs) and what is known of their location and involvement in quiescent vs. regenerative liver tissue.

There are multiple stem cell populations located within the peribiliary glands of the intrahepatic bile ducts in livers of all donor ages [22] and in the ductal plates in fetal and neonatal livers [25, 57, 58]. The ductal plates transition to become canals of Hering in pediatric and adult livers; they consist of small ductules located at each of the portal triads [24, 25, 59, 60]. The canals of Hering give rise to the organ's two specialized epithelial cell types, hepatocytes and cholangiocytes (bile duct cells), via an organized maturational lineage system [3].

Hepatic Stem Cell Isolation and Expansion

We reported several years ago on the isolation of human hepatic stem cells (hHpSCs) from fetal, neonatal, pediatric, and adult human livers by selection with a monoclonal antibody for the surface marker EpCAM [31] (Fig. 15). These cells constitute approximately 1 % (0.5–1.5 %) of the total liver population from early childhood onwards. Unlike mature hepatocytes, they survive extended periods of ischemia, allowing collection even several days after cardiac arrest [61]. The hHpSCs express additional surface markers often found on stem/progenitor cells, such as CD133 (prominin), CD56 (neural cell adhesion molecule, NCAM), and CD44 (the hyaluronan receptor); they also express characteristic endodermal transcription factors SOX9, SOX17, and HES1. They are small (diameter 7–9 μm, which is less than half that of mature parenchymal cells) and express weak or negligible levels of adult liver-specific functions such as albumin, cytochrome P450s, and transferrin. The stem cells display far greater capacity to proliferate in culture than hepatocytes or cholangiocytes and can continue to expand for months with a doubling time of 36–40 h. The colonies that form look remarkably similar to those of embryonic stem (ES) cells or iPS cells [31, 33, 41, 62].

The hHpSCs serve as immediate precursors of hepatoblasts. The hepatoblasts are readily distinguished by the expression of α-fetoprotein and intercellular adhesion molecule-1 (ICAM-1), for which the hHpSCs are negative [25, 31, 33] (Fig. 16). The hepatoblasts, in turn, are precursors of committed unipotent progenitors for hepatocytes and cholangiocytes. When injected into the livers of immune-deficient mice, the hHpSCs give rise to cells expressing characteristic human liver and bile duct proteins, especially after the host's liver has been damaged by treatment such as with carbon tetrachloride.

Whereas there has been limited success to achieve *ex vivo* expansion of hematopoietic stem cells, the stem cell

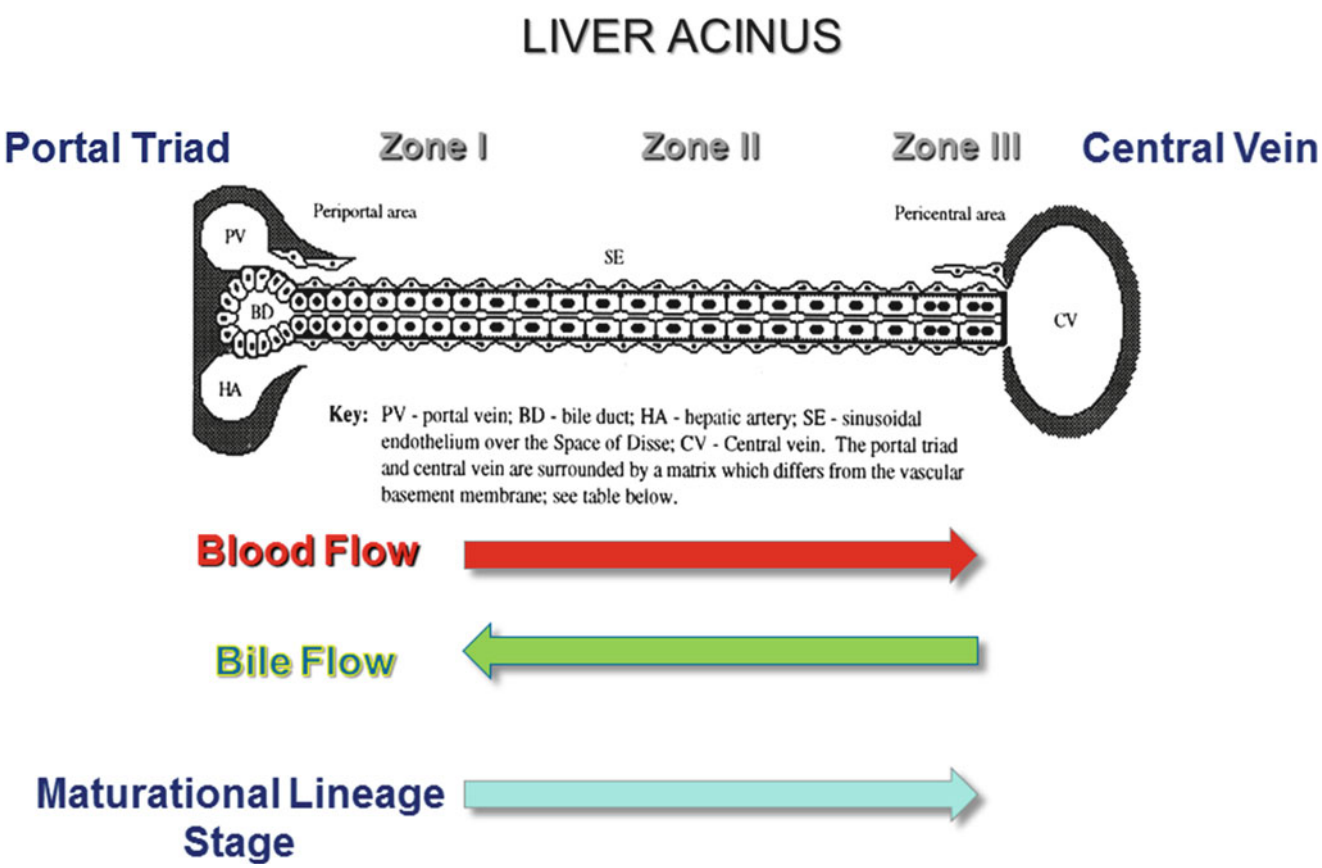

Fig. 13 Schematic of the liver acinus encompassing portal triads to central vein and showing direction of blood flow, bile flow, and maturational lineage. Modified from a figure in Reid et al. (1992)

populations of the hepatic lineages proliferate rapidly and for a sustained period in Kubota's Medium [29] which, as stated previously, contains no additional growth factor or cytokine. Conceivably, pathways important for hepatic stem/progenitor cell survival *in vivo*, such as Hedgehog (Hh) signaling [32], are activated through autocrine loops. The expanded hHpSCs maintain a stable marker phenotype and express the enzyme telomerase, whose mRNA and the protein encoded are localized to the nucleus in the hHpSCs and the hepatoblasts; telomeric enzymatic activity correlated well with both the mRNA and protein and with the protein being found within the nucleus (Fig. 17). However, later lineage stages (committed progenitors to late lineage stage mature cells) have no evidence of synthesis of telomerase but have large amounts of telomerase protein localized cytoplasmically. Telomeric enzymatic activity does not correlate with total telomerase protein levels. We hypothesize that regenerative demands will result in small amounts of the cytoplasmic reserves of telomerase relocating to the nucleus. If we are correct, the enzymatic activity levels should correlate with the amount of telomerase protein in the nucleus [63].

More recently, we have observed that Sal-like protein 4 (SALL4) is strongly expressed in the hBTSCs, hHpSCs, and hHBs but not in committed progenitors of either liver or pancreas [64]. SALL4 is a member of a family of zinc finger transcription factors and a regulator of embryogenesis, organogenesis, and pluripotency. It can elicit reprogramming of somatic cells and is a marker of stem cells. We found it expressed in normal mHBs, normal hHpSCs, hHBs, and hBTSCs, but not in committed hepatocytic or biliary progenitors and not in mature parenchymal cells of liver or biliary tree.

A crucial prerequisite for successful expansion of hHpSCs is to mimic an appropriate microenvironment. When selected for growth *in vitro* on tissue culture plastic and in Kubota's Medium, the hHpSCs grow as colonies with feeders of angioblasts (CD117+, VEGF-receptor+, CD133+, Von Willebrand Factor+); [31, 33, 37] the feeders can be replaced with weakly cross-linked hyaluronans and type III collagen [30, 33, 65]. The cells expand for months under these conditions. By contrast, hHBs survive for only about a week under the same conditions, but they can survive if they are co-cultured with stellate cell precursors (CD146+, alpha-smooth muscle actin+, desmin+, VCAM+, ICAM-1+, GFAP) or feeders of MSCs. The stellate feeder cells (or feeders of MSCs) can be replaced with hyaluronans, type IV collagen, and/or laminin [33, 66, 67]. The medium and matrix conditions

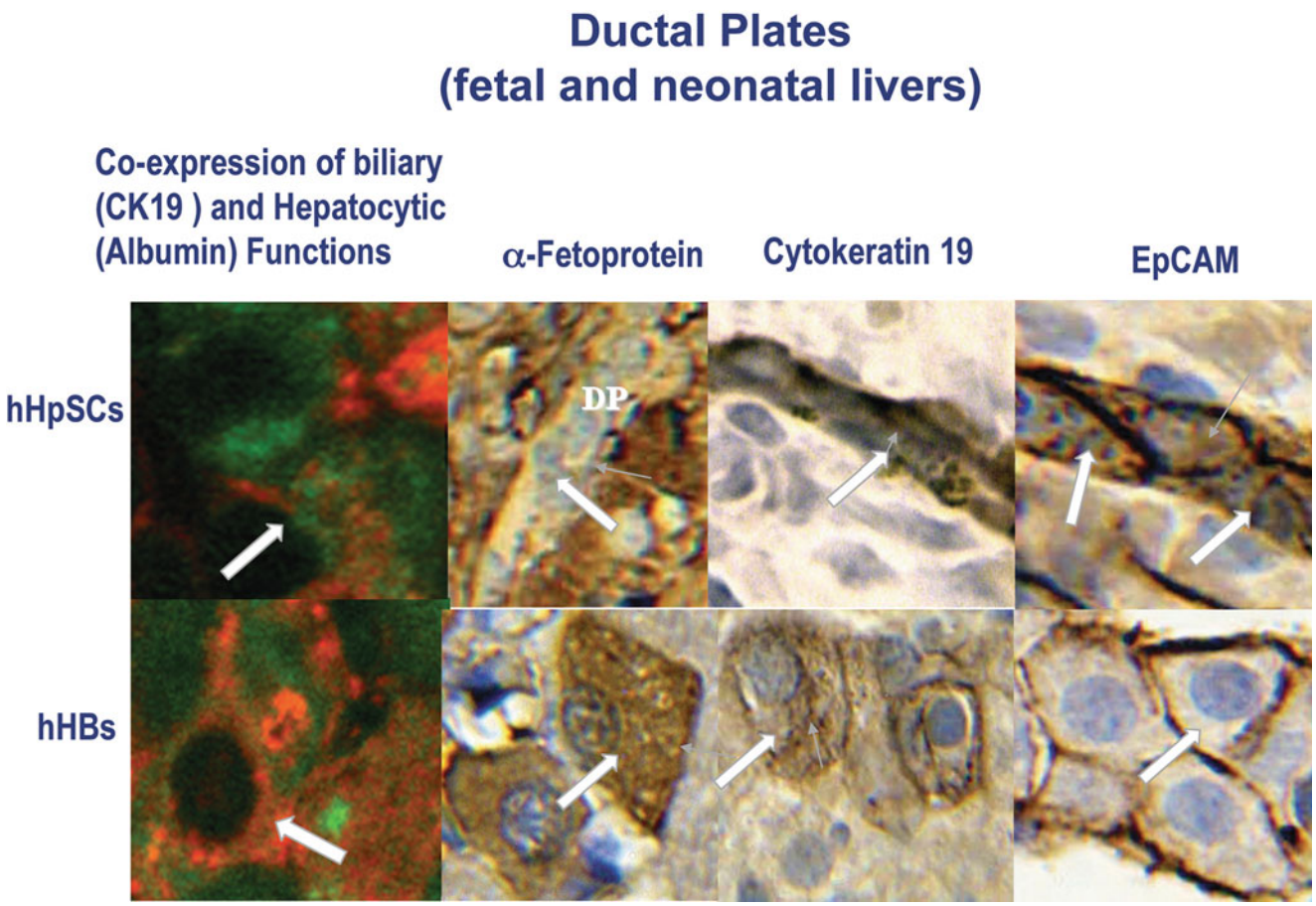

Fig. 14 Ductal plates found in fetal and neonatal livers with the location of hHpSCs and hHBs (see *white arrows*). Both co-express albumin and CK19. The hHBs, but not the hHpSCs, express alpha-fetoprotein (AFP). The expression of CK19 and EpCAM are more intense in hHpSCs. EpCAM in hHBs is confined primarily to the plasma membrane. Published information on the stem cell niches derived from Zhang et al. (2008); figure was first published in a methods review (Wauthier et al. 2008)

described above allow for flow cytometrically purified hHpSCs or hHBs to survive and proliferate in culture and without the need for feeders. Both type III collagen and hyaluronans are constituents of the normal liver stem cell niche [30, 33].

Conversely, 3D cultures in hyaluronans supplemented with other matrix components, and used in combination with serum-free medium supplemented with specific hormones and growth factors (HDM-H or HDM-C), result in differentiation of the cells. The HDM for driving the stem cells to a mature fate consists of Kubota's Medium supplemented with copper (10^{-12} M), calcium (0.6 mM), basic fibroblast growth factor (10 ng/mL), and glucocorticoids (10^{-8} M) and further tailored for hepatocytes with supplementation of glucagon, galactose, triiodothyronine (T3), oncostatin M, EGF, and HGF; an HDM for cholangiocytes contains HGF, vascular epithelial growth factor (VEGF), and EGF [33]. The matrix components used are hyaluronans into which are mixed network collagens (type IV, type VI) and laminin for hepatocytes vs. type I collagen or type I collagen and fibronectin for cholangiocytes.

The hHpSCs also respond to mechanical forces. Initially, it was apparent that hHpSCs grew better on transwells coated with type III collagen rather than hard plastic surfaces with the same coating [30, 62]. A systematic study of hHpSC behavior in 3D cultures using hyaluronan hydrogels of differing stiffness indicated that rigidity of the microenvironment is an important parameter in regulating maintenance of stemness vs. differentiation to more restricted progenitors [68]. This had been studied previously in differentiation of progenitors for bone and other hard tissues, but not for internal organs such as the liver. The optimal expansion of the hHpSCs for clinical applications likely will be achieved in 3D hydrogels containing type III collagen, hyaluronans, and possibly additional matrix components or synthetic mimetics.

The hHpSCs, like human ES cells, grow in tight colonies. Dissociating either type of stem cells has proven to be an

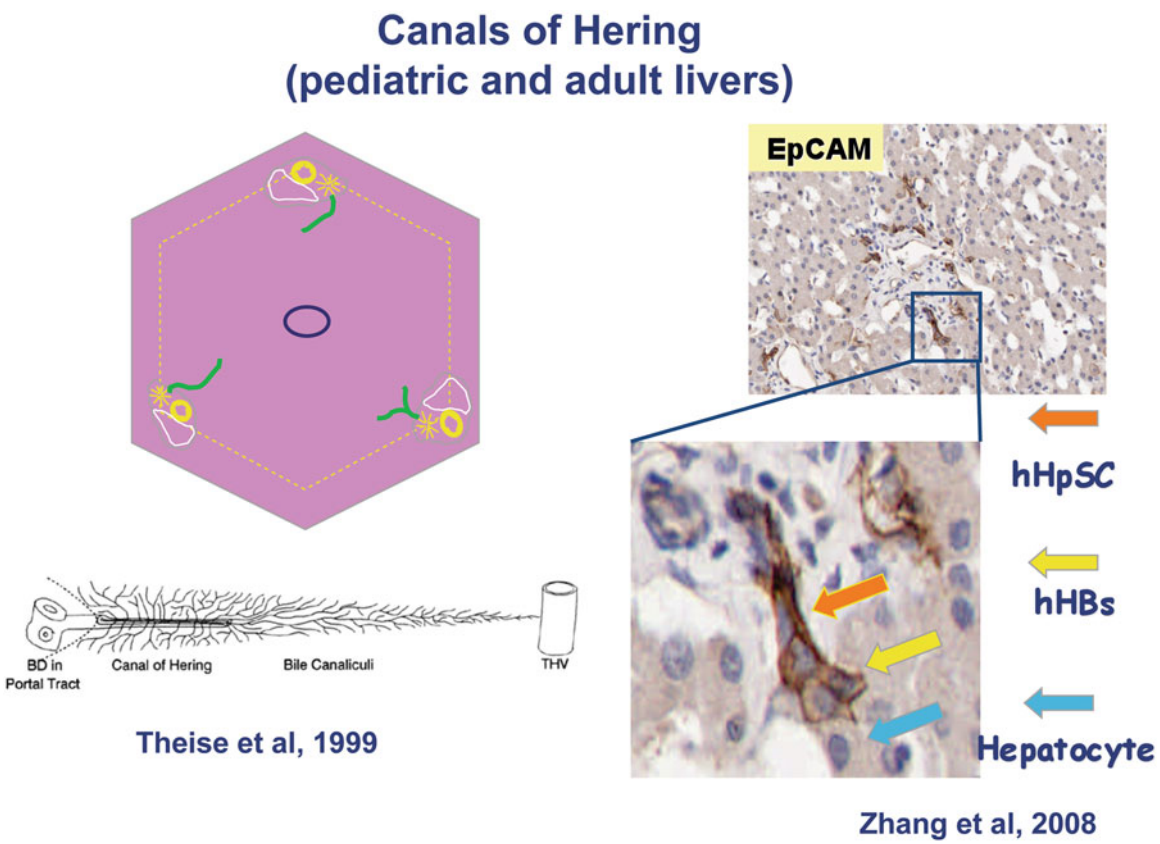

Fig. 15 Canals of Hering in pediatric and adult livers. From Zhang et al. [25] and Theias et al. [24]

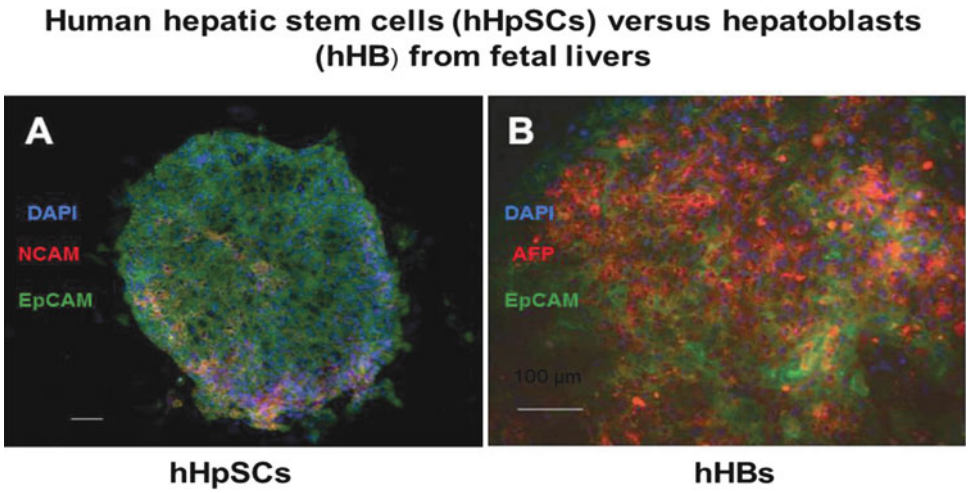

Fig. 16 Colony of hHpSC vs. an hHB colony from human fetal livers. The colonies were stained for EpCAM (*green*) and DAPI (*blue*). The hHpSCs do not express alpha-fetoprotein at all but rather express NCAM (shown in *red*). By contrast, hHBs express alpha-fetoprotein (*red*) and ICAM-1 (not stained). Figure is reproduced from Wang et al. (2010)

important practical problem for their efficient expansion *ex vivo* and for cryopreservation [69]. When treated enzymatically to generate a single cell suspension, both of these stem cell types undergo a high level of cell death. Ding's laboratory screened for chemicals that would enable ES cells to survive enzymatic dissociation and remain pluripotent. They identified two compounds, a 2,4-disubstituted thiazole (Thiazovivin) and a 2,4-disubstituted pyrimidine (Tyrintegin), that met these criteria [70]. They found that Thiazovivin inhibits the Rho-associated kinase (ROCK), a key component of the pathway

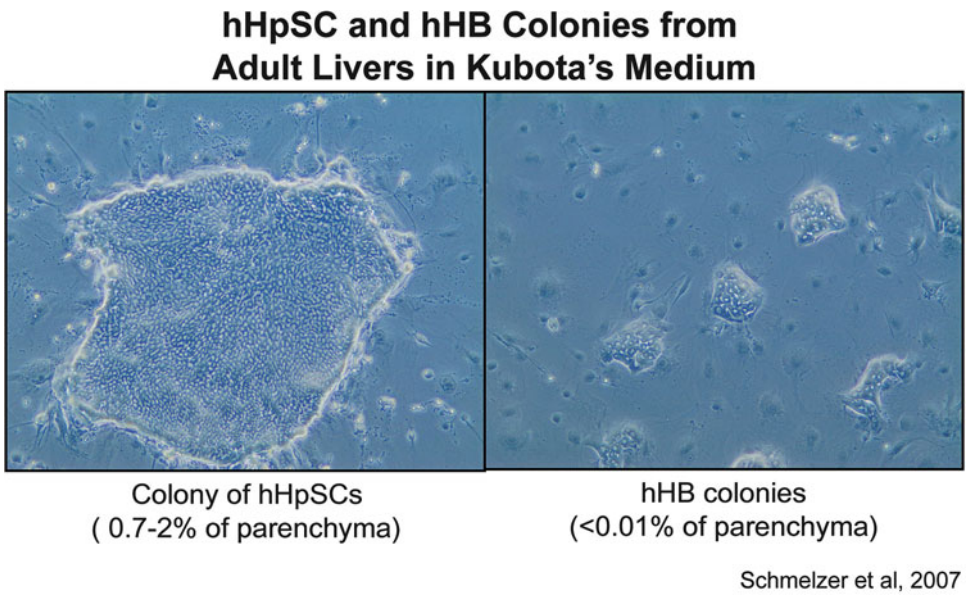

Fig. 17 Phase contrast images of hHpSCs vs. hHBs derived from adult human livers. The hHpSCs form colonies that are similar in appearance to those of ES cell colonies; the cells are relatively uniform in morphology, with high nucleus to cytoplasmic ratio and tightly bound to each other via E-cadherin. The hHB colonies form more cord-like structures interspersed by canaliculi. Image reproduced from Schmelzer et al. (2007). Similar images appear in Wang et al. (2010)

that controls cytoskeleton remodeling, and a likely regulator of cell-ECM and cell-cell interactions. Tyrintegin enhances attachment of dissociated ES cells to ECM and stabilizes E-cadherin. The investigators concluded that ES cell interactions in the normal niche generate signals essential to survival and that small molecules modulating those signals can maintain viability of dissociated cells (Fig. 18).

Likewise, we have observed that hyaluronans, a normal component of most, if not all, stem cell niches, can protect hHpSCs for dissociation and cryopreservation [69]. The addition of hyaluronans was found to protect cell adhesion mechanisms including the hyaluronan receptor, E-cadherin, and certain integrins, markers shared by hepatic and many other stem cell populations [69].

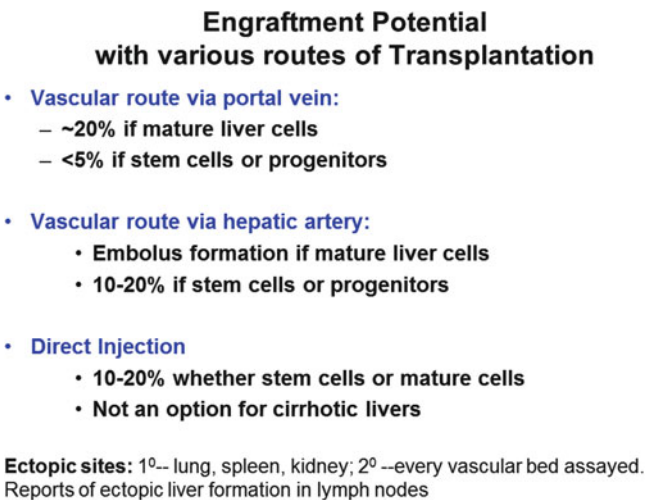

Fig. 18 Summary of engraftment potential of liver cells when transplanted by various routes. Data derive from multiple sources and reviews (Turner et al. 2010; Turner et al. 2012; Puppi et al. 2012)

The Need for Grafting Strategies in Transplantation of Cells from Solid Organs

Transplantation of stem cells into hosts faces challenges applicable to all cell types derived from solid organs. If cells are transplanted via a vascular route, there is inefficient engraftment; the cells disperse to ectopic sites; and emboli may form [65]. Our studies and those conducted by many others have found that mature cells achieve only ~20 % engraftment if injected into the portal vein of the liver [45, 71, 72]. Stem cells are even more challenging, with approximately only 3 % of the cells engrafting if administered via the portal vein (or via the spleen that connects directly to the portal vein). This can be improved to ~20 % engraftment in the liver if stem cells are injected into the hepatic artery [73]. The remaining majority of the cells either die or engraft in ectopic sites, most commonly the lung. Cells that lodge in the vascular beds of ectopic sites can survive for months [74], a finding of unknown significance at this time, but of potential clinical concern.

We have devised grafting strategies for transplantation of hHpSCs embedded into a mix of soluble signals and extracellular matrix biomaterials (hyaluronans, type III collagen, laminin) found in stem cell niches [74]. The hHpSCs maintain a stable stem cell phenotype under the graft conditions. The grafts were transplanted into the livers of immunocom-

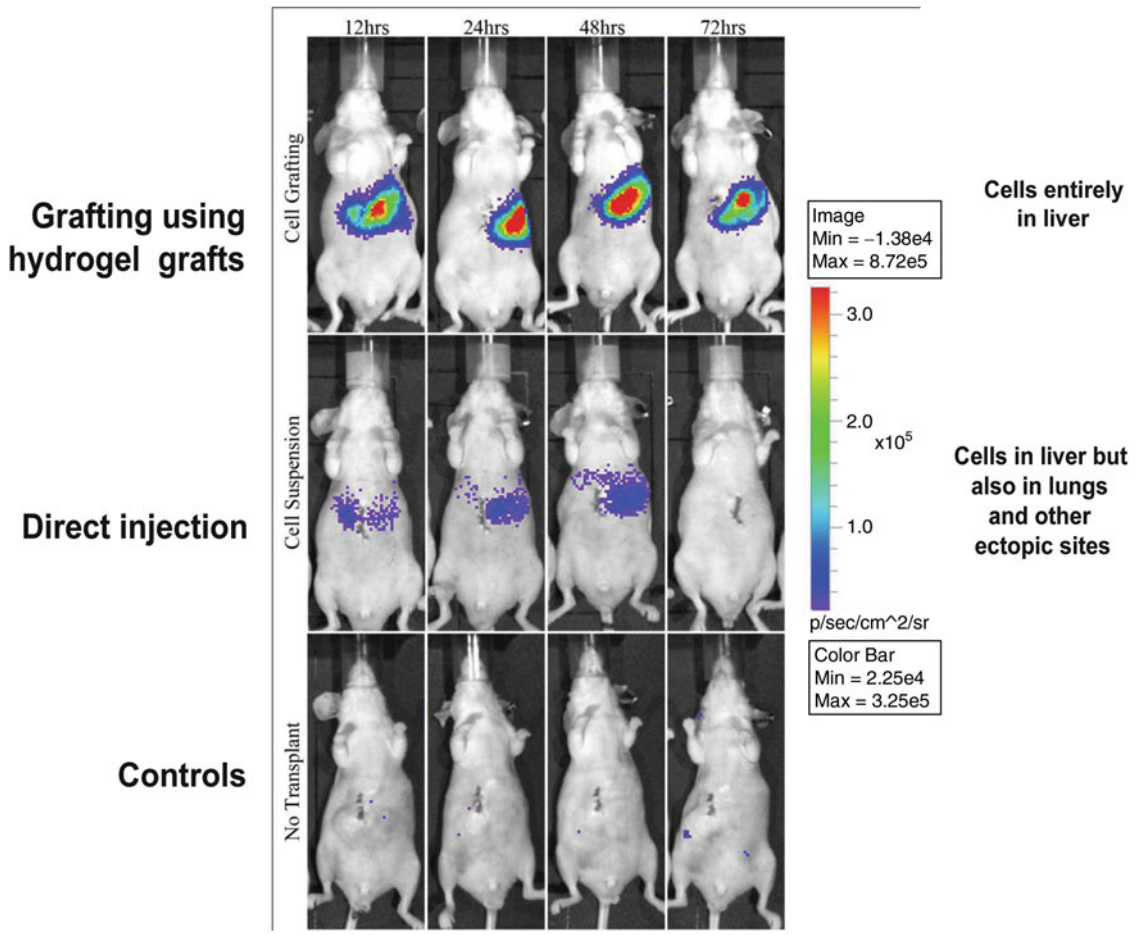

Fig. 19 Grafting strategies dramatically improve engraftment efficiency and minimize ectopic distribution of transplanted cells. Image reproduced from Turner et al. (2012)

promised murine hosts, with and without carbon tetrachloride treatment, to assess the effects of quiescent vs. injured liver conditions. Grafted cells remained localized to the livers, resulting in a larger bolus of engrafted cells in the host livers under quiescent conditions and demonstrated more rapid expansion upon liver injury. We therefore have proposed grafting as a preferred strategy for cell therapies for solid organs such as liver [65, 74] (Fig. 19).

Differentiation

The pharmacology of stem cell differentiation also must encompass both soluble signals (i.e., conventional biologics and/or drugs) and matrix components corresponding to the cells' 3D microenvironments. Cytokines and other soluble factors necessary for liver development and for the maintenance of differentiated hepatocytes have been known for some time [35, 36, 75] (see also Chaps. 4, 22, and 34). However, the specific and efficient directed differentiation of stem or progenitor cells to fully mature hepatocytes and cholangiocytes *ex vivo* has remained a difficult challenge. This, in fact, is a general problem in much of stem cell biology, whether starting with lineage-restricted adult stem cells or pluripotent ES and iPS cells (see Chap. 10).

Biomatrix scaffolds. Approximately 30 years ago, Reid and associates developed a means to provide an environment conducive to maintenance of the differentiated state by presenting cells with ECM components, termed *biomatrices*, prepared by a high-salt extraction procedure [76]. Frozen sections or pulverized liver biomatrices used as cell culture substrata enabled the long-term survival of highly functional hepatocytes, far beyond what could be achieved on plastic or with simple type I collagen gels. Recently, we have established an improved protocol, one involving perfusion strategies and also with high-salt extraction, to prepare decellularized organs. We call the extracts *biomatrix scaffolds*. They are tissue-specific but minimally (if at all) species-specific, and they potently *induce* cell differentiation [41]. The biomatrix scaffolds contain >98 % of the collagens and known collagen-bound matrix components, including most of the fibronectins, laminins, nidogen, entactin, elastin, etc., and essentially all the proteoglycans (PGs). They retain phys-

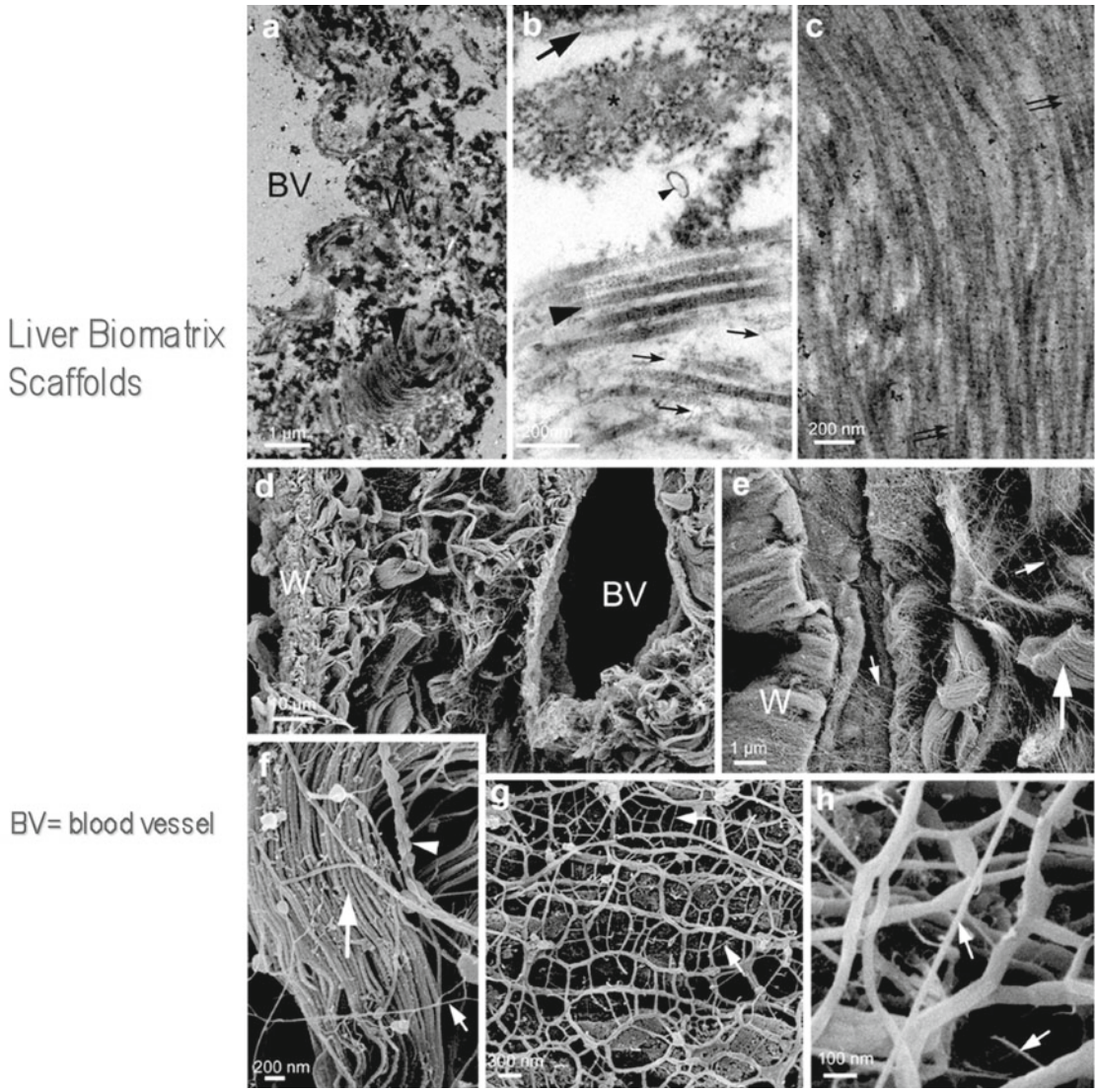

Fig. 20 Transmission and scanning electron microscopy images of rat liver biomatrix scaffolds. (**a**) Low magnification of a blood vessel (BV). probably the portal vein, based on the narrow wall (W) thickness compared to the large diameter of the vessel. The undulations or scalloping of. the vessel (sometimes linked to the internal elastic lamina of an artery) is here probably a partial collapse of the vessel wall. Collagen Type I fibers (*large arrowhead*) are numerous and contains cross-sections of individual fibers that do not take up heavy metal stains (*white dots, small arrowheads*). (A1) Higher magnification of a vessel wall shows basement membrane (*large arrow*), amorphous elastin (*), and associated elastic fibers, a rare membrane vesicle remnant (*small arrowhead*), a collagen Type I banded fiber (*arrowhead*), and small fibrils (*small arrows*). The small fibrils are probably fibrillin (Type VI collagen) that associates closely and helps organize Type I collagen. (**b**) High magnification of Type I collagen with 64 nm banding pattern (*arrows*). (**c**) Low magnification of a vessel with a thin wall (BV) and the wall of a larger vessel (W). (**d**) At higher magnification, the large vessel wall (W) is scalloped, consistent with hepatic artery of a portal triad, see (**a**). Beneath the wall are numerous Type I collagen bundles (*large arrow*) linked by long branching thin, reticular (Type III) collagen fibrils (*small arrows*). (**e**) A large bundle of Type I collagen has characteristic parallel fibers (*large arrow*) associated with a variety of smaller fibers (*arrow*) and nodular or beaded fibers (*arrowhead*). (**f**) 3D meshwork of large/small fibers interlinked in a plane that forms a boundary such as to a liver sinusoid. (F1) Higher magnification of the meshwork showing a variety of fibers (*arrows*): Type III collagen (larger diameter straight), elastic fibers, or Type VI collagen

iological levels of the known matrix-bound cytokines and growth factors found in the tissue. Mature parenchymal cells plated on biomatrix scaffolds in a serum-free HDM remained stable for many weeks and continued to express liver-specific functions equivalent to those of freshly isolated cells.

The hHpSCs seeded onto the liver biomatrix scaffolds in a serum-free defined medium underwent several rounds of cell division, followed by growth arrest and differentiation within approximately a week to mature hepatic parenchymal cells. High levels of specialized hepatocyte and cholangiocyte protein expression and functions could then be maintained for more than 8 weeks [41] (Figs. 20, 21, 22, and 23).

Differentiation of hHpSCs to mature parenchymal cells can be achieved also in 3D hyaluronan hydrogels prepared in

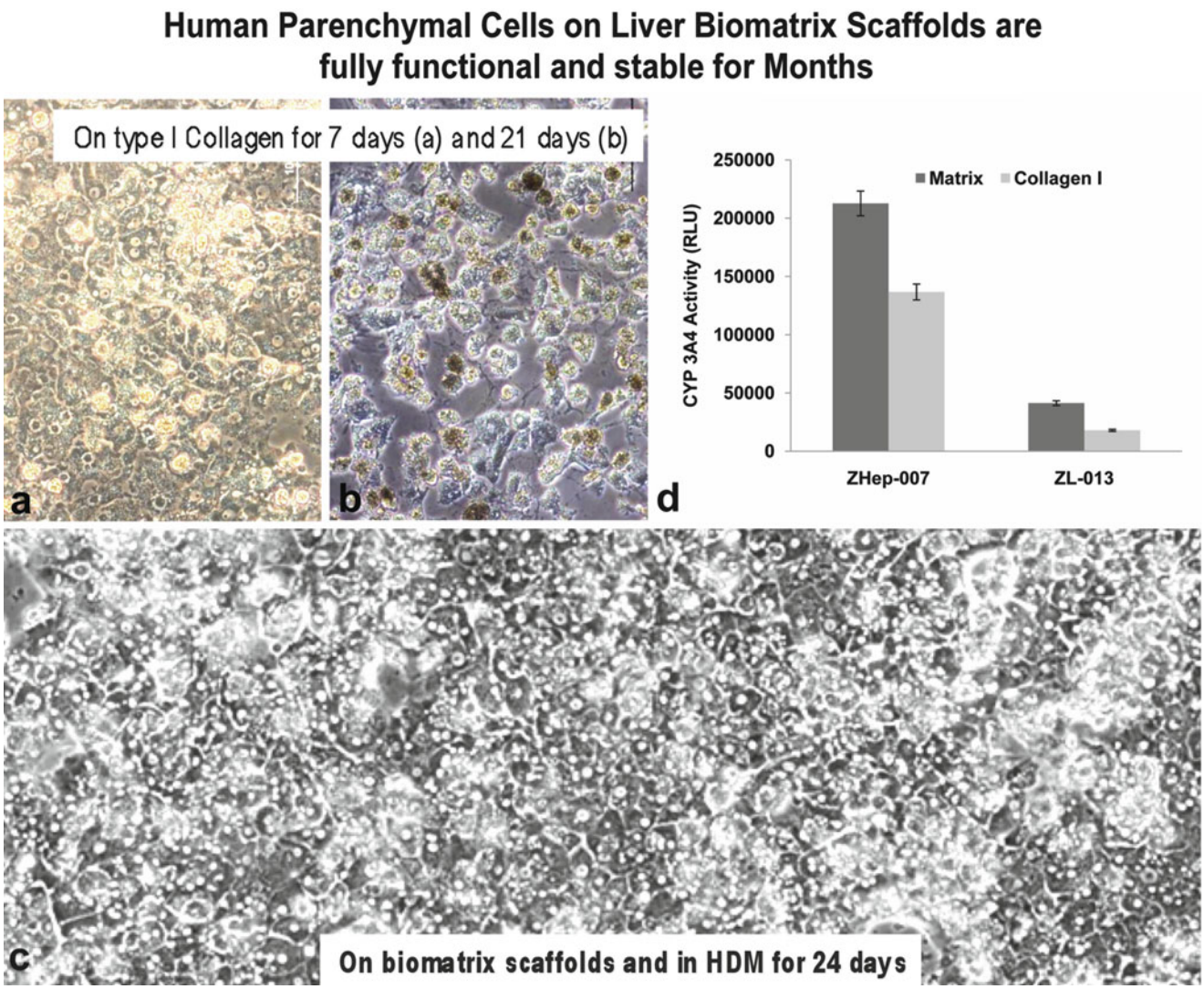

Fig. 21 Cultures of human adult human liver cells, plated in HDM-H and onto either type I collagen gels (**a**, **b**) or onto a frozen section of liver biomatrix scaffolds (**c**). The cultures on collagen gels lasted about 12 days. Those on biomatrix scaffolds lasted more than 8 weeks and with phenotypic traits similar to freshly isolated cells. (**d**) P450 levels in cultures of a "good lot" (ZHep-007) vs. "bad lot" (ZL-013) of frozen human hepatocytes from CellZDirect (RTP, NC) on type I collagen vs. liver biomatrix scaffolds. The "good lots" are ones that will attach on culture plastic; the "bad lots" are those that do not. Reproduced from figure in Wang et al. (2011)

serum-free hormonally defined media (HDM) and supplemented with defined, purified matrix components [41]. As noted above, distinct conditions favor the generation of hepatocytes vs. cholangiocytes. Ultimately, identification of each of the particular tissue-specific matrix molecules necessary for efficient differentiation will be required for mechanistic understanding. It also may be important for clinical translation. The pharmacology of matrix components and their interactions with cytokines and growth factors, the great majority of which bind to the glycosaminoglycan chains of PGs, is a rich, albeit highly complex area that promises to contribute greatly to regenerative medicine [77, 78]. Understanding of the role of complexes of specific growth factors or cytokines bound to defined glycosaminoglycan saccharides [79] in the regulation of cell differentiation and tissue-specific gene expression [80–82] is still in its infancy, but it is likely to become a dominant factor in the maintenance and regulation of stem cells for clinical and non-clinical purposes.

Liver Regeneration

The renowned regenerative capacity of the liver has inspired countless studies on mechanisms associated with the process [43]. It is beyond the scope of this review to summarize that enormous literature, although important aspects of liver regeneration are addressed in other chapters of this book (see

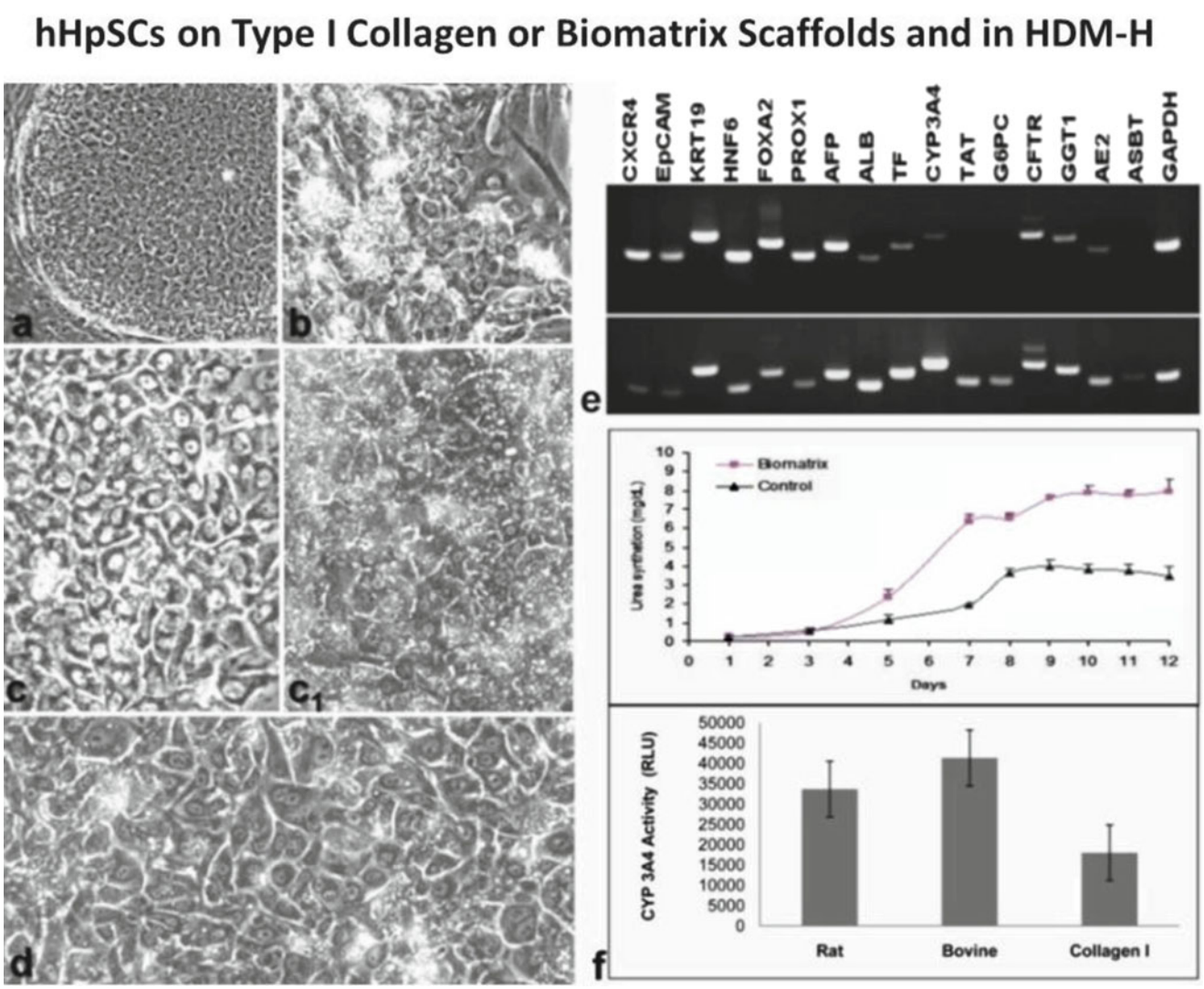

Fig. 22 Lineage restriction of hHpSCs to adult fates is made efficient by culturing the cells on liver biomatrix scaffolds and in HDM-H. (**a**) The hHpSCs on culture plastic and in Kubota's Medium. (**b**) The cells on type I collagen and in HDM-H after 10 days. The cells on liver biomatrix scaffolds and in HDM-H after 10 days (**c**, $\mathbf{c_1}$) and after 21 days (**d**). (**e**) RT-PCR assays of cells on plastic and in Kubota's Medium (*upper panel*) vs. in HDM-H and on liver biomatrix scaffolds (*lower panel*). (**f**) Functional assays (urea and cytochrome P450) of cells on type I collagen vs. liver biomatrix scaffolds. By the time of these assays, the cells on culture plastic have died. The figure is from Wang et al. (2011)

also Chaps. 22 and 34). Here we will note only the known responses of the stem cells and progenitors in two distinct forms of liver regeneration, namely that after partial hepatectomy and that after selective loss of cells in acinar zone 3 (the pericentral zone). (We assume that a parallel process occurs in pancreatic regeneration, though it has been studied in far less detail.)

A key to understanding the responses of the early lineage stage cells, including the stem cells, is recognition of *feedback loop signals*, factors produced by the most mature liver cells, those in zone 3 of the liver acinus, and secreted into the bile. The bile flows from pericentral zone to periportal zone and then into the biliary tree and finally into the gut (Fig. 24).

The signaling molecules include bile acids and salts that affect differentiation [83]; acetylcholinesterase [84], which is produced by mature hepatocytes and serves to inactivate acetylcholine produced by periportal cells [85, 86]; and heparins, which are produced by mature hepatocytes [87] (J. Esko, A. Cadwallader, and L. Reid, unpublished observations) and are relevant in control of stem cells and of tissue-specific gene expression [88, 89]. In addition, the flow of the bile mechanically affects primary cilia on periportal cells and thereby influences signal transduction processes mediated by these organelles [90–92]. In the presence of feedback signals, the stem cells remain in a quiescent state. Diminution or loss of these signals results in disinhibition of the stem/progenitor cell compartments. This leads to hyperplasia of

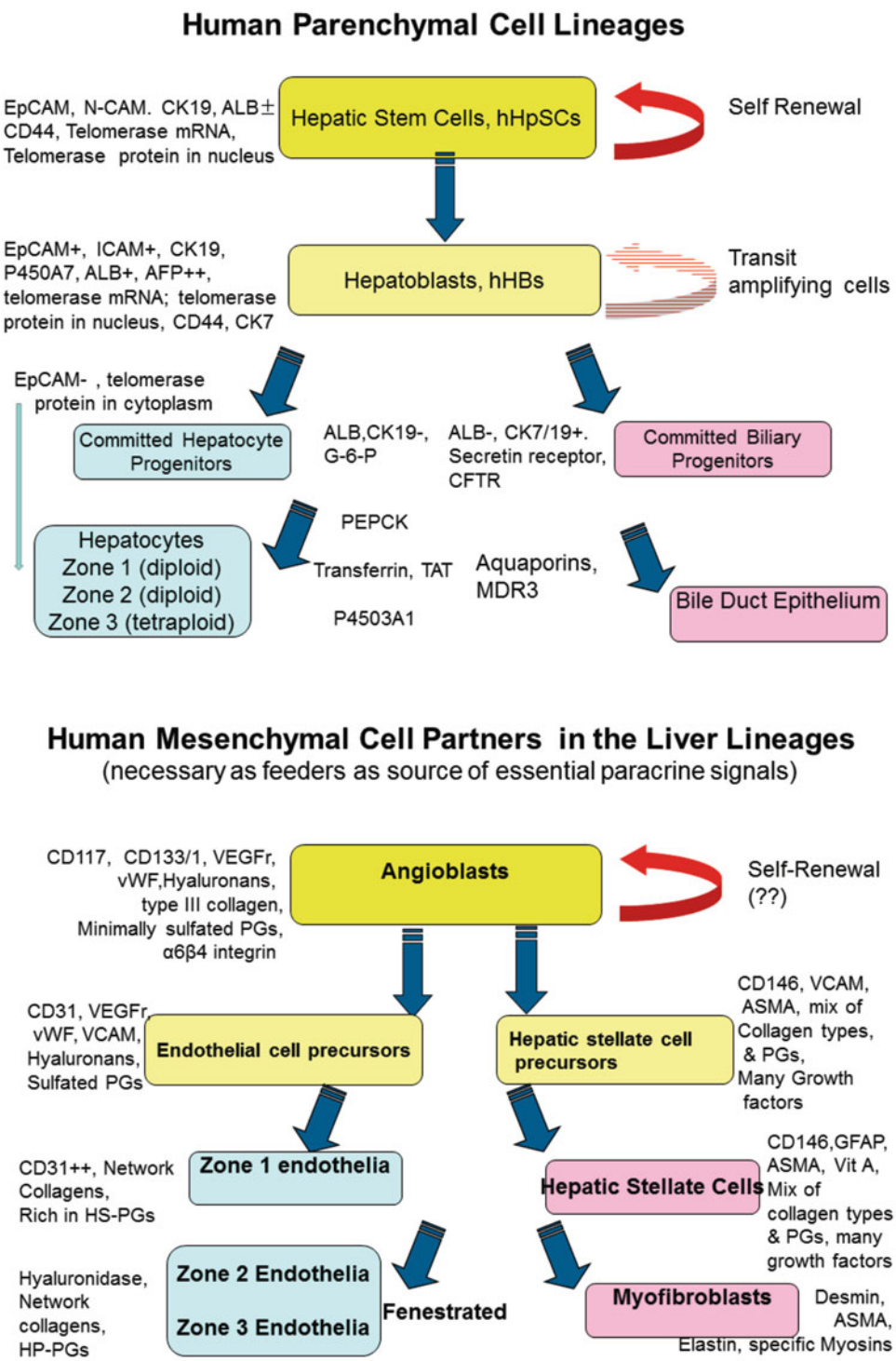

Fig. 23 Epithelial-mesenchymal cell partners in the maturational lineages of the liver. Modified from figure in the online supplement of Turner et al. (2011). *P6s* = proteoglycans; *ASMA* = - α-Smooth muscle actin; *VCAM* = Vascular cell adhesion molecule; *Vit. A* = Vitamin A; *G-6-P* = Glucose-6-phosphate; *GFAP* = Glial fibrillary protein; *CFTR* = Cystic fibrosis transmembrane conductance regulator

the stem cells and other early lineage stage cells. Factors that may release the stem cell compartment from the normal feedback signaling control loops include viruses, toxins, or radiation that selectively kill cells in zone 3, the pericentral zone of the acinus. The hyperplasia transitions into differentiation of the cells. The resulting fully mature cells produce bile, and the restoration or enhancement of feedback loop signals then inactivates the proliferative response.

The regeneration of the liver after partial hepatectomy is distinct from that described above and has been the subject

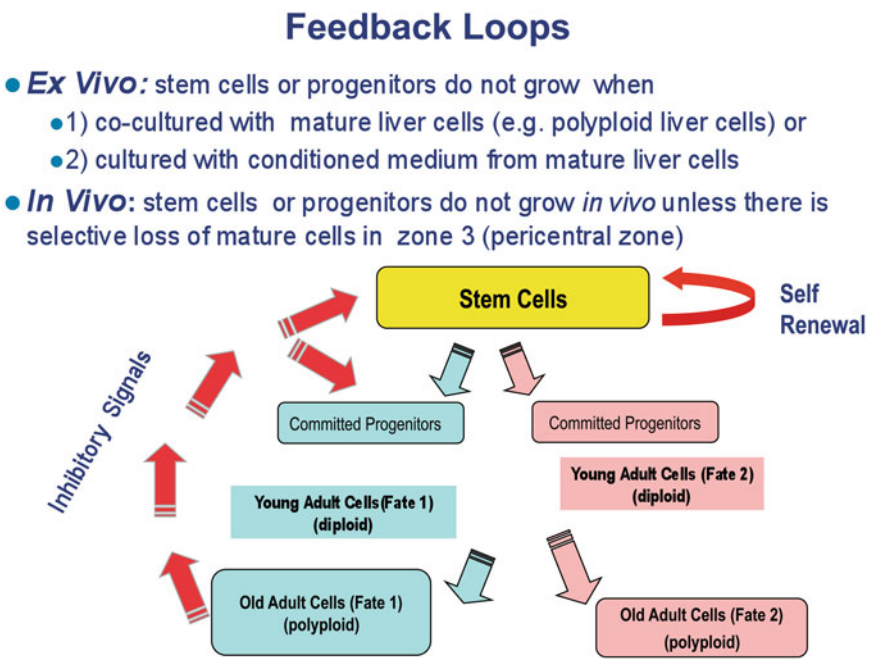

Fig. 24 Feedback loops of signals regulating the quiescent vs. proliferative status of the stem cells and progenitors. The signals *in vivo* are present in bile. In cultures, they are secreted into the medium and so are available in conditioned medium

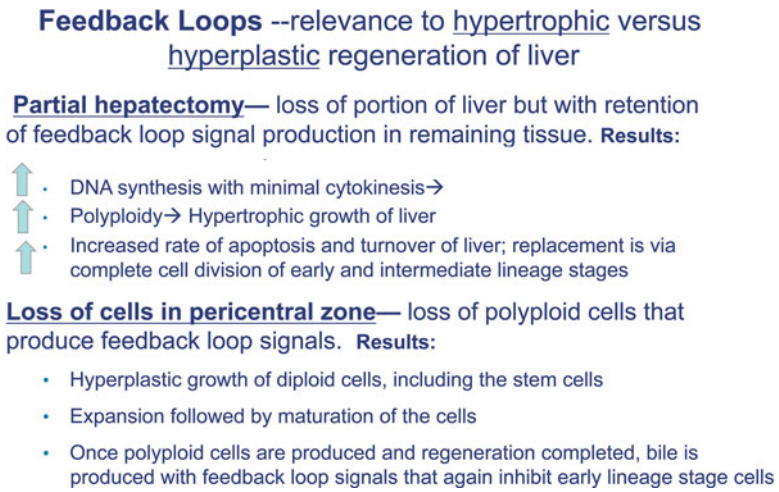

Fig. 25 Feedback loops of signals found in bile (*e.g.*, acetylcholinesterase, bile salts, heparins, and other factors) and the mechanical effects of bile flow influence whether the stem cells and other early lineage stage cells will divide or will assume a quiescent state. The feedback loop signaling processes are the primary mechanisms dictating liver size (i.e., whether the liver is the size for a mouse or human)

of many reviews [43, 93, 94]. The tissue remaining after surgical removal of a portion of the liver (*e.g.*, two-thirds of its mass) continues to have feedback loop signals, and the early lineage stage cells remain competent to respond to these signals. The depletion below threshold levels of various liver functions and secreted products triggers DNA synthesis as a wave across the liver plates [94]. However, the DNA synthesis in most of the cells of the liver (especially those in zones 2 and 3) is not accompanied by cytokinesis [95]. So these cells increase their level of ploidy and demonstrate hypertrophic growth [96]. The polyploidy triggers an increased rate of apoptosis resulting in turnover of the liver. With the loss of the apoptotic cells, there is a low level of proliferation of the stem cells and early lineage stage cells to replace those cells eliminated during apoptotic processes. In mammalian species examined, this turnover occurs in weeks (Fig. 25).

Clinical Programs in Hepatic Stem Cell Therapies

Clinical programs for hepatic stem cell therapies are in their very early stages. To our knowledge the only clinical trials of hepatic stem cell therapies that have been completed to date were carried out in Hyderabad, India, under the management of Dr. Chittoor Habibullah and associates in the Liver

Institute. These investigators found considerable value for hepatic stem cell therapy in treatment of patients with inborn errors of metabolism, cirrhosis, hepatitis B or C, and other liver disorders [73, 97, 98]. They used immunoselected EpCAM+ cells from fetal livers, comprising both hHpSCs and hHBs. Remarkably, immune suppression was not required, although donors and recipients were not matched for histocompatibility antigens. In a portion of the studies that have been published to date, 25 subjects with decompensated liver cirrhosis from various causes received cell infusions into the liver via the hepatic artery. At a 6-month follow-up, multiple diagnostic and biochemical parameters showed clear improvement, and there was a significant ($p < 0.01$) decrease in the mean Mayo End-stage Liver Disease (MELD) score, an accepted metric for clinical severity. The clinical trials, which have been conducted for more than 5 years, were completed in June 2012 and the findings provided the basis to apply for regulatory approval in India, which remains pending.

Future efforts to employ hHpSCs and/or hHBs clinically will be facilitated by large-scale manufacturing of the stem and progenitor cell populations. The sourcing of donor cells may be fetal tissues in those countries that permit their use, as demonstrated by the Habibullah group. However, postnatal tissues also can be used as a source and may have distinct advantages, both ethically and practically. Neonatal tissues, including the liver and biliary tree, adult livers not suitable for whole organ transplantation, and adult biliary tree may serve as the source of stem cells. The cells may be utilized as directly isolated or after expansion in culture (subject to additional levels of regulatory review). It is expected that the grafting strategies discussed above [65, 74], such as transplantation of cells using hyaluronans, possibly in combination with other extracellular matrix components, will greatly improve engraftment, minimize ectopic distribution of cells, and hasten the improvement of liver functions.

Even though immunological issues did not appear limiting in the highly encouraging first trials of fetal liver-derived hepatic stem and progenitor cell therapy by Khan and coworkers [73], it yet may be desirable to match, to the degree possible, the HLA (major histocompatibility) types of donor cells and recipients. Given sufficient expansion, it should be possible to bank large numbers of cells from a modest number of carefully selected donors and achieve a beneficial degree of HLA matching for the large majority of recipients [99] (Figs. 26 and 27).

Acknowledgments Findings from these studies have been included in patent applications belonging to Sapienza University (Rome, Italy) and/or to UNC (Chapel Hill, NC) and licensed to Vesta Therapeutics (Bethesda, MD). The authors do not have equity or a position in Vesta and are not paid consultants to the company. The authors declare no conflicts of interest. Almost all of the figures are reproduced from various publications with permission of the administrators of the journals in which they first appeared. The review was written primarily by Mark Furth and Lola Reid, with input and editing by all of the authors. All of the authors have contributed to the investigations and established the interpretations that are summarized in this review.

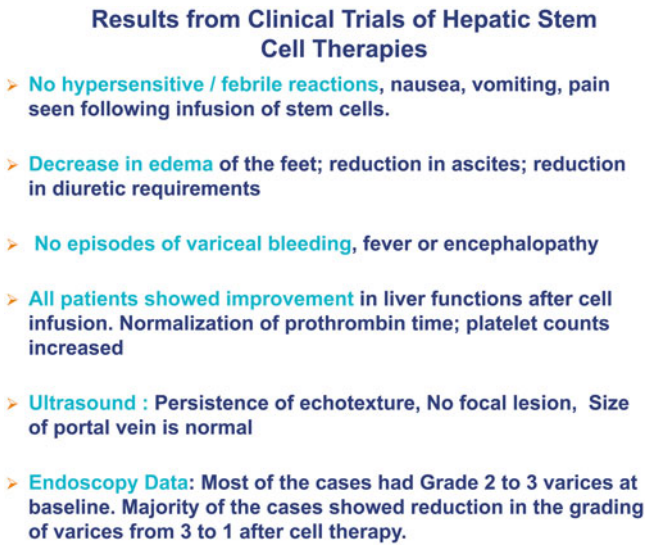

Fig. 26 Summary of the initial findings from clinical trials and that were published [73]. After this and other publications from the initial findings, the clinical trials continued. They ended in the summer, 2012. Filings have been made to regulatory agencies for approval of clinical products based on hepatic stem cell therapies. Therefore, further information on the findings from these trials will soon be available

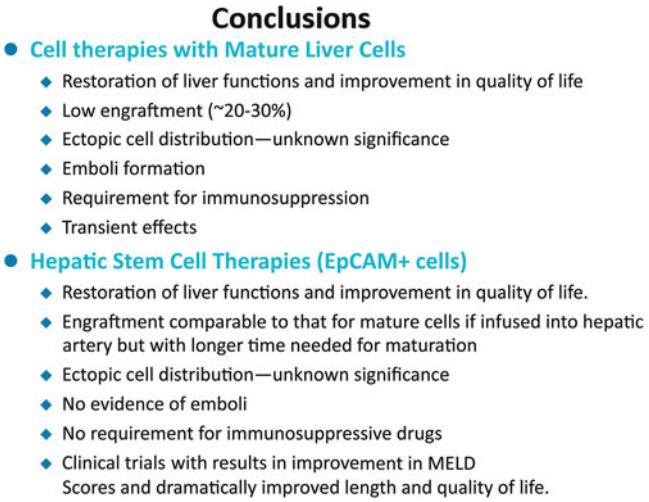

Fig. 27 The conclusions thus far on liver cell therapies with mature cells [45] vs. with hepatic stem cell therapies [73]

Authors' note: As this book goes to press, we acknowledge the publication on "liver buds" (Takebe et al. 2013). The investigators mixed 3 different stem cell populations in culture under appropriate conditions to form the liver buds.

Their findings demonstrate the importance of epithelial-mesenchymal interactions and the resulting paracrine signals in liver formation

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