THE HEPATIC STEM CELL NICHE AND PARACRINE SIGNALING BY MESENCHYMAL CELLS IN SUPPORT OF HUMAN HEPATIC STEM CELLS

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ABSTRACT

Hsin-Lei Yao: The Hepatic Stem Cell Niche and Paracrine Signaling by Mesenchymal Cells in Support of Human Hepatic Stem Cells
(Under the direction of Lola M. Reid)

Paracrine signaling is important for tissues both during embryonic development and in regulation of the tissue's cell lineages in adults. In liver, the mesenchymal cells adjacent to hepatic parenchymal cells secret soluble factors as well as extracellular matrix (ECM) molecules that regulate hepatic parenchymal cells to grow and/or to differentiate in order to form a functional liver. Human hepatic stellate cells (hHpSTCs) with classic stellate cell markers (vitamin A^+, $desmin^+$ and α-smooth muscle $actin^+$) and liver sinusoidal endothelial cells ($VEGFr^+$, von Willebrand $factor^+$ and $CD31^+$) are two major mesenchymal cell types in the liver. They were found present in primary cultures of human hepatic stem cells (hHpSCs) derived from fetal livers and were found to be critical as "companion cells" for survival and expansion of the hHpSCs. Many of the markers are shared by hHpSCs and the other pluripotent hepatic progenitors in human livers, hepatoblasts (hHBs) including expression of albumin, epithelial cell adhesion molecule (EpCAM), cytokeratins 8, 18 and 19, hedgehog proteins, and telomerase but not hematopoietic markers (CD45, CD34, CD38) or mesenchymal markers (those of endothelia or HpSTCs). However, they can be distinquished in that hHpSCs express neuronal cell adhesion molecule (NCAM) and claudin 3 but are negative for intercellular adhesion molecule-1 (ICAM-1) and α-fetoprotein (AFP), whereas

hHBs express intercellular cell adhesion molecule (ICAM-1), AFP, higher levels of albumin but not claudin 3 or NCAM.

The hHpSCs behave differently depending on type of feeder cells are used. Feeders of angioblasts or endothelia resulted in maintenance of hHpSCs as stem cells; feeders replete with HpSTCs resulted in lineage restriction of hHpSCs to hepatoblasts; and STO feeders caused even more differentiation to both hepatoblasts and committed progenitors.

Representative feeders of angioblasts/endothelia versus HpSTCs were characterized for expression of matrix genes and proteins; those matrix components identified were then tested as substrata for effects on the hHpSCs. The hHpSCs remained as stem cells on substrata of type III collagen or culture plastic; they differentiated into hepatoblasts on substrata of laminin or on top of collagen type IV; they differentiated into hepatoblasts and committed progenitors on the surface of type I collagen and into hepatocytes when embedded into type I collagen gel.

STO feeder cells secret inflammatory cytokines and factors, many of them identified in other studies as being produced following liver injury. A number of the factors were induced to be secreted to higher levels by co-culture of STO cells with human hepatic progenitors. These included several members of the interleukin family (e.g. IL-4, IL-5, IL-6, IL-10, IL-11 and IL-13), eotaxin, transforming growth factor-β1 (TGF-β1), interferon-γ (IFN-γ), macrophage inflammatory protein-2 (MIP-2), and others. Some of these factors have been found in preliminary studies to have potent effects on human and rodent hepatic progenitors but have not yet been fully characterized. They are assumed to play complementary roles to those of the matrix components in regulating hHpSCs. Analyses of these role(s) will be part of future studies.

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CHAPTER I

BACKGROUND

A. The Epithelial-Mesenchymal Relationship

Epithelial and mesenchymal cells are distinguished by the morphology of the multicellular structures they form. The epithelium is a sheet of cells with individual epithelial cells contiguous to each other. Cell-cell junctions and adhesions between neighboring epithelial cells hold them tightly together and reduce the movement of individual cells away from the cellular sheet. Epithelial cells have apical, lateral, and basal surfaces, and thus are polarized. Mesenchymal cells have irregular cell shape and tend to be more extended and elongated relative to epithelial cells. Adhesions between mesenchymal cells are weaker than epithelial cells, allowing them to migrate easily (Lee et al., 2006). Epithelial cells can be derived from any of three germ layers whereas mesenchymal cells are originated only from mesoderm. For example, both kidney epithelial and mesenchymal cells are derived from mesoderm, whereas hepatocytes and liver-derived non-parenchymal cells are derived from endoderm and mesoderm respectively. Plasticity issues for stem cells have been much discussed in recent years. The net sum of findings suggests that cells committed to fates of a given germ layer do not cross over to fates of another germ layer. For example, mesodermally-derived epithelial cells can transition to other mesodermal cell types (Perez-Pomares and Munoz-Chapuli, 2002). Precardiac epithelium derived from mesoderm can transition to generate endocardial progenitors (Lough and Sugi, 2000).

1. General Introduction

The epithelial-mesenchymal relationship is an evolutionarily ancient cell-cell relationship known to govern all metazoan tissues. It can be mimicked *ex vivo* by co-culturing of epithelial cells and mesenchymal cells. The direct communication between the epithelium and mesenchyme promotes cellular proliferation and differentiation during development of several organs, such as kidney, lung, intestine, liver and pancreas (Table 1.1). Interruption or inhibition of this relationship leads to impaired organ formation. Mesenchymal cells/stromas affect proliferation and differentiation of their adjacent epithelial cells both *in vitro* and *in vivo*, but the mechanism of interaction is not fully understood. Soluble cytokines and growth factors as well as the extracellular matrix molecules expressed by the mesenchymal cells play important roles in mediating this interaction.

Additional variables mediating the mesenchymal-epithelial interaction include mechanical forces, pH, electromagnetic gradients, and the levels of oxygen and $C0_2$. Differentiation can occur when cells are under different mechanical stresses. Human mesenchymal stem cells were found to express bone morphogenetic protein (BMP-2) and differentiate into osteogenic pathway when stimulated by 10% uniaxial cyclic tensile strain (Sumanasinghe et al., 2006). When subjected to smaller (5%) cyclic strain, the same cell types increased expression of calponin 1, a smooth muscle cell marker, and decreased expression of cartilage markers, such as types X and XI collagens, biglycan and cartilage oligometric matrix protein (COMP) (Kurpinski et al., 2006).

2. Kidney

Development of the kidney is achieved by the reciprocal induction of two mesodermal derivatives: the ureteric bud (epithelium) and the metanephric mesenchyma (mesenchyme). This interaction triggers the process of nephrogenesis and culminates in the formation of the mature kidney. Around the fifth week of gestation in humans, the ureteric bud induces the mesenchyma to form tubular and glomerular epithelia. In turn, the mesenchyma stimulates the ureter to continue to grow, differentiate, and branch into it. The uninduced mesenchyma secretes glial cell line-derived neurotrophic factor (GDNF), a glycoprotein ligand for the c-Ret receptor located on the mesonephric duct. GDNF phosphorylates the Ret receptor and induces branching of the ureteric bud. Appropriate branching of the ureteric bud is obtained by the accurate distribution of these two molecules (Gomez and Norwood, 1999).

In vivo, nephrogenesis is coordinated with renal vascularization. The ureteric bud induces the mesenchyma, which condenses around the ureteric tip and eventually differentiates into the epithelial segment of the nephron (Sakurai and Nigam, 1998). Many of the mesenchymal cells around the condensate are precursors for endothelial, smooth muscle/mesangial, or renin-producing cells. Vascular precursors, epithelial progenitors, and mesenchymal cells communicate with one another in a highly organized fashion and through complex interactions to yield a mature and functional kidney.

3. Mammary Gland

Development of the mammary glands is initiated in the embryo, but the major part of their development occurs in the adult. Mutual and reciprocal epithelial-mesenchymal

interactions are critical for both phases of development. Specific steps such as the formation of the bud, the first appearance of hormone receptors, formation of the primary sprout and ductal elongation have been shown to be governed by epithelial-mesenchymal signaling. In the mouse embryo, mammary buds are formed between embryonic day 10 and 11. At this time, the mesenchyme underlying the mammary bud appears in no way distinct from the rest of the dermis. The mesenchymal cells bordering the mammary buds orient themselves only slowly around the epithelium. By E14, the epithelial gland buds are surrounded by a slightly denser mesenchyme consisting of several concentric layers of fibroblasts oriented around the epithelium. Upon formation of the primary sprout the epithelium pushes through this sheath of mesenchymal cells and penetrates the future fat pad, a secondary stroma of the gland supporting ductal morphogenesis in the late fetal and the entire postnatal period (Robinson et al., 1999).

Primary mammary mesenchyme induces hyperplastic growth in experimentally associated E17 mammary epithelium. Mesenchyme from the prospective fat pad of E14 mice is able to support organotypic development of E14 salivary gland, glandular stomach, intestine and colon and E12 pituitary gland and pancreas. Mesenchyme from the fat pad of older stages gradually loses this supportive capacity (Sakakura et al., 1979 and 1987). Cunha et al. later showed that E13 mouse mesenchyme was able to induce rat midventral or dorsal epidermis to form functional mammary epithelium (Cunha et al., 1995). Although the basis for these differences is unknown these observations may indicate a developmentally regulated expression of growth factors and trophic signals in these mesenchymal cells.

Interactions between epithelium and mesenchyme are also involved in the regulation of ductal outgrowth of the mammary glands in the postnatal period. Activins and inhibins,

members of the transforming growth factor beta family, are important in mammary gland tissue interaction. Mice lacking the gene encoding the β-subunit of activins and inhibins have poorly developed mammary glands and are unable to nurse their pups. These results show that expression of the β-subunit is dispensable in the mammary epithelium but is required in the mesenchyme. The mesenchymal cell-derived activin and/or inhibin β appear to play a paracrine role in development of both the ducts and alveoli (Robinson and Hennighausen, 1997).

4. Placenta

The major components of the mammalian placental membranes are an epithelial surface layer, the trophoblasts, and a heavily vascularized mesenchyme, the allantoic mesenchyme. A placental epithelial-mesenchymal interaction involves paracrine signals from allantoic mesenchyme acting on adjacent trophoblast. The expression patterns of several growth factors and their receptors, including hepatocyte growth factor-scatter factor (HGF-SF) and its receptor, c-met, constitute some of these signals. In nearly all tissues examined, both during development and in the adult, HGF-SF is expressed by mesenchymal tissues and c-met is expressed by adjacent epithelia (Sonnenberg et al., 1993). For example, in the liver, HGF-SF is expressed by nonparenchymal cells and c-met by hepatocytes; in the developing mammary gland, fibroblasts express HGF-SF, whereas the luminal and myoepithelial cells express c-met (Hu et al., 1993; Niranjan et al., 1995). In accordance with these results, the mouse and human HGF-SF promoter sequences are active in mesenchymal, but not epithelial cells (PlascKMe-Schlutter et al., 1995). Other mesenchymal cells expressed cytokines

including insulin-like growth factor II (IGF-II) and colony-stimulating factor a (CSF-1) (Stewart 1996).

5. Anterior Eye

During development of the anterior eye segment, cells that originate from the surface epithelium or the neuroepithelium need to interact with mesenchymal cells,which predominantly originates from the neural crest. In human embryos, migrating mesenchymal cells are seen during the 6^{th} week of development, forming several layers of loosely aggregated cells and filling the space between surface epithelium and lens epithelium. In mouse embryos at E15.5, mesenchymal cells migrate along the inner surface of the primary stroma to form the corneal endothelium. The surface ectoderm that covers the anterior side of the mesenchyme will become the corneal epithelium. Mesenchymal cells between the corneal epithelium and endothelium start to differentiate into corneal stroma fibroblasts or keratocytes, which synthesise the highly specialized extracellular matrix of the corneal stroma. As a result, the cornea finally becomes transparent (Cvekl and Tamm, 2004).

Transcription factors coordinate the complex interaction and communication between the two types of cell populations during morphogenesis of the anterior eye. Pax6, which is expressed in all cell types that contribute to anterior eye development, although at different times and with different intensity, appears to be the critical factor that synchronizes the critical events during formation of the anterior eye (Ashery-Padan and Gruss, 2001; Gehring and Ikeo, 1999). Failure of proper interaction results in anterior eye segment dysgenesis, which comprises a complex of developmental disorders that may critically reduce visual function.

6. Stomach

Vertebrate digestive organs are composed of an epithelial lining that originates from endoderm and a surrounding mesenchyme that is derived from splanchnic mesoderm. During the organogenesis of the vertebrate digestive tract, epithelial-mesenchymal interactions are essential for both epithelial development and mesenchymal differentiation (Fukuda and Yasugi, 2000, 2002 and 2005; Kedinger et al., 1988). In both the chicken and mouse embryos, tissue grafting experiments have revealed that the source of the mesenchyme is important for gut endoderm differentiation (Kedinger et al., 1986; Mizuno and Yasugi, 1990).

Birds are characterized by two stomachs: the rostral proventriculus (PV), which functions in chemical digestion, and the caudally localized gizzard (GZ) for mechanical digestion. The PV epithelium becomes divided into two parts: the glandular and the luminal epithelium. The glandular epithelium gradually forms complex glands and specifically expresses a gene encoding a zymogen of a digestive enzyme, chicken embryonic pepsinogen (ECPg) (Hayashi et al., 1988).

Studies using tissue recombination cultures showed that both PV and GZ epithelia form glands and produce ECPg when cultured with PV mesenchyme. In contrast, epithelial preparations that are cultured with GZ mesenchyme never form glands and do not produce ECPg (Takiguchi et al. 1986). This finding revealed that the PV mesenchyme has inductive effects upon both gland formation and ECPg expression in the epithelium, whereas the GZ mesenchyme is inhibitory to these pathways. In addition, the inhibitory effects of the GZ mesenchyme upon ECPg expression in the epithelium is sufficiently strong that it can be obtained when GZ mesenchymal cells are mixed with PV mesenchyme at a 1:10 ratio (Urase

and Yasugi, 1993). Therefore, as the epithelium of both PV and GZ has the potential for gland formation and ECPg production, the strong inhibitory effects of the GZ mesenchyme may function to form a sharp boundary between the PV and GZ.

Also, ECPg expression is induced in both PV and GZ epithelium cultured in laminin gel, with medium conditioned by PV mesenchyme. These results strongly suggest that both the components of basement membrane (e.g. laminin) and the secreted factors in the conditioned medium from PV mesenchyme are required for the PV epithelium to produce ECPg. It has also been reported by the same group that the GZ mesenchyme produces secreted factors which inhibit ECPg expression in the epithelium. A candidate secreted molecule found in the PV mesenchyme is BMP-2 (Narita et al., 2000). When BMP-2 is overexpressed in the PV mesenchyme using retroviral constructs, the PV epithelium formed more glands and expressed ECPg at a higher level than the control. On the other hand, when the activity of BMP is inhibited by overexpression of noggin in the PV mesenchyme, the formation of glands and the expression of ECPg are completely inhibited.

One of the FGF members, FGF10, is also associated with the formation of the glandular epithelium as a mesenchymal factor in the chicken. FGF10 is expressed in the developing PV mesenchyme, whereas its receptors are expressed in the epithelium (Shin et al., 2005). FGF10 overexpression also results in a dramatic change in the PV epithelium to a highly proliferative epithelial structure with an increase in epithelial cell number. Moreover, inhibition of FGF10 using overexpression of a secreted dominant negative form of FGFR2b, suppresses cell proliferation and gland formation in the PV epithelium. These results suggest that FGF signaling is required for proliferation and gland formation in the PV epithelium.

Epithelial-mesenchymal interactions are essential not only for epithelial differentiation but also for mesenchymal differentiation. The stomach epithelium of the chicken embryo regulates the concentric differentiation of mesenchymal components in the vertebrate gut. BMP4 expression in the mesenchyme is controlled by Shh expressed in the epithelium (Roberts et al., 1995). Shh has an important role in the control of the topology of mesenchyme differentiation.

7. Pancreas

During the development of the pancreas, pancreatic mesenchyme accumulates around the dorsal gut epithelium and induces pancreatic bud formation and branching (Kemp et al., 2003). The appearance of endocrine cells is induced by the actions of morphogenetic factors derived from the lateral plate mesoderm, such as activin and BMPs, on progenitor cells expressing the characteristic transcription factor, Pdx-1 (Soria, 2001; Hill, 2005). Prior to its specification toward a dorsal pancreatic fate, the gut epithelium of the posterior foregut is adjacent to notochord, an axial mesoderm structure. The notochord, through its secreted molecular factors, including the TGF-β family member, activin-β, and FGF-2, is believed to condition the prospective pancreatic mesenchyme and activate pancreatic gene expression by repressing expression of the signaling protein, Sonic hedgehog (Shh) (Hebrok et al., 1998). Inhibition of hedgehog signaling in foregut endoderm and surrounding mesoderm in chick embryo cultures by the steroid alkaloid cyclopamine leads to an extension in pancreatic marker gene expression in the epithelium and formation of pancreatic structures in the distal stomach and duodenal endoderm (Kim and Melton, 1998). Signaling from the mesenchyme to the invading epithelium regulates the appropriate balance of endocrine and exocrine

development, such that removal of the surrounding mesenchyme leads to a disproportionate ratio of the two, by promotion of endocrine cell development and impairment of exocrine development (Gittes et al., 1996).

Lammert et al. showed that signaling from embryonic blood vessel cells, derived from mesoderm, promotes pancreatic bud development (Lammert et al., 2001). Using a combination of mouse $Flk^{-/-}$ embryos lacking endothelial cells and tissue recombination experiments, Yoshitomi and Zaret showed that the initial induction of dorsal endoderm cells positive for the pancreatic and duodenal transcription factor Pdx-1 does not require aorta or endothelial cell interactions, but dorsal pancreatic bud emergence and maintenance of Pdx-1 expression does (Yoshitomi and Zaret, 2004). The aorta itself, apart from the blood supply, causes the induction of Ptf1a in dorsal endoderm explants. Thus, endothelial cell interactions specifically promote early dorsal pancreatic development, at least in part, by inducing $Ptf1a^{+}$ pancreatic progenitors. Signaling by endothelial cells is necessary for the induction of both the insulin and glucagon genes.

Growth factors contribute to endocrine cell proliferation, vascular formation, and islet expansion in the pancreas and originate from multiple tissue compartments including the ductal epithelium, the surrounding mesenchyme, and the vascular endothelium. FGFs are involved in early pancreatic induction from gut endoderm, and the high affinity FGF receptor, FGFR1, has been localized to ductal epithelial cells and to small developing islets (Gonzalez et al., 1996). FGFR4 is expressed in fetal rat islet development *in vitro*. FGF-7, one of the major ligand for FGFR4, is expressed within the mesenchyme adjacent to the pancreatic ducts (Ye et al., 2005). FGF-10 has also been shown to be expressed within pancreatic

mesenchyme in the mouse embryo; its absence causes a failure of ductal branching and an absence of Pdx-1-expressing endocrine progenitor cells (Bhushan et al., 2001).

Vascular endothelial cell growth factor, VEGF, is a potent mitogen for endothelial cells both *in vitro* and *in vivo*. Two of its high affinity tyrosine kinase receptors are Flt-1 and Flk-1. Flk-1 mRNA is expressed within RINm2F islet cells, as well as in fetal rat islets where VEGF is able to induce increased insulin content. Hepatocyte growth factor, HGF, is expressed within tissue mesenchyme in the embryo and fetus, and during postnatal tissue regeneration. Its receptor, the tyrosine kinase called met, is expressed within adjacent epithelial tissues which are target sites for mitogenic and morphogenic actions of hepatocyte growth factor, HGF. In human fetuses, met is found on cells within the pancreatic ducts, while in adult tissues, met mRNA and peptides are localized to islet β–cells. HGF mRNA is abundant in pancreatic stroma and is able to regulate the proliferation and differentiation of adjacent epithelial cells (Wang et al., 2004).

8. Liver

Hepatic genes are first induced in a segment of the definitive endoderm at about 8.5 days of gestation in mouse embryos (Zaret, 2002). Before hepatic induction, the mesenchyme of the septum transversum surrounds the developing cardiac region near the ventral foregut endoderm and is the presumed source of multiple inductive signals triggering liver formation. After exposure to these inductive signals, the hepatic endodermal epithelium becomes more proliferative, and the cells begin to bud into the stromal environment (Zaret, 2002; Zhao and Duncan, 2005). Transplantation of endodermal tissue at the 4-6 somite stage into the precardiac mesoderm results in tissue that morphologically resembles the liver. Interaction

between endodermal and mesodermal tissues is important for hepatic development (Lemaigre and Zaret, 2004).

Zaret et al. searched for locally acting factors expressed in the cardiac mesoderm at this stage and identified a number of members of the FGF family proteins (FGF1, 2 and 8) as potential candidates. In contrast, FGFR-1 and FGFR-4, receptors for these FGF species, were found to be expressed on endodermal cells, indicating that the FGF-FGFR signaling pathway is active between endoderm and mesoderm. Treatment of endodermal cells with FGF-1 and FGF-2 in the absence of the cardiac organ were capable of inducing the expression of albumin mRNA (Jung et al., 1999). Different thresholds of FGFs were needed? for patterning the ventral foregut into liver or lung in the absence of cardiac mesoderm (Serls et al., 2005). Ventral foregut endoderm explants from E8-9.5 mouse embryos respond to exogenous FGF1 and FGF2 in a dose-dependent manner, with lower concentrations activating liver-specific genes and higher concentrations activating lung-specific genes (Serls et al., 2005). Moreover, inhibiting FGF signaling pathway using neutralizing antibodies against FGFR-1 and FGFR-4 strongly inhibited the expression of albumin mRNA. FGF-FGFR signaling, therefore, is critical for the initial process of liver development.

It has been known that the members of the bone morphogentic family of proteins, the BMP family, also play a role in liver organogenesis. Zaret et al. used a mouse BMP4-null mutation and an inhibitor of BMPs to show that BMP signaling from the septum transversum mesenchyme is necessary to induce liver genes in the endoderm and to exclude a pancreatic fate (Rossi et al., 2001). In the BMP4-deficient embryos, the liver bud failed to enlarge and albumin was not expressed. Suppression of BMP signaling by an extracellular antagonist, noggin, inhibited albumin induction in the explants assay. Expression of a liver-enriched

transcription factor, GATA4, was also found to be reduced in liver explants derived from BMP4-null mice. GATA factors are known to regulate the transcription of other liver-enriched factors such as HNF4-α in hepatic cells. Thus, BMPs function by affecting the levels of the GATA4 transcription factor and act synergistically with FGF signals to induce hepatic specification.

B. Stem Cell Niches

1. Introduction

Stem cell niche is a specific location in a tissue where stem cells reside for an indefinite period of time and produce progeny cells while self-renewing (Ohlstein et al., 2004). The cells, matrix glycoproteins and the three-dimensional spaces they form provide ultrastructure for the niche. The contact between these elements allows molecular interactions that are critical for regulating stem cell function. Stem cell niches must ensure that daughter cells differentiate appropriately as they leave the niche.

Currently, the best-characterized stem cell niche at the histological and molecular level is the germline stem cell (GSC) niche in the ovary and testes of Drosophila and Caenorhabditis elegans. Additionally, four mammalian stem cells niches have been described for the bone marrow, central nervous system, intestinal crypt and skin (Naveiras and Daley, 2006). Investigators in the Reid lab have been redefining the stem cell niche for the liver (Schmelzer et al, 2007; Zhang et al, submitted). The studies from this thesis are part of those efforts.

2. Stem cell niches control stem cell activity

Stem cells in different tissues are often associated with specific partner or companion cells. Hematopoietic stem cells (HSCs) reside in niches located in trabecular bone where they are in contact with osteoblasts. Epidermal stem cells with the potential to replenish basal keratinocytes, hair and sebaceous glands are found in the hair follicle bulge with dermal papillae. Neural stem cells are closely associated with other astrocytes, neuroblasts, ependymal cells, endothelial cells and a factor-rich basal lamina.

Cancers have long been recognized as transformed stem cells or early progenitors. The cancers are comprised of aggregates of early progenitors and, therefore, can be used as model stem cell systems (albeit with recognition of critical distinctionswith their normal counterparts). The stem cells giving rise to certain brain tumors have a niche in which the transformed neuronal stem cells are closely associated with vascular cells needed to promote the long-term growth and self-renewal of the malignant neuronal cells. The relationship between transformed stem cells and the endothelial cells may be bidirectional (Yang and Wechsler-Reya, 2007).

Inside the niche, stem cells are often quiescent. The niche's microenvironment, in the quiescent state, is both proliferation- and differentiation-inhibitory. Proliferation and differentiation of stem cells can often be induced *in vitro* or *in vivo* after injury. How stem cells get activated in their niche is an important question in stem cell biology. One theory is that as the niche becomes occupied, excess stem cells are displaced as or shortly after they divide, thereby loosening connections with the niche. In this case, when faced with a new environment, the expelled stem cells or their recently divided progeny progress to differentiate. Alternatively, stem cells might simply remain inactive within the niche until

they have to become functional, for example in response to injury. In this case, an environmental change from the tissue might actively signal to the niche to mobilize their residents (Fuchs et al., 2004).

An alternative hypothesis is that proposed with respect to extracellular matrix components described below. The ability of the niche to retain its stem cells is also likely to play a role in recruiting stem cells, a process referred to as "homing". Although the molecular details of this process are still unknown, it seems likely that niches develop concomitantly with input from both stem cells and the surrounding tissue. Once established, they seem to be able to survive at least transiently as signaling centers to attract stem cells. In vertebrae, transplanted HSCs from one animal can find their way into the irradiated bone marrow of a host animal. Studies in Drosophila showed that when germline stem cell niches are experimentally emptied of their stem cells, the niches still persist and can even signal incoming somatic stem cells to take up foreign residence and at least transiently maintain some of their stem cell features (Kai and Spradling, 2003).

3. The role of extracellular matrix (ECM) as paracrine signals

Components in the extracellular mtrix (ECM), such as the proteoglycans, are critical in dictating fate of the stem cells. Recent reports by Bruce Caterson suggest that stem cell niches in all tissues assayed have a unique extracellular matrix chemistry that includes forms of chondroitin sulfate proteoglycans (CS-PGs) (personal communication with Dr. Bruce Caterson). The proteoglycan chemistry shifts to heparan sulfate proteoglycans (HS-PGs) immediately outside the niche. All sulfated proteoglycans bind growth factors, but CS-PG binds them with less affinity than HS-PGs and has a 3-dimensional structure that results in

many of the sulfated residues internalized within the molecules' rod shape or globular shape. By contrast HS-PGs are structured such that the sulfate groups are externalized and available for binding to growth factors that are then presented to cells as a stable signaling scaffold. Therefore, the stem cell niche has a matrix chemistry that shields the stem cells from growth factor signaling, thereby keeping the cells from signals that would lineage restrict them to a more mature fate. More on aspects of the matrix chemistry of the stem cell niche is given below.

Other extracellular matrix components (ECM) can also regulate stem cells. In skin, β-1 integrins are known to participate in constrained localization of a stem cell population through interaction with matrix glycoprotein ligands (Jensen et al., 1999). In nervous system, absence of tenascin C alters neural stem cell number and function in the subventricular zone (Garcion et al., 2004). Tenascin C seems to modulate stem cell sensitivity to fibroblast growth factor 2 (FGF-2) and bone morphogenetic protein 4 (BMP-4), resulting in increased stem cell propensity to generate glial offspring. In hematopoietic system, deletion of tenascin C has also been shown to affect progenitor cell populations (Ohta et al., 1998). In Drosophila, the posterior mid-gut populations of cells have the capacity to produce two types of daughter cells: enterocytes and enteroendocrine cells. These cells sit on a basement membrane that divides them from surrounding muscle cells. Basement membrane itself might participate in the specialized microenvironment, giving an opportunity for shifting location of the stem cell, sliding along a continuum of the intestine.

A matrix protein, osteopontin (OPN), has been shown to contribute to HSC regulation. OPN interacts with several receptors known to be on HSCs: CD44, and α4 and α5β1 integrins (Nilsson et al., 2005). It is produced by multiple cell types including osteoblasts that

contribute to the HSC niche. Animals deficient in OPN have an increased HSC number. Without OPN, increased stem cell expansion occurred under stimulatory conditions (Stier et al., 2005). Thus, OPN seems to act as a constraint on HSC number, limiting the number of stem cells under homeostatic conditions or with stimulation. These results suggest that matrix components can contribute positively or negatively on the stem cell pool.

4. The soluble paracrine signals

In addition to ECM molecules, soluble signals provided by the niches are also considered to be a major factor regulating stem cell activity. In Drosophila testis, Unpaired (UPD) is secreted by niche (hub) cells and induces stem cell self-renewal via Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling (Kiger et al., 2001; Tulina and Matunis, 2001). In the Drosophila ovary, Decapentaplegic (Dpp) and a BMP analog, Glass bottom boat (Gbb), are produced by niche (cap) cells. These stimulate SMAD signaling in the germline stem cells (GSCs) and result in suppression of differentiation, maintaining the self-renewing stem cell state (Chen and McKearin, 2003; Song et al., 2004; Xie and Spradling, 1998).

Hedgehog (hh) family members have a role in topographic organization and formation of stem cell niches in development. In Drosophila ovary, hh is secreted by cap cells and mediates proliferative effects on not only germ line stem cells, but also the surrounding somatic cells composing the niche itself (Forbes et al., 1996; King et al., 2001). Sonic hedgehog (shh) is essential for hair follicle formation, and distinguishes the localization of specific subsets of epidermal stem cells to either the follicular niche or the interfollicular epidermis (Levy et al., 2005). In the developing intestine, shh and Indian

hedgehog (ihh) contribute to architectural patterning and regularity of the crypts where stem cells reside (Madison et al., 2005). Other paracrine signals that are developmentally critical include TGF-β/BMPs, FGFs, Notch and Wnts. During embryonic development, BMPs and FGFs are secreted by the septum transversum mesenchyme and cardiac mesoderm respectively to initiate liver induction (Zaret, 2002). The Wnt/Frizzled/β-catenin pathway is responsible for intestinal stem cell proliferation. Notch levels fine-tune the fate of the stem cell progeny, thereby determining the lineage of differentiating cells. The ability of one neighbor to activate and the other to repress Notch signaling sets up a boundary that allows the cells to choose between alternative fates. Osteoblasts may maintain HSC self-renewal and suppress differentiation by expressing Notch ligands that stimulate receptors on the HSC surface (Calvi et al., 2003).

5. Niche-regulated symmetric and asymmetric cell divisions

Regulating the balance between symmetric and asymmetric stem cell divisions becomes critical in maintaining proper stem cell number within the niche and in satisfying the demand for differentiated cells within its surrounding tissue. Such niche may participate in orienting asymmetric divisions precisely in order to orchestrate the flow and directionality of the committed daughter cells. In a hypothetical model, interactions between the basal lamina (basal surface) and neighboring stem cells (lateral surface) establish the polarity of the stem cells that in turn leads to the concentration of cell differentiation factors at the apical surface (Perez-Moreno et al., 2003). These factors direct the orientation of the spindle, referred as “spindle-polaring”. Depending upon the relative strength of these spindle-polarizing signals, the ensuing stem cell divisions will either asymmetrically or

symmetrically partition the cell differentiation determinants and/or stem cell factors. If the strongest spindle-polarizing signal localizes at the basal lamina pole, the cell determinants will be asymmetrically partitioned following mitosis. By contrast, if the strongest spindle-polarizing signal localizes at sites of cell-cell contact pole, the determinants are partitioned equivalently, leaving two identical daughter cells. Therefore, the key in assessing whether a division will be asymmetric or symmetric is whether the orientation of the mitotic spindle is parallel to or perpendicular to the major cell polarity axis that aligns the cell fate determinants

6. Germline stem cell (GSC) niche

In Drosophila, the GSC niche is formed by a tight cluster of stromal cells sitting on the apex of gonads. In the testis they are called hub cells, and in the ovary they are termed cap cells. They both intimately contact GSCs, providing the critical membrane-membrane interactions required for self-renewal. The molecular events start with homologous DE-cadherin interactions at the adherens junction connecting stromal and germinal cells; this triggers activation of the Armadillo pathway on the GSCs. Conversely, the nuclear translocation of β-catenin, the vertebrate homologue of Armadillo, has been reported to be sufficient to drive mammalian stem cell self-renewal (Lin, 2002).

A soluble factor in Drosophila, Decapentaplegic (Dpp), is the homologue of BMP2 and BMP4 in mammals and is required for maintaining the GSC population in the ovary through inhibition of Bam (Chen and McKearin, 2003). A gradient of decreasing Dpp and increasing Bam (bag of marbles) indicates GSC differentiation when moving away from the cap cells along the axis of the ovary. A similar mechanism was seen in C. elegans. In the C.

elegans gonad, a gradient of the pro-differentiative factor GLD-1 distal to the tip cells and its inhibitor GLP-1/Notch proximal to the tip are necessary for homeostatic GSC maintenance (Hansen et al., 2004). Hedgehog (hh) signaling appears to regulate the commitment to differentiation of germ cell progeny.

7. Hematopoietic stem cell (HSC) niche

HSC niches are located in the endosteum, the inner surface of the bone that interfaces with the bone marrow. The endosteum is covered with osteoblasts and osteoclasts, which are thought to secret a variety of factors that regulate HSC function. Experiments that increase osteoblast population in mice also increase the number of HSCs in bone marrow (Calvi et al., 2003; Zhang et al., 2003). Some factors, such as angiopoietin, that appear to regulate the maintenance or the numbers of HSCs in bone marrow are expressed by osteoblasts (Arai et al., 2004). Osteoclast activity and the high amounts of calcium generated by bone resorption have also been considered to be involved in promoting HSC maintenance and localization near the endosteum (Adams et al., 2006; Kollet et al., 2006).

The endosteum, however, is not the only niche for HSCs. When osteoblasts are ablated from the bone marrow, HSC population actually increases over time (Visnjic et al., 2004). Moreover, HSCs can be maintained for long periods of time in extramedullary tissues like the spleen and liver, which do not contain endosteum or bone. Therefore, cells other than osteoblasts must be capable of creating HSC niches. The localization of HSCs in hematopoietic tissues showed that about two-thirds of HSCs in the bone marrow and in the spleen are adjacent to sinusoids (Kiel et al., 2005). Sinusoids are surrounded by reticular cells that secrete CXCL12 (also called stromal cell-derived factor (SDF)-1), a chemokine that is

required for HSC maintenance. HSCs localized around sinusoids and to the endosteum are usually associated with CXCL12-secreting reticular cells. The main receptor for CXCL12 is CXCR4 expressed by HSCs (Peled et al., 1999). CXCL12-CXCR4 signaling is required for the colonization of bone marrow by HSC during development and regulates the engraftment of HSCs after transplantation.

8. Neural stem cell (NSC) niche

In the adult rodent brain, neural stem cells (NSCs) are concentrated in subventricular zone (SVZ) of the lateral ventricle wall and the dentate gyrus subgranular zone (SGZ) of the hippocampus. Astrocytes in both regions have been considered as NSCs (Alvarez-Buylla and Lim, 2004; Doetsch, 2003). SVZ astrocytes are in intimate contact with all other SVZ cell types, including the rapidly dividing transient amplifying cells and the committed migratory neuroblasts. Astrocytes, in addition to functioning as primary precursors for the new neurons, also participate in the creation of the microenvironment that stimulates neurogenesis. There is a relationship between vasculogenesis and neurogenesis in the adult brain. In the SGZ, bursts of endothelial cell division are spatially and temporally related to clusters of neurogenesis (Palmer et al., 2000).

Ependymal cells are important regulators in the SVZ and promote a neurogenic niche. These cells produce noggin, an antagonist of BMP signaling, which prevents glial differentiation of SVZ cells induced by BMPs (Lim et al., 2000). In the adult, ependymal cells and SGZ precursors express CXCR4. Its ligand, SDF-1, is required for cerebellar and hippocampal development (Lu et al., 2002; Stumm et al., 2002). SDF-1 acts as an attractant

to hold cerebellar precursors in the external granule layer and cooperates with shh to increase their proliferation (Klein et al., 2001).

Stem cell maintenance and differentiation are often related to their proximity to a basal lamina (BL). Astrocytes are interdigitated with BL and the SVZ BL is rich in laminin, collagen type I, tenascin-C, heparin sulfate and chondroitin sulfate proteoglycans, as well as integrins. The BL tethers factors, anchors cells and provides spatial cues within the stem cell niche. ECM-bound or cell surface-bound molecules can be cleaved to release active ligands or soluble inhibitors. Perhaps the extensive attachment to the BL is important for maintenance of neural stem cell properties.

9. Epidermal and intestinal stem cell niches

In the skin, multipotent stem cells reside in the hair follicle, in a region known as the bulge. The bulge is located below the sebaceous gland and at the juncture of the arector pili muscle. The entire follicle is surrounded by a basement lamina, which is surrounded in turn by a dermal sheath. The bulge also possibly receives inputs from the nearby sensory nerve endings and blood vessels. Bulge stem cells give rise to transient amplifying matrix cells, which proliferate and then differentiate to produce the hair shaft and the channel that surrounds it. The hair follicle bulge (the niche) receives a periodic stimulus from specialized mesenchymal cells, known as dermal papilla (Fuchs et al., 2004). FGF18 and BMP6 have been shown to slow keratinocyte cell growth without inducing terminal differentiation (Christiano, 2004).

The intestine is compartmentalized into crypts. Intestinal stem cells (ISCs) reside near the base of each crypt and give rise to four different progeny: paneth cells, enteroendocrine

cells, goblet cells, and adsorptive villus cells. The crypt epithelium is separated by a basal lamina that is surrounded externally by gut mesenchyme. The mesenchymal cells emit signals that participate in regulating stem cell activity. The pericryptal mesenchyme has been shown to secret various factors such as hepatocyte growth factor (HGF), TGF-β family proteins and keratinocyte growth factor. Corresponding receptors for these factors are located on the neighboring epithelial cells (Rizvi and Wong, 2005; Moore and Lemischka, 2006).

10. Hepatic stem cell (HpSC) niche :

The stem cell niche in liver has long been assumed to be the fetal liver parenchyma, the hepatoblasts, with their signature feature of alpha-fetoprotein (AFP) expression. In the adult tissue it has been thought that there are no stem cells since AFP^+ cells are so rare (<0.01%) and AFP^+ cells do not appear in increased numbers in adult liver except in certain disease states (e.g. cirrhosis). However, recent studies from Dr Reid's laboratory (group) (Schmelzer et al, 2006; Schmelzer et al, 2007; Zhang et al, submitted) have indicated that the stem cell niche in fetal and neonatal livers is the ductal plate (also called limiting plate). This entity is the antecedent to the canals of Hering, the presumptive stem cell niche in adult livers. Given that studies in this thesis are part of that effort to redefine the liver's stem cell niche, the findings and conclusions will be presented as results in the thesis.

The canal of Hering was first described by Ewald Hering who identified a link between the hepatocyte canalicular system and bile ducts in 1867 (Roskams et al., 2004). It is a channel partly lined by hepatocytes and partly by cholangiocytes, and continues into a channel lined entirely by cholangiocytes, called the ductule. Cells of morphology and immunophenotype intermediate between hepatocytes and cholangiocytes are found at

canal of Hering, but not in other regions of the liver (Theise et al., 1999). These cells are considered as bipotent liver progenitor cells with a diameter larger than 6 microns but less than 40 microns. They have been reported to express some biliary antigens (cytokeratins 19 and 7 and OV-6), some hepatocyte antigens (e.g. albumin, α1-antitrypsin and occasionally AFP, and neuronal cell adhesion molecule or NCAM/CD56) (Roskams et al., 2003).

One of the earliest cellular responses to chemical hepatocarcinogens includes the proliferation of epithelial cells that are different in morphology and biochemistry than mature hepatocytes (Factor et al., 1994). These highly proliferative stem-like cells are called oval cells due to the shape of their nuclei, and are able to give rise to a new system of oval cell ducts spreading into the acinus and to differentiate into hepatocytes inside the ducts in the periportal areas in mouse livers. Several injury models have been developed to study the origin and properties of oval cells. Factor et al. injected the DNA-alkylating agent Dipin (1,4-bis[N,N'-di(ethylene)-phosphamide]-piperazine) intraperitoneally followed by two-thirds partial hepatectomy to induce generation of oval cells in mouse livers (Factor et al., 1994). Wang et al. used Nycodenz gradient centrifugation to separate the oval cells from the mature hepatocytes after DDC-(3,5-diethoxycarbonyl-1,4-dihydrocollidine) treatment to the wild-type (WT) adult mice, and subsequently transplanted the purified oval cells into the *Fah*-/- (fumaryl-acetoacetate hydrolase deficient) recipient mice followed by a long-term NTBC (2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione) withdraw (Wang et al., 2003). The recipient mice had significant donor-derived hepatocyte repopulation and phenotypic rescue. It has been reported that mouse A6-positive hepatic oval cells expressed AFP and several

hematopoietic stem cell markers, such as CD34, CD45, and Sca-1 (Petersen et al., 2003). Since these highly proliferative stem-like cells were induced by carcinogens, long-term monitor of these oval cells following hepatocarcinogen treatment to adult mice should be conducted to investigate the possibility of transformation of these stem-like cells to the hepatic cancer cells. It is still unclear whether oval cells found in the mouse liver after carcinogen-induced injury are normal hepatic stem cells or are heterogenically composed of normal as well as tumorigenic progenitor cells.

The matrix chemistry within the liver has been defined broadly and for the entire liver acinus but not yet for the stem cell niche. The matrix components are present in the Space of Disse, located between the parenchyma and the endothelia. They form a gradient in the matrix chemistry that begins at the portal triads and transitions to that near the central vein (Reid et al., 1992; Sigal et al, 1992; Martinez-hernandez, 1993; Zern and Reid, 1993). The periportal zone contains hyaluronans, collagen types III, IV and V, laminin, and fetal forms of proteoglycans, such as chondroitin sulfate proteoglycan (CS-PG) and heparin sulfate proteoglycan (HS-PG). It transitions to a matrix chemistry in association with the most mature parenchymal cells near the central vein and comprised of type I collagen, fibronectin, and heparin proteoglycan (HP-PG).

C. Companion Cells Located in or near the Hepatic Stem Cell Niche

The major non-parenchymal cells that are companion cells to the HpSCs and in or near the niche include hepatic stellate cell precursors and angioblasts. The liver acinus, outside of the niche, contains sinusoidal endothelial cells, mature hepatic stellate cells, and hemopoietic cells, particularly the Kupffer cells, a form of macrophage. These “companion”

cells regulate development, proliferation and differentiation of hepatic parenchymal cells by secretion of cytokines as well as synthesis and remodeling of extracellular matrix molecules. Although characterization of these companion cells in the mature liver has been well-documented, little is known of the biology of the companion cell precursors found in the stem cell niche.

1. Hepatic Stellate Cells (HpSTCs)

Hepatic stellate cells (HpSTCs) are mesenchymal cells in the subendothelial space of Disse, separating the apical surface of hepatocytes from the ablumenal side of sinusoidal endothelial cells. These close cell-cell contacts between neighboring cells are important for efficient paracrine transport of growth factors and cytokines. HpSTCs play a pleiotrophic role in regulation of liver growth via extracellular matrix production and remodeling, growth factor and cytokine synthesis and changes in sinusoidal porosity. HpSTCs are thought to be fibroblastic mesenchymal cells based on their significant induction of α-smooth muscle actin (α-SMA), matrix molecules, and matrix metalloproteinases during activation. They also exhibit many neuroendocrine features, including the expression of synaptophysin, glial fibrillary acidic protein (GFAP), neural cell adhesion molecule (NCAM), nestin, neurotrophins, dopamine-β-hydroxylase (DBH), and tyrosine hydrozylase (Sicklick et al., 2005).

HpSTCs express at least three major hepatocyte mitogens: transforming growth factor-α (TGF-α), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (Ankoma-Sey and Friedman, 1998; Bachem et al., 1992; Maher, 1993; Mullhaupt et al., 1994). HGF is a heparin-binding protein stored within the extracellular matrix. During

regeneration after partial hepatectomy, HGF mRNA is increased in the liver reaching its peak level at 10-12 hours after surgery (Selden et al., 1990). HpSTCs also synthesize and release a variety of inhibitory growth factors including TGF-β which also promotes liver fibrosis via induction of MMP-2 and TIMPs. Activin A, a member of the TGF-β superfamily, is produced by HpSTCs after CCl_4 treatment (Date et al., 2000). Expression of Activin A receptors is down-regulated maximally at 24 hours after toxic injury and returns to normal by 96 hours. Down-regulation of Activin A receptors prevents Activin A from transducing its anti-proliferative stimulus. Activin A also promotes fibronectin synthesis in HpSTCs. Bone morphogenetic protein-6 (BMP-6), also a member of the TGF-β superfamily, is expressed in HpSTCs and Kupffer cells (KCs) (Knittel et al., 1997).

Stem cell factor (SCF) and acidic fibroblast growth factor (FGF-a) have been identified in HpSTCs in rat livers during regeneration after partial hepatectomy (Fujio et al., 1994; Marsden et al., 1992). Insuline-like growth factor I and II (IGF-I and –II), their binding proteins and their receptors, are also produced by HpSTCs (Pinzani et al., 1990; Zindy et al., 1992). Expression of heparin-binding growth factor-2 is localized to HpSTCs in CCl_4-treated rat livers (Charlotte et al., 1993). Using transgenic animals expressing a reporter gene controlled by the erythropoietin gene, Maxwell et al. reported expression of the erythropoietin gene in HpSTCs (1994). Pro-inflammatory mediators MCSF and monocyte chemotactic peptide 1 (MCP-1) are also secreted by HpSTCs (Marra et al., 1993; Pinzani et al., 1992). MCSF production is stimulated by PDGF and FGF-b. MCP-1 production is stimulated *in vitro* by interleukin-1α (IL-1α) and interferon-γ (IFN-γ). IL-6 mRNA is expressed in primary HpSTC and HpSTC lines derived from normal rat livers (Greenwel et al., 1993).

HpSTCs express hedgehog (hh) ligands and multiple components of the hh pathway including Sonic hedgehog (shh), Indian hedgehog (ihh), Ptc, Smo, Gli1, Gli2, and Gli3 (Sicklick et al., 2005). Freshly isolated HpSTC fraction from adult mouse livers had a 313-fold higher expression of Shh and a 7-fold higher expression of ihh than total non-parenchymal cell fraction. Clonally derived HpSTC line 5H from adult rat livers had significantly higher apoptotic (caspase 3/7) activity and lower cell viability in the presence of Shh-neutralizing antibody (5E1) (10 μg/ml) for 48 hours. This finding suggests that hh pathway activity prevents apoptosis of HpSC and modulates HpSTC activation. HpSCs can be activated *in vitro* and *in vivo* after liver injury. Previously reported markers of the activated HpSTCs include actin-α_2 (ACTA2), collagen types I-α_1 and -α_2 and IV-α_1, -α_2, -α_5 and -α_6, galectin 1, laminin-α_5 chain, tissue inhibitor of metalloprotease-2 and -4 (TIMP-2 and -4), TGF-β1 and IL-10 receptor (Kannangai et al., 2005).

2. Endothelial cells (ECs)

Endothelial cells (ECs) forming the fenestrated walls of the liver sinusoids are characterized by a thin cytoplasm, flattened nuclei and, in zone 2 and 3 (but not zone 1) of the liver acinus, numerous fenestrae grouped in sieve plates, enabling direct communication between the sinusoid and space of Disse (Winwood and Arthur, 1998). During embryonic development of the liver and in hepatic regeneration, the formation of hepatic sinusoids by ECs is a relatively late event. A network of continuous capillaries with a basement membrane feeds differentiating hepatocytes. The liver development is induced in a step-wise process with the signals from cardiac mesoderm and then from the ECs (Lammert et al., 2003). Endodermal cells are induced by cardiac mesoderm to differentiate into hepatic stem cells

within the ventral endoderm at E8.5 of mouse gestation (Gualdi et al., 1996). Initial stages of hepatogenesis require FGFs secreted from pre-cardiac mesoderm and BMPs from the septum transversum mesenchyme (STM) (Duncan, 2003). At E9.5, newly specified hepatic cells delaminate and migrate into the surrounding STM and intermingle with ECs. ECs remain in contact with hepatic cells throughout development. In flk-1 mutant mouse embryos, which lack ECs, initial hepatic induction occurs, but no hepatic cells proliferate into the surrounding STM at E9.5, indicating the importance of ECs for normal hepatic outgrowth during early stages of liver organogenesis prior to blood vessel function (Matsumoto et al., 2001; Shalaby et al., 1995).

ECs in normal liver express TGF-β (Braun et al., 1988). TGF-β mRNA was enriched in the EC fraction isolated from adult rat livers, but at much lower levels than the message detected in Kupffer cells (KCs). TGF-β inhibits EGF-induced DNA synthesis *in vitro* in hepatocytes from normal or regenerating liver after partial hepatectomy. HGF has been reported to be produced by ECs during liver regeneration (Maher, 1993). In normal liver, HGF and its mRNAs were abundant in HpSTCs, with lesser amounts detected in sinusoidal KCs and ECs (108.8, 17 and 6.2 U/million cells respectively). In regenerating liver, HGF gene expression increased exclusively in ECs. HGF mRNA levels rose 6-fold in ECs, peaking at 6 hours after carbon tetrachloride treatment, and returning to near normal by 24 hours. The rise in HGF mRNA was accompanied by a 5.4-fold increase in HGF secretion as indicated by enzyme-linked immunosorbent assay (ELISA) experiment.

ECs have the potential to synthesize matrix components. Jarnagin et al. examined the cell-specific expression of two fibronectin isoforms, EIIIA and EIIIB, during experimental hepatic fibrosis induced by ligation of the biliary duct (1994). Both isoforms were

undetectable in normal liver at mRNA level. EIIIA-containing fibronectin mRNA increased rapidly in sinusoidal ECs within 12 hours of injury. In contrast, EIIIB-containing isoform appeared only after a lag of 12-24 hours and was localized to hepatic stellate cells. At the protein level, EIIIA-containing fibronectin increased significantly within two days of injury and distributed at sinusoidal region. Secretion of this form by sinusoidal ECs accelerated the activation of normal hepatic stellate cells (HpSCs) to myofibroblast-like cells. Pretreatment of matrices with monoclonal antibody against the EIIIA segment blocked this activation of HpSTCs.

3. Kupffer cells (KCs)

Kupffer cells (KCs) are located in the hepatic sinusoids and are involved in the metabolism of various compounds, immunological responses and inflammatory reactions. Primitive macrophages first develop in the murine and human yolk sac and then differentiate into fetal macrophages and migrate into the fetal liver. The resident macrophages expand quickly and transform into KCs in the late stage of ontogeny and after birth. Hepatocytes provide a microenvironment for the development, differentiation and proliferation of macrophages in fetal liver by producing colony-stimulating factors (CSFs) (Naito et al., 1997). Human fetal liver (13-19 weeks of gestation)-derived KCs have been isolated using density gradient separation method and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) on plastic (Kutteh et al., 1991). Human fetal KCs are able to phagocytose 0.8μm latex beads and respond to bacterial lipopolysaccharide (LPS) with production of TNF-α, IL-6 and IL-1β. Both dexamethasone and cortisol inhibited the production of LPS-stimulated IL-6 (Kutteh et al., 1991). Similar to human KCs, cultured KCs isolated from

adult rat livers have been shown to produce IL-1, IL-6, IL-8 and prostaglandin E_2 (PGE_2) *in vitro* in serum-supplemented media (Goss et al., 1993; Maher, 1995).

Both pro-proliferative and anti-proliferative cytokines are produced and released from KCs. Boulton et al. showed that following KC depletion the early phase of liver regeneration after partial hepatectomy was enhanced, as indicated by a greater proportion of hepatocytes undergoing DNA synthesis, and a higher mitotic index (1998). KCs were selectively eliminated in rat livers by liposome encapsulated dichlormethylene bisphosphonate (Cl_2MBP). HGF and TGF-β mRNA were reduced in KC-depleted animals, and IL-1β mRNA was absent. In the control rats without partial hepatectomy, depletion of KCs increased proliferation of hepatocytes in resting liver undergoing DNA synthesis. Conditioned medium prepared from LPS-stimulated KCs induced time-dependent increase in IL-8 mRNA levels in SK-hepatoma cells over a 24-hour period (Thornton et al., 1991).

IL-10 is produced by KCs. In KC-depleted rat livers, IL-10 expression was decreased significantly and TNF-α was constitutively overexpressed compared with normal animals (Rai et al., 1997). Less IL-10 from KCs allows more sustained TNF-α release from other cells, leading to increased hepatocyte proliferation. During liver regeneration after partial hepatectomy, TNF-α activates TNF receptor-1 which then increases hepatic levels of the transcription factor NFκβ at 1-4 hours, followed by an increase in IL-6 at 2-6 hours (Malik et al., 2002).

KCs may play a role in remodeling the newly deposited matrix during liver regeneration. Human and rat KCs synthesize and release gelatinase B/type IV collagenase in primary culture (Winwood et al., 1995). Gelatinase B is a member of the family of matrix metallopropeinases that has specific degradative activity against denatured collagens

(gelatin), collagen types IV and V and elastin. The physiological function of KC-derived gelatinase B is unknown. It is thought to facilitate migration and proliferation of KCs at space of Disse in normal liver during injury.

D. Use of Feeders (Co-cultures) for *ex vivo* Maintenance of Stem Cells

1. Early experiments on feeder effects

In 1955, Puck and Marcus published the first "feeder" paper in which they provided a method of clonal growth of HeLa cells with the aid of irradiated HeLa cells, the feeder cells (Puck and Marcus, 1955). Unirradiated HeLa cells tend more often to form closely packed colonies in the presence of a "feeder" system than in its absence. Consequently, the need for the feeders should decrease as growth proceeds. Feeder cells may help to promote growth of dependent cells by providing necessary diffusible factors and/or by removing toxic actions of some metabolites in the media (Fish and Puck, 1956; Puck et al., 1956). However, they reported that medium previously conditioned by the growth of unirradiated cells was not effective in promoting colony formation.

Stoker and Sussman set up a half-feeder cultures with irradiated mouse embryonic (ED17) fibroblasts occupying only one side of a petri dish and found that the dependent cells, BKM21 (hamster fibroblasts) cells, formed large colonies confined to the half of the dish with attached feeder cells (Stoker and Sussman, 1965). A few small colonies were formed on the bare side, and there was a very sharp boundary at the edge of the feeder cells. They also found that the feeder layer had no effect on attaching and spreading of added cells. The feeder effect, once established, depends on the continued integrity of the feeder cells. It is

maximal within 0.2 mm of the feeder cells and is detectable with dimishing effect up to 1.5 mm from the feeder cells.

2. Culture of keratinocytes.

Culture of keratinocytes on a feeder-layer of murine fibroblasts has been reported (Rheinwald and Green, 1975; Tenchini et al., 1992). The stromal cell line, NIH 3T3, derived from murine embryos, turned out to be the most effective for this purpose. The most widely used procedure to obtain non-proliferating NIH 3T3 cells is to expose them to high dose (6000 rads) of gamma rays. Alternatively, treatment with Mitomycin-C can be used, but since this compound is a mutagen, irradiation is generally preferred. The 3T3 cells have also been used as feeder cells to generate various epithelial cell sheets, such oral mucosal cells, corneal cells, and retinal pigment epithelial cells (Hata et al., 1995; Kobayashi et al., 2005; Pellegrini et al., 1997)

3. Cultures of primordial germ cells

Most available culture methods for primordial germ cells (PGCs) rely upon the presence of feeder cell layers, such as irradiated STO and mouse Sertoli cell-derived cell lines (De Felici and Dolci, 1991; Donovan et al., 1986). Isolated PGCs recovered from 11.5 and 12.5 day post-cotium (dpc) embryonic mouse gonads did not survive at 37°C without somatic cell support, and undergo rapid apoptotic degeneration (De Felici and McLaren, 1983; Farini et al., 2005). The addition of single growth factors to the culture medium only slowed down PGC apoptosis and did not substitute for the feeder cell layers (Pesce et al., 1993). Lee and Piedrahita compared the numbers of colonies of porcine PGCs after 8-10

days of culture on different feeder cells including STO (mouse embryonic fibroblast cell line), TM_4 (mouse Sertoli cell-derived cell line), Sl/Sl^4 m220 (mouse homozygous null Sl/Sl^4 embryonic cell line), PEF (porcine embryonic fibroblasts isolated from day 25-30 fetuses), and COS-7 cells (African green monkey kidney cell line) (Lee and Piedrahita, 2000). STO, TM_4, and Sl/Sl^4 m220 supported survival of porcine PGCs during culture and there were no differences among them. However, the numbers of AP-positive colonies cultured on PEF and COS-7 cells were significantly lower than the other three cell lines. The three supportive feeder cells were known to express membrane-bound SCF (stem cell factor) while the other two were not. This result indicates that colony formation ability of PGCs is feeder cell-specific.

Kubota and Brinster developed a culture system for mouse spermatogonial stem cells using a serum-free defined medium and growth-arrested STO feeder cells (Kubota and Brinster, 2006). The cultured mouse spermatogonial stem cells express Oct-4 and alkaline phosphatase, markers similar to undifferentiated embryonic stem cells. The serum-free medium contains glial-cell-line-derived neurotrophic factor (GDNF), soluble GDNF-family receptor α1 and basic fibroblast growth factor (FGF-b). These three factors are required for self-renewal of both murine and rodent spermatogonial stem cells *in vitro* (Ryu et al., 2005).

4. Culture of hematopoietic stem cells (HSCs) and neuronal progenitors.

Use of endothelial cells as feeder cells for supporting HSCs has been reported. Auerbach et al. derived 3 endothelial cell lines (C165, C166, and C167) from murine embryonic (ED12) yolk sacs and irradiated them with 25Gy using a ^{137}Cs source (Auerbach et al., 1996; Auerbach et al., 1998; Lu et al., 1996; Wang et al., 1996). HSCs isolated from

mouse embryonic (ED10) yolk sacs (YS-HpSCs) were dispensed on top of the yolk sac-derived endothelial cell (YS-EC) monolayers (Huang and Auerbach, 1991). YS-EC line C166 permitted a 16- to 36-fold increase in the total number of nonadherent YS-HpSCs generated after 1 week with an antigenic profile of Sca-1^+ and major histocompatibility complex (MHC) class I^+. These expanded YS-HpSCs are able to differentiate into B-cells, T-cells, and various myeloid cells *in vitro* and to reconstitute hematopoietic lineages in mildly irradiated BALB/c scid mice when transplanted. These results showed that expansion with retention of stem cell properties can be accomplished by using a co-culture system consisting of a cloned endothelial cell line derived from the yolk sac of mid-gestation (12-day) embryos. However, this endothelial feeder failed to maintain the more primitive hematopoietic stem cell phenotype from day-10 embryonic yolk sac with an antigenic profile of Sca-1-negative and MHC class I- and II-negative.

Feeder cells have also been utilized to promote neurogenesis. Hermann et al. cocultured human adult hippocampal neuroprogenitor cells (hNPCs) with fetal cortical cultures containing astrocytes and neurons, mouse embryonic fibroblasts, or PA6 stromal cells, and showed increases of neurogenesis of hNPCs on all three types of feeders (Hermann et al., 2006).

5. Transformation assays

In addition to promoting growth of dependent cells, feeder cells have also been used in transformation assays. Marquardt and Heidelberger plated irradiated rat or mouse embryonic fibroblasts 24 hours prior to the plating of G23 (mouse ventral prostate fibroblasts) cells, which poorly metabolize carcinogenic hydrocarbons (Marquardt and Heidelberger,

1972). The feeder cells increase the yield of malignant transformation because of their ability to metabolize and activate the hydrocarbons. Feeder cells have also been shown to increase colony forming efficiency of X-irradiated human colon cancer cells in tissue culture dishes (Leith et al., 1991). However, feeder cells do not affect the radiation survival parameters of those tumor cell lines. The question of whether some types of cancer cells may be altered in their post-irradiation survival responses by feeder cells must relate to the conditioning factors.

6. Use of feeder cells for human embryonic stem (ES) cell growth.

The human ES cells were first isolated in 1994 (Bongso et al., 1994). The first continuous human ES cell lines were established in 1998, and were derived and propagated by direct contact on murine embryonic fibroblasts (Thompson et al., 1998). Since then various feeder systems have been developed for the growth of human ES cells (Cowan et al., 2004; Reubinoff et al., 2000; Rose-John, 2002). Although mouse embryonic fibroblasts allow long-term culture of human ES cells, xeno-coculture introduces complications for the delivery of safe therapies for clinical programs in the future. The human ES cells might be infected by some murine viruses possibly active in the animal feeders. Certain mouse glycoprotein molecules, such as Neu5Gc, has been found to be incorporated metabolically into human ES cells and derived embryoid bodies co-cultured with mouse embryonic fibroblasts (Martin et al., 2005). It is also possible that cell fusion might occur between human and murine cells (Stacey et al., 2006).

Approaches to use human feeder cells have also been reported. Richards et al. compared 11 different human adult, fetal, and neonatal feeders as well as mouse embryonic fibroblasts which were Mitomycin-C-inactivated for human ES cell support and ranked them

as supportive and non-supportive (Richards et al., 2003). Fibroblast cell lines established in-house from human fetal skin, human adult abdominal skin biopsies, and human fetal muscles supported prolonged undifferentiated human ES cell growth for over 25, 30, and 50 weekly passages respectively in serum-free medium supplemented with 20% serum-replacement. Compared with human feeders, human ES cells growing on murine embryonic fibroblasts showed lower percent of completely undifferentiated colonies generated after 8 days of culture (55%-75% on mouse vs. 85%-90% on human). Other human feeder cells shown to supported long-term growth of human ES cells include newborn foreskin fibroblasts, placental fibroblasts, adult uterine endometrial cells, adult breast parenchymal cells, embryonic fibroblasts, and adult bone marrow-derived stromal cells (Amit et al., 2003; Cheng et al., 2003; Choo et al., 2004; Genbacev et al., 2005; Hovatta et al., 2003; Lee et al., 2004, 2005).

E. Feeder-Free Conditions

The contribution of feeder cells in stem/progenitor cell culture is not entirely understood. It has been suggested that feeder cells provide both a suitable attachment substrate and important soluble factors for the maintenance and differentiation of undifferentiated stem/progenitor cells *in vitro*. However, the soluble factors as well as the extracellular matrix (ECM) molecules produced by the feeders remain poorly defined components of co-culture of feeder cells and supported cells. Feeder-free cultures would eliminate the difficulties of standardization and optimization procedures, thus making analyses of stem/progenitor cell properties easier.

1. Human embryonic stem (ES) cells

Traditionally, human embryonic stem (ES) cells have been cultured with mouse embryonic fibroblast (MEF) feeder layers, which allow them to grow continuously in an undifferentiated state (Amit and Itskovitz-Eldor, 2006). Mouse ES cells maintain their stem cell features and remain undifferentiated when grown on gelatin without MEF feeder and within medium supplemented with LIF (Smith et al., 1988; Williams et al., 1988). However, LIF failed to support feeder-free culture of hESCs (Thomson et al., 1998). The mechanism for self-maintenance of human ES cells is still unclear. The first feeder-free culture for human ES cells was reported by Xu and coworkers (2001). Four human ES cell lines have been successfully cultured on Matrigel in MEF-conditioned medium supplemented with 20% serum replacement and 4 ng/ml of hFGF-b. The longest culture has been maintained for over 130 population doublings (180 days). All long-term cultured cells retained morphology and karyotype of human ES cells, and expressed markers such as SSEA-4, Tra 1-81, OCT-4 and hTERT.

A combination of the BMP signaling antagonist Noggin and high concentration of FGF-b has been reported to be sufficient to maintain undifferentiated human ES cells in feeder-free conditions (Xu et al., 2005). BMPs have previously been shown to induce human ES cell differentiation into trophoblast (Xu et al., 2002). In contrast, in mouse ES cells, BMP4 synergizes with LIF to maintain self-renewal of mouse ES cells in the absence of serum (Ying et al., 2003). DMEM/F12 supplemented with 20% serum replacement, 1 mM L-glutamine, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, 500 ng/ml noggin and 40 ng/ml FGF-b sustained undifferentiated proliferation of human ES cells. The same group recently reported a detail protocol of serum-free condition used for culturing human ES cells

(Ludwig et al., 2006). Cloned zebrafish FGF-b (100 ng/ml), TGF-β1 (0.6 ng/ml), insulin (20 μg/ml) and bovine holo-transferrin (11 ug/ml) were included in the medium and Matrigel was used as matrix to coat the plates.

Although feeder-free conditions have been developed for culturing human ES cells, it has been reported that human ES cells cultured in feeder-free conditions may gain chromosomal changes (Draper et al., 2004). When a subline of H7 human ES cells was cultured on Matrigel without MEF feeders or MEF-conditioned medium for over 25 passages, a clone of these feeder-free H7 cells was found to carry an isochromosome, i(12p), the characteristic karyotypic marker of human embryonic carcinoma cells. It was hypothesized that enzymes used in passaging may push cells to too demanding culture conditions that may induce abnormalities (Skottman and Hovatta, 2006).

2. Neural stem cells and adult skin-derived multipotent cells

Neural stem/progenitor cells derived from embryonic and adult central nervous system have been cultured as neurospheres in the presence of EGF and FGF-basic in feeder-free condition (Jesseberger et al., 2007; Uchida et al., 2000). Neurosphere assay has been used as a tool for isolating and understanding the biology of neural stem cells (NSCs) (Reynolds and Rietze, 2005). Uchida et al. used flow cytometry to sort $CD133^+$ NSCs from human fetal spinal cord and brain tissues and cultured them in neurosphere initiation medium consisting of *Ex Vivo* 15 medium with N2 supplement, FGF-b (20 ng/ml), EGF (20 ng/ml) and LIF (10 ng/ml). Neurosphere formation and clonal expansion from single $CD133^+$ cells were observed in 6-8 weeks. Progeny of single cell-derived neurospheres can be differentiated into neurons (β-tubulin$^+$) and astrocytes (GFAP (glial fibrillary acidic protein)$^+$)

in the medium with growth factors, brain-derived neurotrophic factor (10 ng/ml), glial-derived neurotrophic factor (10 ng/ml) and laminin (10 μg/ml) on polyornithine-coated chamber slides (Uchida et al., 2000).

Ray and Gage isolated NSCs from whole brain tissues of adult mice and cultured these cells in serum-free N2 medium containing FGF-b (20 ng/ml), EGF (20 ng/ml) and heparin (5 μg/ml) with seeding density of 20,000 cells/cm^2 on uncoated tissue culture plastic plates (Ray and Gage, 2006). Proliferating clusters of cells, attached to the plates, started to appear by 2 weeks. Similar condition has also been used for culturing rat NSCs except that EGF and heparin were not added. Proliferating clusters of cells, attached to the plates, started to appear in about 1-2 weeks. This result showed that NSCs can be cultured to form monolayers instead of neurosphere. Cells within neurospheres are tightly packed. Properties of these cells (morphology, proliferation and differentiation) can only be examined after dissociation and replating of neurospheres. Culturing cells to form monolayers can eliminate these limitations.

Multipotent stem cell-like cells has been isolated from adult trunk skin of mice and humans. These cells were cultured in DMEM/F12 containing B-27 supplement, FGF-b (20 ng/ml), EGF (10 ng/ml) and LIF (10 ng/ml) with high seeding density 100,000-160,000 cells/cm^2 on uncoated cell culture flask (Wong et al., 2006). Formation of floating spheres from these cells was observed within 4-7 days and the expression of neural crest stem cell (NCSC) markers, p75 (low affinity neurotrophin receptor) and Sox10 (transcription factor), was detected on these cells. On dishes coated with fibronectin, neurogenesis, gliogenesis, and smooth muscle formation were observed after 3-7 days. Chondrocyte formation was observed after 9 days in DMEM containing 10% FCS (fetal calf serum), ascorbic acid (50

μg/ml) and FGF-b (10 ng/ml), followed by DMEM containing 10% FCS, ascorbic acid (50 μg/ml) and BMP-2 (10 ng/ml) for another 3 days. Adipocytes were occasionally observed when cultured in DMEM/F12 containing B-27 supplement and BMP-2 (10 ng/ml). Melanocytes were observed when cultured in DMEM containing 10% FCS, murine SCF (50 ng/ml) and endothelin-3 (100 nM).

3. Hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs)

Suspension cultures of hematopoietic stem cells (HSCs) without bone marrow stroma has been reported to be relatively ineffective in sustaining HSCs for long term *in vitro*. This may be due to the absence of natural three-dimensional topography and poorly defined characteristics of the bone marrow environment *in vivo*. Rosenzweig et al. used a tantalum-coated porous biomaterial to culture $CD34^+$ bone marrow-derived HSCs and showed that this material may help in maintaining HSCs, multipotency, long-term survival, and retroviral transduction *in vitro*. The number of $CD34^+/CD38^-$ cells was relatively higher on this material compared with bone marrow stroma (Ehring et al., 2003; Rosenzweig et al., 1997). They also found that addition of exogenous cytokines, IL-3 (100 ng/ml), IL-6 (20 ng/ml) and SCF (100 ng/ml), to cultures significantly inhibited T cell differentiation. A serum-free condition with recombinant human GM-CSF (100 ng/ml), TNF-α (50 U/ml) and SCF (20 ng/ml) has also been reported to be capable of maintaining human $CD34^+$ HSCs (Strobl et al., 1996). The same condition with addition of TGF-β1 (0.5 ng/ml) strongly induces dendritic cell differentiation.

$CD133^+/VEGFR2^+$ human endothelial progenitor cells (EPCs) can be isolated from peripheral blood, cord blood and human fetal livers at 14 to 16 weeks of gestation, and have

been cultured in feeder-free condition with high serum (20% FBS) and high seeding density (500,000 cells/cm^2) on fibronectin- or collagen type I-coated plates (Peichev et al., 2000; Powell et al., 2005). EPC colony-forming units, defined as a central core of rounded cells surrounded by elongating and spindle-shaped cells, were observed in one week on human fibronectin-coated plates and were positive for endothelial markers, such as CD31, vWF (von Willebrand factor) and VE-cadherin. When supplied with recombinant human FGF-b (5 ng/ml) and heparin (5 units/ml) on collagen type-I-coated dishes, monolayers of mature endothelial cells appeared in 2 weeks. Reyes et al. isolated multipotent adult progenitor cells (MAPCs) from human bone marrow and induced these cells to differentiate into vWF^+ endothelial cells in a serum-free defined medium with VEGF (10 ng/ml) on fibronectin-coated culture dishes without feeder cells (Reyes et al., 2002). When replated in Matrigel with VEGF, vascular tube formation was observed from the MAPC-derived endothelium within 6 hours.

4. Hepatic stem cells and hepatoblasts

It has been reported that hepatic stem cells (HpSCs) as well as hepatoblasts can be isolated from human fetal livers obtained from mid-gestational stages (16-20 weeks) and cultured under feeder- and serum-free defined condition (Schmelzer et al., 2006;). Human fetal liver-derived HpSCs and hepatoblasts were expanded on tissue culture plastics for more than 3 months in a hormonally defined medium (HDM) supplemented with insulin (5 μg/ml), iron-saturated transferrin (10 μg/ml), hydrocortisone, nicotinamide (4.4 mM), selenium (30 nM), L-glutamine, mixture of six free fatty acids (7.6 μM), 2-mercaptoethanol (50 μM) and bovine serum albumin (0.1%). The hHpSCs have an antigenic profile positive for EpCAM

(epithelial adhesion molecule), NCAM (neuronal adhesion molecule), albumin, cytokeratin-19 (CK19), CD133/1 (also known as prominin in mouse) and claudin-3 and negative for ICAM-1 (intercellular adhesion molecule-1) and AFP (alpha-fetoprotein). They can give rise to more differentiated bipotent hepatoblasts with an antigenic profile positive for EpCAM, ICAM, AFP, albumin, CK19 and CD133/1 and negative for NCAM.

Although feeder cells are not necessary for *in vitro* expansion of human HpSCs, non-parenchymal cells, such as endothelial cells and hepatic stellate cells, have been found to exist in culture with HpSC colonies. These companion cells possibly form aggregates with HpSCs during isolation process and survived in the feeder- and serum-free condition. Perhaps these non-parenchymal cells are meant to co-exist with HpSCs as naturally designed feeders to support growth of HpSCs *in vitro* and as components of liver stem cell niche to maintain HpSCs *in vivo* during quiescent state. Human hepatoblasts have also been maintained in three-dimensional hyaluronan hydrogel scaffold in feeder- and serum-free condition (Turner et al., 2007).

5. Germline stem cells (GSCs)

Murine spermatogonial stem cells can be cultured for extended periods in the presence of serum and feeder cells (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). Spermatogonial stem cells from neonatal testis were able to proliferate for more than 5 months on mouse embryonic fibroblasts (MEFs) in the medium supplemented with GDNF (glial cell line-derived neurotrophic factor), FGF-b, EGF, LIF and FCS (fetal calf serum). Recently, Kanatsu-Shinohara et al. reported feeder-free condition that can support mouse GSCs to expand in the presence of recombinant rat GDNF (10 ng/ml), mouse EGF (20

ng/ml), mouse LIF (1000 U/ml), human FGF-b (10 ng/ml) and 1%FCS on laminin-coated plates (Kanatsu-Shinohara et al., 2005). They grew exponentially for at least 6 months and produced normal, fertile progeny following transplantation into infertile mouse testis. However, the murine GSCs could not expand when both serum and feeder cells were absent.

F. Cytokines/Growth Factors/Hormones Important in Normal Liver Development

The possible paracrine signals secreted from mesenchymal cells in septum transversum mesenchyme and cardiac mesoderm to induce hepatic specification in ventral endoderm in the embryos include fibroblast growth factor-1 (FGF-1 or FGF-acidic), FGF2 (or FGF-basic), FGF8, bone morphogenetic protein-2 (BMP-2), and BMP-4 (Jung et al., 1999; Rossi et al., 2001; Zaret, 2002). Although the exact mechanisms through which bipotential hepatoblasts decide to become hepatocytes or cholangiocytes are still unclear, it has been hypothesized that transforming growth factor-beta (TGF-β) and hepatocyte growth factor (HGF) may orchestrate to control bipotency and cell-fate decisions in the early liver. Oncostatin M (OSM), glucocorticoid, epidermal growth factor (EGF), and activins are also possible factors regulating cell-fate decision of hepatoblasts. Hedgehog (Hh) signaling is required for endodermal commitment and hepatogenesis.

1. Stem cell factor (SCF)

Stem cell factor (SCF), also termed Kit ligand, steel factor or mast cell growth factor, is the ligand of the c-kit protooncogene product. It is a glycoprotein existing in both soluble and membrane bound forms after alternative splicing and proteolytic cleavage (Reber et al., 2006). Binding of SCF homodimers to c-kit induces homodimerization and intermolecular

tyrosine phosphorylation of the receptor, creating docking sites for a number of Src-homology2 (SH2)-containing signal transduction molecules. Kit has the potential to participate in multiple signal transduction pathways as a result of interacting with several enzymes and adaptor proteins (Roskoski Jr., 2005). Growth factor receptor-bound protein-2 (Grb2) is an adaptor protein that associates with the son-of-sevenless protein (sos). This complex interacts with and activates the small G-protein Ras which leads to activation of Raf-1, MAP kinase p38, ERK1/2 and c-JunN-terminal kinase (JNK).

SCF also induces phosphatidyl-inositol-3 kinase (PI3-kinase) pathway. PI3-kinase recruitment leads to Akt activation and to the subsequent phosphorylation of the pro-apoptotoc factor Bad, which promotes cell death. Src and Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathways can also be activated by SCF. Src kinase and PI3-kinase signaling pathways converge to activate Rac1 and JNK after SCF stimulation in bone marrow-derived mast cells (BMMC), promoting cell proliferation. SCF-induced JAK/STAT activation is associated with proliferation and differentiation of fetal liver hematopoietic progenitor cells. Protein kinase C (PKC), protein phosphatase SHP-1 as well as suppressors of cytokine signaling (SOCS)-1 and -6 can downregulate c-kit signaling.

2. Hedgehog Signaling

During embryonic development, the Hedgehog (Hh) signaling pathway regulates proliferation and differentiation in a time- and position-dependent fashion so that developing tissues reach their correct size with appropriate cell types. The Hh ligands and their signaling pathway components have been found to be expressed in murine and human hepatic progenitor cells (Sicklick et al., 2006). Indian Hh (Ihh) but not Sonic Hh (Shh) was expressed

in the stem cell compartments of human fetal livers and colocalized with CK19, EpCAM and α-fetoprotein (AFP). Freshly isolated EpCAM+ cells from neonatal and adult human livers expressed Ihh, Shh and Patched (Ptc). In both murine and human hepatic progenitor cells, manipulation of Hh activity influences their survival.

Hh proteins are secreted signaling proteins that were first discovered in Drosophila. They are highly hydrophobic molecules which, once secreted, can diffuse and establish gradients in tissues. Three Hh homologues have been identified in human: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). The Hh signaling pathway is initiated by Hh binding to the Patched (Ptc) protein. In the absence of Hh ligans, Ptc repressed the activity of Smoothened (Smo), a G-protein-coupled receptor (GPCR)-like receptor, presumably by preventing its localization to the cell surface. It is thought that an endogenous intracellular small molecule that acts as an agonist for Smo is transported outside the cell by Ptc so that it is unavailable to bind to Smo (Rubin and Sauvage, 2006). Upon binding an Hh ligan, Ptc is internalized and destabilized and is no longer capable of transporting the Smo agonist molecule outwards. This allows the Smo agonists to accumulate intracellularly and activate Smo, which itself translocates to the plasma membrane, concentrating in cilia in at least some types of cells.

Hh signal is transmitted via a protein complex that includes Costal 2 (Cos2), Fused (Fu), Suppressor of Fused (SuFu) and the zinc-finger transcription factor cubitus interruptus (Ci). The activator and repressor forms of Ci have been divided between three separate zinc-finger proteins in vertebrates, with Gli1 and Gli2 functioning as activators and Gli3 as a repressor (Fuccilo et al., 2006). Ci is regulated at several levels via phosphorylation through kinases such as protein kinase A (PKA), glycogen synthase kinase 3β (GSK3β) and casein

kinase 1α (CK1α), which have a crucial role in processing, activity and nuclear localization of Ci. In the absence of Hh signaling, Ci is cleaved and its amino-terminal fragment acts as an inhibitor of Hh target gene transcription. After initiation of Hh signaling, cleavage of Ci is prevented and the ful-length molecule becomes an activator of Hh target gene transcription.

3. Epidermal growth factor/Transforming growth factor-α (EGF/TGF-α)

Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) are two potent mitogens for hepatocytes that stimulate hepatocellular DNA synthesis. Accumulation of pro-TGF-α has been found in human and rat hepatocyte nuclei suggesting that pro-TGF-α plays a role in mitosis (Breuhahn et al., 2006). EGF and TGF-α bind EGF receptor (EGF-R) and activate its downstream signaling pathways. The mammalian EGF/TGF-α family includes EGF, TGF-α, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), beta-cellulin (BTC), epiregulin (EPR) and epigen (EPI). Each of the mature peptide growth factors is characterized by a consensus sequence consisting of six spatially conserved cystine residues that form three intracellular disulfide bonds. This EGF motif is critical for binding members of the EGF-R tyrosine kinase family (Singh and Harris, 2005). EGF-R is a receptor tyrosine kinase (RTK) whose intrinsic kinase upon ligand binding is activated. EGF-R tyrosyl phosphorylates itself and many effector molecules.

The downstream signaling pathways of EGF-R include those that involve phospholipase C-γ (PL C-γ), Ras, MAP kinase, small GTPases such as Rho and Rac, multiple STAT isoforms, heterotrimeric G proteins, PI3 kinase and phospholipase D (PLD). In polarized epithelial cells, EGF-R is localized at the basolateral surfaces, allowing for epithelial-mesenchymal communication from mesenchyme-derived TGF-α and other matrix-

associated EGF-R ligands. Following ligand binding, EGF-R receptors are rapidly internalized from the cell surface. Ligand-induced internalization and degradation results in signal reduction with removal of both ligand and EGF-R or ligand alone. Different ligands can dictate the strength and lifespan of EGF-R signals. *In vitro* studies on fetal (E13) rat liver-derived hepatoblasts and adult rat liver-derived hepatocytes showed that the colony size of the hepatoblasts increased in the absence of EGF, whereas adult, diploid hepatocytes gave rise to large colonies in the presence of EGF (Kubota and Reid, 2000). EGF is a strong mitogen for mature hepatocytes, but has negative effects on the growth of embryonic hepatoblasts. It is thought to lineage restrict the hepatoblasts into hepatocytes.

4. Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) is the most potent growth factor for hepatocytes. It binds to high affinity receptor tyrosine kinase (RTK) c-met, resulting in receptor autophosphorylation and paraphosphorylation of adaptor proteins (e.g. Shc, Gab-1 and Grb2), followed by activation of downstream effectors (e.g. PLC-γ, STATs, PI3 kinase and ERK1/2). Overexpression of the adaptor protein, Shc, results in increased cellular proliferation and migration in response to HGF (Pellicci et al., 1995). GTP-binding proteins Ras, Rac and Rho are involved in the regulation of HGF responses pathway (Stuart et al., 2000). HGF is secreted in an inactive proform that needs proteolytic cleavage for full biological activity. Availability of pro-HGF-activating proteins such as HGF activator inhibitor type 1 and uPA are required for ligand-induced onset of the HGF signaling. HGF increases Raf and mitogen-activated peptide (MAP) kinase activity in hepatocytes through a protein kinase C (PKC) and calcium-dependent pathway. MAP kinase subsequently phosphorylates several nuclear

transcription factors, including c-myc and CCAAT/enhancer binding protein (C/EBP) β, as well as $pp90^{rsk}$ and RSK (S6 kinase).

5. Fibroblast growth factors (FGFs)

It is widely accepted that human ES cells require exogenous FGF-2 to sustain self-renewal and the ability to differentiate into variety of somatic cell types. FGF-2 pathway is one of the most significant regulators of human ES cell self-renewal and cancer cell tumorigenesis. Jung et al. searched for locally acting factors expressed in the cardiac mesoderm at this stage and identified FGF family proteins (FGF-1, -2 and -8) as potential candidates (Jung et al., 1999). In contrast, FGFR-1 and FGFR-4, receptors for these FGF species, were found to be expressed on endodermal cells, indicating that the FGF-FGFR signaling pathway is active between endoderm and mesoderm. Treatment of endodermal cells with FGF-1 and FGF-2 in the absence of the cardiac organ were capable of inducing the expression of albumin mRNA (Jung et al., 1999).

FGF-10 has been reported to prevent the differentiation of the proximal pancreas and liver into hepatic and pancreatic cells respectively (Dong et al., 2007). In zebrafish mutants deficient in FGF-10, the hepatopancreatic ductal epithelium is severely dysmorphic. Cells of the hepatopancreatic ductal system and adjacent intestine misdifferentiate toward hepatic and pancreatic fates. These results suggest that FGF-10 signaling from the mesenchyme adjacent to foregut endoderm is crucial for refining the boundaries between the hepatopancreatic duct and organs.

Different thresholds of FGFs for patterning the ventral foregut into liver and lung in the absence of cardiac mesoderm have been reported (Serls et al., 2005). Ventral foregut

endoderm explants from E8-9.5 mouse embryos respond to exogenous FGF1 and FGF2 in a dose-dependent manner, with lower concentrations activating liver-specific genes and higher concentrations activating lung specific genes. Moreover, inhibiting FGF signaling pathway using neutralizing antibodies against FGFR-1 and FGFR-4 strongly inhibited the expression of albumin mRNA. FGF-FGFR signaling, therefore, is critical for the initial process of liver development.

Binding of FGF-2 to its receptor is extracellularly modulated by non-signaling heparin/heparan sulfate (HP-/HS-) proteoglycans involved in the processing of FGF-2. The signaling pathway downstream of FGFRs involves tyrosine phosphorylation of the docking protein FRS2 followed by recruitment of multiple Grb2/Sox complexes, leading to activation of the Ras/MAPK signaling pathway (Dvorak et al., 2006; Eswarakumar et al., 2005). Targeted disruption of the FRS2α gene causes severe impairment in mouse development and is embryonic lethal at E7-7.5. In addition to MAPK pathway, FGFs can also induce recruitment of PI3K by Gab1, resulting in activation of the Akt dependent anti-apoptotic pathway.

6. Bone morphogenetic proteins (BMPs) and transforming growth factor-β (TGF-β) superfamily

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily, which includes TGF-βs, activins/inhibins, Nodal, myostatin, anti-Mullerian hormone (AMH) and more than 20 BMP-related proteins (Miyazono et al., 2005). BMP ligands and their signaling molecules are expressed in the extraembryonic tissues of the mouse embryos, contributing to the embryonic patterning (Kishigami and

Mishina, 2005). BMP-4 is highly expressed in the extraembryonic ectoderm and primitive streak before and during gastrulation. BMP-4 mutant embryos showed a ventrally projected bulge in the posterior region of the primitive streak (Fujiwara et al., 2002). BMP-2 starts to present at E6 in the extraembryonic region and then in the extraembryonic mesodermal cells lining the chorion and amnion after gastrulation. BMP-2 mutant embryos can gastrulate, but they develop abnormally in the extraembryonic region and die by E8.5 (Zhang and Bradley, 1996). BMP antagonist, activin A, induced endodermal differentiation of human and mouse ES cells *in vitro* (D'Amour et al., 2005; Gouon-Evans et al., 2006; Yasunaga et al., 2005). These findings suggest that BMPs are important cytokines during embryogenesis as well as fate determination *in vitro*.

The BMP family of proteins bind to two different receptors, type II and type I serine/threonine kinase receptors, both of which are required for signal transduction. After binding, the type II receptor phosphorylates the type I receptor, resulting in the recruitment of Smads (R-Smads, Smads 1, 5 or 8). After phosphorylation, the R-Smads are released from the receptor and recruit the common mediator Smad (Co-Smad, Smad 4) into the complex. This complex migrates into the nucleus and activates the transcription of specific target genes. In addition to Smad pathways, BMPs are believed to activate several kinase pathways including MAPK, PI3K and PKC. The signaling of the BMPs is modulated by numerous proteins. BMP antagonists, such as Cerbarus, Dan, Gremlin and Noggin, bind only specific BMPs and regulate the initiation of the signaling cascade. At the receptor level, the oligomerization status of the receptors determines the specificity of the activation of the signaling pathway. Inside the cells, the signal can be modulated by the activation of inhibitory Smad proteins such as Smad 6 and Smad 7 (Nohe et al., 2004).

7. Interleukin-6 (IL-6) family

Interleukin-6 (IL-6) is a critical component of the regenerative response in liver. Mice deficient in IL-6 gene are developmentally normal but have impaired liver regeneration after hepatectomy, indicated by liver necrosis and failure (Cressman et al., 1996). Blunted DNA synthetic responses were observed in hepatocytes from the mutant animals but not in non-parenchymal cells. Absence of STAT3 activation and depressed AP-1, Myc and cyclin D1 expression suggested cell cycle abnormalities in G1 phase. Moreover, supplying a single preoperative dose of IL-6 to the mutant mice returned STAT3 binding, gene expression and hepatocyte proliferation to near normal and prevented liver damage.

The propagation of mouse ES cells is dependent on leukaemia inhibitory factor (LIF) or related cytokines (Williams et al., 1988). LIF can be provided by a feeder layer of embryonic fibroblasts or as a recombinant protein. LIF is a cytokine of IL-6 family which includes IL-6, IL-11, LIF, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC). Receptors involved in signaling of IL-6 type cytokines include non-signaling α-receptors (IL-6Rα, IL-11Rα and CNTFRα) and the signal transducing receptor (gp130, LIFR and OSMR). IL-6 and IL-11 are the only IL-6 type cytokines that signal via gp130 homodimers. The rest of the IL-6 family signal via heterodimers of either gp130 and LIF-R (LIF, CNTF, CT-1 and CLC) or gp130 and OSMR (OSM) (Heinrich et al., 2003).

The complex of LIF, LIF-R and gp130 activates associated Janus-associated (JAK) tyrosine kinases that phosphorylate the receptor chains. The phosphorylated tyrosines then act as docking sites for proteins containing Src homology 2 (SH2) domains that can also be

phosphorylated by the JAK. The signal transducer and activator of transcription (STAT) family of transcription factors are key substrates for JAK. Recruitment and activation of STAT3 is required for self-renewal of EC cells. Inhibition of STAT3 in ES cells forces differentiation. STAT3 activation is not only necessary but also sufficient to block differentiation of ES cells (Matsuda et al., 1999). Activation of this chimaeric molecules sustains ES cell self-renewal without LIF.

Signaling downstream of IL-6 family receptor, gp130, is not limited to activation of STAT3 but includes stimulation of the Ras/ERK/mitogen-activated protein kinase (MAPK) pathway. ERK activation has a pro-differentiation effect and is antagonistic to ES cell self-renewal. The overall balance of activation of STAT3 and ERKs might determine the efficiency of ES cell self-renewal.

Activation of gp130 also results in activation of phosphatidylinositol-3 phosphate kinase (PI3K). The PI3K signaling pathway is important during ES cell propagation. Mouse ES cells deficient in PTEN (phosphatase and tensin homolog) accelerated proliferation and viability (Sun et al., 1999). PTEN is a lipid phophatase and a tumor suppressor functioning as a negative regulator of the PI3K pathway by removing phosphate from the 3' position of 3-phospoinositides. In addition to JAK-STAT3, SH2-ERK and PI3K signaling downstream from gp130, Src family of non-receptor tyrosine kinases (SFK) Yes and Hck are regulated by LIF and serum and are downregulated following differentiation (Anneren et al., 2004). Inhibition of Yes decreases growth and expression of marker genes for self-renewal, such as Oct-3/4, alkaline phosphatase and Nanog (Kristensen et al., 2005).

8. Signaling crosstalk in the control of stem cell fate

Wnt signaling is detected in undifferentiated mouse ES cells and is subsequently downregulated on differentiation (Sato et al., 2004). The Wnt and TGF-β pathways can interact directly to maintain human ES cells *in vitro* (Labbe et al., 2000; Varga and Wrana, 2005). LIF activates the pro-self-renewal JAK-STAT pathway, but also pro-differentiation MAPK pathway. BMPs activate the MAPK pathway and the induction of Id genes which block neurogenesis. Together LIF and BMP can drive ES cell self-renewal in a serum-free medium. LIF alone is insufficient to drive self-renewal as it blocks differentiation of mesoderm and endoderm but only weakly blocks differentiation into neuroectoderm. BMP signaling blocks neurogenesis. However, its potential to induce differentiation into other lineages is blocked by LIF/STAT3 signaling. The interplay between these two pathways leads to self-renewal (Friel et al., 2005; Ying et al., 2003).

TABLE

Table 1.1 Known Cellular Components in Stem Cell Niches (with focus on endodermal tissues)

Germ Layer	Representative Tissue Types	Cellular Components			
			Mesenchymal Cells		
		Epithelial Stem Cells*	Blood Vessel Precursors	Pigmented/ steroidogenic "Nurse" Cells**	Other
Ectoderm	Skin	Epidermal stem cells	Angioblasts	Melanocytes	---
	Brain	Neuronal stem cells	Angioblasts	Ependymal Cells	---
Endoderm	Gut	Intestinal stem cell	Angioblasts	Paneth Cells	---
	Pancreas	Pancreatic stem cells	Angioblasts	Pancreatic Stellate Cells	---
	Liver	Hepatic stem cells	Angioblasts	Hepatic Stellate Cells	Hemopoietic Stem Cells
	Lung	Lung stem cells (Clara cells)	Angioblasts	Pulmonary Neuroendocrine cells	---
Mesoderm	Bone Marrow	Hemopoietic Stem Cells	Angioblasts	Adipocytes	Osteoblasts
Mixed germ layer origins	Ovary	Oogonia	Angioblasts	Granulosa cells	----
	Testis	Spermatogonia	Angioblasts	Sertoli cells	----

* All of the stem cells are “label retaining cells” that retain label in pulse chase labeling studies indicating that they divide very slowly. They do not respond to mild or moderate injuries to the tissue but rather only to massive loss of mature cells in the tissue. The cells responding to mild or moderate injuries are “transit amplifying” cells that are the immediate descendents of the stem cells. In the liver these are the hepatoblasts with their signature feature of α-fetoprotein.

** It is unknown if the pigmented cells found in stem cell niches are themselves stem cells. Typically they produce steroidogenic signals, retinoids, a variety of growth factors and matrix molecules.

CHAPTER II

METHODS AND MATERIALS

Methods and Materials for Chapter III

Rats

Pregnant Fisher 344 rats were obtained from the Charles River Breeding Laboratory (Wilmington, MA). The morning on which the plug was observed was designated day 0. Male Fisher 344 rats (200-250g) were used for isolation of adult HpSTCs. All animal experiments were conducted under the institutional guidelines. The University of North Carolina Institutional Animal Care and Use Committee approved all experimental procedures.

Cell preparation

Fetal livers were isolated from 13~14 dpc rats and digested with 800 U/ml collagenase (Sigma) followed by further digestion with Trypsin-EDTA solution (Sigma). Subsequently, the cell suspension was treated with 200 U/ml DNase I (Sigma) (Kubota and Reid, 2000). Two litters of fetal rats were used to isolate cells, and average cell number obtained from 13 dpc or 14 dpc fetal livers was 4.5×10^5 cell/liver (mean, n=302) or 2.4×10^6 cell/liver (mean ± SEM, n=130), respectively. Isolation of HpSTCs from adult livers was performed using a standard protocol described elsewhere (Hellerbrand et al., 1996).

Cell culture

Fetal liver cells were cultured on STO cell feeders and in a serum-free hormonally defined medium (HDM) as described previously (Kubota and Reid, 2000). HDM was consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12, Invitrogen) to which was added 2 mg/ml bovine serum albumin (Sigma), 5 μg/ml insulin (Sigma), 10^{-6} M dexamethasone (Sigma), 10 μg/ml iron-saturated transferrin (Sigma), 4.4 x 10^{-3} M nicotinamide (Sigma), 5 x 10^{-5} M 2-mercaptoethanol (Sigma), 7.6 μeq/l free fatty acid (Chessebeuf and Padieu, 1984), 2 x 10^{-3} M glutamine (Invitrogen), 1 x 10^{-6}M $CuSO_4$, 3 x 10^{-8}M Na_2SeO_3 and antibiotics (penicillin and streptomycin) (Kubota and Reid, 2000). STO feeder cells were prepared as previously described using STO5 transfected with pEF-Hlx-MC1neo (Kubota and Reid, 2000). For long-term culture of sorted vitamin A-specific autofluorescent cells, cells were cultured on STO feeders and in HDM supplemented with 10 ng/ml human leukemia inhibitory factor (LIF; Boehringer Mannheim) and 10 ng/ml epidermal growth factor (EGF; Collaborative Biomedical Product). Medium was changed every other day, and cells were subcultured to fresh STO feeders every week**.**

Immunocytochemistry

Staining procedures for cultured cells were described previously (Kubota and Reid, 2000). For nestin or desmin expression, cells were stained with anti-nestin antibody (Rat-401, Developmental Studies Hybridoma Bank, The University of Iowa) or anti-desmin antibody (D33, Dako) followed by Alexa488-anti-mouse IgG (Molecular Probes).

Flow cytometry

Cells were analyzed and sorted by a FACStar Plus cell sorter (BD Biosciences) equipped with dual Coherent I-90 lasers. To detect vA-specific autofluorescence, cells were excited at 351 nm, and fluorescence emission was detected with the use of 450DF20 filter (Omega Optical Inc, Brattleboro, VT). Fluorescence-conjugated antibodies were excited at 488 nm, and their fluorescence emission was detected by standard filters. All antibodies were obtained from BD biosciences unless otherwise indicated. Monoclonal antibodies used for analysis of rat cells were FITC-anti-RT1A$^{a, b, l}$ (rat MHC Class I antigen; B5), phycoerythrin (PE)-anti-rat ICAM-1 (1A29), anti-rat VCAM-1 (5F10, Babco), anti-rat CD44 (OX-49), PE-anti-rat VCAM-1 (MR109), PE- or biotin-anti-rat β3-integrin (2C9.G2), biotin-anti-rat PECAM-1 (TLD-3A12), biotin-anti-rat Thy-1 (OX-7). For staining with unconjugated anti-VCAM-1 antibody (5F10), fetal liver cells were incubated with the anti-VCAM-1 antibody followed by staining with biotin-anti-mouse IgG_{2a} monoclonal antibody (R19-15). Streptavidin-Cy-Chrome (BD biosciences) was used to detect biotin-conjugated antibodies. For the experiments of fluorescence-activated cell sorting (FACS) to isolate long-term cultured cells derived from sorted vA-specific autofluorescenct cells, all cells in the culture were harvested and stained with biotin-anti-mouse CD98 (H202-141) followed by streptavidin-Cy-Chrome to separate cultured rat cells and STO feeder cells (Kubota and Reid, 2000). To block non-specific antibody binding, cells were incubated with 20% goat serum (Invitrogen), 1% teleostean gelatin (Sigma), and anti rat CD32 antibody (D34-485) solution prior antibody staining in FACS experiments.

Colony forming assay (CFA)

The protocol for a CFA was described for hepatoblasts previously [10]. Briefly, sorted cells were placed on STO feeders in triplicate at 500 or 2500 cells/well (3.8cm^2) in a 12-well plate and cultured in HDM for 14~15 days with medium changes every other day. To identify if the hepatoblasts are pluripotent, double immunofluorescence staining of albumin and CK19 was performed. The colonies were stained by Diff-Quick (Baxter) to count the number of the colonies per well.

Cell proliferation assay.

Vitamin A-specific autofluorescenct cells isolated by FACS were placed in triplicate at 500 cells/well in a 96-well plates with HDM supplemented with laminin (Collaborative Biomedical Products) at the final concentration of 8 μg/ml. LIF and EGF were added at concentrations indicated. Five days after plating cells cultures were rinsed twice to remove floating cells and added fresh medium with the tetrazolium salt WST-1 (Boehringer Mannheim) to measure the number of viable adherent cells (Kubota and Reid, 2000). After 4 hours, the absorbance was determined according to the manufacturer's protocol.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The primer sequences used for PCR are shown in Supplemental Table. The procedure of RT-PCR for sorted cells by FACS was described previously (Kubota et al., 2002). cDNAs synthesized from total RNAs of sorted cells were normalized by the cell number..

Immunohistochemistry

Frozen sections (8μm) were prepared of whole rat fetuses obtained from Dr. M. Dabeva (Albert Einstein) and were mounted on slides and stored at -80°C. For immunostaining, sections were air dried, fixed with cold acetone, and stained with primary antibodies. Primary antibodies used were mouse anti-rat VCAM-1 (MR109) and Armenian hamster anti-rat β3-integrin (2C9.G2). Goat Texas-Red-anti-Armenian hamster IgG and goat Alexa488-anti-mouse IgG_1 were used for secondary antibodies, respectively. The sections were counter stained with 4',6-diamino-2-phenylindole (DAPI) for visualization of cell nuclei and analyzed by confocal microscopy.

Methods and Materials for Chapter IV

Human Liver Sourcing.

Fetal Livers. Liver tissue was provided by an accredited agency (Advanced Biological Resources, San Francisco, CA) from fetuses between 18-22 weeks gestational age obtained by elective terminations of pregnancy. The research protocol was reviewed and approved by the IRB for Human Research Studies at the UNC.

Postnatal Livers. Intact livers from cadaveric neonatal, pediatric and adult donors were obtained through organ donation programs via UNOS. Those used for these studies were considered normal with no evidence of disease processes. Informed consent was obtained from next of kin for use of the livers for research purposes, protocols received Institutional Review Board approval, and processing was compliant with Good Manufacturing Practice.

Mice

C57Bl/6 SCID/nod mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed in a barrier facility on the campus of the University of North Carolina-Chapel Hill (UNC-CH), NC . Animals received care according to the Division of Laboratory Animal Medicine, UNC-CH guidelines, ones approved by AALAC.

Liver Processing.

Fetal livers. All processing and cell enrichment procedures were conducted in a cell wash buffer composed of a basal medium (RPMI 1640) supplemented with 0.1% bovine serum albumin (BSA Fraction V, 0.1%, Sigma, St. Louis, Mo.), insulin and iron saturated transferrin both at 5 ug/ml (Sigma St Louis MO) trace elements (selenious acid ,300 pM and ZnSO4, 50 pM), and antibiotics (AAS, Gibco BRL/Invitrogen Corporation, Carlsbad, California). Liver tissue was subdivided into 3 mL fragments (total volume ranged from 2-12 mL) for digestion in 25 mL of cell wash buffer containing type IV collagenase and deoxyribonuclease (Sigma Chemical Co. St Louis, both at 6 mg per mL) at 32 EC with frequent agitation for 15 – 20 minutes. This resulted in a homogeneous suspension of cell aggregates that were passed through a 40 gauge mesh and spun at 1200 RPM for five minutes before resuspension in cell wash solution. Erythrocytes were eliminated by either slow speed centrifugation (54, 55) or by treating suspensions with anti-human red blood cell (RBC) antibodies (Rockland, #109-4139) (1:5000 dilution) for 15 min followed by LowTox Guinea Pig complement (Cedarlane Labs, # CL4051) (1:3000 dilution) for 10 min both at 37°C. Estimated cell viability by trypan blue exclusion was routinely higher than 95%. See supplemental data for further details.

Postnatal livers. The livers were perfused through the portal vein and hepatic artery

for 15 min with EGTA-containing buffer and then with 600 mg/L collagenase (Sigma) for 30 min at 34^{o}C. The organ was then mechanical dissociated in either collection buffer; the cell suspension passed through filters of pore size 1,000, 500, and 150 microns; the single cells collected and then live cells fractionated from dead cells and debris using density gradient centrifugation (500 x g for 15 min at room temperature) in Optiprep-supplemented buffer in a Cobe 2991 cell washer. The resulting hepatic cell band residing at the interface between the OptiPrep/cell solution and the RPMI-1640 without phenol red was collected.

Magnetic Immunoselection

Isolation of cells expressing EpCAM from human liver cell suspensions was carried out using monoclonal antibody HEA-125 coupled to magnetic microbeads, and separated using a a miniMACS™ , a midiMACS ™, an autoMACS™ or CliniMACS® magnetic column separation system from Miltenyi Biotec (Bergisch Gladbach, Germany), following the manufacturer's recommended procedures. Similar protocols were used for sorts for NCAM^{+}, CD117^{+}, VEGFR2 (KDR)$^{+}$, CD34^{+} and CD146^{+} cells.

Colony Formation

Cells were plated in serum-free, hormonally defined medium, "Kubota's Medium (KM), in 6-well tissue culture dishes seeded with monolayers of Mitomycin-treated STO feeder cells, as described by Kubota and Reid (Kubota and Reid, 2000; Macdonald et al., 2002). Medium was changed every 2 to 4 days. Colonies were observed within a 7-10 days and were followed for up to six months using an inverted microscope.

Histology

Human or mouse liver specimens were fixed in 4% buffered paraformaldehyde for 12 hours and subjected to immunohistochemical staining by the methods noted in supplemental data.

Flow Cytometry

For cell surface markers 1 x 10^6 cells were labeled, following standard procedures, with 1 μg of each monoclonal antibody directly conjugated with fluorescein (FITC) or phycoerythrin (PE). For intracellular markers, cells first were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature and then incubated in permeabilization/blocking buffer (2% Triton X-100, 10% goat serum, 2% teleostean fish gel in PBS) for an additional 30 to 60 min, prior to incubation with a monoclonal antibody, washing, and incubation for 30 min with a fluorescent-labeled secondary antibody. Analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Inc., Palo Alto, CA). Nonspecific binding with isotype-matched control antibodies (BD Pharmingen, San Jose, CA) was used to establish gating. Monoclonal antibodies, labeled with fluorescein (F) or R-phycoerythrin (PE), were: EpCAM-F (clone Ber-EP4, DakoCytomation); CD133/1-PE (clone AC133, Miltenyi Biotec); CD34-PE (BD Pharmingen); CD45-PE (BD Pharmingen); CD14-PE (BD Pharmingen); CD38-PE (BD Pharmingen); CD4-PE (BD Pharmingen). For detection of intracellular antigens, primary mouse monoclonal antibodies of IgG_{2a} isotype to human serum albumin (clone HAS-11, Sigma Aldrich), *a*- fetoprotein (clone 4A3, Biodesign), or cytokeratin 19 (CK19, clone A53B/A2, Zymed) were utilized and detected using secondary Alexa Fluor® 647-goat anti-

mouse IgG_{2a} (InVitrogen, Carlsbad, CA), which does not react with the IgG_1 EpCAM-F monoclonal antibody.

In vivo Engraftment

C57Bl/6 NOD/*scid* mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used at 5 weeks of age. Mice were anesthetized with Ketamine-HCl (Vedco Inc, St. Joseph, Mo) and Xylazine-HCl (ProLab LTD., St. Joseph, Mo) and injected intrasplenically with 8 x 10^5 cells. The spleen was exteriorized through a small left flank incision (5 – 10 mm), and 70 µl of cell suspension was injected slowly into it using a 26-gauge needle on a Hamilton syringe. The spleen was returned to the abdominal cavity and the incision site was closed. Seven days following transplantation animals were euthanized under sedation and the livers were recovered. In parallel, half the animals were treated with carbon tetrachloride at 0.6 ul/gm body weight. In some experiments the cells were injected after CCl_4 treatment, and in others were injected prior to CCl_4 treatment. A portion of tissue was frozen in liquid N_2 and stored at -80° C for isolation of RNA. The remainder was fixed for histological analysis.

Fluorescent Staining of Cultured Cells

Cells were fixed with acetone/methanol (1:1) for 2 hrs at ambient temperature, rinsed with Hank's Balanced Salt Solution (HBSS), incubated with 20% goat serum in HBSS for 4 – 6 hours, and rinsed. Fixed cells were incubated with monoclonal antibodies to albumin and CK19 for 6 – 12 hrs, washed, incubated for 4 – 6 hrs with labeled isotype-specific secondary antibodies, and washed, always at 4º C. Stained cells were preserved with 2%

formaldehyde in HBSS and viewed using an inverted fluorescence microscope.

Confocal Microscopy

Immunofluorescence was observed using a Zeiss 510 Meta Laser Scanning Confocal Microscope (Zeiss; Jena, Germany) or Leica SP2 Laser Scanning Confocal Microscope (Leica; Wetzlar, Germany).

Immunhistochemistry

Fetal livers (16 – 20 weeks of gestation) were fixed in 4% para-formaldehyde over night and stored in 70% ethanol. Tissues were embedded in paraffin and cut into 5 μm sections. Sections were de-paraffinized with xylene and re-hydrated with decreasing alcohol series. Antigens were retrieved by boiling sections for 25 min in a pressure cooker in Retrieval Buffer (Dako, Carpinteria, CA). Endogeneous peroxidases were blocked by incubation for 30 min in 0.3% H_2O_2 solution. Sections were blocked in Serum Block (Dako); primary antibody was applied in Diluent (Dako), using rabbit anti-human telomerase reverse transcriptase (Calbiochem/EMD Biosciences, San Diego, CA). Secondary antibody and ABC-staining were performed using the RTU Vectastain Kit (Vector Laboratories, Burlingame, CA). DAB (Dako) was used as substrate and sections were counterstained with hematoxylin QS for nuclei staining (Vector Laboratories). Sections were de-hydrated with increasing alcohol series, fixed in xylene and embedded in Eukitt Mounting Media (Electron Microscopy Sciences, Hatfield, PA). Sections were analyzed using a Leica DMIRB inverted microscope and pictures were taken with a MicroPublisher camera (Q-Imaging, Burnaby, BC, Canada) controlled by SimplePCI (Compix Imaging Systems) software.

Telomerase Activity Measurement

Measurement of telomerase activity was done using a modified, real-time PCR Telomeric Repeat Amplification Protocol (TRAP) adapted for the use in the Roche LightCycler and Roche SYBR Green DNA kits (Roche). Collected cell pellets were resuspended in 20 µl ice cold CHAPS buffer (Chemicon, Temecula, CA) containing 20 U RNase out (Invitrogen), tissue was homogenized in 150 µl ice cold buffer using glas potter and pestle. Extracts were incubated for 30 min on ice. After centrifugation (20 min, 4°C, 20,000 g) supernatants were frozen on dry ice immediately and kept at -80°C. Total protein measurement was done using the DC-protein assay (Bio-Rad Laboratories, Hercules, CA). Extracts from cultured HeLa cells (American Type Culture Collection, Manassas, VA) were used as positive controls, CHAPS buffer with RNase out served as negative control, and heart tissue was used as reference. HeLa extracts were used to create standard curves in quantitative real time PCR, using the LightCycler (Roche) and SYBR Green Fast Start Master DNA kit (Roche). Extracts containing 0.2 µg total protein were incubated with SYBR green mix and oligonucleotide sequences (primer TS: 5' AAT CCG TCG AGC AGA GTT 3'; ACX: 5' GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3') and incubated for 20 min at 25°C in the dark. Two step PCR was performed with 10 min denaturation at 95°C, and 35 cycles at 95°C for 20s and at 60°C for 90s. Standard curves were linear applying extracts from 1, 10, 100, 1,000 and 10,000 HeLa cells.

Quantitative RT-PCR

The assays were done as described previously (Van Den Heuvel et al., 2001). Gene

specific primers used included the following:

Gene	Gene Bank Acc. No.	Forward Primer (5'→3')	Reverse Primer (5'→3')	Tm F/R Primer (°C)	Product length (bp)
AFP	NM_001134	accatgaagtg ggtggaatc	Tggtagccag gtcagctaaa	59.64/58.53	148
ALB	NM_000477	gtgggcagca aatgttgtaa	tcatcgacttcc agagctga	59.59/59.66	188
C3A4	NM_017460	gcctggtgctc ctctatcta	ggctgttgacca tcataaaagc	57.11/60.86	187
CK19	NM_002276	ccgcgactac agccactact	gagcctgttccg tctcaaac	60.47/59.85	152
c-kit	NM_000222	gatgacgagtt ggccctaga	caggtagtcga gcgtttcct	60.22/59.50	233
EpCAM	NM_002354	ctggccgtaaa ctgctttgt	agcccatcattg ttctggag	60.30/60.07	182
GAPD	NM_002046	atgttcgtcatg ggtgtgaa	gtcttctgggtg gcagtgat	59.81/60.12	173

Methods and Materials for Chapters V and VI

Kubota's Medium (KM)

All cultures were put into serum-free Kubota's Medium (KM) (Kubota and Reid, 2000), a serum-free medium tailored for hepatic progenitors. It was prepared as described previously (Macdonald et al., 2002).

Sourcing of Cell Lines

Bone marrow-derived human mesenchymal stem cells (hMSCs) were obtained from a 26-year-old male donor (Cambrex Bio Science, Walkersville, MD). The human umbilical vein endothelial cells (HUVECs) were obtained from Dr. Cam Patterson (University of North Carolina; Chapel Hill, NC). A clone of murine embryonic stromal cells, STO cells, was prepared from STO cells obtained from the ATCC.

Sourcing of Human Liver Tissue

Human fetal liver tissues, 16-20 weeks gestational age, were obtained from Advanced Biological Resources (ABR, San Francisco, California). Protocols for procurement were approved by the IRB for Human Studies at the UNC.

Isolation and culture of hHpSCs

Human fetal livers were processed as previously described (Schmelzer et al., 2006 and 2007; Sicklick et al., 2006). The freshly isolated parenchymal cells were put into KM and on culture plastic or on top of pre-plated feeders of hUVECs, hMSCs, STO cells, or primary cultures of human fetal liver-derived cells at a plating density of 5,000 cells/cm^2. The cells were in KM plus 2% FBS overnight and then switched to serum-free KM thereafter. The cultures on plastic and in serum-free KM yield colonies of hHpSCs surrounded by angioblasts and hepatic stellate cell (hHpSTCs) precursors that are not activated (Schmelzer et al., 2007).

Preparation of feeders

*A*ll stocks of mesenchymal feeders were cultured on culture plastic and in Endothelial

Growth Medium, EGM-2 (Cambrex, Walkersville, MD) with 2% FBS, conditions optimal for endothelial and mesenchymal cells (Bagley et al., 2003; Lin et al., 2000; Santhanam et al., 2007). The exceptions were the hMSCs and the adult liver-derived HpSTCs grown as described below. All were grown to confluence, growth arrested with Mitomycin-C, and then switched to serum-free KM for use in co-cultures with hHpSCs. Below are the variationused:

- Human bone marrow-derived mesenchymal stem cells (hMSCs) were plated onto tissue culture dishes with DMEM plus 1% antibiotics, ascorbic acid, 2mM L-glutamine and 10% FBS (Invitrogen).
- Purified preparations of HpSTCs from adult rat and adult human livers were prepared by Dr. YiWei Rong (Core service for Center for Gastrointestinal and Biliary Disease Biology at UNC) by protocols published previously (Schnabl et al., 2001; Schwabe et al., 2003). The stocks of feeders were cultured on plastic and in KM + 5% FBS.
- STO5 feeders were prepared as described previously (Kubota and Reid, 2000; Macdonald et al., 2002). Cloned subpopulations of STO cells, obtained from the ATCC, were tested for their efficacy on rodent hepatic progenitors. STO5 proved the most active, were expanded, aliquoted, and the stocks frozen. The STO5 stocks were thawed and grown in KM to which 5% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) was added.
- Primary cultures of human fetal liver-derived mesenchymal cells: The livers were enzymatically digested using 0.45 mg/ml collagenase type IV and 0.3 mg/ml deoxynuclease (Sigma, St. Louis, MO) and then mechanically dissociated into single cell suspensions by cross scalpels. After washing away excess enzymes, the cells were put through 3 rounds of slow-speed centrifugation (20 X g) for 5 minutes followed by collection of the supernatant and resuspension of the cells with RPMI-1640 plus selenium

(10^{-9}M), 1% antibiotics and 0.1% BSA. The cells were then plated onto culture plastic and in KM supplemented with 10% FBS. The mesenchymal cells attached within minutes to hours and quickly transitioned into stromal feeders comprised of activated hHpSTCs recognizable by having high levels of desmin, CD146 and αSMA.

- KDR^+ or $CD31^+$ cells were isolated from the fetal liver cell suspensions by immunoselection with the magnetically activated cell sorting (MACS) system using monoclonal anti-human KDR mouse IgG1 (Cell Sciences, Canton, MA), goat anti-mouse IgG coupled to magnetic microbeads or using monoclonal anti-human CD31 mouse IgG1 conjugated to magnetic microbeads. Plating density for KDR^+ and $CD31^+$ cells was 20,000 cells/cm^2.
- Feeders depleted of stromal cells were prepared by negative selection for fibroblasts using monoclonal anti-human fibroblast mouse IgG2a conjugated to magnetic microbeads for depleting fibroblasts according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The plating density for fibroblast-depleted supernatant cells was 500,000 cells/cm^2.

Purified Matrix Substrata

Fibronectin: Fibronectin (Sigma, F0895) was prepared at three different concentrations. The dishes were precoated with concentrations of 0.5, 1.0, or 2 ug/cm^2 and neutralized to pH 7.4.

Laminin: Laminin (Sigma, L2020) was prepared at two different concentrations. The dishes were coated with concentrations of 0.52 or 1.0ug/cm^2 at pH 7.4.

Collagen Types III and IV: Collagen coatings were prepared on dishes utilizing 1 of 5 different protein concentrations (2.1, 4.2, 6.3, 8.3, and 10.4 μg/cm^2)**.** The matrix attached during a 10-hour period with conditions of 37°C and 5% CO_2. Matrix components were added in acidic buffers in dishes. After 10 hours, the matrix molecules attach and stabilize, are sterilized by UV irradiation for 2 hours, and then rinsed 3 times with PBS (Gibco, #14190-144). Collagen III (Sigma, C-3511) was formed with pH 3 acetic acid and Collagen IV (Sigma, C-5533) with 0.5M acetic acid.

Collagen Type I*:* Vitrogen 100 (Cohesion Technologies, Palo Alto, CA) was modified into liquid collagen type I by adding specific ratios of 10x DMEM and 0.1M NaOH. It was important to prevent air bubble formations within the collagen I suspension, because air bubbles make the gel unstable. The collagen type I was used for monolayers of cells or as a sandwich to embed cells between two layers of collagen.

Monolayers of Cells on Collagen type I: Liquid collagen type I was maintained at 4°C prior to distributing 0.4mls into each well of the 6-well plates. After coating, the collagen was gelled at 37°C and 5% CO_2 for 1 hour. Cells were seeded on top of the gelled collagen.

Sandwich (Embedded cells) Model: Cells were sandwiched between layers of collagen. After the 10-hour cell attachment period, unattached cells were removed, and a second 0.4ml layer of collagen type I added. The system was allowed to gel at 37°C and 5% CO_2 for 1hour to solidify the top collagen layer.

Immunohistochemistry on human hepatic progenitor cells and human fetal liver-derived feeder cells

After 1-2 wks of culture, the cells were fixed with 4% paraformaldehyde for immunostaining procedures. The antibodies used were as follows: FITC-conjugated anti-human vWF sheep IgG (US Biologicals, Swampscott, MA), PE-conjugated anti-human CD56 (NCAM) mouse IgG1, anti-human CD31 mouse IgG1, PE-conjugated anti-human CD54 (ICAM-1) mouse IgG1 (BD, San Jose, CA), anti-human αSMA mouse IgG2a, anti-human type I collagen mouse IgG1, anti-human type III collagen mouse IgG1, anti-human laminin mouse IgG1, anti-chondroitin sulfate proteoglycan mouse IgM (Sigma, St. Louis, MO), anti-human fibronectin mouse IgG1 (Oncogene Research Products, Cambridge, MA), rabbit anti-human type IV collagen IgG (Research Diagnostics Inc., Flanders, NJ), rat anti-human perlecan IgG2a (Lab Vision, Fremont, CA), rabbit anti-human AFP IgG (Zymed-Invitrogen, South San Francisco, CA), anti-human KDR mouse IgG1 (Cell Sciences, Canton, MA), Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-mouse IgG1 and Alexa Fluor 488 goat anti-mouse IgG2a (Molecular Probes-Invitrogen, Eugene, OR).

The markers for the non-parenchymal cells included: KDR, (VEGFR2), expressed on angioblasts and endothelia); CD31, also called PECAM, a marker on endothelia; it is expressed at low levels on angioblasts. CD146, also called MCAM, A32, MUC18, Mel-CAM , or S-endo , and was found on endothelia, melanomas, smooth muscle, intermediate trophoblast, and a subpopulation of activated T cells. Desmin, an intermediate filament molecule expressed by hepatic stellate cells (HpSTCs) and by smooth muscle cells; α-Smooth Muscle Actin (αSMA), a signature feature of HpSTCs; its expression becomes

greatly elevated in activated HpSTCs.

Quantitative Real-Time PCR

Total RNA was extracted from each of three cell types, HUVECs and human fetal liver-derived $CD31^+$ endothelial and αSMA^+ fibroblast-like cells, using RNeasy Mini (Qiagen, Valencia, CA). The extracted RNA was then reverse-transcribed into cDNA using SuperScript II RT (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using sequence specific primers and probes shown in the table below and analyzed by the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Ribosomal RNA (18S) from each cell type was used as internal control. The mRNA expression levels relative to 18S were determined and the fold changes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primers used are as given below:

Primers used for RT-PCR:

Genes	ABI assay number
Ribosomal RNA (18S)	4308329
type I collagen-α1 chain (COL1A1)	Hs00164004_m1
type III collagen-α1 chain (COL3A1)	Hs00164103_m1
type IV collagen-α1 chain (COL4A1)	Hs00266237_m1
type V collagen-α1 chain (COL5A1)	Hs006090088_m1
fibronectin module 1 (FN1)	Hs01549972_m1
laminin-α2 chain (LAMA2)	Hs00166308_m1
laminin-α4 chain (LAMA4)	Hs00158588_m1
laminin-α5 chain (LAMA5)	Hs00245699_m1

laminin-β1 chain (LAMB1)	Hs00158620_m1
laminin-γ1 chain (LAMC1)	Hs00267056_m1
syndecan-1 (SDC1)	Hs00174579_m1
syndecan-2 (SDC2)	Hs00299807_m1
glypican-3 (GPC3)	Hs00170471_m1
glypican-5 (GPC5)	Hs00270114_m1

Methods and Materials for Chapter VII

Preparation of mesenchymal cell conditioned media

Each of different feeder cell types was plated onto tissue culture dishes at a density of 16,000 cells/cm^2 in the media suitable for attachment of corresponding cell types. On the next day, the media were changed to serum-free hormonally-defined medium (HDM) for every cell type with an amount of 5 ml per million cells seeded. HDM comprises of RPMI 1640 basal medium (Invitrogen, Carlsbad, CA) plus fatty acid-free bovine serum albumin (BSA, 0.1%), insulin (5 μg/ml), holotransferrin (10 μg/ml), free fatty acid mixture (7.6 μeq/L, Sigma, St. Louis, MO), selenous acid (300 pM, Johnson Matthey Chemicals, London, UK) and antibiotics (Invitrogen). The culture was then incubated for 2 days, followed by collection of used media. The collected media were centrifuged at 400xg for 10 minutes and the supernatant were filtered through a 0.2 μm pore size Acrodisc. The filter media were then diluted 1:2 with fresh serum-free KM to make conditioned media. For the purpose of enzyme-liked immunoabsorbent assay (ELISA), used and filtered serum-free HDM were not diluted before sending to the Cytokine Core Lab.

Sources, maintenance and derivation of feeder cells

Feeder cell types used for producing conditioned media include mouse embryonic fibroblast cell line (STO cells), human fetal lung fibroblast cell line (MRC5 cells), immortalized human stellate cell line (h-tert-HSCs) and primary human fetal liver-derived stromal cells. STO cell line was derived by Dr. Hiroshi Kubota. H-tert-HSCs were produced by retrovirally infecting the primary adult human hepatic stellate cells and provided by Dr. David Brenner. The primary human liver stromal cells were derived from human fetal livers in mid-gestation stage (16-18 weeks) obtained from Advanced Bioscience Resources Inc. (San Francisco, CA). After enzymatic digestion of the human fetal liver samples, the cell suspension was centrifuged at 50xg for 5 minutes and the supernatant was collected and plated onto tissue culture dishes to produce primary human fetal liver stromal feeder. Both STO cells and human fetal liver-derived stromal cells were maintained in RPMI 1640 (Invitrogen) plus 10% FBS (fetal bovine serum, Hyclone, Logan, UT) and antibiotics (Invitrogen). H-tert-HSCs were maintained in RPMI 1640 plus 10% FBS and G418 (200μg/ml, Invitrogen). MRC5 cells were maintained in RPMI 1640 plus 20% horse serum (Hyclone).

Colony formation assay for rat hepatic progenitor (rter6) and human hepatoblastoma (HepG2) cells

Rter6 cells were derived from E13 Fisher 344 rat fetal liver (Kutoba and Reid, 2000). For testing the effect from STO conditioned medium, rter6 cells were plated at seeding densities of 400, 200 and 100 cells/cm^2 on pre-prepared growth arrested STO or MRC5

feeder cell layer (250,000 cells/cm^2). On the next day, the medium was changed to STO conditioned medium and the culture was incubated for 10 days with medium change every 3 days. For testing the effect from cytokines, rter6 cells were plated at seeding densities of 250 cells/cm^2 on pre-prepared growth arrested STO feeder cell layer. On the next day, the medium was changed to serum-free hormonally-defined medium (HDM) supplemented with test cytokines with concentration of manufacturers' suggested ED_{50} and the culture was incubated for 10 days with medium change every 3 days. Concentration for each of tested cytokines is: 0.5 ng/ml for leukemia inhibitory factor (LIF), 10 ng/ml for interleukin-11 (IL-11), 0.05 ng/ml for transforming growth factor-β1 (TGF-β1), 0.01 ng/ml for IL-6, 1 ng/ml for IL-13, 10 ng/ml for growth related oncogene (GRO), 0.05 ng/ml for tumor necrosis factor-α (TNF-α) and 50 ng/ml for macrophage inflammatory protein-α (MIP-1α).

HepG2 cells were propagated in RPMI 1640 (Invitrogen) plus 10% FBS (Hyclone) and antibiotics (Invitrogen). Cells with subconfluent density were trypsinized, centrifuged, and then resuspended with the same medium. The resultant cell suspension was then filtered through a sterile 30 μm pore size cell strainer, followed by cell counting and plating onto uncoated tissue culture plastic with a seeding density of 50-100 cells/cm^2. On the next day, the medium was removed and the dishes were washed with phosphate-buffered saline (PBS) (Invitrogen), followed by addition of various test media (0.2 ml/cm^2). To test the effect from cytokines and candidate growth stimulating molecules individually, serum-free hormonally-defined medium (HDM) was supplemented with insulin-like growth factor-II (IGF-II, 2 ng/ml, Sigma), human growth hormone (10 μU/ml, Sigma), epidermal growth factor (EGF, 10 ng/ml, Collaborative Research, Waltham, MA), transforming growth factor-β1 (TGF-β1, 5 ng/ml), interleukin-11 (IL-11, 10 ng/ml), IL-6 (10 ng/ml), IL-13 (1 ng/ml), tumor necrosis

factor-α (TNF-α, 10 ng/ml), or growth related oncogene (GRO, 10 ng/ml).

HepG2 cells were incubated with the test media for 10 days and the media were changed every 5 days. Colony formation was scored at day 10 by staining with Diff-Quick and counting manually.

Enzyme-liked immunoabsorbent assay (ELISA)

Human fetal liver cells were seeded at 400,000 cells/cm^2 on plastic or on STO feeder cells in HDM for three days. The used media were then collected , centrifuged to remove cell debris and stored at -80°C until ELISA analysis. HDM incubated with STO feeder cells only for three days were also collected for ELISA test. The test media were sent to the Cytokine Core Lab at The University of Maryland at Baltimore.

CHAPTER III

IDENTIFICATION AND CHARACTERIZATION OF VITAMIN A-STORING CELLS IN FETAL LIVER: IMPLICATION OF FUNCTIONAL IMPORTANCE OF HEPATIC STELLATE CELLS IN DEVELOPMENT AND HEMATOPOIESIS

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Liver development requires paracrine signals from the neighboring mesenchymal cells. Endothelial cells, hepatic stellate cells and Kupffer cells are three major non-parenchymal cell types in the liver. Endothelial cells have been proven to be necessary in hepatic progenitor cell outgrowth during liver organogenesis. Hepatic stellate cells capable of producing several cytokines, growth factors and extracellular matrix (ECM) molecules, as well as remodeling ECM in the liver are important during development and regeneration of liver. Most studies on biology of hepatic stellate cells were done using adult liver-derived cells. However, hepatic stellate cells derived from adult livers did not support growth of human hepatic stem cells (hHpSCs). A hypothesis is that developmental paracrine signals are provided by mesenchymal precursors in the embryos. Hepatic stellate cells derived from fetal livers may possess different property than those derived from adult livers. To test this hypothesis, successful isolation and propagation of fetal liver-derived hepatic stellate cells are important. This chapter described the key efforts and findings in purification and clonal expansion of fetal rat liver-derived hepatic stellate cells.

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ABSTRACT

Hepatic stellate cells (HpSTCs), or "vitamin A-storing lipocytes", are major regulators of hepatic fibrogenesis in adults. However, their early development in fetal liver is largely unknown. To characterize fetal HpSTCs in the liver in which hepatic development and hematopoiesis occur in parallel, we determined the phenotypic characteristics of HpSTCs from rat fetal livers utilizing a strategy focused on vitamin A. Storage of vitamin A in the cytoplasm is a unique characteristic of HpSTCs, permitting identification of them by vitamin A-specific autofluorescence (vA^+) when excited with UV light utilizing flow cytometry. A characteristic vA^+ cell population was identified as early as 13-day post coitum liver and having a surface phenotype of RT1A$^-$ ICAM-1$^+$ VCAM-1$^+$ β3-integrin$^+$. Although non-specific autofluorescent cells were found with the antigenic profile of RT1A$^-$ ICAM-1$^+$ VCAM-1$^+$, they were β3-integrin$^-$ and proved to be hepatoblasts, bipotent hepatic parenchymal progenitors. In addition to expression of classical HpSTC markers, the vA^+ cells were able to proliferate continuously in a serum-free hormonally defined medium containing leukemia inhibitory factor, which was found as a key factor for their replication. These results demonstrated that the vA^+ cells are fetal HpSTCs with extensive proliferative

activity. Furthermore, the vA+ cells strongly express hapatocyte growth factor, stromal derived factor-1α, and Hlx, homeobox transcriptional factor, indicating that they play important roles for hepatic development and hematopoiesis. The ability to isolate and expand fetal HpSTCs enable further investigations into their roles in early liver development and facilitate identification of possibly novel signals of potential relevance for liver diseases.

INTRODUCTION

Hepatic stellate cells (HpSTCs) are liver-specific mesenchymal cells in the space of Disse, located between the plates of parenchymal cells and the flanking endothelia. A prominent characteristic of HpSTCs is the presence of cytoplasmic lipid droplets containing vitamin A. Indeed, a major role of HpSTC is uptake, storage, and release of vitamin A compounds, which are requisite for embryonic development and, in adults, for vision and reproduction (Blaner, 1994; Geerts et al., 1994; Blomhoff et al., 1990). In mammals, about 50 to 80% of the total body vitamin A is stored in HpSTCs (Blomhoff et al., 1990).

HpSTCs are key effectors in hepatic fibrogenesis in adult liver tissue (Geerts et al., 1994; Ankoma-sey and Friedman, 1998). Although HpSTCs have been studied in adult livers, little is known about their embryonic origin and early development (Geerts, 2004; Cassiman et al., 2006). In early liver development, endodermal cells in the foregut give rise to hepatic diverticulum around 10 days post coitum (dpc) of rats (Shiojiri, 1997). Subsequently, the hepatic diverticulum develops into the surrounding mesoderm called the septum transversum and forms hepatic cords. HpSTC are thought to derive from mesenchymal cells of the septum transversum, because histological analyses have suggested that mesenchymal cells in the septum transversum become trapped in the subendothelial space of the hepatic cords and are

hypothesized to develop into HpSTCs (Enzan et al., 1997; Wake, 1980). In rat liver at 13 dpc, the walls of sinusoids consist of two layers, endothelial cells and pericytes. The latter are dendritic cells and contain lipid droplets, and their cytoplasmic processes extend between parenchymal cells consisting of hepatoblasts, bipotent hepatic progenitors (Enzan et al., 1997; Wake, 1980; Kubota and Reid, 2000). Although these dendritic cells are believed to be fetal HpSTCs, neither biological characteristics of the dendritic pericytes nor any HpSTC-specific marker expression has been studied to confirm that.

In the fetus, liver is a primary site for hematopoiesis. Therefore, fetal HpSTCs may have unique roles to support fetal liver hematopoiesis as well as hepatic development. Although the antigenic profiles of hematopoietic and hepatic cells in fetal livers have been studied (Kubota and Reid, 2000; Morrison et al., 1995; Suzuki et al., 2002), those of fetal HpSTCs have not. In this study, we determined the antigenic phenotype of fetal HpSTCs in the rat and revealed the unique phenotypic and biological characteristics.

RESULTS

Identification of vitamin A-specific autofluorescent cells in fetal liver

Vitamin A-rich lipid droplets in the cytoplasm has been used as a unique characteristic for identification of HpSTCs. Vitamin A specifically produces a green-blue fluorescent when excited with light of 330-360 nm (ultra violet, UV) laser. Flow cytometric analysis (FCA), using a UV laser, is able to detect the vitamin A-specific green-blue fluorescent in the cytoplasm of HpSTCs in adult liver (Geerts et al., 1998). To identify fetal HpSTCs, we sought cells with the vitamin A-specific green-blue fluorescent characteristic in

fetal liver by flow cytometry. In a previous study, we identified hepatoblasts as RT1A^{-} (rat major histocompatibility complex class Ia^{-}) OX18^{low} ICAM-1^{+} side scatter (SSC)^{high} cells in 13 dpc liver of rat fetus (Kubota and Reid, 2000). Hepatoblasts showed relatively high SSC characteristics among the cell population of the fetal liver. Therefore, an autofluorescent signal produced by hepatoblasts must be distinguished from vA-specific autofluorescent signal in FCA. Figure 3.1A shows the patterns of FCA of fetal liver cells at 13 dpc followed staining with antibodies against RT1A and ICAM-1. Based on the SSC signal, two gates, R1 and R2, were created (Figure 3.1A ALL), and the expression patterns of RT1A and ICAM-1 were analyzed (Figure 3.1A R1 and R2). As expected, the majority of cells of R2 (SSC^{high}) were RT1A^{-} ICAM-1^{+} cells (Figure 3.1A R2, lower right), identified previously as hepatoblasts (Kubota and Reid, 2000).

Subsequently, we analyzed autofluorescent activities in the 13 dpc fetal liver cells. The vA-specific blue-green autofluorescent signal was measured by detecting the emission light with a 450 nm filter by excitation of a UV laser (351nm) (see Materials and Methods). To detect a non-UV laser-specific autofluorescent signal, a 488 nm laser and 530/30 nm bandpass filter was used. NADH, riboflavines, and flavin coenzymes commonly cause intrinsic cellular autofluorescence (Aubin, 1979; Nokubo et al., 1988), and the peak autofluorescence emission of these molecules after 488 nm excitation overlaps with the detection region of FITC, which peak emission is 518 nm. Patterns of the autofluorescent signals, the UV laser-specific and non-UV laser specific autofluorescence, of whole fetal liver cell population as well as two subpopulations (R1 and R2 gates of Figure 3.1A) were shown in Figure 3.1B. In the pattern of the whole cell population (Figure 3.1B ALL), two distinct subpopulations with high autofluorescent characteristics were identified. One had an

autofluorescent signal specific for UV light (Figure 3.1B ALL, upper left), which is referred to here as vA^+, whereas cells locating diagonally in the upper right quadrant indicate non-specific autofluorescence, because the autofluorescent signals were detected with the 530 nm filter and the 450 nm filter when excited by the 488 nm laser and the UV laser, respectively. The subpopulation with non-specific autofluorescent characteristic (designated as ns-$autoflu^+$) exclusively derived from the SSC^{high} gate (Figure 3.1A R2 and Fig.3.1B R2) while vA^+ cells (Figure 3.1B, upper left) were detected in both R1 and R2. Figure3.1C shows the pattern of vA-specific autofluorescent signal and RT1A expression, which was detected by a FITC-conjugated antibody against RT1A. FCA indicated that vA^+ cells as well as ns-$autoflu^+$ cells had no RT1A expression, because those two populations did not shift in the stained sample (Figure 3.1C) compared to the control sample (Figure 3.1B). In addition, FCA indicated that the $RT1A^-$ $ICAM\text{-}1^+$ SSC^{high} cells (Figure 3.1A R2, lower right), which represents the hepatoblast population, and $RT1A^-$ ns-$autoflu^+$ cells (Figure 3.1C R2, arrow) are an overlapping population by this FCA (data not shown).

To determine whether these autofluorescent signals were specific in fetal liver, fetal lung cells from the 13 dpc fetuses were isolated and analyzed by flow cytometry. The FCA showed there were neither ns-$autoflu^+$ cells nor vA^+ cells in the lung cells (Figure 3.1D), indicating that the autofluorescent signals in particular subpopulations in the fetal liver represent unique phenotypic characteristics. These results clearly indicate that flow cytometry was able to detect characteristic vA^+ cells in rat fetal liver as early as 13 dpc and that the vA^+ cells were $RT1A^-$.

Vitamin A^+ cells express VCAM-1 and β3-integrin

VCAM-1 (CD106) has been identified as a unique surface marker of mature HpSTCs in adult liver (Knittel et al., 1999). Therefore, we analyzed VCAM-1 expression in fetal liver cells to investigate whether the vA^+ cells express VCAM-1. By FCA, it appeared that about 15 % of cells were VCAM-1^+ in the 13 dpc fetal liver (Figure 3.2A). We next analyzed the pattern of autofluorescence and RT1A expression of the VCAM-1^+ cells. Interestingly, the VCAM-1^+ cells contained essentially all vA^+ cells as well as the entire ns-autoflu$^+$ cell population (Figure 3.2A), indicating that HpSTCs and hepatoblasts express VCAM-1. FCA of two monoclonal antibodies against rat VCAM-1 (5F10 and MR109) showed an identical pattern of VCAM-1 expression (data not shown). In addition, fetal liver VCAM-1^+ cells were RT1A$^-$ ICAM-1^+ cells because the R1 gate in Figure 3.2B included the VCAM-1^+ cell population. These results indicate that fetal liver RT1A$^-$ ICAM-1^+ VCAM-1^+ cells consist of vA^+ cells, hepatoblasts, and some autoflu$^-$ cells.

We next investigated surface antigens to distinguish the two autofluorescent populations, the vA^+ cells and the hepatoblasts, both of which were RT1A$^-$ ICAM-1^+ VCAM-1^+ cells. Because it has been reported that β3-integrin (CD61) is expressed on adult HpSTCs (Zhou et al., 2004), two-color FCA of VCAM-1 versus β3-integrin were performed (Figure 3.3B). The vA^+ RT1A$^-$ cells expressed β3-integrin, whereas ns-autoflu$^+$ RT1A$^-$ cells were clearly β3-integrin$^-$. Autoflu$^-$ RT1A$^-$ cells contained some VCAM-1^+ β3-integrin$^+$ cells. The remaining major population (Figure 3.3B, R4) was VCAM-1^- and appear to be correspond to R2 cell population in Figure 3.2B. These cells were non-adherent when they were cultured on plastic dishes (data not shown). We also analyzed expression of PECAM-1 (CD31), which is known as an endothelial cell marker. FCA indicated that PECAM-1 expression in vA^+ RT1A$^-$ cells and ns-autoflu$^+$ RT1A$^-$ cells is negligible, while PECAM-1^+ cells were detected in the

autoflu$^-$ RT1A$^-$ and non-adherent cell populations (Figure 3.3B). In addition, we analyzed expression of Thy-1 (CD90). Thy-1 is a surface marker for oval cells that appear in adult livers after oncogenic insults (Petersen et al., 1998). Oval cells share some characteristics with hepatoblasts in fetal liver (Sigal et al., 1992; Shiojiri et al., 1991). Nonetheless, FCA showed that ns-autoflu$^+$ RT1A$^-$ cells are Thy-1$^-$. By contrast, vA$^+$ RT1A$^-$ cells, autoflu$^-$ RT1A$^-$ cells and non-adherent cells express Thy-1 heterogeneously. We found that ns-autoflu$^+$ RT1A$^-$ cells were CD44^{lo} whereas vA$^+$ RT1A$^-$ cells were CD44$^-$ (data not shown). CD44 is a cell adherent molecule, and its ligand, hyaluronans, is a basic component of embryonic extracellular matrices (Aruffo et al., 1990). CD44 appeared to be expressed differentially in the vA$^+$ RT1A$^-$ cells and ns-autoflu$^+$ RT1A$^-$; however, the expression on the cell surface was rather weak. Together, these data suggest that only β3-integrin antibody staining among all antibodies examined, facilitates distinguishing the vA$^+$ cells and ns-autoflu$^+$ cells, both of which were RT1A$^-$ VCAM-1$^+$ ICAM-1$^+$ cell population in the fetal livers.

To gain insight into in situ localization of vA$^+$ cells in fetal liver, immunohistochemistry for VCAM-1 and β3-integrin was performed. Whereas VCAM-1 was detected throughout the liver parenchyma (Figure 3.4A), we observed only a few β3-integrin$^+$ in the same field (Figure 3.4A). The β3-integrin$^+$ co-expressed VCAM-1, and the VCAM-1$^+$ β3-integrin$^+$ cells showed a dendritic morphology with cytoplasmic processes extending between parenchymal cells. In addition, some of them located along the sinusoids, suggesting that they are fetal HpSTCs.

Non-specific autofluorescent$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^-$ cells are hepatoblasts

In a previous study, we developed an *in vitro* CFA for hepatoblasts, which can differentiate to either hepatocytes or biliary epithelial cells depending upon the microenvironment (Kubota and Reid, 2000). In the CFA, a single hepatoblast can generate a colony of cells with both hepatocytic and biliary markers. Using the assay, we proved that almost all hepatic cells at 13 dpc of the rat are indeed bipotent (Kubota and Reid, 2000). To examine whether vA^+ cells have any potential to generate hepatoblast colonies, the CFA was performed. Four cell populations were isolated by FACS and subjected to the CFA for hepatoblasts: 1) ns-autoflu$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^-$, 2) vA^+ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$, 3) autoflu$^-$ RT1A$^-$, and 4) VCAM-1$^-$ non-adherent cells. The CFA clearly indicated that hepatic colonies were generated exclusively from group 1, ns-autoflu$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^-$ cells (Table 3.1). More than 95% of the hepatic colonies derived from the group 1-sorted cells contained both hepatocytic (albumin$^+$ CK19$^-$) and biliary epithelial (albumin$^-$ CK19$^+$) cells (Figure 3.4B). The colony forming efficiency was approximately 31 % (Table 3.1). A hepatic progenitor cell line (rhel4321) established in a previous study (Kubota and Reid, 2000) had a colony efficiency in the CFA of 42.5 ± 1.8 %. Therefore, the result of CFA in this experiment indicates that the group 1-sorted cells are nearly pure hepatoblasts, because the colony efficiency by established cell lines without sorting is assumed much higher than that of freshly isolated cells. These results clearly demonstrated that, while the ns-autoflu$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^-$ cells are hepatoblasts, the other groups of sorted cells, including the vA^+ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ cells, do not contain hepatoblasts.

Gene expression of freshly isolated vitamin A^+ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ cells

We next analyzed the gene expression pattern of the vA^+ $RT1A^-$ $VCAM\text{-}1^+$ β3-$integrin^+$ cells to examine whether they express various markers for HpSTCs. Five population were isolated by FACS, and RNAs were isolated from the five populations. RT-PCR for HpSTC markers was performed using cDNAs synthesized from the RNAs. The five populations were: 1) ns-$autoflu^+$ $RT1A^-$ $VCAM\text{-}1^+$ β3-$integrin^-$, 2) vA^+ $RT1A^-$ $VCAM\text{-}1^+$ β3-$integrin^+$, 3) $autoflu^-$ $RT1A^-$ $VCAM\text{-}1^+$, 4) $autoflu^-$ $RT1A^-$ $VCAM\text{-}1^-$, and 5) $VCAM\text{-}1^-$ non-adherent cell population. HpSTCs in adult liver express intermediate filaments, desmin and nestin (Yokoi et al., 1984; Niki et al., 1999), which are not expressed in other cell types in the liver. Vimentin is expressed broadly in mesenchymal cells, while smooth muscle α-actin (SMαA) is expressed in myogenic cells. Expression of vimentin and SMαA increase after activation of HpSTCs (Geerts et al., 1994; Ankoma-sey and Friedman, 1998). RT-PCR analyses showed that vA^+ $RT1A^-$ $VCAM\text{-}1^+$ β3-$integrin^+$, $autoflu^-$ $RT1A^-$ $VCAM\text{-}1^+$, and $autoflu^-$ $RT1A^-$ $VCAM\text{-}1^-$ cells expressed all four intermediate filaments (Figure 3.4C). ns-$autoflu^+$ $RT1A^-$ $VCAM\text{-}1^+$ cells express albumin as well as Prox1 (Figure 3.4C), which is a transcriptional factor expressing specifically in hepatoblasts (Sosa-Pineda et al., 2000). This result was consistent with the data obtained from the CFA, which demonstrated they were hepatoblasts (Table 3.1). There was no expression of nestin, SMαA, or vimentin in the hepatoblasts. A weak expression of desmin in the hepatoblasts (Figure 3.4C) agree with the results of the prior immunolocalization study for desmin with confocal microscopy (Vassy et al., 1993). The expression of HpSTC specific intermediate filaments strongly suggests that vA^+ cells are fetal HpSTCs.

Subsequently, expression of hepatocyte growth factor (HGF), stromal cell-derived factor-1 alpha (SDF-1α), and divergent homeobox transcriptional factor Hlx were

investigated using RT-PCR. HGF is required for normal hepatic development, especially for proliferation and differentiation of hepatoblasts in the mouse (Schmidt et al., 1995). In adult liver HpSTCs are major producers of HGF (Schirmacher et al., 1992). SDF-1α is a potent chemokine for hematopoietic progenitors, and hematopoietic stem cells in fetal liver migrate in response to the chemokine (Christensen et al., 2004). Hlx is expressed in mesenchymal cells in developing fetal liver and plays an indispensable role in fetal liver hematopoiesis and hepatic development (Hentsch et al., 1996). Interestingly, vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells expressed HGF, SDF-1α and Hlx transcripts most strongly among all cell fractions examined (Figure 3.4C).

Ex vivo clonal expansion of vitamin A^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells.

Although HpSTCs isolated from adult liver can proliferate *in vitro* in serum-supplemented media, they usually transform to myofibroblast cells and show only limited proliferative activity (Geerts et al., 1994). Fetal HpSTCs may have extensive proliferative potential, when cultured in an appropriate condition. Therefore, we investigated the *ex vivo* growth capability of the vA^+ cells in fetal livers. When vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells isolated by FACS were cultured in HDM at a cell density of 500 cells/well of 96-well plates for 5 days in the presence of LIF, a pleiotrophic growth factor for different types of cells including embryonic stem cells or myogenic cells (Austin and Burgess, 1991; Smith et al., 1992), the cells expanded in a dose-dependent manor (Figure 3.5A). In addition, EGF potenciated the proliferation of fetal HpSTCs induced by LIF, but did not support the expansion on its own (Figure 3.5A).

The proliferation, however, did not persist in the culture conditions making use of plastic culture plates. We next placed the sorted vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells on STO feeders (Kubota and Reid, 2000). Although STO cells produce LIF, exogenous LIF and supplementation of EGF supported colony formation from sorted vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells dramatically (Figure 3.5B). Proliferating cells in the culture expressed desmin and nestin, whereas STO feeders did not express either (Figure 3.5C). Three single colonies were picked and placed on fresh STO feeders. The single colony-derived cells continued to proliferate in the co-cultures with STO feeders supplemented with LIF and EGF for 2 months, indicating that they have extensive growth potential. Expression of desmin and nestin were maintained in the proliferating cells (Figure 3.5D). To compare further the characteristic phenotypes of 2 month-cultured cells with freshly isolated vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells, RT-PCR was performed. Single colony-derived cells (A428-3) that were maintained for 2 months in culture were separated from STO feeder cells by FACS, and the RNA was extracted for RT-PCR analysis. RNA was isolated from STO feeder cells that were sorted simultaneously as a control. In addition, RNA was isolated from adult HpSTCs to compare with those from A428-3 and STO cells. The results demonstrated that A428-3 expressed desmin, nestin, SMαA, vimentin, β3-integrin, SDF-1α, HGF, and Hlx, indicating the expression pattern was a similar to fresh vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells (Figure 3.5 and Figure 3.6A). Furthermore, VCAM-1 expression was confirmed by FCA (Figure 3.6B). RT1A expression appeared to be induced in vitro culture (Figure 3.6B). The RT-PCR results of adult HpSTCs agreed with previous reports, in which the phenotype of normal adult HpSTCs is $desmin^+$, glial fibrillary acidic protein $(GFAP)^+$, HGF^+, but $SM\alpha A^{lo/-}$. The results also showed that adult HpSTCs express SDF-1α, β3-integrin, and Hlx. There was neither

expression of GFAP in A428-3 cells (Figure 3.6A) or in any fractions tested in fetal liver (data not shown).

DISCUSSION

Vitamin A-storing cells in fetal liver were identified by flow cytometry using the specific auto-fluorescence generated by cytoplasmic vA rich lipid droplets. The surface phenotype of vA^+ cells appeared to be uniform and consisted of cells that are $RT1A^-$ $ICAM\text{-}1^+$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ $PECAM\text{-}1^-$. VCAM-1 has been shown as a unique surface marker to distinguish HpSTCs from myofibroblasts in adult liver (Knittel et al., 1999). ICAM-1 and β3-integrin expression on HpSTCs also have been demonstrated in previous studies (Hellerbrand et al., 1996; Zhou et al., 2004). In addition to those surface markers, vA^+ cells express intermediate filaments specific for HpSTCs including desmin, vimentin, SMαA, and nestin. These molecular markers have been used to identify HpSTCs in adult liver (Geerts et al., 1994; Ankoma-sey and Friedman, 1998; Yokoi et al., 1984; Niki et al., 1999). These results strongly supports that the vA^+ cells are fetal HpSTCs.

VCAM-1, an immunoglobulin gene superfamily of adhesion molecules, plays a key role in supporting adhesion of hematopoietic progenitors to bone marrow stromal cells (Miyake et al., 1991). Although fetal liver is a major hematopoietic organ in fetus, hematopoietic cells originally are generated in yolk sac and the aorta-gonad-mesonepheros region. Shortly after that, they colonize to fetal liver and produce large numbers of hematopoietic progenitors until birth (Dzierzak and Medvinsky, 1995). In the later developmental stage *in utero*, hematopoietic stem cells move to bone marrow, which becomes the primary hematopoietic organ throughout the postnatal life (Morrison et al.,

1995). Adhesion molecules expressed on endothelial cells are important for homing process of hematopoietic stem/progenitor cells (Quesenberry and Backer, 1998). Studies using blocking antibodies for VCAM-1 or VLA-4, which is expressed on hematopoietic stem/progenitors, demonstrated that hematopoietic stem/progenitor cells failed to colonize to bone marrow when the VCAM-1/VLA-4 adhesion pathway was blocked (Papayannopoulou et al., 1995; Vermeulen et al., 1998). Therefore, VCAM-1 molecule in fetal liver is likely to involve establishment of fetal liver hematopoiesis.

While FCA indicated that high VCAM-1 expression was detected on hepatoblasts and fetal HpSTCs, the later may play more important roles for hematopoietic cells, because they express SDF-1α as well. SDF-1α is a potent chemoattractant for hematopoietic stem cells, which express CXCR4, the receptor for SDF-1α (Wright et al., 2002). The chemokine plays a central role during the migration of hematopoietic stem/progenitor cells to bone marrow (Lapidot, 2001). It was shown that SDF-1α up-regulates VLA-4 dependent adhesion to VCAM-1 (Hidalgo et al., 2001). Therefore, it is possible that SDF-1α and VCAM-1 expression on fetal HpSTCs are crucial to recruit hematopoietic stem/progenitor cells into fetal liver.

Interestingly, our study reveals that VCAM-1 is expressed by hepatoblasts, as well as HpSTCs. In addition to the surface antigenic profile and the cells' mRNA expression, the culture assays clearly demonstrated that VCAM-1^+ cells are hepatoblasts. This finding is unexpected because VCAM-1 is known as a surface marker for mesenchymal cells such as endothelial cells, myogenic cells, or HpSTCs (Knittel et al., 1999; Osborn et al., 1989; Rosen et al., 1992). The expression appears to be developmentally controlled because adult hepatocytes are VCAM-1^- by FCA (data not shown), which agrees with previous studies (van

Oosten et al., 1995). Several lines of evidence demonstrated that VCAM-1 and VLA-4 interaction occurs on myogenic cells and controls myogenesis in addition to the adhesion pathway between hematopoietic stem cells and endothelial cells; so, it is worthwhile to elucidate the biological functions of VCAM-1 on hepatoblasts. VCAM-1 expression has been found on mouse hepatoblasts as well (Kubota, unpublished data), suggesting that the physiological role might be conserved across species.

Our data indicates that fetal HpSTCs are major HGF producers in the fetal liver. HGF is a crucial growth factor for hepatic development (Schmidt et al., 1995), and the factor is responsible for liver parenchymal cell growth during liver regeneration as well (Michalopoulos and DeFrances, 1997). In addition, it has been shown that HpSTCs, but not parenchymal cells, endothelial cells, or Kupffer cells, express HGF in adult liver (Schirmacher et al., 1992). Therefore, our data and previous studies suggest that HpSTCs are main HGF producers from fetuses to adults in the liver. Interestingly, a recent study indicated that HGF potentiated SDF-1α-mediated recruitment of hematopoietic progenitors to the liver (Kollet et al., 2003). The study suggested HGF produced from HpSTCs in injured adult liver is important for homing of hematopoietic cell into the livers. Likewise, fetal HpSTCs may play a crucial role for hepatic and hematopoietic development in the fetal liver because the cells are main producers for HGF and SDF-1α.

A divergent homeobox protein, Hlx, is expressed in septum transversum and mesenchymal cells in fetal liver (Lints et al., 1996). A previous study of Hlx knockout mice demonstrated that the mutant mice have impaired hepatic development and fetal liver hematopoiesis (Hentsch et al., 1996). Transplantation experiment indicated that the hematopoietic defect was caused by the fetal liver microenvironment, but not by the

hematopoietic progenitors per se. Thus, Hlx^+ cells are a crucial cell population in fetal liver for supporting hepatic and hematopoietic development. Our data indicated that fetal HpSTCs in the rat expressed Hlx strongly, and we have found that a cell population in the mouse fetal liver express desmin, nestin, VCAM-1, HGF, SDF-1α, and Hlx as well (H Kubota, unpublished data). Therefore, it is interesting to examine whether Hlx knockout mice have the cell population, which is a counterpart of rat fetal HpSTCs. Although relationship between Hlx expression and HpSTC development is not clear, loss of Hlx expression in fetal HpSTCs may cause defects in the biological function of HpSTCs in the developing liver of the mutant mice. Furthermore, considering the unique phenotypic and functional characteristics of fetal HpSTCs including expression of VCAM-1 and Hlx and production of HGF and SDF-1α, the cells might comprise a stem cell niche for hematopoietic stem cells, hepatic stem cells, or both in the liver.

GFAP is a marker used to identify HpSTCs in adult liver (Gard et al., 1985). However, we did not detect GFAP mRNA by RT-PCR in any cell fractions examined as well as in the whole fetal liver sample. In addition, even after culture of isolated fetal HpSTCs, GFAP expression was not induced, whereas desmin and nestin expression were sustained in the cultured cells. This result suggests that differentiation of fetal HpSTCs will result in GFAP expression in a later developmental stage. We cannot, however, exclude another possibility, in which $GFAP^+$ cells are derived from different precursors that do not exist in the 13 dpc fetal livers. Circulating cells in blood flow may be a source of the alternative cellular origin (Baba et al., 2004). However, the majority of HpSTCs express GFAP in adult liver; therefore, the minor contribution of circulating cells from the blood is unlikely to

become a dominant population in the liver. Thus, it seems more likely that acquisition of GFAP expression happens during maturation of HpSTC.

Fetal HpSTCs that were purified by FACS proliferated under a serum-free condition supplemented with EGF and LIF. With the support of STO feeders, fetal HpSTCs replicated continuously more than 2 months. The phenotypes of fresh and *in vitro* cultured fetal HpSTCs were similar, indicating the culture condition did not transform HpSTCs to myofibroblast cells, which commonly happen in serum-supplemented conditions. Until now, HpSTCs from adult livers have been cultured in medium supplemented with animal serum. HpSTCs cultured in the serum-supplemented medium give rise to myofibroblastic cells, which acquired fibroblastic characteristics and lose the original HpSTC phenotypes. Although the myofibroblastic cells can replicate and expand in culture, they eventually cease proliferation. Therefore, the serum-supplemented medium conditions are not appropriate for the culture of HpSTCs. Recently, LIM homeobox protein Lhx2 was identified as a negative regulator for myofibroblastic transformation of HsSCs (Wandzioch et al., 2004). In addition, Lhx2 was found as a molecule important for maintenance of hair follicle stem cells, in which the transcriptional factor maintains the undifferentiated status of the stem cells (Rhee et al., 2006). Thus, Lhx2 might play a similar role to maintain a fetal stage of HpSTCs. The culture system developed in this study will facilitate to investigate molecular mechanisms of proliferation and differentiation of HpSTCs.

An interesting question is whether fetal type HpSTCs are also evident in adult livers. Given the surface markers we have identified, it is possible to try to immunoselect these cells from adult livers and then further able to characterize them using the serum-free culture system developed and that was found to maintain the unique characteristics of fetal HpSTCs

ex vivo. Transplantation of fetal liver-derived HpSTCs or fetal type HpSTCs is a plausible cell therapy to study to try to alter some of the aberrations described for adult fibrogenic livers. In addition, the fetal HpSTCs are likely to express novel factors that may prove regulatory of hematopoiesis or hempoiesis and therefore offer new directions for novel therapeutic approaches for liver diseases.

TABLE

Table 3.1. The frequency of hepatic colonies from sorted rat fetal liver cells.

	Inoculated cell number	Hepatic colony number	Colony efficiency (%)
ns-autoflu$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^-$ §	250 (6)	77.0 ± 6.7	30.8 ± 2.7
vA$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ ¶	2500 (6)	3.3 ± 0.9	0.1 ± 0.0
autoflu$^-$ RT1A$^-$	2500 (6)	5.5 ± 0.2	0.2 ± 0.1
VCAM-1$^-$ †	2500 (3)	0.0 ± 0.0	0.0 ± 0.0

Gates for fractionation of ns-autoflu$^+$ RT1A$^-$, vA$^+$ RT1A$^-$ and autoflu$^-$ RT1A$^-$ cells were created as shown in Figure 3 R3, R1, and R2, respectively.

§: VCAM-1$^+$ β3-integrin$^-$ cells from the Figure 3 R3 were sorted.

¶: VCAM-1$^+$ β3-integrin$^+$ cells from the Figure 3 R1 were sorted.

†: VCAM-1$^-$ cells from the Figure 3 R4 were sorted.

Flow cytometrically sorted cells were cultured on STO feeders at indicated cell numbers per well in a 12-well plate. The hepatic colony number is the average per well. Colony efficiency is expressed as the percentage of cells inoculated in culture and that went on to form colonies after 15 days of culture. Values are mean ± SEM. Number of total wells inoculated sorted cells is enclosed in parentheses.

FIGURE LEGENDS

Figure 3.1. Flow cytometric analysis for autofluorescent cells in 13 dpc rat fetal liver and lung

(A) The pattern of forward scatter (FSC) and side scatter (SSC) of the entire population (ALL). Based on the value of SSC, R1 and R2 gates were created and represented high (SSC^{hi}) and low (SSC^{lo}) SSC, respectively. Expression patterns of RT1A and ICAM-1 in the R1 and R2 are also shown. $RT1A^{-}$ $ICAM\text{-}1^{+}$ SSC^{hi} cells (R2, lower right) are hepatoblasts in the rat fetal liver [10]. The number indicates percentage of each quadrant. (B) The autofluorescent pattern of entire population (ALL), R1, and R2 were analyzed with UV laser and 488 nm laser. UV laser specific autofluorescent signal was detected with a 450 nm filter, while non-specific autofluorescent signal excited with a 488 nm laser was measured with a 530/30 bandpass filter. UV laser-specific autofluorescent cells were detected in R1 and R2 (upper left). (C) Expression of RT1A and UV laser specific autofluorescent signal was studied. UV laser-specific autofluorescent cells were $RT1A^{-}$. ns-$autoflu^{+}$ $RT1A^{-}$ cells (allow) were identified and were correspond to rat hepatoblast population. (D) UV laser specific autofluorescent signal was analyzed in 13 dpc fetal lung cells. There are no UV specific autofluorescent cells in the lung cell population. Most of the cells are $RT1A^{-}$, and no non-specific autofluorescent cells (comparable to the hepatoblast population in the fetal liver) were detected.

Figure 3.2. VCAM-1 and ICAM-1 expression on vA^{+} cells

(A) Histogram of flow cytometry for VCAM-1 expression on 13 dpc fetal liver. Approximately 15% of the cells express VCAM-1 on the cell surface. Closed and open

histograms represent stained cells and unstained cells, respectively. VCAM-1$^+$ and VCAM-1$^-$ cells were analyzed by flow cytometry for their autofluoresent signals. All vA$^+$ cells and ns-autoflu$^+$ cells are VCAM-1 positive. The numbers represent the percentage of each quadrant. (B) Two color analysis of 13 dpc fetal liver cells for RT1A and ICAM-1. R1 cell population (RT1A$^-$ ICAM-1$^+$) contains all vA$^+$ cells and ns-autoflu$^+$ cells. These results indicate that vA$^+$ and ns-autoflu$^+$ cells are VCAM-1$^+$ RT1A$^-$ ICAM-1$^+$.

Figure 3.3. Antigenic profiles of vA$^+$, ns-autoflu$^+$, and autoflu$^-$ RT1A$^-$ cells in 13 dpc fetal liver

(A) Flow cytometric analysis for UV-autofluorescence and RT1A expression. In the RT1A$^-$ cell population, three gates, vA$^+$ RT1A$^-$ (R1), autoflu$^-$ RT1A$^-$ (R2) and ns-sutoflu$^+$ RT1A$^-$ (R3) were created based on the autofluorescent signals. R4 gate covered remaining cells in the fetal liver, which were non-adherent cells. (B) Two color analysis of VCAM-1 versus β3-integrin, PECAM-1, or Thy-1 expression for each gated cell population (R1-R4). The numbers represent the percentage of each quadrant. Primarily R1 cells are VCAM-1$^+$ β3-integrin$^+$, while R3 cells uniformly express VCAM-1, but none of β3-integrin, PECAM-1, or Thy-1.

Figure 3.4. Characterization of VCAM-1$^+$ cells in fetal liver

(A) Conforcal microscopy of a rat 14 dpc fetal liver section immunostained with anti-VCAM-1 (green) and anti-β3-integrin (red) antibodies. Nuclear DNA was counterstained with DAPI (blue). Scale bar is 50 μm. (B) Immunocytochemistry of a bipotent hepatblast colony. ns-autoflu$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^-$ cells were isolated by FACS and placed on

STO feeder cells in HDM at a clonal cell density (250 cells in a well of 12-well plate; 66 cells/cm^2). After 15 days in culture, the cells were fixed and stained with antibodies against albumin (red) and CK19 (green). Each colony was generated from a single sorted cell. More than 95% (95.7 ± 0.4 %; mean ± SEM, n = 3) of hepatic colonies contained albumin$^+$ CK19$^-$ and albumin$^-$ CK19$^+$ cells, which represent hepatocytic and biliary differentiation, respectively. Scale bar is 500 μm. (C) RT-PCR analysis of 14 dpc fetal liver cells fractionated by FACS. lane 1, ns-autoflu$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^-$; lane 2, vA$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$; lane 3, autoflu$^-$ RT1A$^-$ VCAM-1$^+$; lane 4, autoflu$^-$ RT1A$^-$ VCAM-1$^-$; lane 5, remaining VCAM-1$^-$ cell population; lane 6, no cDNA. vA$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ cells (lane 2) express SDF-1α and HGF strongly. The vA$^+$ cells are positive for HpSTC markers (desmin, nestin, vimentin, SMαA) and negative for hepatoblast markers (albumin and Prox1).

Figure 3.5. In vitro culture of vA$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ cells isolated by FACS

(A) Effect of LIF and EGF on *in vitro* proliferation of vA$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ cells. Five hundred vA$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ cells isolated by FACS were placed in a well of 96-well plate with HDM plus laminin supplemented LIF and/or EGF at the concentration indicated. After 5 days-culture, degree of cell proliferation was measured by the tetrazolium salt WST-1. LIF support proliferation of the vA$^+$ cells at as low as 0.1 ng/ml. EGF slightly improved the vA$^+$ cell proliferation. (B) Effect of LIF and EGF on vA$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ cells cultured with STO feeders. Two hundred fifty vA$^+$ RT1A$^-$ VCAM-1$^+$ β3-integrin$^+$ cells isolated by FACS were seeded on STO feeder cells in HDM with EGF and/or LIF. Twelve-well plates were used. The cultures were stained with

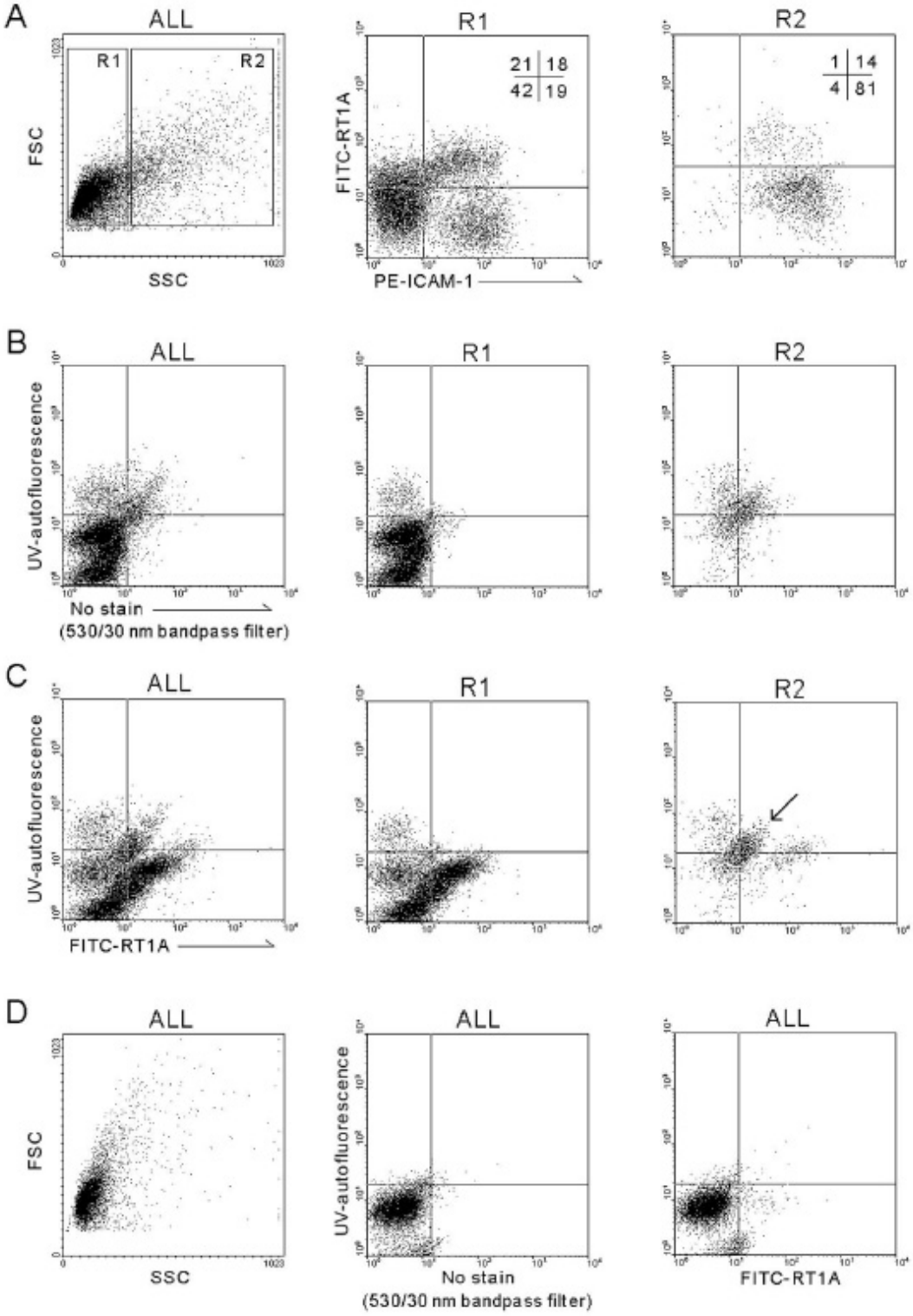
Diff-Quick™ after 2-week culture period. Although STO cells express LIF, the amount of the production was not adequate to support clonal expansion of the cells in the absence of exogenous LIF supplementation. Exogenous LIF and addition of EGF dramatically improved clonal expansion of the vA^+ cells. (C) Immunocytochemistry of colonies derived from vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells isolated by FACS. Cells were placed on STO feeders in HDM supplemented with EGF and LIF. Fifteen days after in vitro culture, cultures were stained with antibodies for desmin or nestin. Colony forming cells express nestin and desmin, whereas STO cells do not express either. Scale bar is 200 μm. (D) Immunocytochemistry of 2-month cultured vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells isolated by FACS. Sorted cells were placed on STO feeders in HDM supplemented with EGF and LIF. Proliferating cells were subcultured 5 times on fresh STO feeders. Cultured cells were stained with antibodies for desmin or nestin. Proliferating cells maintain the expression of nestin and desmin during the entire culture period. Scale bar is 200 μm.

Figure 3.6. Phenotypic characteristics of 2-month cultured vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells

(A) RT-PCR analysis of cultured vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells. Cells were isolated by FACS and cultured on STO feeders in the HDM with EGF and LIF. After 2-month culture cells were fractionated by FACS. Proliferating rat cells and mouse STO feeder cells were fractionated by FACS following antibody staining of mouse CD98 monoclonal antibody. CD98 is expressed on mouse STO cells, and the monoclonal antibody reacts specifically mouse CD98, but not rat CD98. RNAs were isolated from vA^+-derived rat cells and STO cells. Normal rat HpSTCs were also used and isolated the RNA for a control.

cDNAs were synthesized from those RNAs and subjected to PCR with primers specific for various transcripts that expressed in HpSTCs. (B) Flow cytometry for cultured vA^+ $RT1A^-$ $VCAM\text{-}1^+$ $\beta3\text{-}integrin^+$ cells. Cells used for RT-PCR were stained with anti-VCAM-1 or anti-RT1A antibody and mouse CD98 antibody. The CD98 negative fraction was analyzed for VCAM-1 or RT1A expression. Continuously proliferating cells derived from vA^+ cells in rat fetal livers express VCAM-1 and RT1A uniformly under the culture condition examined.

Figure 3.1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R1
R2
FSC
SSC
R1
21 | 18
42 | 19
FITC-RT1A
PE-ICAM-1
R2
1 | 14
4 | 81
B
ALL
UV-autofluorescence
No stain
(530/30 nm bandpass filter)
R1
R2
C
ALL
UV-autofluorescence
FITC-RT1A
R1
R2
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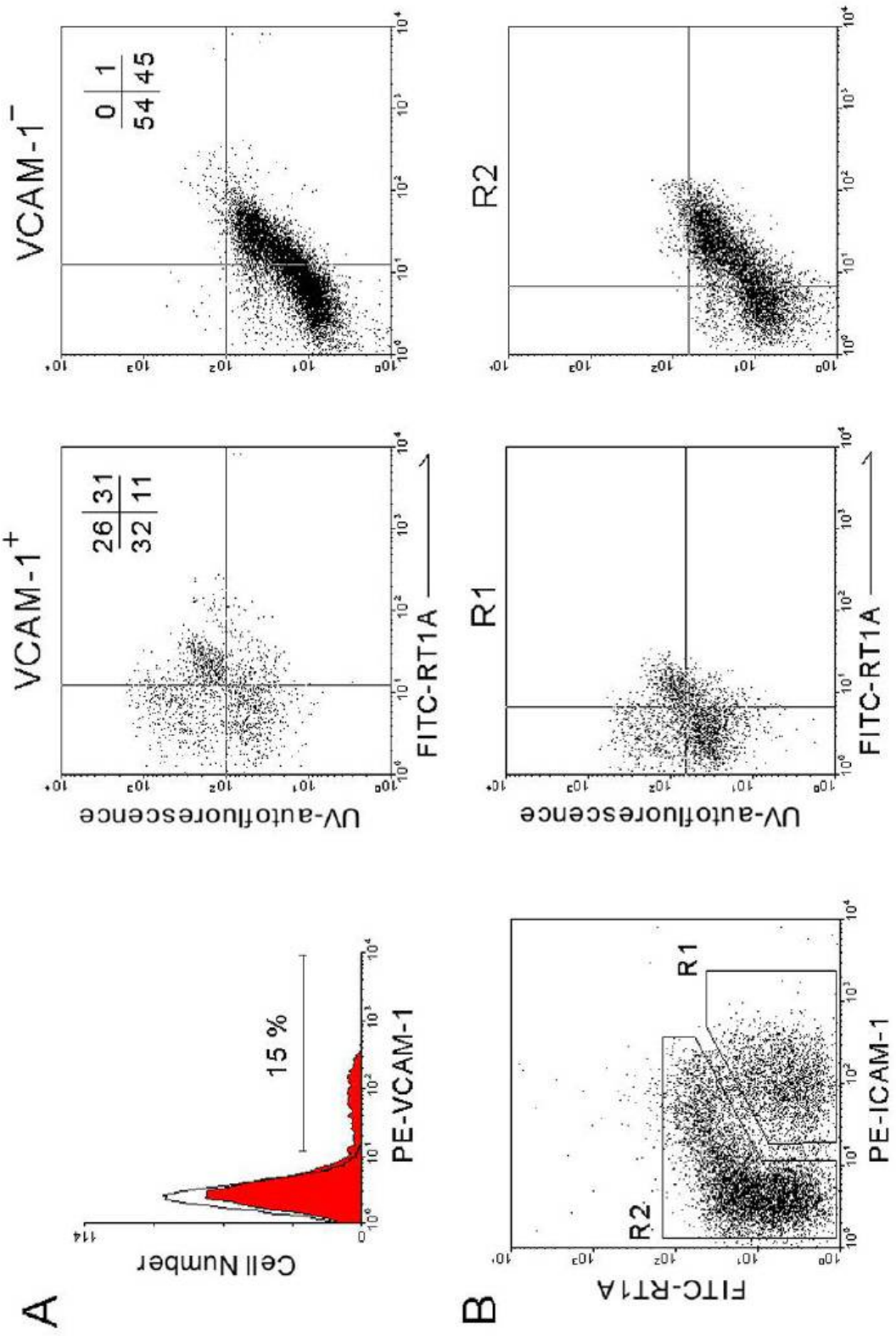
FSC
SSC
ALL
UV-autofluorescence
No stain
(530/30 nm bandpass filter)
ALL
FITC-RT1A

Figure 3.2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VCAM-1+
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32 | 11
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VCAM-1−
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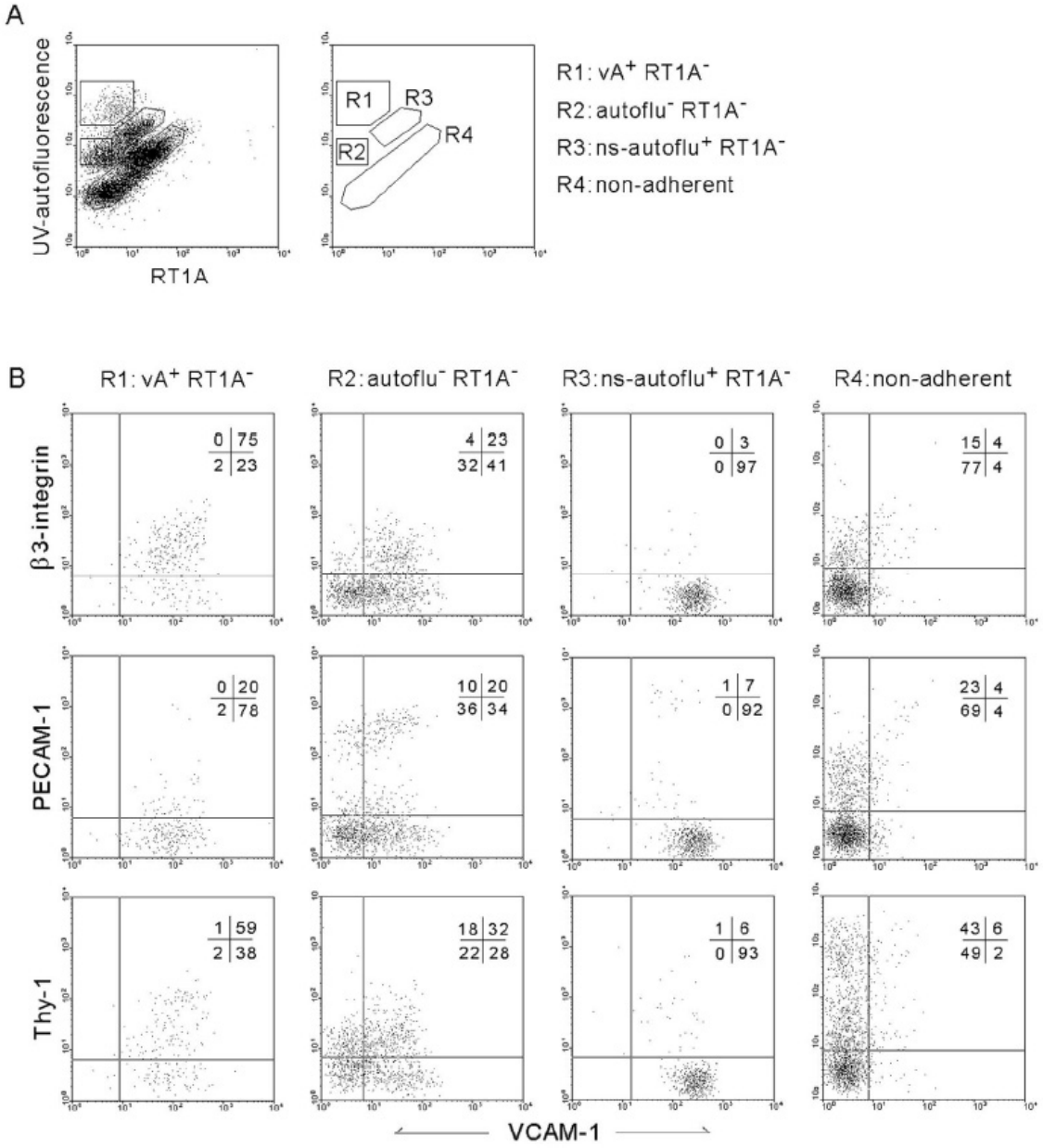
54 | 45
B
R1
R2
PE-ICAM-1
FITC-RT1A
UV-autofluorescence

Figure 3.3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R1
R2
R3
R4
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R4: non-adherent
B
R1: vA+ RT1A-
R2: autoflu- RT1A-
R3: ns-autoflu+ RT1A-
R4: non-adherent
β3-integrin
PECAM-1
Thy-1
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4 23 32 41
0 3 0 97
15 4 77 4
0 20 2 78
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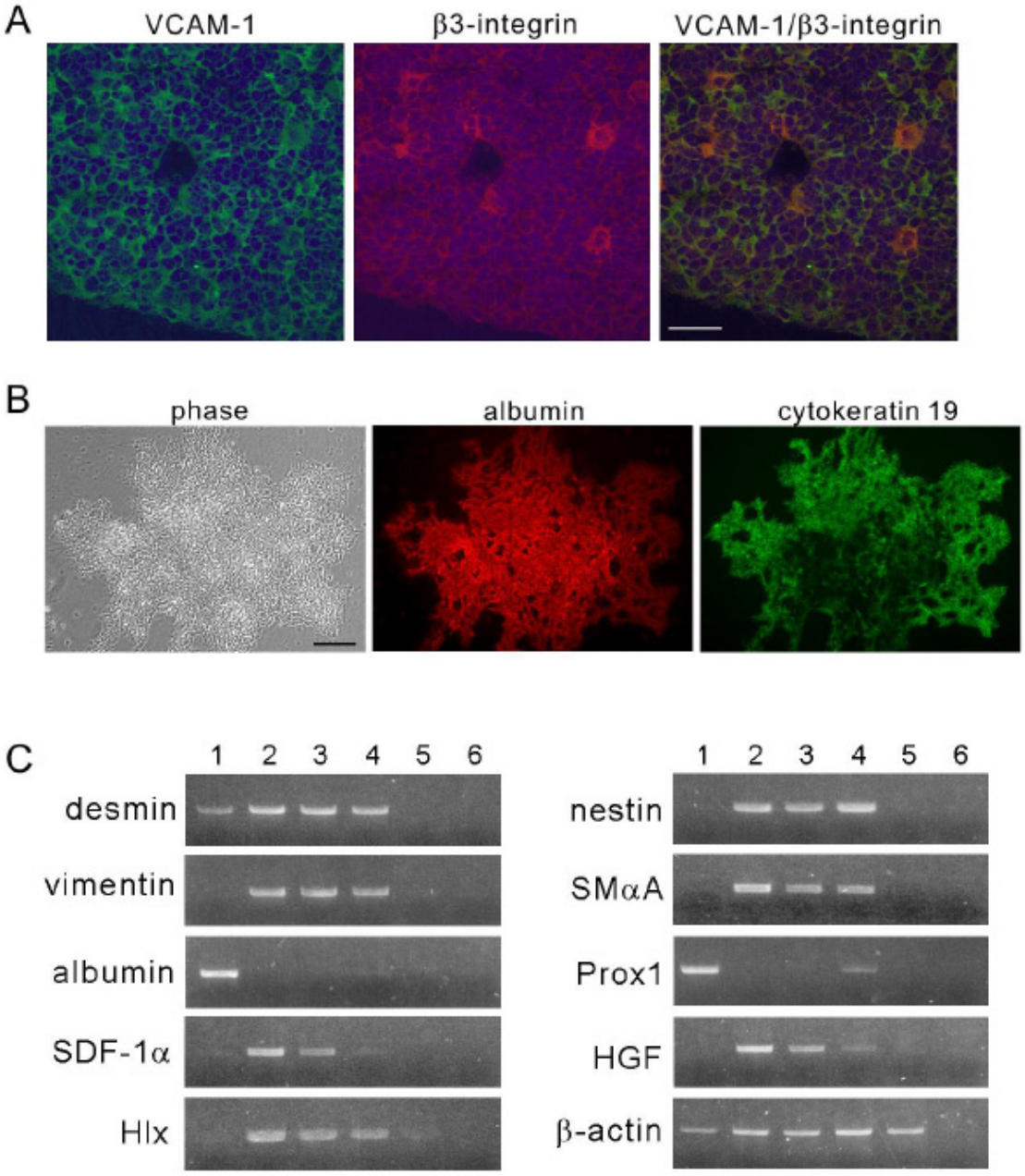
VCAM-1

Figure 3.4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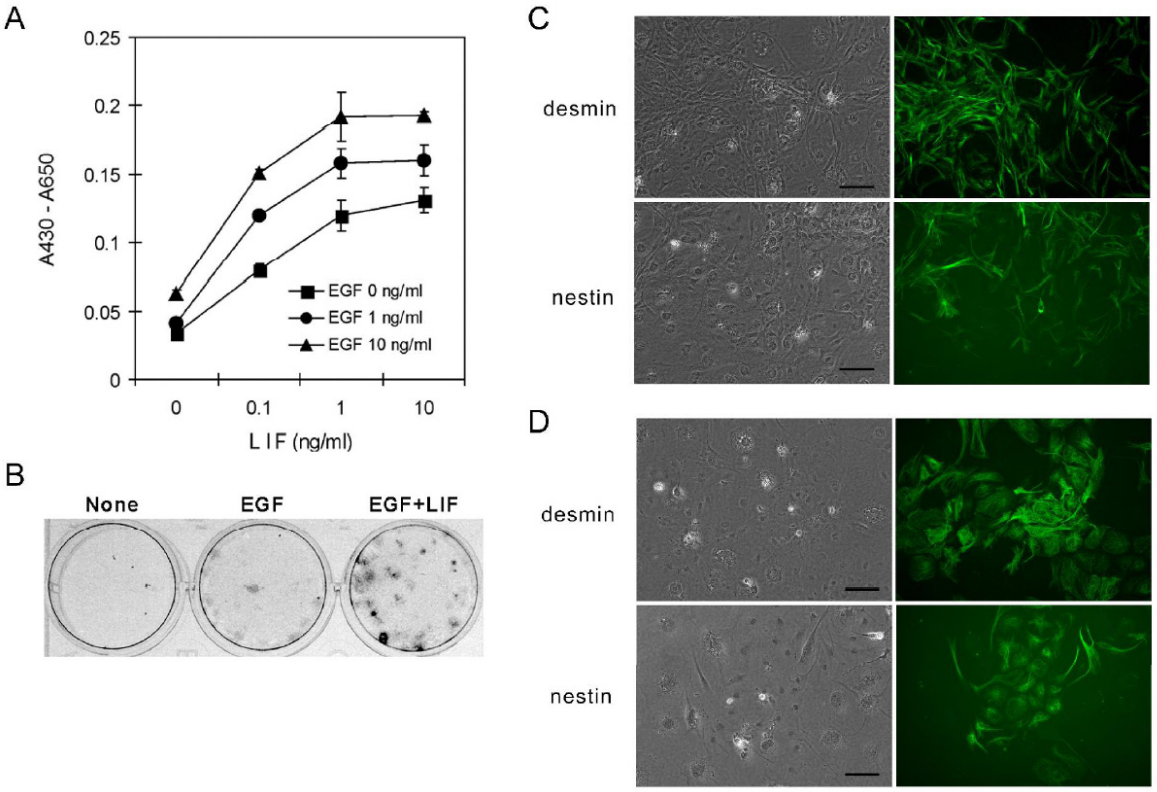
nestin
SMαA
Prox1
HGF
β-actin

Figure 3.5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EGF 1 ng/ml
EGF 10 ng/ml
B
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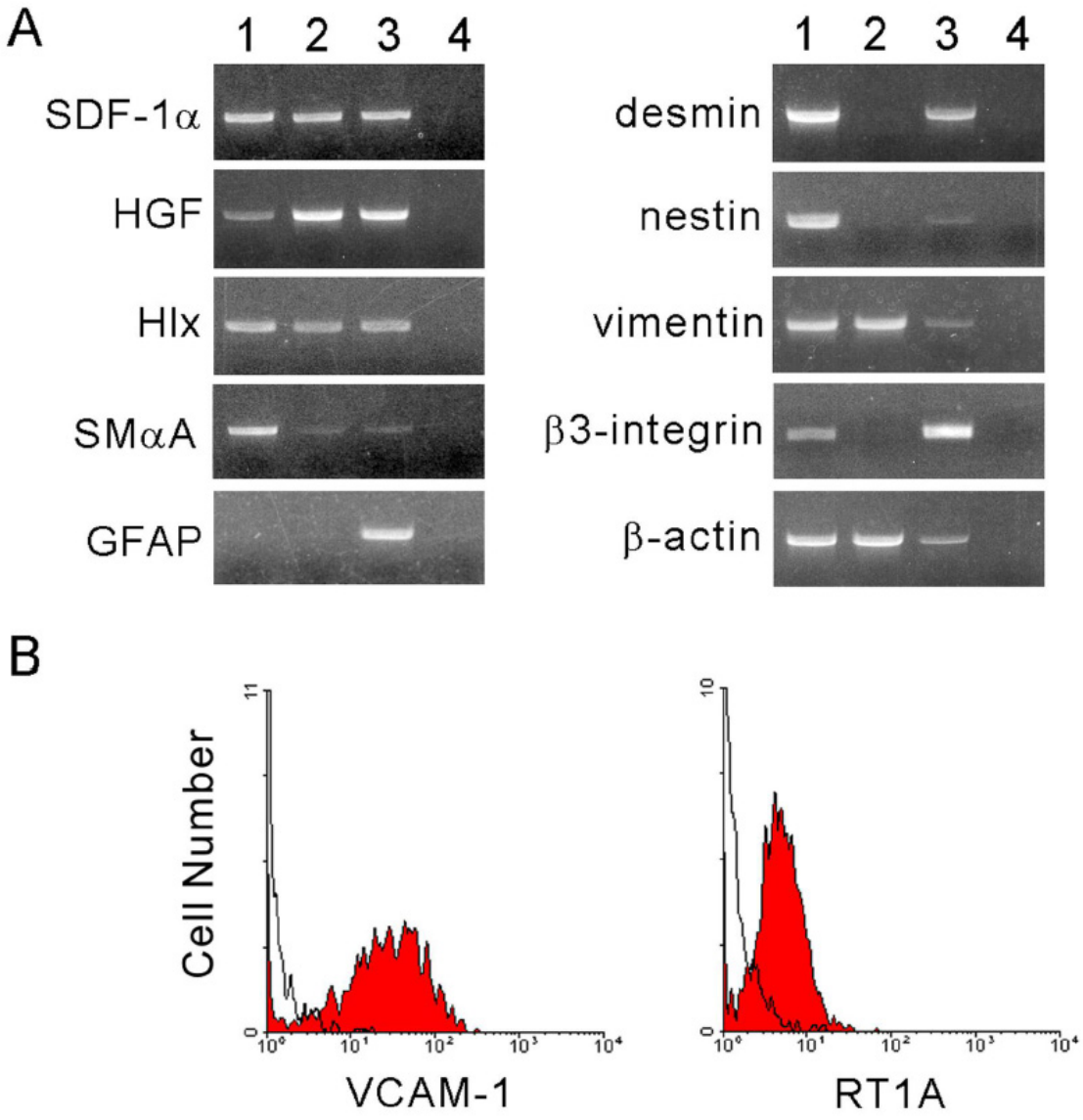
EGF
EGF+LIF
C
desmin
nestin
D
desmin
nestin

Figure 3.6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GFAP
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desmin
nestin
vimentin
β3-integrin
β-actin
B
Cell Number
VCAM-1
RT1A

CHAPTER IV

HUMAN HEPATIC STEM CELLS FROM FETAL AND POSTNATAL DONORS

(Refereed article in press in the Journal of Experimental Medicine, 2007)

In order to understand paracrine signaling between hepatic mesenchymal and epithelial cells, identification and characterization of both cell types are critical. Several methods have been developed to purify the highly proliferative hepatic cells from fetal and adult mice. In animal models, only one pluripotent hepatic progenitor cell type, hepatoblasts, has been identified, and expresses both albumin and alpha-fetoprotein. It was long assumed that this was the only pluripotent progenitor in human livers also. In these studies we show that there are two pluripotent progenitors, the hepatic stem cells and their immediate descendents, hepatoblasts, found in fetal livers and in all ages of postnatal livers. This chapter details the identification and comprehensive characterization of two bipotent hepatic progenitor cell types found within human fetal, neonatal, postnatal and adult livers: hepatic stem cells or HpSCs ($EpCAM^+$, $NCAM^+$, claudin 3^+, $CK19^+$, albumin$^{\pm}$, P450 $3A7^-$, AFP^-) and hepatoblasts or HBs ($EpCAM^+$, ICAM-1^+, $albumin^+$, $CK19^+$, AFP^+, P450 $3A7^+$).

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ABSTRACT

Human hepatic stem cells (hHpSCs), pluripotent precursors of hepatoblasts and thence of hepatocytic and biliary epithelia, are located in ductal plates in fetal livers and in canals of Hering in adult livers. They can be isolated by immunoselection for $EpCAM^+$ cells and constitute ~ 0.5-2.5% of liver parenchyma of all donor ages. Self-renewal capacity of hHpSCs is indicated by phenotypic stability after expansion for more than 150 population doublings in a serum-free, defined medium and with a doubling time of ~36 hours. Survival

and proliferation of hHpSCs require paracrine signaling by hepatic stellate cells and/or angioblasts that co-isolate with them. The hHpSCs are ~9 μm in diameter, express cytokeratins 8, 18 and 19, CD133/1, telomerase, CD44H, claudin 3, and albumin (weakly). They are negative for α-fetoprotein, ICAM-1, and for markers of adult liver cells (e.g. cytochrome P450s), hemopoietic cells (e.g. CD45), and mesenchymal cells (e.g. VEGFr, desmin). If transferred to STO feeders, hHpSCs give rise to hepatoblasts, recognizable by cord-like colony morphology and up-regulation of α-fetoprotein, P450A7 and ICAM-1. Transplantation of freshly isolated $EpCAM^+$ cells or of hHpSCs expanded in culture into NOD/SCID mice results in mature liver tissue expressing human-specific proteins. The hHpSCs are candidates for liver cell therapies.

INTRODUCTION

The role of hepatic stem cells (hHpSCs), particularly in the maintenance and regeneration of the adult liver, has been a subject of debate without clear consensus (Michalopoulos and DeFrances, 1997; Thorgeirsson et al., 2004; Alison et al., 2004; Schmelzer et al., 2006; Oh et al., 2002). During embryonic development, endodermal cells in the mid-region of the embryo bulge into the cardiac mesenchyme, are affected by critical signaling from endothelia forming vasculature, and form the liver bud (Lemaigre and Zaret, 2004; Matsumoto et al., 2001). The cells within the liver bud are recognized as hepatoblasts due to expression of a signature marker, α-fetoprotein (AFP) and are bipotent giving rise to hepatocytes and bile duct epithelial cells, cholangiocytes (Saxena and Theise, 2004). We and others have described the isolation and expansion in culture of AFP^+ cells from fetal and adult livers of several species (Kubota and Reid, 2000; Tanimizu et al., 2003; Haruna et al.,

1996). Clonogenic expansion assays of rodent hepatoblasts under wholly defined conditions have demonstrated that hepatoblasts are capable of extensive expansion *ex vivo* as well as differentiation to both hepatocytic and biliary lineages (Kubota and Reid, 2000). The findings from investigations of liver organogenesis as well as the *ex vivo* studies of hepatoblasts have led to a long-standing assumption that hHpSCs correspond to hepatoblasts, and that hHpSCs would express AFP. However, AFP^+ cells are rare in normal adult livers (<0.01%) except in livers with severe injury or disease (Saxena and Theise, 2004; Kubota et al., 2002; Abelev, 1971). In addition, the renowned replicative capacity of hepatocytes *in vivo* (Overturf et al., 1999) has led to the opinion that adult livers do not have hHpScs and that all regenerative responses are from mature parenchymal cells except in certain disease states (Michalopoulos and DeFrances, 1997).

Here we define a novel class of AFP-negative cells in fetal and adult human livers, that are precursors to hepatoblasts, and have properties consistent with hHpSCs. The hHpSCs are negative for AFP but positive for epithelial cell adhesion molecule, EpCAM (CD326, C017-1A antigen, GA733-2). This protein, encoded by the tumor –associated calcium signal transducer 1 gene, is expressed by many carcinomas and serves a regulatory function in certain normal epithelia, including all of those derived from endoderm (e.g. liver, lung, pancreas, intestine) (Balzar et al., 1999; Balzar et al., 1998). By immunohistochemistry, deBoer and colleagues observed that hepatoblasts in embryonic human liver are $EpCAM^+$, whereas mature hepatocytes are $EpCAM^-$ (Balzar et al., 1999). In adult livers, most (but not all) bile duct epithelia are $EpCAM^+$. Also, expanded ductular structures, seen in cases of focal nodular hyperplasia or biliary cirrhosis, contain numerous $EpCAM^+$ cells (Balzar et al., 1999).

We have reported previously that $EpCAM^+$, AFP-negative cells from human livers are hHpSCs and have compared their pattern of gene expression with that of hepatoblasts and mature liver parenchyma (Schmelzer et al., 2006). We now show that the hHpSCs are located in ductal plates in fetal and neonatal livers and in the proximal branches of the intrahepatic biliary tree, the Canals of Hering, in pediatric and adult livers of all donor ages, with the frequency of hHpSCs remaining relatively constant throughout life. We further document the immunoselection of these cells using monoclonal antibodies to EpCAM and test whether they meet the defining criteria for stem cells, namely, self-renewal and pluripotency.

RESULTS

In Vivo Localization of $EpCAM^+$ Hepatic Stem Cells (hHpSCs)

Sections of fetal and adult livers were stained for EpCAM and for liver-specific markers (albumin, AFP, and CK19) (Figure 4.1). We found that ductal plates, a band of tissue encircling each of the portal triads in fetal and neonatal livers, have small cells (7-10 μm) with a paucity of cytoplasm, and stained intensely both cytoplasmically and at the surface for CK19, EpCAM, and weakly for albumin but are negative for AFP. In non-diseased, postnatal (pediatric and adult) livers, cells staining positively for EpCAM by immunohistochemistry appear exclusively in the canals of Hering in the vicinity of the portal triads of acini. Theise and colleagues (Theise et al, 1999) reported that cells lining these ductules express cytokeratin (CK) 19, present in biliary epithelia but not hepatocytes. Our data confirm their report and demonstrate that the $CK19^+$ cells within the canals of Hering express EpCAM and that subpopulations of them also express albumin (Figure 4.1; yellow color is due to overlap of CK19 and albumin expression). The co-expression of CK19 and

albumin is consistent with hHpSCs and corroborates the hypothesis of others that the Canals of Hering comprise a niche for hHpSCs (Theise et al, 1999). Below are data from *ex vivo* studies of $EpCAM^+$ cells supportive of the interpretation that they include hHpSCs.

Most parenchymal cells of fetal and neonatal livers consisted of hepatoblasts, slightly larger cells (10-12 μm in diameter) that stained positively for albumin, AFP, and CK19. In hepatoblasts the distribution of CK19 is more particulate and less intense than that in ductal plate cells. EpCAM expression in AFP^+ cells, both in fetal and postnatal livers, occurred at the membrane only. In pediatric and adult livers, hepatoblasts were found as individual cells or small clusters of cells tethered to the ends of the canals of Hering (Figure 4.1). The hepatoblasts in non-diseased, postnatal livers constitute fewer than 0.01% of cells and express AFP weakly.

Flow Cytometry of $EpCAM^+$ Cells

By flow cytometry we observed $EpCAM^+$ cells in human liver cell suspensions of all donor ages (Figure 4.2A). Suspensions from fetal livers from which hemopoietic cells had been purged contained, on average, 12% $EpCAM^+$ cells. However, the percentage could be as high as 20% depending on the gestational age of the fetus. Most of the $EpCAM^-$ cells in fetal livers were of non-hepatic lineage and were predominantly hemopoietic. The vast majority (>90%) of the $EpCAM^+$ cells in fetal livers co-express AFP, albumin (ALB), and CK19 (Figure 4.2). They could be subdivided into two subpopulations: 1) Hepatoblasts showed expression of ICAM-1, AFP, ALB, CK19, CD133/1, P450A7 and CD44H (hyaluronan receptor); 2) hHpSCs, constituting ~5% of $EpCAM^+$ cells from fetal livers, had

an overlapping but distinct profile: they were positive for ALB (weak), CK19, CD44H, CD133/1, NCAM, and claudin 3, but negative for AFP and for P450A7.

Cell suspensions from adult human livers averaged 1.3% $EpCAM^+$ cells, and those from neonatal and pediatric donors two to three-fold higher. Preparations having $EpCAM^+$ cell populations at levels above 1.5% were obtained typically from livers subjected to ischemia (cold or warm) prior to organ procurement and/or during transportation. This suggests that the $EpCAM^+$ liver cells are more resistant to ischemia than mature liver cells. The postnatal $EpCAM^+$ liver cells also co-express CK19 and ALB (data not shown) but have no detectable AFP^+ cells (by flow cytometry), except in rare cases of overt hepatic disease (e.g. cirrhosis; data not shown). Neonatal livers, including some from premature births, showed rapidly decreasing levels of AFP^+ cells as a function of age, falling below detection level by a few months after birth. Based on considerations detailed below and from our previously published work (Schmelzer et al., 2006), we identify the $EpCAM^+$ cells from pediatric and adult livers as almost exclusively hHpSCs, not hepatoblasts.

Culture Selection on Plastic and in Serum-free KM isolates hHpSCs but not Hepatoblasts

Suspensions of liver cells plated in Kubota's Medium (KM), a serum-free medium optimized for *ex vivo* expansion of hepatic progenitors (Kubota and Reid, 2000), either on tissue culture plastic or on embryonic stromal cell feeders, yielded parenchymal cell colonies with two distinct morphologies. Type I colonies consisted of cells forming a cord-like morphology interspersed with clear channels and expressing EpCAM, albumin, CK19, ICAM, and AFP but not NCAM (Figures 4.9-12). Type 2 colonies consisted of densely packed, morphologically uniform cells, strongly expressing EpCAM, NCAM, CD44H,

claudin 3, weakly expressing or negative for ALB, and negative for AFP and ICAM-1 (Figures 4.3 and 4.11). We interpret the type I colonies as corresponding to hepatoblasts and the type 2 colonies as corresponding to hHpSCs (Table 4.1).

In cultures on plastic, by 5-7 days (mean 5.2 ± 1.6; maximum of days), the hepatoblast colonies disappeared. However, if cultured on STO feeders, hepatoblasts survived for up to two months, continuing to show co-expression of ALB, AFP and CK19 (Figures 4.9 and 4.10). The hepatoblast colonies typically contained fewer than ~100 cells.

By contrast to hepatoblasts, hHpSC colonies on plastic continued to expand. A time lapse sequence of a growing hHpSC colony is shown in Figure 4.3A-E in which the expansion of hHpScs seeded at very low density is shown on day 1, day 3 and day 8. The hHpSCs can be subcultured after mechanical disaggregation and continue to multiply extensively. Their doubling time on plastic is ~36 hours. That doubling time decreased to less than 24 hours if they are plated on specific extracellular matrix substrata (McClelland et al., submitted). By 2-3 weeks, hHpSC colonies typically contained many thousands of cells.

The hHpSC colonies were assessed for expression of lineage markers by immunofluorescent staining. The expression pattern closely resembled that of ductal plate cells *in vivo*. They were positive for CK19, NCAM, EpCAM, and CD44H (Figure 3F-L). In addition, they were positive for ALB (weak), E-Cadherin, N-Cadherin, CK8 and 18, CD133/1, integrin β-1 (CD29), claudin 3, and telomerase (data not shown). They were negative for AFP, any form of cytochrome P450, hemopoietic markers (CD34, CD45, CD38, CD14, CD90, and glycophorin A), endothelial cell markers (VEGFr, von Willebrand factor, and PECAM or CD31), and mesenchymal markers such as those for hepatic stellate cells (CD146, desmin, and α-smooth muscle actin). The expression of NCAM by the hHpSCs is

significant, since previous studies have shown that this marker is present on the ductal plate in fetal livers and evident on liver cell populations proliferating under various disease states (Libbrecht et al., 2001; Roskams et al., 1998; Fujimoto et al., 2001; Van Den Heuvel et al., 2001).

Immunoselection using EpCAM Isolates Hepatoblasts from Fetal and Neonatal Livers; Immunoselection Using EpCAM or NCAM Isolates hHpSCs from Livers of All Donor Ages

To enrich for hepatic progenitors from liver cell suspensions, we explored a number of fractionation strategies, including separation by buoyant density on Ficoll gradients (Table 4.3) and by immunoselection. The most satisfactory results were obtained using magnetic immunoselection. While fluorescence activated cell sorting (FACS) was able to yield highly purified cellular subpopulations, the shear forces and the use of buffers (e.g. phosphate buffered saline) that are not optimal resulted in low yields of viable cells. We used magnetic microspheres conjugated with monoclonal antibody to EpCAM (Miltenyi Biotec, Auburn, CA) to immunoselect $EpCAM^+$ cells from liver cell suspensions and obtained robust, highly viable sorted cells that survived and expanded well when cultured (Figures 4.4 and 4.9-12, Table 4.4). From postnatal livers up to 10 billion viable cells were processed in a single pass using the CliniMACS apparatus (Miltenyi Biotec) and yielded over 100 million $EpCAM^+$ cells. Purity of the enriched $EpCAM^+$ cells was typically 75 to 90% and recovery usually exceeded 90%. Representative fractionations of a fetal liver and of a postnatal liver are depicted in Figure 4. A cell suspension from the liver of a two year-old donor was found to contain 0.7% $EpCAM^+$ cells. The immunoselected population contained 81% $EpCAM^+$ cells,

while the flow-through fraction was almost entirely depleted of $EpCAM^+$ cells. The majority of hepatic $EpCAM^+$ cells were of 8-10 μm diameter, as judged by Coulter Counter analysis, in contrast to 18-20 μm for diploid hepatocytes, the predominant population in the initial liver cell suspension. A small peak of presumptive tetraploid cells also is evident, measuring ~25 μm in diameter. Light scatter ("Side Scatter") profiles indicate that the $EpCAM^+$ liver cells are considerably smaller and less granular than the bulk of the parenchymal cell population.

Magnetic immunoselection for $NCAM^+$ cells from fetal livers enriched for cells capable of forming only hHpSC colonies (Figure 4.4). The majority of $EpCAM^+$ cells from fetal liver co-expressed NCAM, whereas only ~40% of those from adult liver were also $NCAM^+$. Therefore, sorting for $NCAM^+$ cells proved useful for isolation of hHpSCs from fetal livers but less so from adult livers. It is unknown at this time whether NCAM and EpCAM co-expression is a definitive property of hHpSCs. An alternative hypothesis, now being tested, is that NCAM is present on angioblasts or other mesenchymal companion cells tightly bound to the hHpSCs such that immunoselection for it results in co-selection of the two cell types (see below for more on this theme). Sorts for KDR (VEGFR2) resulted mostly in angioblasts (Figure 4.4). However, these sorts also yielded an increase in hHpSC colonies due, we assume, to co-selection of hHpSCs and angioblasts.

Proteins and Genes Expressed by $EpCAM^+$ Cells

Immunoselected $EpCAM^+$ cells from fetal and postnatal livers were examined by flow cytometry for expression of lineage markers characteristic of various cell types that reside in the liver (Figures 4.2 and 4.4). As judged by double label flow cytometry, about

95% of the immunoselected EpCAM$^+$ cells expressed CK19, and comparable percentages expressed ALB and CD133. Evaluations of many preparations indicated that over 90% of the EpCAM+ cells are positive for CD133, detected with monoclonal antibodies to two distinct epitopes (CD133-1 with monoclonal antibody AC133; CD133-2 with monoclonal antibody AC141). Virtually all CD133-1$^+$ cells in adult liver cell suspensions were found in the EpCAM$^+$ selected fraction, and mature hepatocytes were clearly negative. However, it appeared that ~40% of liver cells with light scatter profiles consistent with mature hepatocytes were positive for CD133-2. Examination by immunofluorescent microscopy showed that staining for CD133-1 clearly outlines cell membranes, whereas that for CD133-2 shows a more diffuse pattern (data not shown). It is likely that the staining of many more liver cells by CD133-2 results from a known cross-reactivity with CK18 (Potgens et al., 2002) which is expressed by hepatocytes and reportedly can be found on the cell surface (Wells et al., 1997). Based on the more specific CD133-1 antibody, we conclude that EpCAM and CD133 (prominin) are co-expressed by the vast majority of hHpSCs.

Neural cell adhesion molecule, NCAM (CD56), shown previously to be expressed by glia, muscle cells and by neurons (Goridis and Brunet, 1992), was found on the majority of hHpSCs derived from fetal and neonatal livers, but only ~40% of the EpCAM$^+$ cells from adult livers. In our prior studies NCAM mRNA was enriched strongly in EpCAM$^+$ cells from both fetal and postnatal livers, but expression at the protein level was variable (Schmelzer et al., 2006). NCAM staining was most evident at the borders of the hHpSC colonies (Figure 4.3).

Less than 1% of the enriched EpCAM$^+$ cells stained for the hemopoietic marker CD45 (leucocyte common antigen), which is found on Kupffer cells (tissue macrophages)

and lymphocytes in the liver. The $EpCAM^+$ cells were negative for expression of other hemopoietic markers assayed (CD34, CD14, CD38, CD4, CD90, and glycophorin A), for endothelial cell markers (CD34, VEGFR2 or KDR, von Willebrand factor, and CD31), and for mesenchymal markers, especially those associated with hepatic stellate cells (CD146, also called Mel-CAM, desmin, α-smooth muscle actin) (data not shown). Finally, we found AFP expression at RNA and protein levels in $EpCAM^+$ cells from fetal and neonatal livers, but not from pediatric or adult livers. As noted above, small numbers of cells weakly positive for AFP, as judged by immunohistochemistry, were observed tethered to the ends of the canals of Hering in sections from pediatric and adult livers (Figure 4.1E). From our experience, these cells are too few and express AFP too weakly to permit recognition as a defined subpopulation by flow cytometry.

Assessment by RT-PCR of RNA expression in the $EpCAM^+$ liver cells (Figure 4.2) gave results consistent with the flow cytometry data. Further details of these findings are reported elsewhere (Schmelzer et al., 2006). In brief, $EpCAM^+$ selection from fetal livers more than doubled the expression levels of albumin, AFP and CK19. Immunoselection for $EpCAM^+$ cells from postnatal livers strongly enriched for transcripts encoding EpCAM, CK19, CD133 and CD117 (c-Kit); these transcripts were barely detectable in the EpCAM-negative cells. AFP transcripts were not detectable in $EpCAM^+$ or $EpCAM^-$negative cells from postnatal livers.

Although immunoselected cells are enriched for relative expression of CD117 mRNA, we have not observed the corresponding protein by immunostaining of freshly isolated cells from fetal or postnatal livers nor on cultured cells from postnatal livers. However, we occasionally have observed low levels of CD117 staining on cells at the periphery of hHpSC

colonies from fetal livers, located in regions where hHpSCs overlap with mesenchymal companion cells.

Cytochrome P450 3A4 (CYP3A4), a protein expressed by mature hepatocytes, was not found at all in parenchymal cells, either $EpCAM^+$ or $EpCAM^-$, from fetal livers in terms of both mRNA and protein level of it. The level of mRNA for cytochrome P450 in $EpCAM^+$ cells was twenty-fold lower relative to EpCAM-negative cells from postnatal livers. The small amount of CYP3A4 RNA in the $EpCAM^+$ cell fraction from postnatal livers could be accounted for by residual hepatocyte contaminants. By contrast, the hepatoblasts, but not the hHpSCs, were found to express P450A7, a protein found in fetal livers (data not shown). $EpCAM^+$ cells from postnatal livers also showed eight-fold lower relative expression of albumin RNA than the flow-through (EpCAM-negative) population. Again, some transcripts can be attributed to incomplete removal of hepatocytes. However, the detection of albumin protein in $EpCAM^+$ cells by flow cytometry, taken together with the transcript data, demonstrates that these progenitor cells express the albumin gene, albeit at a significantly lower level than differentiated hepatocytes.

Assays for telomerase activity indicate significant levels in freshly isolated $EpCAM^+$ cells from livers of all donor ages and in cultures of colonies of both hHpSCs and hepatoblasts. Full characterization of telomerase activity and its regulation in various fractions of human liver cells from fetal and postnatal donors is presented elsewhere (Schmelzer E and Reid L, submitted) as are studies on the effects of purified matrix substrata on telomerase activity in cultures of hHpSCs (McClelland et al, submitted).

Ex Vivo Clonogenic Expansion—Evidence for Self Renewal

Colony formation by committed hepatic progenitors or diploid adult parenchyma involves a limited number of divisions (typically 5-7 divisions) over a relatively short period of time (e.g. 2 to 3 weeks) (Kubota and Reid, 2000). By contrast, self-renewal involves clonogenic expansion that can go on for more than 100 population doublings with phenotypic stability, properties associated with stem cells. We found previously that rat hepatoblasts multiply far more extensively in KM with STO feeder cells than on tissue culture plastic (Kubota and Reid, 2000). However, STO feeders and KM were not permissive for clonogenic expansion of human hepatoblasts. Under these conditions the hepatoblasts survived for a few months but demonstrated limited growth. By contrast, hHpSCs from livers of all donor ages could undergo clonogenic expansion for more than 6 months (>150 population doublings) in culture on tissue culture plastic and in KM with only the native feeders (the companion cells) (Table 4.2). The cells maintained phenotypic stability as assessed by morphology and by antigenic and biochemical profiles (Tables 4.1 and 4.2). hHpSC colonies starting from 1-3 cells grew to cover 4.9$\pm$0.3 mm^2 in area and contained an average of 1400 $\pm$ 520 cells (three independent counts of total cells from 50 dispersed colonies). Thus, the cells in this representative experiment had gone through 10-11 population doublings in 2 weeks corresponding to an average doubling time of 31-34 hours (Figure 4.3, Table 4.2).

Mesenchymal Companion Cells Provide Critical Paracrine Signaling for hHpSCs

The tightly packed colonies of hHpSCs have a prominent ridge at the perimeter (Figures 4.3, 4.4 and 4.6) at which we have identified mesenchymal companion cells (Figure 4.5). As the colonies grow, the companion cells penetrate the colonies and become found

throughout them. When removed from a culture dish, the attachment to the plastic surface is evident only at the edge of the colonies, not in the center. This suggests that attachment to the plastic is mediated either by mesenchymal cells or by cooperative interactions between the hHpSCs and the mesenchymal cells.

Phenotypic analyses of the companion cells indicates at least two distinct populations: angioblasts (KDR^+, $CD133/1^+$, $CD117^+$, von Willebrand $factor^+$, $CD31^{weak}$); and hepatic stellate cells ($desmin^+$, α-smooth muscle $actin^+$, $CD146^+$) (Figure 4.5). A comparison of their morphological and antigenic phenotypes is given in Figure 5. Cells rigorously purified away from the companion cells (e.g. by repeated immunoselection for $EpCAM^+$ cells) did not survive on culture plastic but only on STO feeders (data not shown). Immunoselection of $CD117^+$ cells yielded angioblasts but neither hepatic stellate cells nor hHpSCs (data not shown). Immunoselection for other markers found on the companion cells (e.g. VEGFr) resulted mostly in selection of the companion cells alone, though we did find co-selection for hHpSCs to occur at low and variable frequency (Figure 4.4). We still cannot rule out that the consistent enrichment of hHpSCs from fetal liver by immunoselection for NCAM could actually result from co-selection of the stem cells via tight association with $NCAM^+$ companion cells.

Proof of Pluripotency of hHpSCs

Passaging (transfer) of colonies of hHpSCs (whether derived from fetal or adult livers) from culture plastic onto feeder layers of STO cells resulted within hours in eruption of hepatoblasts from the periphery of hHpSC colonies (Figure.6 and 4.7; Table 4.1). After the transfer, the morphology and antigenic profile of the cells within the hHpSC colonies proper

did not change in most of the cells, though there were occasional cells with distinct gene expression within the colony (Figure7). Rather the colonies of hHpSCs gave rise to cord-like eruptions from their edges, yielding cells with morphology, antigenic and biochemical profiles identical to that of hepatoblasts. The cells in these erupted areas strongly expressed AFP, ICAM-1, ALB, and were positive for cytochrome P450-A7 (data not shown), but were negative for NCAM (Figure7). In addition, committed progenitors biliary progenitors were sometimes observed erupting from a colony of hHpSCs, as shown by staining for CK19 but not albumin (Figure7).

In cultures of cells from postnatal livers, in colonies stained by double label immunofluorescence for CK19 and ALB, we have observed distinct sectors positive for one or the other marker but not both (Figure12). This was found most frequently in colonies of hepatoblast morphology. We interpret such sectors as deriving from unipotent cells, corresponding to committed progenitors for biliary and hepatocytic lineages, respectively. The sectoring could occur if at division a bipotent cell gives rise to a daughter cell restricted to the biliary or to the hepatocytic lineage. Occasional small colonies showed expression of only one of the lineage markers (CK19 or albumin) and are assumed to have arisen from committed progenitors for the corresponding cell type.

EpCAM$^+$ Cells and Colonies of hHpSCs Give Rise to Human Liver Tissue in vivo

Transplantation of freshly isolated EpCAM$^+$ cells or of hHpSC colonies, from either fetal or postnatal livers, into livers of NOD/SCID mice resulted in engraftment and the formation of human liver tissue (Figure8). Islands of cells staining positive for human ALB, CK19 and AFP were found within 2 days of transplantation (Figure 8A, C, and E) and

persisted within the livers for weeks (Figure 4.8F). The extent of engraftment and expansion of human liver cells *in vivo* was enhanced by treatment of the mice with carbon tetrachloride, CCl_4, a poison for the pericentral zone of the liver acinus, often used to create a cellular vacuum in transplanted hosts (Figure 4.8B, D and G). Human-specific DNA sequences were found in the liver of animals that received the human cell transplants, but not in other tissues, nor in control animals that did not receive human cells (data not shown). Prior to transplantation, as expected, the cells were shown to express EpCAM and CK19 strongly and ALB weakly, but were negative for AFP at both RNA and protein levels. The liver sections from mice transplanted with hHpSCs contained cells strongly expressing human-specific forms of ALB, CK19, and AFP but were negative for EpCAM. However, human cytochrome P450 3A was not detectable. Therefore, it appears that after transplantation and expansion in recipient livers, the human cells lost expression of a marker (EpCAM) found only on stem and progenitor cells, and, acquired some, but not yet all, of the functions specific to mature hepatocytes. We think it logical that transferrin is expressed by not yet P450 3A in transplanted cells given that in mature liver, transferrin is expressed by zone 2 parenchymal cells, whereas P450 3A by zone 3 parenchymal cells within the acinus. Thus, P450 3A is a late gene produced by cells at the end of the liver's maturational lineage.

As an independent test of engraftment, we assessed expression of the human transferrin gene, encoding a protein characteristic of mature hepatocytes. We found by quantitative RT-PCR analysis with human-specific primers that the livers of mice sampled one week after injection of the $EpCAM^+$ cells derived from postnatal human livers contained significant levels (2100 ± 1140 strands per 100 ng) of human transferrin RNA. Such sequences were undetectable in RNA from livers of control mice (<100 strands per 100 ng

RNA). Although prior to transplantation over 80% of cells in the test cell population expressed EpCAM and CK19, cells in recipient animals were positive for human ALB and were negative for both of the progenitor cell markers. Taken together, the data suggest that within seven days *in vivo*, the engrafted hHpSCs gave rise to mature human liver cells.

DISCUSSION

Cells in the ductal plates in fetal and neonatal livers and in the Canals of Hering in pediatric and adult livers are hHpSCs. They can be isolated efficiently by selective culture conditions and by immunoselection for EpCAM (CD326) and/or NCAM (CD56). The hHpSCs have features typical of stem cells including Sonic and Indian Hedgehog signaling (Sicklick et al., 2005) and high telomerase activity (Schmelzer and Reid, submitted). They are capable of self-renewal, as shown by clonogenic expansion for over 150 population doublings, and are pluripotent, with the ability to give rise directly to committed biliary progenitors and to hepatoblasts and thence to hepatocytic and biliary lineages, as well as to other endodermal cell types (our unpublished data). The hHpSCs express certain markers of both hepatocytic and the biliary lineages but lack expression of mature liver functions (Schmelzer et al., 2006). They yield human liver tissue when transplanted intrahepatically in immune deficient mice. Hepatoblasts, expressing AFP, ALB and CK19, and emerging in newly forming liver tissue, have long been considered hepatic stem cells (Fausto et al., 1993). However, AFP-negative hHpSCs are actually precursors to hepatoblasts.

Recognition of the ductal plate as the liver's stem cell niche provides a new insight into organogenesis. The specification of foregut cells to hepatic fate is associated with expansion of endoderm into the surrounding cardiac mesenchyme, a process leading to ductal

plate formation (Sicklick et al., 2005). Our data suggests that ductal plates are directly antecedent to the Canals of Hering, which have been identified as the reservoir of stem cells in postnatal livers (Theise et al, 1999; Roskams et al., 2004).

The liver at all ages displays a remarkable capacity to regenerate after physical or toxic injury (Michalopoulos and DeFrances, 1997). Two forms of regenerative response are known. The first is a hypertrophic cellular response by mature hepatocytes that undergo DNA synthesis with minimal cytokinesis, the predominant mechanism of regeneration after partial hepatectomy in postnatal livers (Forbes et al., 2002; Sigal et al., 1999). The other is a hyperplastic response by both progenitor cells and diploid hepatocytes following significant loss of liver cells in zones 2 and 3, as detailed in a number of review articles (Thorgeirsson et al., 2004; Grisham and Thorgeirsson, 1997; Thorgeirsson and Grisham, 2003). Contributions of progenitors to regeneration after partial hepatectomy have been presumed negligible based on assumptions that they should be AFP^+ (Michalopoulos and DeFrances, 1997). Since hHpSCs are AFP-, their role in liver regeneration in adults requires re-evaluation by tracking the involvement of $EpCAM^+$ and $NCAM^+$ cells. The frequency of $EpCAM^+$ cells in suspensions prepared from postnatal human livers is consistent at all ages beyond a few months, in the range of 0.5 to 2.5%. We infer that a substantial pool of hHpSCs is maintained throughout life. The number of hHpSCs is much higher than estimates based on the frequency of AFP^+ cells (<0.01%). Some authors have argued that the mature liver contains only "facultative" stem cells, activated in response to pathological states and injuries that invoke a hyperplastic response (Alison et al., 1996; Alison et al., 2004; Theise et al, 1999; Thorgeirsson and Grisham, 2003). We have previously raised the alternative hypothesis that hHpSCs function routinely to replenish the liver as mature cells are lost slowly through

terminal differentiation (Schmelzer et al., 2006; Sigal et al., 1992; Sigal et al., 1999). The presence of a much larger pool of hHpSCs than previously suspected in normal adult livers provides a further rationale to examine this possibility more carefully.

During development a limited number of hHpSCs are associated with developing portal tracts and steadily give rise to hepatoblasts that we hypothesize are the liver's transit amplifying cells. The hepatoblasts, in turn, are precursors to committed hepatocytic and biliary progenitors. Further evidence for hepatoblasts in normal adult livers is given elsewhere (Zhang et al., submitted). In the findings reported here, we show the generation of hepatoblasts and unipotent progenitors from hHpSC colonies in culture. This occurs spontaneously from discrete regions at the periphery of hHpSC colonies and may correspond to a localized signal that triggers a rapid burst of expansion from one or more cells. As noted below, cell-cell interactions are keys to both maintenance and differentiation of hHpSCs. STO feeder cells promote differentiation of hHpSCs, whereas they contribute primarily to survival and expansion of rodent hepatoblasts, and may offer a tool to identify some of the differentiation-promoting signals for hHsPCs.

Functions of Cell Surface Markers of hHpSCs

Characteristic cell surface antigens of hHpSCs and hepatoblasts overlap extensively, with both populations expressing EpCAM, E-Cadherin, integrin β-1 (CD29), and CD133. The hHpSCs and hepatoblasts are negative for markers of hematopoietic (CD34, CD45, CD38, CD14, CD90, glycophorin A), endothelial (VEGFRr, von Willebrand factor, CD31) and mesenchymal (CD146, desmin, and α-smooth muscle actin) cell lineages.

EpCAM is present on proliferating epithelial cells in most, if not all, organs derived from endoderm (e.g. liver, lung, pancreas, intestine). The extracellular domain of EpCAM is thought to generate a relatively weak homotypic bond between adjacent cells (Balzar et al., 1999). Conversely, the cytosolic domain of EpCAM is believed to diminish the effectiveness of E-cadherin binding through impairment of the interaction between beta-catenin and the actin cytoskeleton (Balzar et al., 1998). This role may be physiologically relevant for hHpSCs and hepatoblasts, as E-cadherin was expressed with the same distribution as EpCAM.

The role of NCAM in the biology of hHpSCs requires further elucidation. We found that the hHpSCs from fetal and neonatal liver consistently show strong NCAM expression and that immunoselection for NCAM enriches for hHpSCs. However, only ~40% of hHpSCs from adult livers are positive for this antigen. In cultures of hHpSCs, NCAM expression is observed in a characteristic scalloped pattern located most prominently at the borders of the colonies. Thus far we have not been able to ascertain unequivocally whether NCAM is expressed by hHpSCs, by tightly associated mesenchymal companion cells, or both. Ultrastructural studies by electron microscopy are needed to resolve this point. NCAM is a member of the Ig superfamily, with more than 20 alternatively spliced mRNAs encoding multiple protein isoforms (Fujimoto et al., 2001; Cunningham et al., 1987). It is the only sialated cell adhesion molecule and forms homotypic cell/cell attachments that are inversely proportional to the degree of sialation; an increase in sialation results in muted cell-cell adhesion and consequent increase in migration and invasion (Fujimoto et al., 2001). Several groups have reported that NCAM is expressed by ductal plate cells within the fetal liver and, interestingly, also by proliferative ductular cells that characterize pathologies collectively

termed ductal plate malformations, such as primary biliary atresia (Libbrecht et al., 2001; Fabris et al., 2000; Van Den Heuvel et al., 2001; Neubauer et al., 1996; Anatskaya et al., 1994). Thus, positive staining for NCAM, in addition to ALB, CK19 and CK8/18, supports the interpretation of these cells as ones derived from the ductal plate.

We found consistent expression of CD133 (prominin-1) by hHpSCs cultured on both plastic and on STO substrata, and by >90% of $EpCAM^+$ cells immunoselected from adult livers. Although angioblasts also are $CD133^+$, the staining in hHpSC colonies was associated with most or all cells, indicating that CD133 is expressed by the hHpSCs and not only by companion cells. This pentaspan transmembrane glycoprotein was first identified on hematopoietic stem cells, and its expression also has been observed on stem/progenitor cells of a variety of lineages, including endothelia, muscle, neural, prostatic, epidermal, and others (Jiang et al., 2002; Bhatia, 2001). The role, if any, of CD133 in the self-renewal and differentiative capacity of hHpSCs is not yet understood. However, it may be significant that an isoform of CD133 specifically associated with stem cells was found in cells of the basal layer of human neonatal epidermis, and co-expressed there with integrin β-1, which also is expressed by hHpSCs and hepatoblasts. Furthermore, CD133 expression was lost as the epidermal cells stopped proliferating and acquired a differentiated phenotype in culture (Yu et al., 2002).

Association of Mesenchymal "Companion" Cells with hHpSCs

The specific association of multiple adhesion molecules with hHpSCs and hepatoblasts suggests that they play important regulatory functions in modulating interactions with cells that comprise local inductive environments and/or stem cell niches. A critical,

enabling event, required for formation of the liver, is that angioblasts from the septum transversum induce the hepatic bud to form (Matsumoto et al., 2001). Such key interactions are now amenable to study *in vitro* using hHpSCs. We observed that colony expansion and cell outgrowth of hHpSCs depends on mesenchymal companion cells prominent at the periphery of hHpSC colonies and identified by antigenic profiles as angioblasts (positive for VEGFR , CD31 or PECAM, CD117, CD 133) or hepatic stellate cell precursors (positive for CD146 (MEL-CAM, MCAM), desmin and α-smooth muscle actin). These findings parallel our prior work defining hepatic stellate cell precursors supportive of rodent hepatic progenitors (Kubota et al., 2007).

CD146 forms homotypic cell-cell connections that were first localized on melanoma cells and subsequently at cell junctions within endothelial cell layers (Bardin et al., 2001). CD146 has now been identified on many different cell types including keratinocytes and hair follicle epithelia, stromal cells in adipose tissue and bone marrow, and most cell types in the thymus (Gronthos et al., 2001; Seftalioglu and Karakoc, 2000). The expression of CD146 on cells at the periphery of colonies of hHpSCs, and extending to the innermost cells of aging colonies on plastic, is consistent with the known anti-cohesive activity of CD146. In this capacity CD146 functions as an outside-in transducer that suppresses gap junction connections, inhibits β-1-mediated integrin binding, and disturbs E-cadherin-based adherens junctions (Bardin et al., 2001; Johnson, 1999). Furthermore, the strong expression of ICAM-1 by more differentiated cells emerging from hHpSC colonies may also reflect modulation of cell-cell interactions. ICAM has been shown to act in conjunction with CD146 to disturb E-cadherin-based cell junctions (Benveniste et al., 1999). The dramatic increase in expression of CD146 in association with differentiation of the hHpSCs into hepatoblasts is assumed to

model angiogenesis in the forming liver, an interpretation supported by the findings of Sonic and Indian Hedgehog signaling and the Patched receptor in the ridge formed by the angioblasts and the hepatic stellate cells (Sicklick et al., 2005).

The possibility of co-isolation of hHpSCs with mesenchymal cells may account for some apparent differences between the hHpSCs described here and candidate hHpSCs reported by others (Dan et al., 2006). For example, liver-derived, stem-like cells have been reported to express markers shared with hematopoietic progenitors, including CD45 (leucocyte common antigen), CD34, and CD117, and/or to be capable of giving rise to both hepatic and endothelial cells (Crosby et al., 2002). Another report describes candidate hepatic stem cells found in regeneration after massive hepatic necrosis as "lymphoid blastlike" cells that express CD133 and CD117 but not CD45 (Craig et al., 2004). We suspect that the multipotent stem cells found in fetal liver and reported to give rise to liver and also mesenchymal lineages (e.g. cartilage) and that co-express EpCAM and various mesenchymal cell markers are also an example of co-selection (Macdonald et al., 2002).

The hHpSC populations from both fetal and adult livers appear essentially negative for CD45, CD34 and CD117. However, CD34 and CD117 are expressed by angioblasts, which we have found in companion cells associated with hHpSCs. Based on immunofluorescent staining CD117 was variably present in the border zone between the companion cells and some (but not all) hHpSC colonies cultured on plastic. Also, transcript analysis revealed a slight enrichment in CD117 mRNA in $EpCAM^+$ cell populations from fetal livers and a significantly greater enrichment in $EpCAM^+$ cells from postnatal livers (Schmelzer et al., 2006). Nonetheless, immunoselection for CD117 or CD34 yielded

angioblasts, not hHpSCs. In summary, our data remain inconclusive; we cannot rule out that a minor subpopulation of hHpSCs cells express CD117.

It is conceivable that phenotypic differences between hHpSCs obtained by different isolation procedures reflect varying stages within a common lineage and/or subtle effects of *in vitro* selection protocols. However, it is clearly important to be aware of the physical and functional interaction of hepatic (endodermal lineage) and mesenchymal lineage cells both in the developing liver and the adult organ, and the possibility of ascribing properties to a single cell type that actually correspond to a mixed population. In any case we argue that the relative frequency and anatomical location of $EpCAM^+$, $CD133^+$. CD34-negative cells, and the growth and differentiation capacity of these cells, provide strong evidence that the hHpSCs described here are authentic stem cells in fetal and postnatal livers.

Purified $EpCAM^+$ cells, from fetal or postnatal livers, are able to engraft the livers of immunodeficient adult mice, with or without prior injury, yielding mature human liver tissue. The engrafted cells lose expression of stem cell markers (e.g., EpCAM, CD133, and CK19) and show enhanced expression of mature human proteins and mRNAs characteristic of hepatocytes (e.g. albumin and transferrin). The use of human-specific antibodies and sequence probes confirmed that these were made by donor-origin cells. The extent of humanization of the murine livers was greatly enhanced by treatment of mice with CCl_4, known to selectively kill mature parenchymal cells and, thereby, to create a cellular vacuum in the host.

The efficiency with which $EpCAM^+$ cells can be isolated from human livers, their ability to clonogenically expand *ex vivo*, their pluripotency, and the evidence that they yield mature liver tissue after transplantation encourage consideration of their clinical utility.

Potential applications include cell-based therapies of liver disease and generation of cells for bioartificial livers.

TABLES

Table 4.1. **Antigenic Profiles of Human Hepatic Stem Cell (hHpSC) and Hepatoblasts**

Markers	**hHpSC on Plastic and in KM[1]**	**hHpSC on STO feeders within KM[1]**	**Hepatoblasts**
α- fetoprotein[2]	Negative		+++ in those from fetal liver; ± in those from postnatal livers
Albumin[2]	Negative or ±		++
P450-3A4[2]	Negative		
P450-A7[2]	Negative		Positive
CK 8/18, CD29, CAM 5.2	+++		
CK19[2]	+++		+ found as particular staining in cytoplasm
EpCAM[2]	+++ cytoplasmically and at membrane		++ at membrane surface but not cytoplasm
NCAM[2]	Strongly positive especially at edge of colonies		Negative
Indian and Sonic Hedgehog and Patch[3]	+++; Sonic is at periphery and Indian in center of cells		++
ICAM-1[2]	Negative		Positive (see **Figure 6**)
Claudin 3[2]	+++		Negative
CD44H [4]	+++		
CD133/1[2]	+++		
Telomerase[5]	+++		
CD117[2]	Debatable**		Negative

Mesenchymal[6] Cell Markers	Negative
Endothelial Cell Markers[7]	Negative
Hemopoietic Markers[8]	Negative

**It is not detected on freshly isolated $EpCAM^+$ cells from fetal or postnatal livers; RNA for it is enriched in $EpCAM^+$ cells (Balzar et al., 1998) ; it is variably found in HpSC colonies from fetal but not adult livers and when found, is always near or overlapping with the companion cells; immunoselection for it does not yield hHpSCs. We suspect it is on angioblasts but not the hHpSCs (and for certain is not on hepatoblasts).

[1]Kubota's Medium. (Tanimizu et al., 2003)

[2]Phenotypic characterization of more than 20 genes by RT-PCR and Western blot analyses was done on hHpSCs, hepatoblasts and hepatocytes from livers from varying donor ages (Balzar et al., 1998).

[3]More extensive studies on hedgehog signaling are presented elsewhere (Van Den Heuvel et al., 2001)

[4]More extensive studies on hyaluronan receptors and their relevance to use of hyaluronan hydrogels for ex vivo maintenance of hHpSCs are given elsewhere

[5]Telomerase activity has been measured in hHpSCs, hepatoblasts versus in mature liver cells (Schmelzer E. and Reid L submitted)

[6]Mesenchymal markers: CD146, desmin, α-smooth muscle actin

[7]Endothelial cell markers: VEGFR2 (also called KDR), von Willebrand factor, CD31

[8]Hemopoietic cell markers: CD45, CD34, CD14, CD38, Thy 1 (CD90), Glycophorin A

Table 4.2. Evidence for Self-Renewal

	hHpSCs	**Hepatoblasts**
Minimum Conditions for survival	KM[1] + Culture plastic	KM[1] + STO Feeders
Life-span of cells	Culture plastic:>6 months STO Feeders: indefinite	Plastic: No survival after ~5-8 days STO Feeders: ~2 months
Doubling Time on Plastic or feeders[2]	Plastic : 1.5-2 days STO Feeders: 12-24 days (<24 hours on certain matrix substrata[4])	Plastic: No survival STO Feeders: essentially no growth
[2]Cell number/colony after 2 weeks ("clonogenic" expansion)	Plastic: $1.4X10^3 \pm 5.2 \times 10^2$ (derived from a single hHpSC partnered with a single companion cell—see movies)	Plastic: No survival
Phenotype of hHpSCs after > 150 divisions (>6 months in culture)	Identical to that of cells after initial plating; characterization summarized in Table 1	Within the ~ 2 months of survival time on STO feeders, cells retained expression of albumin, *a*-fetoprotein and CK19
Ability to form liver tissue after transplantation [3]	Capable after 1-2 months in culture on plastic and in KM[1]	Only if transplanted within ~7 days of culture on plastic (not tested with cells on STO feeders)

[1]KM =Kubota's Medium, serum-free RPMI 1640 with no copper, low calcium (0.3 mM) and supplemented with zinc, selenium, insulin, transferrin, HDL and lipids (Kubota et al., 2002).

[2]Clonogenic expansion occurs but requires each hHpSC to be partnered with at least one companion cell; the companion cells proved to be angioblasts or hepatic stellate cell precursors (see Figure 4.5; see also the movies in supplemental data for colony formation at low seeding densities and on days 1-8).

[3]In Figure 4.8 are shown images from liver sections from animals transplanted with human hepatic stem cells.

[4]Elsewhere we report that plating the stem cells onto specific forms of extracellular matrix, found in abundance in embryonic or fetal tissues enables them to go for months through rapid divisions with doubling times of <24 hours for months (McClelland et al., submitted).

Table 4.3. Antigenic profile of freshly isolated cells in the Ficoll interface fraction versus Ficoll pellet fraction derived from suspensions of human fetal liver cells

Marker*	Interface cell fraction	Pellet cell fraction
α- fetoprotein	26.0 ± 7.6	72.3 ± 3.9
Albumin	33.7 ± 5.4	79.8 ± 9.2
CD117 (c-kit)	5.4 ± 3.0	2.2 ± 1.7
E-Cadherin	57.9 ± 6.2	70.8 ± 1.5
NCAM	5.7 ± 1.8	2.0 ± 0.9
EpCAM	63.9 ± 9.4	61.2 ± 9.5
CD146	7.5 ± 1.5	0.6 ± 0.2
CD133	4.9 ± 1.3	3.0 ± 1.9
CD31	11.0 ± 1.9	3.7 ± 2.0
ICAM-1	33.2 ± 15.3	22.6 ± 7.5
N-Cadherin	0.9 ± 0.4	1.0±0.3
CD45	10.5 ± 1.8	2.2 ± 1.2
CD14	3.0 ± 2.6	0.3 ± 0.2
CD34	12.2 ± 0.8	2.6 ± 0.8
HLA	28.4 ± 8.1	6.4 ± 3.9

Mean + SEM. N=3

*All data are calculated for the glycophorin A-negative population (gated out by flow cytometric analyses). Glycophorin A was present at 57.5 ± 7.6% in the interface fraction and at 39.5 ± 7.0% in the pellet. The pellet fraction was found to be predominantly hepatoblasts.

Table 4.4. Colony forming Cells in Postnatal Human Livers

Donor Age (yr)	Samples Analyzed	EpCAM$^+$ Cells (%)	Hepatoblast Colonies	HpSC Colonies
	[1]Unfractionated	1.8	1209	2
0.75	[2]EpCAM$^+$	84.7	18	199
	[3]Flow-Through	< 0.05	1293	0
	Unfractionated	0.5	339	0.5
3	EpCAM$^+$	75.0	237	76.2
	Flow-Through	< 0.05	308	0
	Unfractionated	0.73	169	1.3
9	EpCAM+	80.9	1.3	52.3
	Flow-Through	< 0.05	101	0.3
	Unfractionated	3.9	> 500	11
36	EpCAM+	93.2	1.7	27.7
	Flow-Through	< 0.05	> 500	3.3

[1] Unfractionated: starting cell suspension after cell isolation

[2] EpCAM$^+$ cells: positive fraction from immunoselection

[3] Flow-Through: cells not retained during magnetic selection for EpCAM

Data are means of triplicate wells seeded with 20,000 live cells.

FIGURE LEGENDS

Figure 4.1. Immunohistochemical Studies on Human Fetal Livers

Confocal microscopic images on 5 μm liver sections. The antigenic profiles are given in the Table. Sections were stained for:

Human Fetal Livers: A. EpCAM (green) and CK19 (red)**;** B. EpCAM (green) and AFP (red)**;** C. CK19 (green) and albumin (red)**;** D. CK19 (green) and AFP (red)

Adult Livers: E. EpCAM. Probable hepatoblasts at the end of a canal of Hering; F. Canals of Hering around portal triad with EpCAM (green) and CK19 (red)**;** G. A canal of Hering showing $EpCAM^+$ cells (green) some of which also express albumin (red).

Figure 4.2. Flow Cytometric Characterization of $EpCAM^+$ Cells

A. The % of $EpCAM^+$ cells in livers of varying donor ages. The numbers for fetal livers have been reported previously (Fausto et al., 1993) but are presented here for comparison to findings in livers from older donors. B. Analyses of $EpCAM^+$ cells from fetal livers (similar findings occur with $EpCAM^+$ cells from adult livers except that few cells express AFP). C. Quantitative RT-PCR Assays on freshly isolated $EpCAM^+$ versus $EpCAM^-$ cells from fetal versus postnatal livers. These data are compared to the findings from colonies of hHpSC grown on plastic and in serum-free KM for 30-60 days.

Figure 4.3. hHpSCs in culture

A-E show a stem cell colony forming at 2 days (A), 4 days (B), 7 days (C), 10 days (D), and 14 days (E) in culture on plastic and in KM. Scale bar: 20 um. Phase contrast

coupled with image of cells with staining for NCAM (H and I), CK19 (F and GH), EpCAM (J and K), and CD44H (L).

Figure 4.4. Magnetic immunoselection

A-F. Flow cytometry on human fetal liver cells stained for EpCAM (Panel D; Panel A is the isotype control for D used for setting the gate shown in pink) indicated 20.7 % of the cell suspension were positive for EpCAM. The cells were subjected to magnetic bead sorting and yielded a suspension enriched for EpCAM to 54.6% of the cells (Panel B is the isotype control used for the data in panel E). The flow-through cells (Panel F; Panel C is the isotype control for Panel F) were depleted in EpCAM yielding 7.15% of the cells. G-I. Flow cytometry on cells from adult livers. EpCAM expression (Y axis) vs side scatter (X axis) . In the original, unfractionated cell suspension were found 0.73% EpCAM+ cells. H. a single pass through Miltenyi microbead sorting resulted in enrichment for EpCAM+ cells to 80.9%. I. In the flow through were found only 0.06% EpCAM+ cells. J. The EpCAM+ cells were ~9-10 μm in diameter vs 18-22 μm for mature parenchyma and had low side scatter (K) relative to that found for mature cells in the unfractionated mixture (UMIX) of liver cells (L). **Table:** summary of profiles of cells immunoselected for EpCAM, NCAM, KDR (VEGFr) or for $KDR^-/EpCAM^-$ cells. **Phase micrograph images** are of an hHpSC colony from an $EpCAM^+$ sort and one from an $NCAM^+$ sort.

Figure 4.5. Companion cells to the HpSC Colonies comprise Hepatic Stellate cells and Angioblasts

hHpSCs are associated with mesenchymal companion cells with distinct antigenic profiles. See movies in the Supplement. Two types of companion cells are evident: angioblasts (positive for VEGF-R, CD133/1, CD117, weakly positive for CD31 and von Willebrand factor) and hepatic stellate cells (positive for desmin and α-smooth muscle actin (αSMA)).

Figure 4.6. hHpSCs shifted to STO Feeders Erupt Hepatoblasts

Passage of hHpSCs from plastic to STO feeders results in cord-like eruptions that morphologically and antigenically are identical to hepatoblasts. A. An hHpSC colony shortly after passaging. B-E. A small group of passaged stem cells appears as a tightly compacted group of cells that erupts cords of hepatoblasts at the periphery of the colonies. Shown is a colony and the steady eruption of hepatoblasts by the end of day 1 (B), day 3 (C), day 5 (D) and day 7 (E) after passaging.

Figure 4.7. Shift in Antigenic Profile from hHpSCs to Hepatoblasts when on STO Feeders

The border between the hHpSC colony and hepatoblast outgrowths is marked by arrowheads. A and B. The antigenic profile of the cords of cells erupting from the parent colony is identical to that of hepatoblasts and includes a shift from expression of NCAM (green) to ICAM (red). C and D. Lineage restriction to committed biliary progenitors (CBPs) indicated by staining for CK19 (green) and albumin (red). E and F expression of AFP (green) and albumin (red) indicates that erupting cells are hepatoblasts.

Figure 4.8. Transplantation of EpCAM$^+$ Cells (or Colonies of Stem Cells in culture) results in Engrafted Liver Tissue in NOD/scid mice

NOD/scid mice were transplanted with 10^6 cells of either freshly isolated and immunoselected EpCAM$^+$ cells or colonies of hHpSCs from culture on plastic for 30-60 days (>~40 population doublings). Similar results were obtained with both populations. After transplantation, half the mice were treated with carbon tetrachloride (CCL4) (representative results shown in Fig. 8B, D, and F; representative results from transplanted mice not treated with CCL4 are shown in Fig. 8A, C, E and F). In Fig 8A, C, and E are shown sections of murine livers stained for human-specific proteins two days (Fig. A, C, and E), 8 days (Fig. 8F) or 8 days after transplantation and 7 days after CCL4 treatment (Fig. B, D, G). Control is a section of mice not transplanted (H). Control for albumin indicated some cross-reactivity between human and mouse albumin.

Figures 4.9 and 4.10. Hepatoblasts cultured on tissue culture plastic and in Kubota's Medium.

The cells form cords and intensely co- express alpha- fetoprotein, albumin and cytokeratin 19. The cells survive only for 7-10 days. Hepatoblasts colonies do not survive on culture plastic (Fig. 9) but only on embryonic stromal feeders (Fig. 10). Cultures in Figs A-H are plated onto tissue culture plastic and those on I-N are on feeders of murine embryonic stromal cells, STO cells. Figs A, C. E and G are cells after 1 day in culture; those in B, D.F and H are after 5 days; all Figs on STO cells are after 14 days in culture. A, B, J, and N are phase micrographs. Those in C, D and I are stained for albumin; those in E, F and K are stained for *a*- fetoprotein; those in G, H, and M are stained for CK 19. On day 1 the cells are

tightly aggregated and show strong immunofluorescent staining for ALB, *a*- fetoprotein and CK19. By day 5 the cells have disaggregated and died. Immunostaining for albumin and *a*-fetoprotein is reduced to background levels; however residual cells still stain strongly for CK19. Scale bar = 20 μm.

Hepatoblasts plated directly onto a STO feeder layer, fixed, immunostained and imaged at day 14. Upper panels Phase contrast images of three groups of hepatoblasts. Lower panels: Immunofluorescent staining of ALB, *a*- fetoprotein, and CK19. Note how morphology and immunogenic profile is sustained on STO feeders, unlike the cultures on plastic. The cells survived for more than 2 months and grew very slowly when on STO feeder cells. Table in Fig. 9 is a survey of markers on the cells on culture plastic; Table in Fig. 10 is a survey of the markers on the hepatoblasts on STO feeders.

Figure 4.11. Colonies of hepatic progenitors

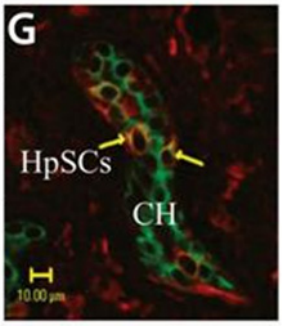
Phase microscopy shows the colonies of hepatic progenitors from postnatal livers that include hepatoblasts (A) and hHpSCs (B, C). Immunochemistry studies show expression in HpSC colony of albumin (D), CK19 (E), and merged image (F).

Figure 4.12. Cell culture of hepatoblasts and unipotent progenitor colony

Hepatoblasts (A-F), sectored colonies (G, H, K, L) and unipotent progenitor colony (I, J) shown in phase microscopy (A, E, G, I, K) or for immunochemistry for albumin (green) and CK19 (red). Note that CK19 in hepatoblasts are filamentous and in hHpSCs (Fig. 11) are particulate; in parallel, albumin expression is also distinct in hHpSCs and hepatoblasts, implicating distinctions in processing of these proteins in these two lineage stages.

Figure 4.1

Antigenic Profile of hHpSCs vs Hepatoblasts (*in vivo*)		
Marker	**hHpSCs**	**Hepatoblasts**
AFP	**Negative**	**+++**
Albumin	**Negative or ±**	**++**
CK19	**+++**	**++**
EpCAM	**+++**	**++**
NCAM	**Fetal/Neonatal: ++ Pediatric/Adult: ~40% ++**	**Negative**
CD133/1	**++**	**++**
ICAM1	**Fetal/Neonatal: Negative Pediatric/Adult: - or ±**	**++**
CD45	**Negative**	**Negative**



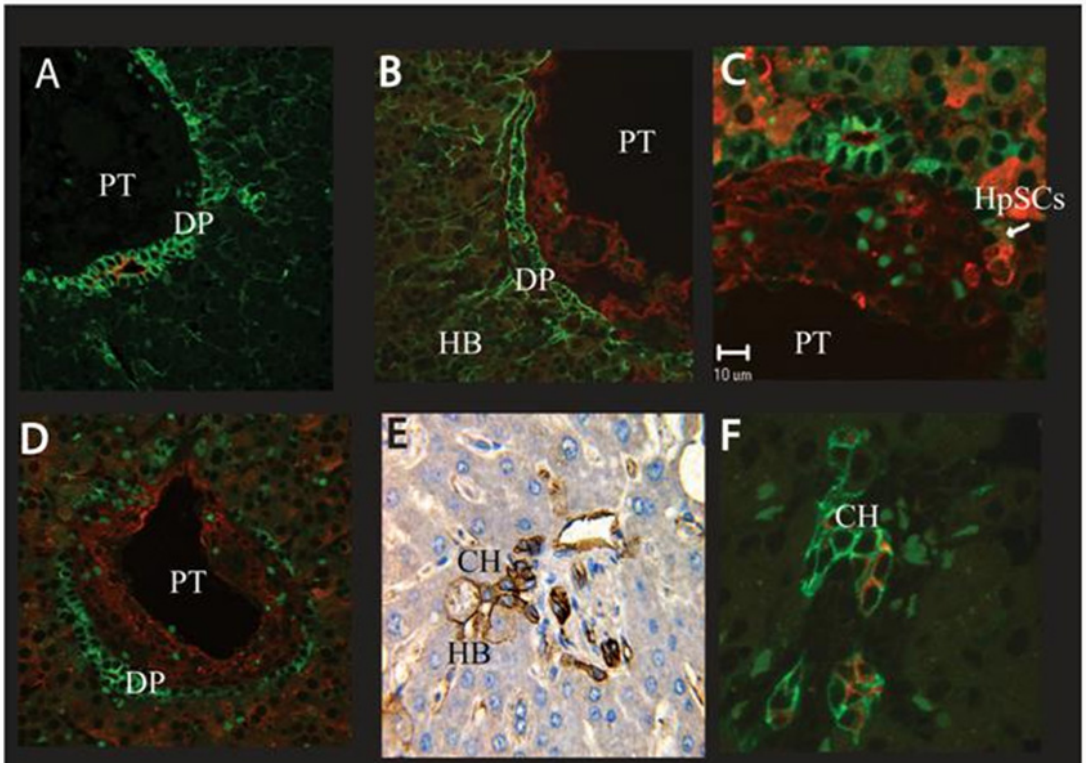
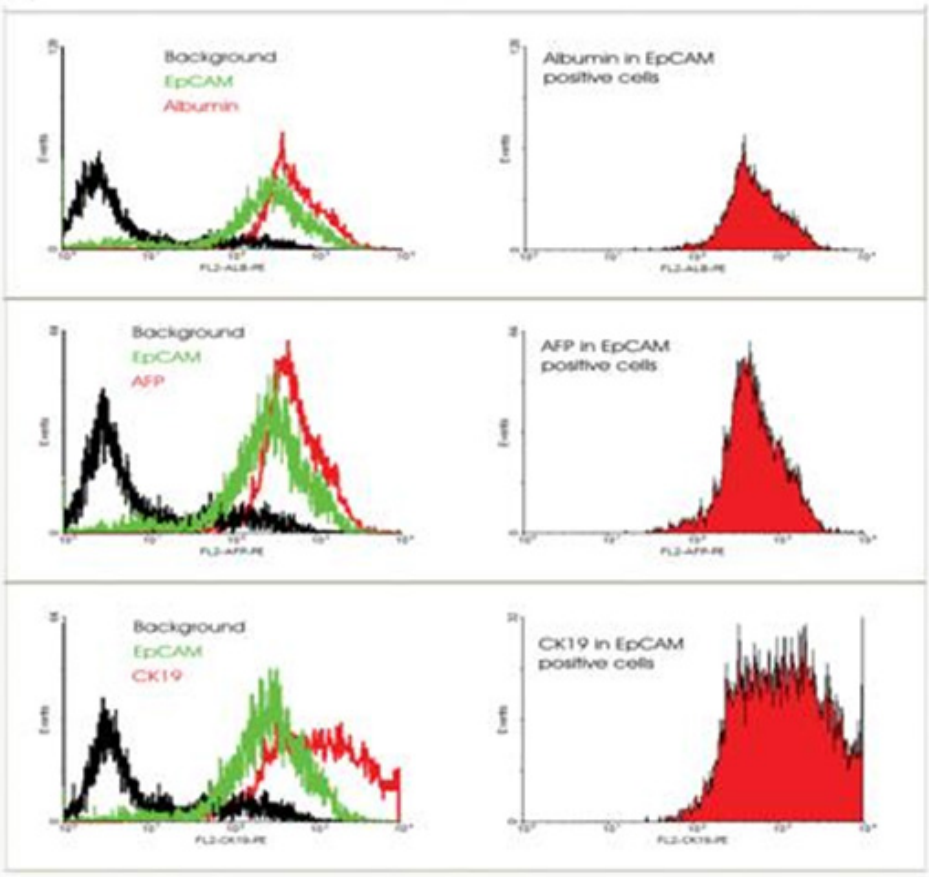


Figure 4.2

A. Percentage of EpCAM+ Cells in Livers from Donors of Varying Age				
Age Group	**# samples**	**% EpCAM+ Cells**	**% EpCAM+ Cells that are**	
			hHpSCs	**Hepatoblasts**
Fetuses (16-20 wks)	**10**	**12.1 ± 2.3**	**~5%**	**~95%**
Neonates (0-1 yr)	**7**	**2.7 ± 2.4**	**~50%**	**~50%**
Pediatric (2-13 yrs)	**17**	**2.1 ± 1.6**	**>99%**	**<0.01%**
Adult (19-81 yrs)	**38**	**1.3 ± 1.0**	**>99%**	**<0.01%**
hHpSCs =positive for EpCAM, NCAM and/or claudin 3 but not AFP **Hepatoblasts**=positive for EpCAM, ICAM, AFP but not NCAM or claudin 3				

B



C. Gene Expression in EpCAM+ versus EpCAM- Cells in fetal vs Postnatal Livers			
Gene	**Fetal Livers**	**Postnatal Livers**	**hHpSC Colonies**
	Ratio of # of Transcripts in EpCAM+ Cells/EpCAM- Cells		**# Transcripts**
AFP	2.0	0/0	0.0 ± 0.0
Albumin	2.9	0.14	4.0±1.6
CK19	1.7	433.3	97.2± 18.0
CD117 (ckit)	0.01/0.0	23.3	0.1±0.03
P450 3A4	0/0	0.045	0.0±0.0

Figure 4.3

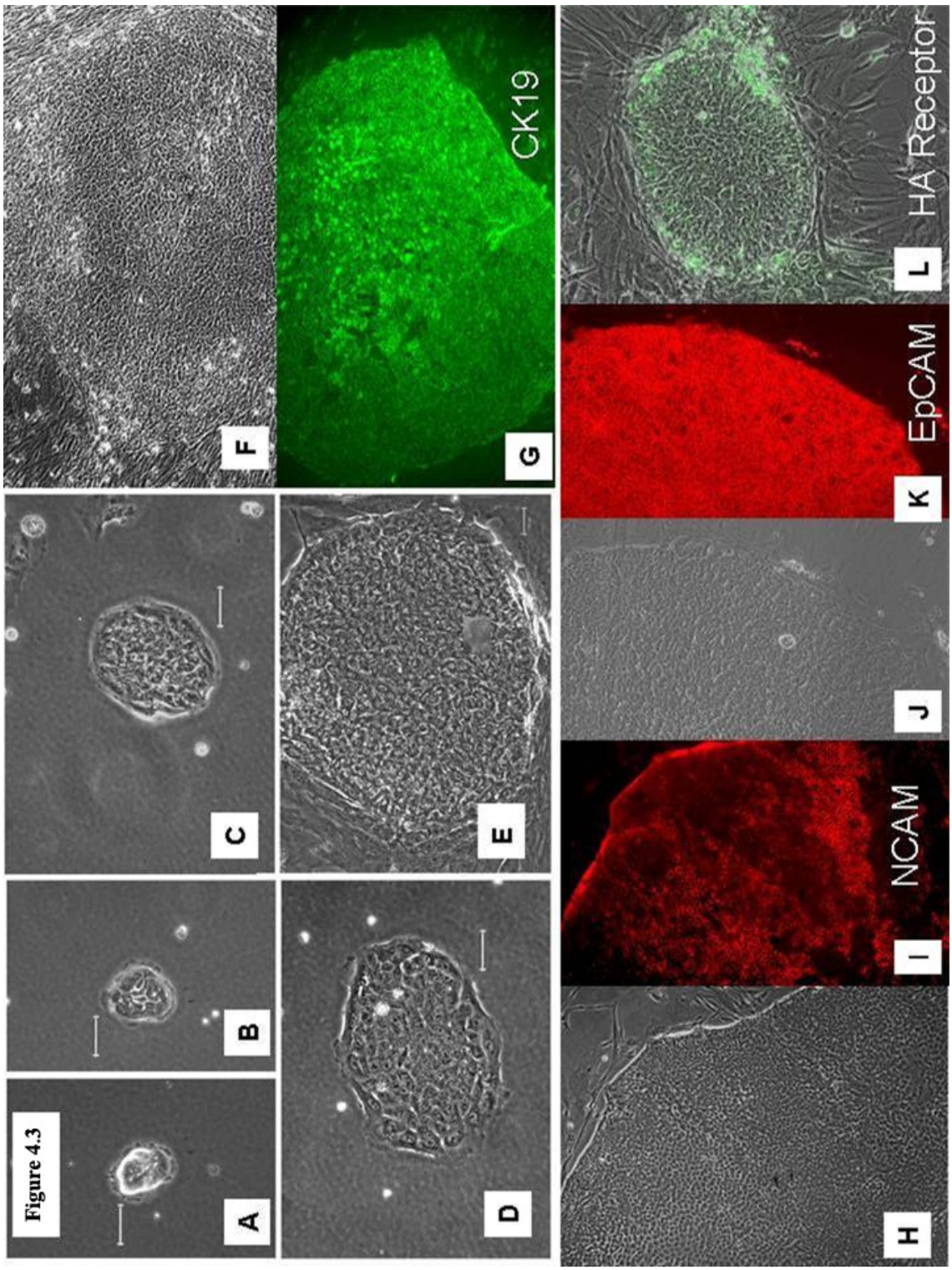
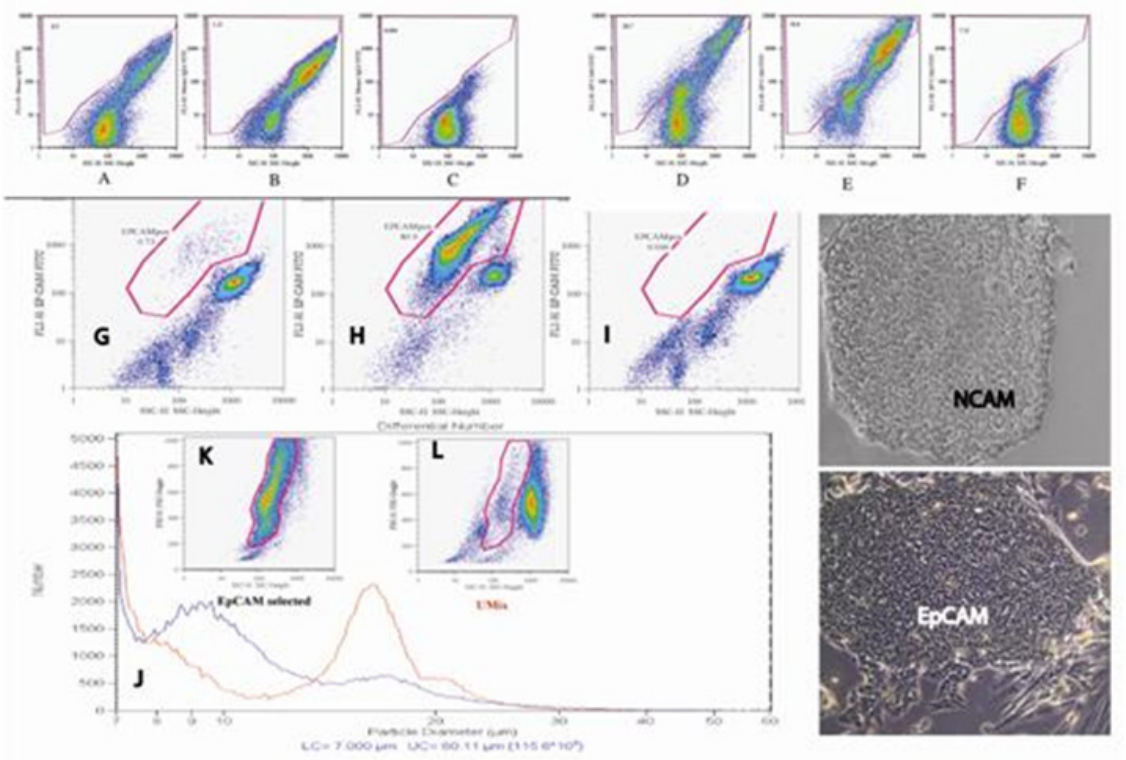


Figure 4.4



M. Antigenic Profiles of Immunoselected Cells				
Markers	**Cells Immunoselected for:**			
	EpCAM+	**NCAM +**	**VEGFr (KDR)+**	**EpCAM-/KDR-**
AFP	+++ (fetal/neonatal) Neg (pediatric/adult)	Neg	Neg	Neg
Albumin	++	Weak	Neg	Neg
CK19	+++	+++	Neg	Neg
EpCAM	+++	+++	Neg	Neg
Claudin 3	Mixed + and Neg (fetal/neonatal) +++ (pediatric/adult)	+++	Neg	Neg
CD133/1	+++	+++	+++	Neg
Epithelial	+++	+++	Neg	Neg
Endothelial	Neg	Neg	+++	Neg
Hemopoietic	Neg	Neg	Neg*	Mixed + and Neg
Stellate Cell	Neg	Neg	Neg	+++
Epithelial markers: CK 8 and 18, CD29, CAM 5.2 Endothelial Markers:CD133/1, VEGFr, CD31, Von Willebrand Factor; *some with CD34 Hemopoietic Markers: CD45, CD34, CD38, CD14, CD90, Glycophorin A Hepatic Stellate Cell Markers: Desmin, CD146, α-smooth muscle actin				

Figure 4.5

Figure 4.5 Antigenic Profiles of Mesenchymal "Companion" Cells to hHpSCs

Markers	Angioblasts	Hepatic Stellate Cell Precursors
Parenchymal cell markers*	Neg	Neg
CD133/1, CD31 (PECAM), VEGFr (KDR)	+++	Neg
CD146	Not tested	+++
CD45, CD38, CD14, Glycophorin A	Neg	Neg
CD34	Variable (some positive)	Neg
CD117	+++	Variable
Desmin, α-smooth muscle actin (ASMA)	Neg	+++
*Parenchymal cell markers: albumin, AFP, EpCAM, CK19, CK8 and 18		

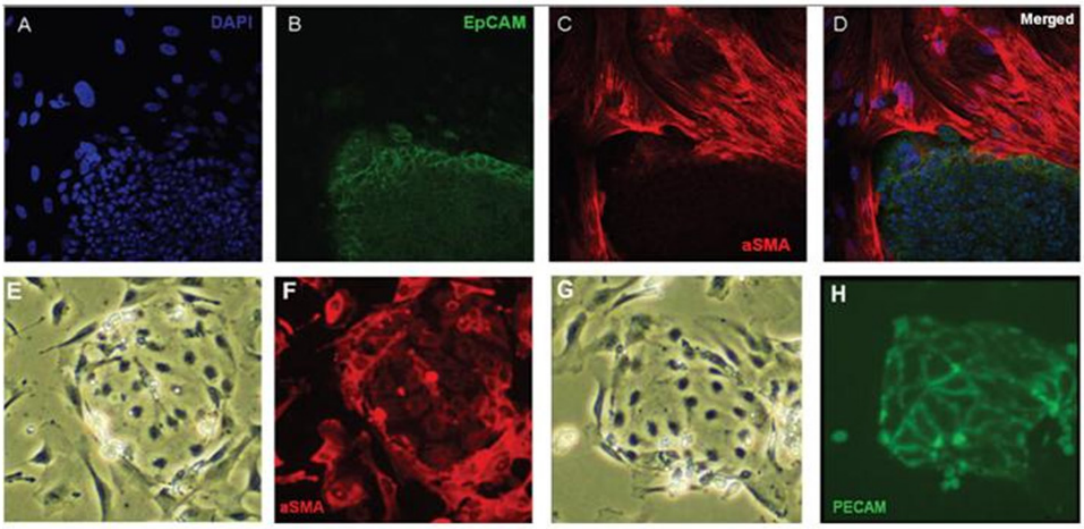


Figure 4.6

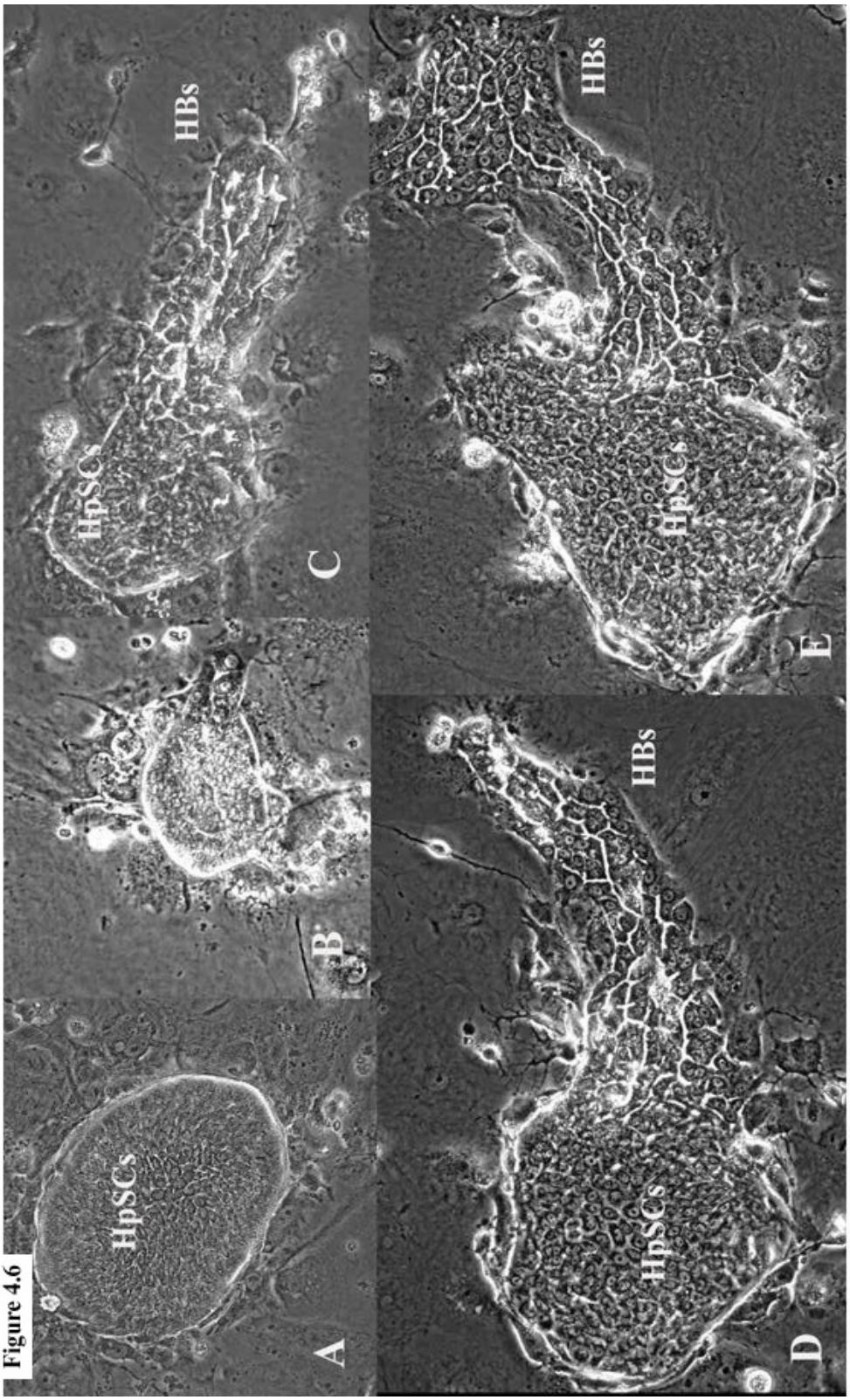


Figure 4.7

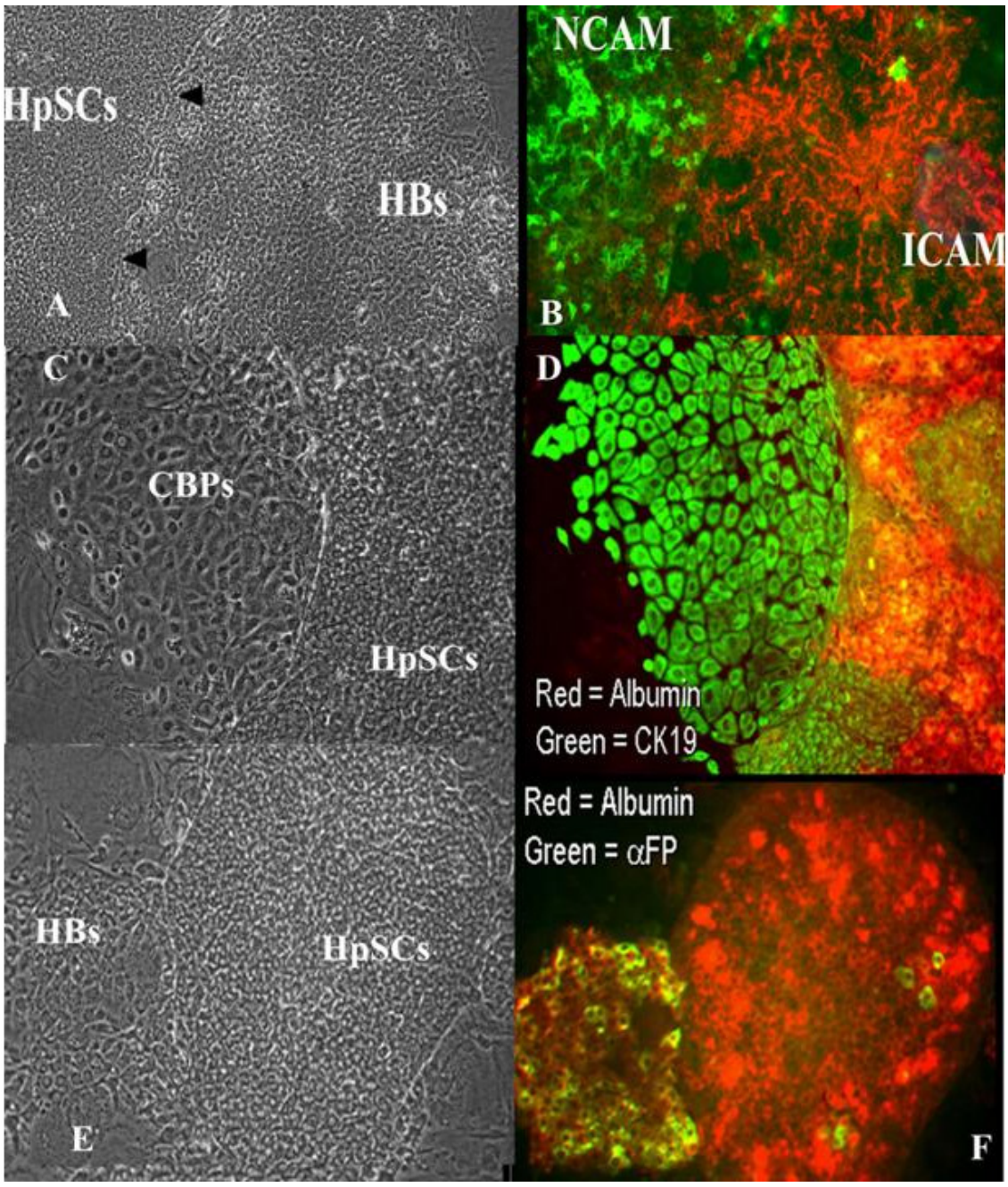

HpSCs
HBs
A
NCAM
ICAM
B
C
CBPs
HpSCs
D
Red = Albumin
Green = CK19
HBs
HpSCs
E
Red = Albumin
Green = αFP
F

Figure 4.8

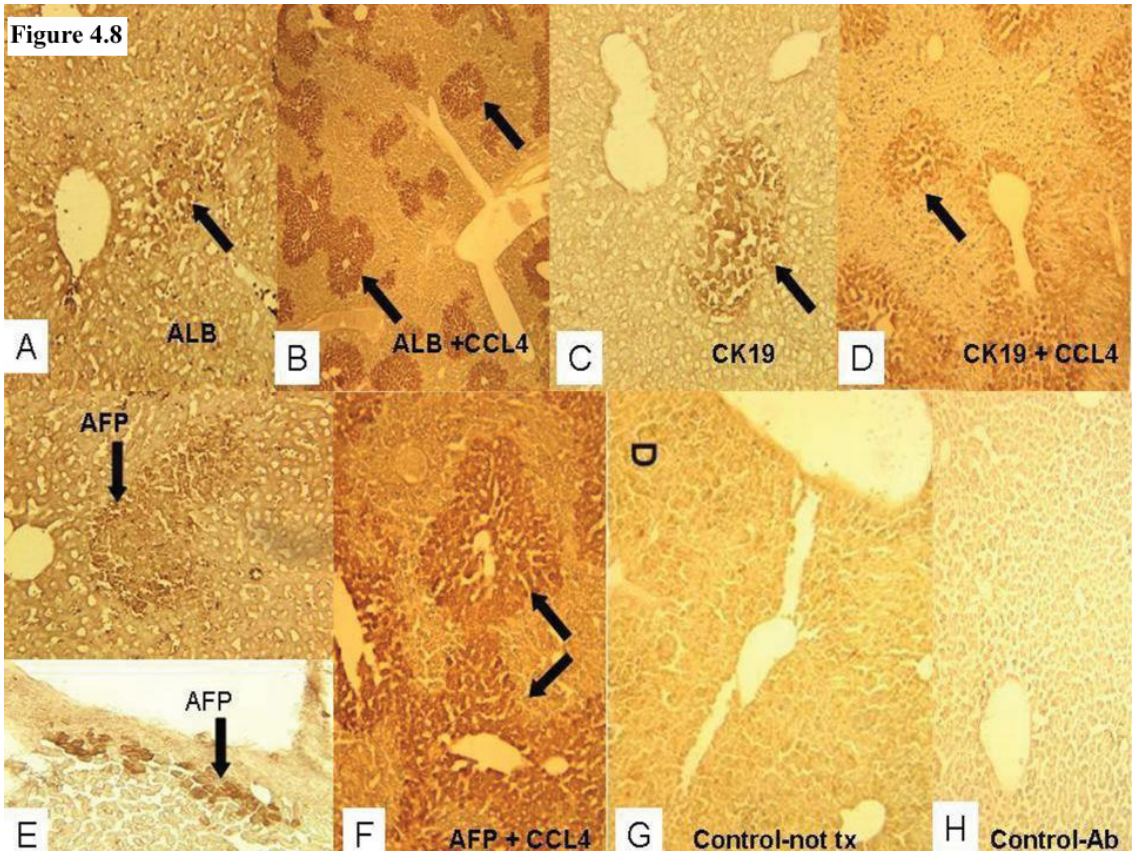


Figure 4.8
A
ALB
B
ALB +CCL4
C
CK19
D
CK19 + CCL4
AFP
AFP
E
F
AFP + CCL4
G
Control-not tx
H
Control-Ab

Figure 4.9

Hepatoblasts from Fetal Livers on Culture Plastic

Markers	Expression
Albumin	+++ (rapid decline within a week)
AFP	
CK 8/18, CK19, CD29, Ep-CAM	+++
N-CAM	Negative
I-CAM	+++
CAM 5.2	+
CD133/1	Negative
CD146	Negative
CD117	Negative
KDR	Negative
CD45, CD38, CD34, CD14	Negative

CD29= β1-integrin

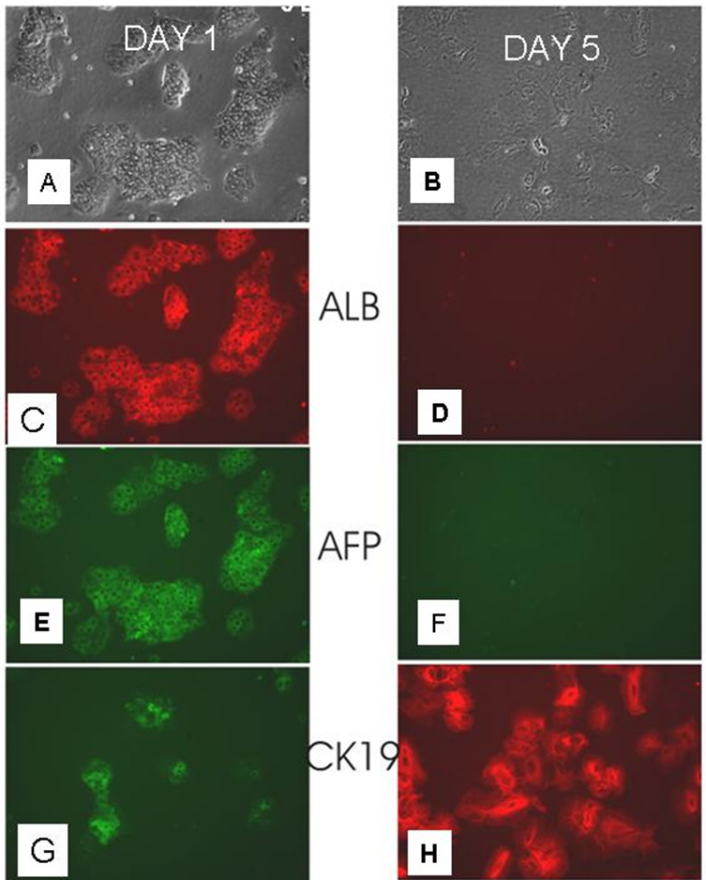


Figure 4.10

Hepatoblasts from Fetal Livers on STO Feeder Cells

Markers	Expression
Albumin	+++ for more than 2 months
AFP	
CK 8/18, CK19, CD29, Ep-CAM	+++
N-CAM	Negative
I-CAM	+++
CAM 5.2	+
CD133/1	Negative
CD146	Negative
CD117	Negative
KDR	Negative
CD45, CD38, CD34, CD14	Negative

CD29= β1-integrin

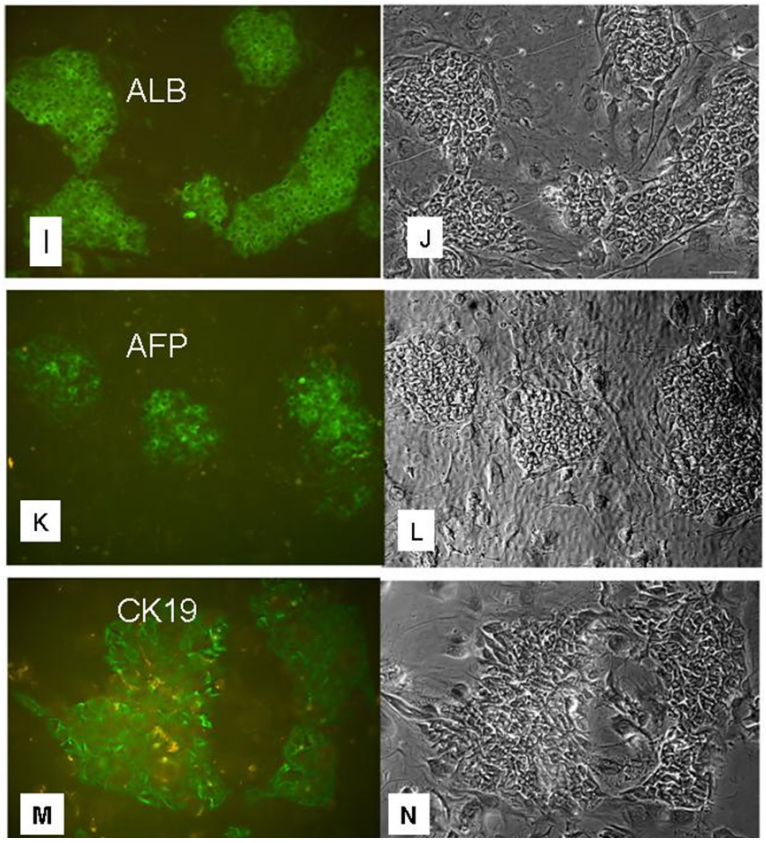


Figure 4.11

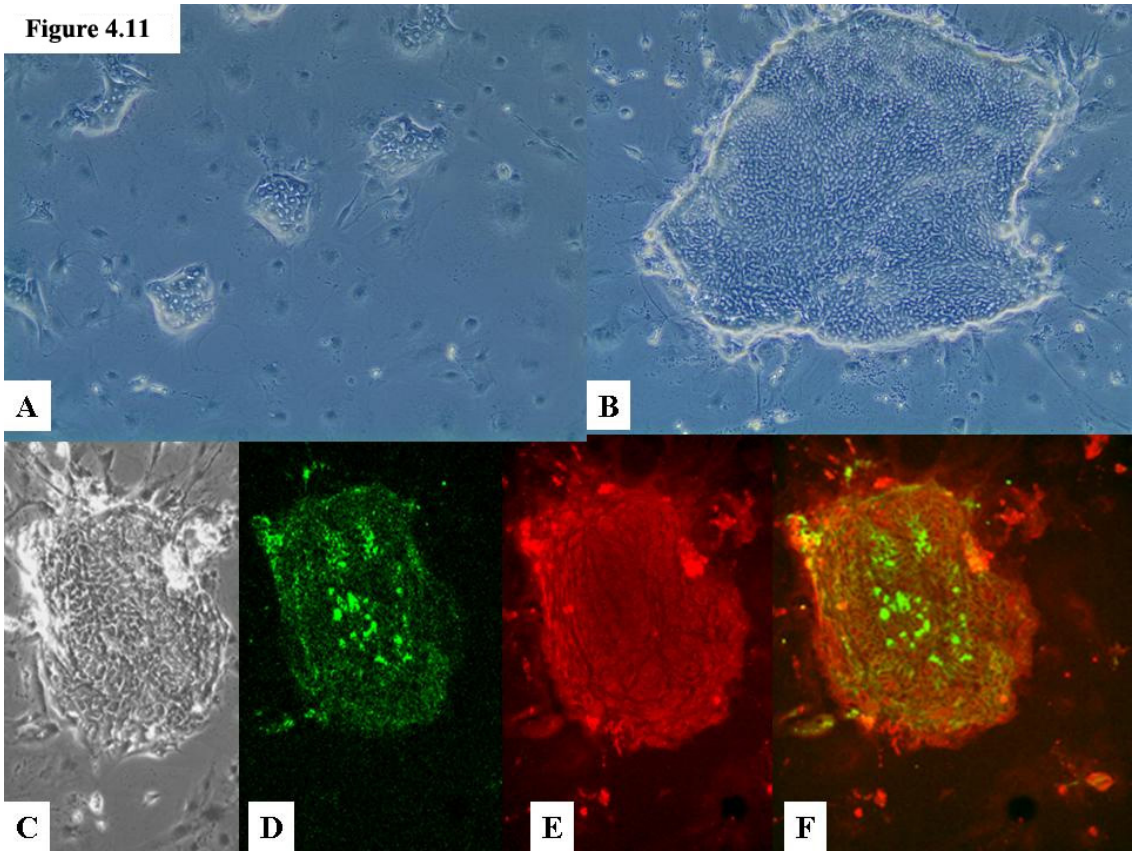
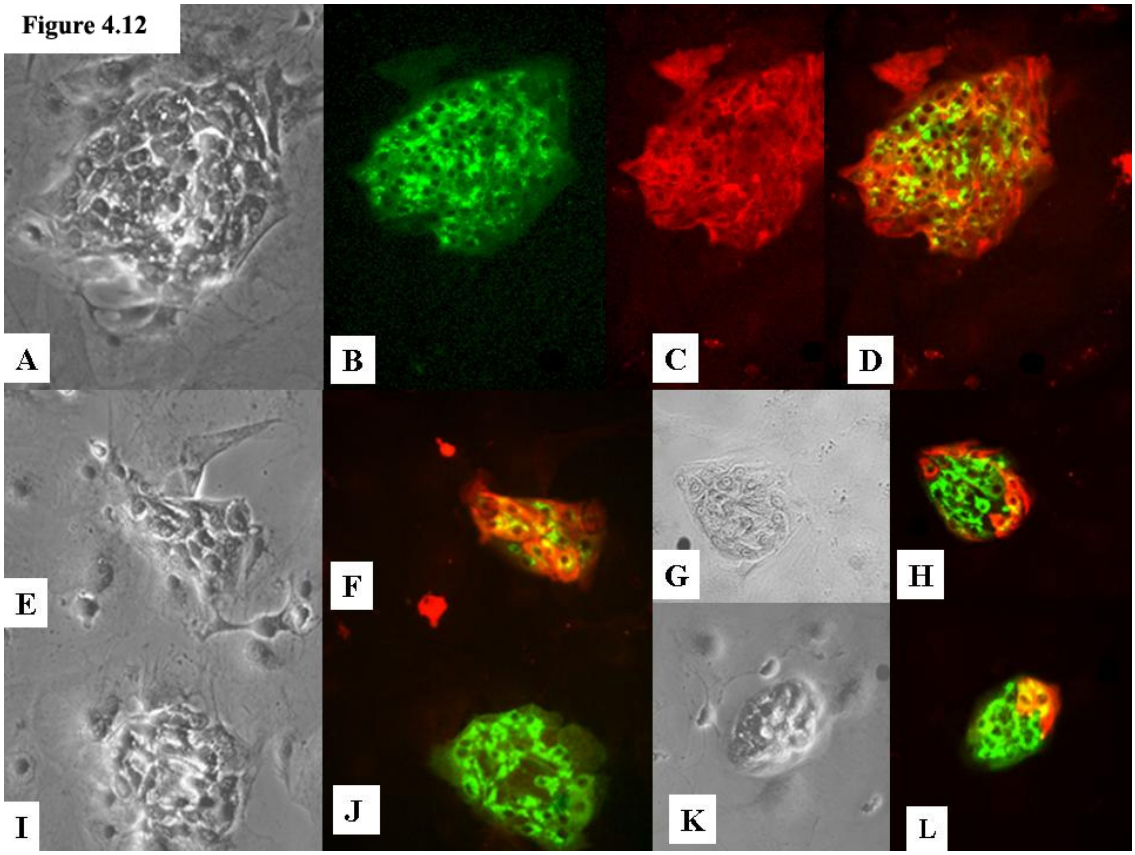


Figure 4.12



CHAPTER V

HUMAN HEPATIC STEM CELLS SELF-REPLICATE ON FEEDERS OF ANGIOBLASTS/ENDOTHELIA AND LINEAGE RESTRICT TO HEPATOBLASTS ON FEEDERS OF ACTIVATED HEPATIC STELLATE CELLS

(Refereed article submitted to Tissue Engineering)

In vivo tissue engineering (TE) involving transplantation of purified hepatic cells into the liver and the *ex vivo* tissue engineering using an extracorporeal liver support system (bioartificial liver) have been considered as alternative therapeutic approaches for patients with fulminant hepatic failure. Both approaches require substantial numbers of functional hepatic cells. The pluripotent human hepatic progenitors, especially hepatic stem cells (HpSCs), cells that can self-renew, should be an ideal cell source for liver TE. Understanding the mechanisms increasing cell growth of HpSCs and/or inducing differentiation of these cells into functional hepatocytes or cholangiocytes would help to accelerate success of protocols for liver TE. Paracrine signaling from adjacent mesenchymal cells is thought to govern the proliferation/differentiation state of the hepatic epithelial cells. This chapter identifies distinct categories of mesenchymal feeders,

implicating overlapping but also distinct paracrine signals among them. The feeders are being used to identify the paracrine signals so that eventually one can expand and differentiate the HpSCs in the absence of feeder cells.

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Key Words: human, liver, hepatic stem cells, stem cell niche, feeders, paracrine signaling, extracellular matrix, collagens, laminin, fibronectin, integrins, proteoglycans.

Abbreviations: **AFP=**α-fetoprotein**; ALB=** albumin; **CK=** cytokeratin; **EC**= endothelial cell; **hHpSCs**= human hepatic stem cells; **HpSTCs**= hepatic stellate

cells; **HUVEC**= human umbilical vein endothelial cells; **hMSCs**= human mesenchymal stem cells from bone marrow; **KM**= Kubota's Medium, a serum-free, hormonally defined medium tailored for hepatic progenitors; **STO** cells= murine embryonic stromal feeder cells; **TE**= tissue engineering; **ECM**= extracellular matrix.

ABSTRACT

Human hepatic stem cells, hHpSCs ($NCAM^{+}$, $Claudin3^{+}$, $albumin^{\pm}$, AFP^{-}) *in vivo* or on plastic and in a serum-free medium tailored for hepatic progenitors, Kubota's Medium (KM), are closely associated with angioblasts ($VEGFR2^{+}$, $CD31^{+}$, $CD117^{+}$, von Willebrand Factor $(vWF)^{+}$) and hepatic stellate cells (hHpSTCs) ($ICAM\text{-}1^{+}$, $desmin^{+}$, vitamin A^{+}, α-smooth muscle actin $(\alpha SMA)^{+}$). Various embryonic/fetal mesenchymal feeders were tested with KM for influence on hHpSCs. The hHpSCs exhibited minimal differentiation on plastic or with feeders of angioblasts/endothelia or of human umbilical vein endothelial cells (HUVECs); they lineage restriction to hepatoblasts ($ICAM\text{-}1^{+}$, Claudin 3^{-}, Albumin $^{++}$, AFP^{++}) on feeders dominated by activated hHpSTCs; they slowed in growth and underwent differentiation when on STO feeders. All feeders produced multiple families of matrix components (collagens, basal adhesion proteins, proteoglycans) with the angioblasts/endothelia producing the lowest levels; hHpSTCs feeders producing intermediate levels; and STO cells the maximum levels and also being

the only feeders to produce heparan sulfate proteoglycans. Specific matrix components, used in combination with KM, could mimic, in part, the feeder effects. Type III collagen resulted in minimal differentiation; laminin or type IV collagen yielded expansion with restriction to hepatoblasts; and embedding in type I collagen resulted in slowing of growth and more extensive differentiation.

INTRODUCTION

Tissue engineering of human liver necessitates expansion of the available liver cells to yield sufficient numbers of cells for bioartificial livers or for cell therapy programs (Gerlach, 1996; Gupta et al., 1999; Kulig and Vacanti, 2004; Selden and Hodgson, 2004; Strom et al., 1997; Susick et al., 2001). The hope has become to make use of human hepatic stem cells (hHpSCs) ($EpCAM^+$, $NCAM^+$, Claudin 3^+, cytokeratin 19^+, $albumin^{\pm}$, alpha-fetoprotein $(AFP)^-$) that can be expanded and then lineage restricted to mature liver cells. In our recent studies, we reported on the antigenic profile permitting immunoselection of hHpSCs from livers of all donor ages and on conditions for *ex vivo* expansion of the cells (Schmelzer et al., 2006 and 2007; Sicklick et al., 2006). In these reports we showed that survival and *ex vivo* expansion of the hHpSCs is dependent on paracrine signals from mesenchymal companion cells defined to be angioblasts ($CD31^+$, von Willebrand Factor $(vWF)^+$, $CD133/1^+$, $CD117^+$, Vascular Endothelial Growth Factor

Receptor 2 (VEGFR2)$^{+}$), and/or hepatic stellate cell precursors (ICAM-1^{+}, VCAM-1^{+}, vitamin A^{+}, desmin^{+}, α-smooth muscle actin (αSMA)$^{+}$, CD146^{+}) (Schmelzer et al., 2007).

Liver development is induced in a step-wise process with signals from cardiac mesoderm and then from endothelia (Jung et al., 1999; Lemaigre et al., 2004; Zaret, 1998). During liver organogenesis, endodermal cells are induced by cardiac mesoderm to differentiate into hHpSCs within the ventral endoderm (Gualdi et al., 1996; Lammert et al., 2003). Subsequently, newly specified hepatic cells delaminate and migrate into the surrounding septum transversum mesenchyme and intermingle with endothelia which remain in contact with hepatic cells throughout development. A receptor for VEGF, flk-1, is essential for endothelia to form. Flk-1 mutant mouse embryos, lacking endothelia, show initial hepatic induction but without the proliferation of hepatic cells into the surrounding septum transversum mesenchyme, indicating the importance of endothelia for liver organogenesis (Matsumoto et al., 2001; Roberts et al., 2004; Shalaby et al., 1995 and 1997).

The stem cell niche in human livers has recently been determined to be the ductal plates in fetal and neonatal livers that proved to be the antecedents to the canals of Hering in pediatric and adult livers (Schmelzer et al., 2007; Zhang et al., 2007). The canals of Hering have long been considered to be the probable stem cell compartment in the liver (Roskams et al., 2004; Saxena and Theise, 2004; Theise et al., 1999). The cell

subpopulations found within ductal plates and canals of Hering include hHpSCs, angioblasts, and hHpSTC precursors. Culture conditions that select for the hHpSCs co-select for these same populations (Schmelzer et al., 2007). Little is known about the paracrine signaling between these stem cell niche cell populations, but it is presumed to be important. Therefore, we sought to establish model systems for defining the paracrine signals.

We show that feeders representative of angioblasts/endothelia cause hHpSCs to remain as stem cells, whereas those replete with hHpSTCs result in lineage restriction to hepatoblasts, effects that can be mimicked, in part, by purified paracrine matrix signals produced by the feeders. A separate report is being prepared on the soluble signals from the feeders (Yao et al., in preparation).

RESULTS

Native Mesenchymal Companion Cells for Human hHpSCs (hHpSCs)

Freshly isolated hHpSCs survived *ex vivo* on culture plastic in KM, when the colonies of hHpSCs are surrounded by companion cells defined to be angioblasts (VEGFR2$^+$, CD31$^+$, CD133/1$^+$, CD117$^+$) and quiescent hHpSTCs (low levels of CD146, desmin and αSMA) (Figures 5.1-3, Table 5.1). The hHpSC colonies consist of cells very tightly bound to each other on their lateral borders but with minimal attachment to the

culture dish except at the perimeter of the colonies, the site at which the mesenchymal companion cells were located. Thus, the attachment to the dish was either by the mesenchymal companion cells alone or by the combination of them with the hHpSCs. When passaging, a colony could be detached at its perimeter and lifted off as a sheet of cells. Efforts to disperse the sheet into single cells were not successful with the customary enzymes (e.g. trypsin, chymotrypsin, dyspase, collagenase IV) that caused variable extent of dissociation but with rapid loss of viability of the cells. Mechanical passaging, as used for human ES cells in culture (Reubinoff et al., 2000), resulted in efficient passaging of hHpSCs.

Feeder Cell Lines and Primary Cultures used to Model the Native Feeders

Several forms of embryonic mesenchymal cells, either primary cultures or cell lines, were prepared as models of the native feeders, the angioblasts and hHpSTCs, and were used in combination with serum-free KM. Minimizing exposure to serum was essential, given its profound effects on the phenotype of the hHpSCs but proved a challenge for handling the mesenchymal feeders with requirements for serum factors. The strategy finally adopted was to grow the stocks of mesenchymal feeders in a serum-supplemented medium such as EGM-2 with 2% serum before switching them to serum-free KM for the assays in co-cultures of feeders with the hHpSCs. When maintained in serum-free

medium (Figures 5.1-3), any hHpSTCs present were found to be quiescent with low levels of CD146, desmin and α-smooth muscle actin (αSMA). Exposure to serum, even at low levels (1-2%) or for short periods (a few days), resulted in activation of the hHpSTCs as evidenced by very high levels of CD146, desmin and αSMA) (Figure 5.4). Exposure to serum also induced primary cultures of fetal liver cells, or immunoselected cells (e.g. KDR^+ or $CD31^+$ cells), to transition to mesenchymal cell populations dominated by hHpSTCs. Efforts are still ongoing to develop serum-free conditions for mesenchymal cells to obviate this problem for future studies.

The cell lines included human umbilical vein endothelial cells (HUVECs) (Figure 5.5B), human bone marrow derived mesenchymal stem cells (hMSCs) (Figure 5.5A), and murine embryonic stromal cells or STO cells, used routinely for maintenance of ES cell cultures. Primary cultures were prepared from cell suspensions of 16-20 wks human fetal livers by immunoselection using magnetically-activated cell sorting (MACS) for: 1) cells expressing KDR (flk-1/VEGFR2); 2) cells expressing CD31 (PECAM); or 3) cells left behind after negative sorting for fibroblasts to reduce or eliminate stromal cells. The percentages of sorted KDR^+ and $CD31^+$ cells within the whole liver sample were found to be about 0.5% and 1% respectively.

Immunoselected KDR^+ cells changed rapidly in culture. In the first week, the cells morphologically and antigenically were angioblasts or endothelia (Figure 5.3D). However,

by the second week, all markers for angioblasts/endothelia disappeared, and the cells remaining were dominated by HpSTCs. Even if the cell suspension was negatively fractionated to eliminate stroma, the same phenomenon occurred (Figure 5.5C-E and G). After 11 days of culture, the cultures were completely confluent, and most of the cells were strongly positive for αSMA, a marker for hHpSTCs and negative for vWF, an intracellular marker for endothelial cells (Figure 5.5F and H and 5.6I and K).

$CD31^+$ cells appeared as cobblestone-like cells in morphology in EGM-2 medium before day 5 and were positive for CD31 and vWF, indicating a phenotype of endothelial cells. After 5-7 days of culture, fibroblast-like cells that proved to be hepatic stellate cells started to dominate the dish and quickly reached complete confluency by day 9-10 in EGM-2 medium with strong expression of αSMA and desmin (data not shown). The culture conditions (EGM-2), though specifically designed for endothelial cells are nevertheless permissive for outgrowth of hHpSTCs that eventually dominate the cultures.

An alternative interpretation is that the immunoselected angioblasts (KDR^+) and endothelial cells ($CD31^+$) lineage restricted to hHpSTCs, since they share certain markers (e.g. VCAM1, ICAM1, CD146) , and this hypothesis is suggested also by the findings that there were clusters of the endothelia surrounded by a ring of hHpSTCs. The data to date cannot distinguish between the two interpretations of culture selection for a minor subpopulation of preexisting hHpSTCs or lineage restriction of angioblasts or endothelia

to hHpSTCs.

Effects of Feeders in co-Cultures with hHpSCs

Preliminary studies were done with primary cultures of mesenchymal feeders from adult rat livers or adult human livers and of purified HpSTCs prepared from adult rat livers and adult human livers by previously published protocols (Hellerbrand et al., 1996). However, these feeders in combination with KM showed minimal, if any, effects on the hHpSCs in terms of survival, growth or gene expression (data not shown). This complements prior studies in which feeder cells had to derive from embryonic livers for support of rat hepatoblasts (Agelli et al., 1997; Brill et al., 1995; Kubota and Reid, 2000; Reid et al., 1994; Sigal et al., 1994 and 1995). All of the biologically active feeders utilized in these studies are from fetal tissues.

hHpSCs on feeders of Liver-derived Angioblasts or HUVEC cells remain as stem cells

Isolation and clonogenic expansion of hHpSCs on culture plastic and in close association with angioblasts (Figure 5.3) resulted in cells that remained as hHpSCs with minimal differentiation (Schmelzer et al., 2007). The hHpSC colonies can be seen 2 weeks after plating and are positive for NCAM, EpCAM, albumin, CK19 and CLDN-3 and negative for AFP. Plating of cells in KM and on top of HUVEC cells or on feeders of

KDR^+ cells immediately after sorting and plating also resulted in maintenance of the stem cell colony phenotype (Figure 5.7B) with an antigenic profile of $EpCAM^+$, $NCAM^+$, $ICAM\text{-}1^-$, AFP^-, $CLDN\text{-}3^+$ (Table 5.1).

The hHpSCs cultured on feeders of activated hepatic stellate cells lineage restrict to hepatoblasts.

Feeders replete with activated hHpSTCs (high levels of expression of desmin, αSMA and CD146) caused rapid transition, within hours, of hHpSCs to hepatoblasts (Figure 5.4). This included the hMSCs, the primary cultures of human fetal liver stroma, the primary cultures of human fetal liver stroma depleted of fibroblasts by immunoselection, and the primary cultures of KDR^+ cells or of $CD31^+$ cells that were maintained in culture for more than a week. After 8-9 days of co-culture with one of these feeders, the hepatic progenitor colony morphology consisted of cord-like structures interspersed with clear channels, the presumptive biliary canaliculi (Figures 5.7 and 5.8) and with an antigenic profile indicative of hepatoblasts ($EpCAM^+$, $NCAM^-$, $ICAM\text{-}1^+$, AFP^+) (Table 5.1, Figure 5.1). Moreover, the morphology of hepatoblast colonies was more 3-dimensional causing them to be refractile when evaluated by bright field (Figures 5.7 and 5.8) possibly caused by multiple layers of cells and/or accumulation of extracellular matrix.

The hHpSCs plated onto STO Feeders

The feeder model system resulting in the maximum differentiation proved to be STO feeders, used routinely for embryonic stem (ES) cell cultures. The hHpSCs plated onto these feeders significantly slowed their growth and then gave rise to eruptions of hepatoblasts and committed progenitors from the edges of the colonies, findings corroborating our earlier reports (Schmelzer et al., 2007).

Expression of Genes Encoding Matrix Molecules by the different Feeders

Three feeder cell types were chosen to represent feeders that sustained the hHpSC phenotype (HUVEC cells) versus two that resulted in lineage restriction to hepatoblasts (primary cultures of human fetal liver mesenchymal cells and $CD31^+$ cells, fetal liver-derived endothelia, cultured for more than a week, both assayed at time points at which hHpSTCs were the dominant cell population). Using quantitative real-time PCR, it was found that fibronectin mRNA encoding the type I module of the fibronectin molecule was the highest-expressed matrix component among the three feeders assayed, especially in HUVECs (Figure 5.9). The HUVEC feeders, supportive of maintenance of the hHpSC phenotype produced collagen type IV, laminin (α4, β1 and γ1 chains), and little or no collagen types I and III, other laminin chain isoforms, or proteoglycan core proteins. Those that induced lineage restriction to hepatoblasts (human fetal liver-derived αSMA^+

fibroblast-like and $CD31^+$ cells) produced type I, III and IV collagen, laminin (β1, γ 1), but not collagen type V, other laminin chains or any of the proteoglycan core protein genes assayed (Glypican-3 and -5 and Syndecan-1 and -2).

Expression of Extracellular Matrix Proteins by different Feeders

Immunohistochemistry (IHC) was done on different feeders for 7 different matrix molecules: types I, III and IV collagens, laminin, fibronectin, and heparan sulfate proteoglycans (HS-PG, perlecan) and chondroitin sulfate proteoglycans (CS-PG). All feeders produced a mix of extracellular matrix molecules, but the lowest levels observed were in the primary cultures of angioblast/endothelia; intermediate in the HUVECs cell line or in primary cultures in which hHpSTCs had been culture selected, and with the highest levels of all found in the STO cells (Table 5.2). The basal adhesion molecule, fibronectin, was found in all of them and with the highest levels by protein assays found in STO cells. By IHC, collagen type III was found only in STO cells. Since it was found in other feeders by quantitative real-time PCR and there are few reports of matrix components expressing mRNA but not the protein, it is likely that the epitope recognized by the antibody is buried or masked by other matrix molecules and not available for binding in the IHC assays. HS-PGs were absent in all of the feeders by IHC and by quantitative real-time PCR except for STO cells.

The distinctions appear to be that the feeders supportive of the hHpSC phenotype produce an extracellular matrix containing forms of laminin (with integrins α4 and, by inference, β6), type IV collagen, CS-PG, and no forms of HS-PGs. Those that induced lineage restriction to hepatoblasts have elevated levels of type I, III and IV collagens, laminin (no α4 and an increase in β1), CS-PG and no HS-PGs. The STO feeders that induced the most striking differentiation also produced all of the matrix components assayed but at the highest levels of them observed by immunohistochemistry, and were unique among the feeders assayed in making HS-PGS.

Effects of purified Matrix Molecules on hHpSCs versus Hepatoblasts

The hHpSCs were cultured in serum-free KM and on each of 5 different types of matrix components that were coated onto plastic dishes: fibronectin, laminin and type I, III or IV collagens. Few of the hHpSCs cells attached to fibronectin, and those that attached did not grow, surviving for only a few days (data not shown). hHpSCs lineage restricted to hepatoblasts if cultured on laminin, type IV collagen or if plated on the surface of type I collagen gels (Figure 5.10). Alternatively, the conditions were not permissive for hHpSCS and resulted in selection of hepatoblasts. Lineage tracking studies are required to decide between these two interpretations.

When embedded into type I collagen, the hHpSCs differentiated the most, with the

morphology and antigenic profile of the colonies resembling mature hepatocytes The data also complement and corroborate the findings of McClelland et al (submitted) that notes the response of hHpSCs to matrix components found *in vivo* in the periportal versus pericentral zone of the liver acinus.

DISCUSSION

Human hHpSCs remain as stem cells, undergo lineage restriction to hepatoblasts or more extensively differentiate depending on the mesenchymal feeder cells used in co-cultures. Three distinct classes of feeders have been identified: 1) minimal differentiation, that is maintenance of the stem cell state, occurs with feeders of endothelia or angioblasts without hHpSTCs or with quiescent hHpSTCs; 2) feeders replete with activated hHpSTCs yield lineage restriction to hepatoblasts; and 3) murine embryonic stroma, STO cells, yield more differentiated parenchymal cells. The behavior of the co-cultures parallels that observed during liver development governed by paracrine signals from mesenchyme adjacent to the epithelium (Lemaigre and Zaret, 2004; Matsumoto et al., 2001; Zaret, 2002) and is paralleled by our findings of the effects of specific and purified matrix factors used for cultures of hHpSCs.

Interactions between endodermal and mesodermal tissues are important for hepatic development (Lammert et al., 2003; Matsumoto et al., 2001). Prior to hepatic induction,

septum transversum mesenchymal cells surround the developing cardiac region near the ventral foregut endoderm and are thought to be the source of various embryological inductive signals (Zaret, 2002). After these inductive signals, the hepatic endodermal epithelium becomes more proliferative, and the cells begin to bud into the mesenchymal environment. Mouse embryonic stem (ES) cells co-cultured with chick cardiogenic mesoderm could be induced to express endodermal transcription factors and several early liver-specific genes (Fair et al., 2003). We reported that mesenchymal "companion" cells, including angioblasts and quiescent hHpSTCs, are required for the survival and growth of hHpSC colonies *in vitro* (McClelland et al., 2007) and also reported that hedgehog signaling, long known to be important in angiogenesis and to regulate liver development, is important for *ex vivo* maintenance of hHpSCs derived from livers of all donor ages (Sicklick et al., 2006).

Hepatic stellate cells have only recently been determined to be different in fetal versus adult tissues (Kubota et al., 2007), though this can be inferred by the studies on $Lhx2^{-/-}$ (Wandzioch et al., 2004) and those with striking biological activity on hHpSCs are from fetal tissues. The known distinctions are that HpSTC precursors found in fetal or embryonic tissues do not express GFAP, have low content of lipids, and express very low levels of desmin, vitamin A, and αSMA. By contrast, the HpSTCs from adult livers are $GFAP^{+}$, have much higher levels of desmin, vitamin A, αSMA, and lipids; indeed it is the

lipid content of adult liver-derived hHpSTCs that has been utilized as a key feature in protocols for their purification (Geerts, 2001). Efforts are ongoing to discern distinctions in soluble paracrine signals from the fetal tissue-derived versus adult tissue-derived cells.

Attempts to establish primary cultures of purified angioblasts or endothelia (KDR^+, $CD31^+$) from human fetal liver under serum-free conditions to enable them remain as angioblasts/endothelial cells were minimally successful. The cultures were positive for endothelial cell markers for ~5-7 days and then rapidly converted thereafter to stromal/stellate cultures uniformly expressing high levels of desmin and αSMA and losing entirely the endothelial cell markers (CD31, KDR, vWF). Although we cannot rule out culture selection for a pre-existing, initially minor subpopulation, we suspect that the hHpSTCs are lineage restricted from angioblasts based on shared markers (e.g. VCAM-1, β3 integrin) and on observations of hHpSTCs encircling clusters of angioblasts.

It has been reported that HpSTCs (both rat and human) express low levels of αSMA in a quiescent state versus high levels of αSMA and collagen type I when activated during inflammation processes *in vitro* or *in vivo* (Abergel et al., 2006; Geerts, 2001; Shi et al., 2006). Our culture conditions for the immunoselected cells from human fetal liver cell suspensions are hypothesized to have activated the hHpSTCs, leading to significant growth, and high αSMA and collagen type I expression.

Signals produced by "activated hHpSTCs" overlap with but also are distinct from

those produced by the "quiescent stellate cells" surrounding the hHpSCs in our cultures. Brenner et al. found that activated hHpSTCs upregulated adhesion molecules such as ICAM-1 and secrete cytokines such as IL-6, IL-8, and RANTES (regulated on activation, normal T-cell expressed, and presumably secreted), and increase NF-κB activation (Hellerbrand et al., 1996; Paik et al., 2006; Schwabe et al., 2003). Other cytokines such as IL-10 and MCP-1 (monocyte chemo-attractant protein-1) also have been reported to be upregulated in activated hHpSTCs (Kisseleva and Brenner, 2006; Wang et al., 1998). It is hypothesized that signals produced by the hHpSTCs in the activated status dictate lineage restriction of the hHpSCs to hepatoblasts, an hypothesis now under investigation.

Matrix chemistry has long been known to be relevant to embryonic development (Corda et al., 2000; Davis and Senger, 2005; Dou and Levine, 1995). Fibronectin proved to be the matrix component that was expressed by all the feeders and the one produced at the highest levels by HUVECs. Given its universality among the feeders, it cannot be the explanation for the distinctions among them in their effects on hHpSCs *in vitro.* Moreover, the hHpSCs did not bind to fibronectin to any significant extent in the assays utilizing purified matrix components. Those that did bind did not grow. Indeed, fibronectin may be a critical matrix molecules needed by the feeders themselves. Fibronectin is widely used as a substratum for culturing HUVECs (Baudin et al., 2007).

By contrast, the cells were able to bind to laminin and to several types of collagens

(type I, III and IV). We found that the HUVECs make laminin and type IV collagen, corroborating an earlier report that HUVECs produce collagen type IV and laminin *in vitro* (Corda et al., 2000).

The αSMA^+ fibroblast-like, human fetal liver-derived cells expressed collagen type I at both mRNA and protein levels suggesting these cells may be activated hHpSTCs. HpSTCs are known to express αSMA, collagen types I, III and IV, fibronectin, and laminin α1 and γ1 chains (Kikkawa et al., 2005; Schlaf et al., 2004; Shi et al., 2006; Uemura et al., 2005). TGF-β1-stimulated hHpSTCs upregulate collagen types I and III, fibronectin and αSMA. Expression of collagen type III was detected in the human fetal liver-derived $CD31^+$ and αSMA^+ cells, suggesting possible ECM remodeling events occurred in the culture of these two cell types.

Chondroitin sulfate proteoglycan (CS-PG) was present at protein levels in both human fibroblast-like, fetal liver-derived cells and bone marrow-derived mesenchymal stem cells (hMSCs). The two feeders found to have CS-PGs caused lineage restriction of hHpSCs into hepatoblasts. So, CS-PGs are candidate signals for that process. It has been reported that CS-PGs can interact with chemokines, and their structure can be modified in TGF-β1-treated fibroblasts (Kawashima et al., 2002; Tiedemann et al., 2005). Therefore, CS-PGs, like heparan sulfate proteoglycans, are scaffolds for growth factors and other signals dictating how the factors are presented to the cells (Bernfield et al., 1992;

Kawashima et al., 2002; Ruoslahti and Yamaguchi, 1991; Vongchan et al., 2005). Interestingly, there appear to be distinct forms of CS-PGs present in many types of stem cell niches, ones with little to no sulphate groups (Caterson et al., personal communication). Caterson hypothesizes that the stem cell niche is dominated by GAGs with little to no sulfation such as hyaluronans and these minimally sulphated CS-PGS and could, therefore, act as a barrier minimizing the presentation of signals to the stem cells. As the stem cells are pushed out of the niche, they come into contact with GAGs and proteoglycans with more extensive sulfation and bound growth factors that could influence the stem cells either with respect to growth or with respect to lineage restriction to various differentiated cell fates.

The culture conditions used for growing HUVECs, typically containing serum, are not permissive for the expression of collagen type III at mRNA and/or in protein level. It has been reported that HUVECs have no expression of collagen type III at mRNA level (Hitraya et al., 1995). However, other studies showed positive expression of type III collagen in mRNA or protein level by endothelial cells (Maher and McGuire, 1990; Sales et al., 2006). Maher et al. showed that mRNA amount of type III collagen in rat sinusoidal endothelial cells was 15-fold higher than in hepatocytes, and was downregulated 45% 5 days after bile duct ligation and upregulated 25% after tetrachloride administration (Maher and McGuire, 1990). Immunohistochemistry on normal human fetal livers

revealed positive staining of type III collagen at perisinusoidal endothelium (Couvelard et al., 1998). Endothelium is known to be heterogeneous in different tissues. It is not surprising that HUVECs may have different cellular characteristics than human fetal liver-derived endothelial cells.

The most extensive differentiation, accompanied by a loss in growth potential, was observed in hHpSCs plated onto STO feeder cells; the hHpSCs went into growth arrest and erupted hepatoblasts and unipotent progenitors (committed biliary and hepatocytic progenitors). The STO feeders produced the highest levels of extracellular matrix and were unique in producing HS-PGs, forms of proteoglycans renown for operating as high affinity chemical scaffolds for growth factors (Bernfield et al., 1992; Kallunki and Tryggvason, 1992; Kim et al., 1994). HS-PGs have been purified and characterized from rodent livers by Gebhardt and associates (Pierce et al., 1992) and from human liver by Linhardt and associates (Vongchan et al., 2005). Maturation of the parenchymal cells is associated with heparan sulfates with higher degrees of sulfation (Geerts et al., 1986; Pierce et al., 1992; Roskams et al., 1995). Indeed, a form has been isolated that is a true heparin proteoglycan and is located in association with the most mature cells in the liver (Pierce et al., 1992; Vongchan et al., 2005).

The matrix substrata tested that induced such differentiation comprised ones with type I collagen. The extent of differentiation was found to differ depending on whether the

cells were plated on top of or embedded into the type I collagen. Indeed, cells morphologically similar to mature hepatocytes were found in those cultures embedded in the collagen (Figure 5.10). This is likely due both to a direct effect of type I collagen and also an indirect effect via stabilization of HS-PGs by type I collagen, an effect reported years ago by Bernfield and associates (Bernfield et al., 1992). Mature hepatocytes are known to bind directly to collagens, especially type I collagen, via an integrin unique to the hepatocytes, αVβ1 and not on biliary epithelia (Couvelard et al., 1998). Therefore, the differentiative response of the hHpSCs on or in type I collagen is likely to occur through direct effects and not through another ligand. More detailed characterizations of the cells on substrata of purified matrix components are given in two separate reports (McClelland et al., submitted).

In Figure 5.11, we summarize the known sequential changes in matrix components and matrix receptors that occur with the transitions from hHpSCs through hepatoblasts and ultimately to mature parenchymal cells and that combine our own data with that in the literature on mature parenchymal and non-parenchymal cells (Couvelard et al., 1998; Eichmann et al., 1997; Fornaro et al., 1998; Kikuchi et al., 2005). Our studies to date allow us to begin to transition to feeder-free cultures at least with respect to matrix requirements. Completion of these efforts will be achieved with the identification of the soluble signals and assessment of synergies between them and the matrix signals.

ACKNOWLEDGEMENTS

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TABLES

Table 5.1. Effects of feeders/substratum on human hepatic stem/progenitors

Substratum/Feeder		Colony Morphology/Antigenic Profile of Cells Maintained for Feeders for a Week or more
Plastic* **		hHpSC
HUVECs		hHpSC
hMSCs		Hepatoblasts
Liver Cell Suspensions immunoselected for:	**KDR^+ Cells in first week**	hHpSCs
	KDR^+ cells after 7-10 days	Initially mixture of hHpSCs and hepatoblasts that transitions rapidly to only hepatoblasts
	$CD31^+$ Cells	Hepatoblasts
	Depleted of stromal cells	Hepatoblasts
STO cells		Growth-arrested hHpSCs with eruptions of hepatoblasts and committed (unipotent) progenitors
Fibronectin		Few cells attached; those that did rapidly lost viability
Laminin		Hepatoblasts
Type III collagen		hHpSCs
Type IV collagen		Hepatoblasts
		Growth-arrested cells that are a mixture of

On surface of Type I collagen	Hepatoblasts and committed (unipotent) progenitors
Embedded in Type I collagen	Mature hepatocytes

All cultures were in serum-free KM.

***Note:** when cells are on culture plastic, the hHpSC colonies survive only when they are in association with companion cells comprised of angioblasts and/or hepatic stellate cell precursors.

***Culture Morphology and antigenic profile:**

hHpSC=colonies are monolayers with cells of uniform morphology, high nucleus to cytoplasmic ratio, ~7-9 μm in diameter, tightly packed and surrounded by companion cells that include angioblasts and hHpSTCs. Unique Antigenic profile: $NCAM^{+}$, Claudin 3^{+}, albumin$^{\pm}$, AFP^{-}.

Hepatoblasts= colonies that are more 3-dimensional, with cord-like structure interspersed by clear channels (bile canaliculi) and with cells that are slightly larger (10-12 μm) in diameter. Unique antigenic profile: $ICAM^{+}$, Claudin 3-, albumin^{++}, AFP^{++}.

Shared antigenic profile between hHpSCs and hepatoblasts: positive for EpCAM, CK-8, -18, and -19, Sonic hedgehog (shh), Indian hedgehog (ihh), telomerase; negative for hemopoietic markers (CD34, CD45, CD38, glycophorin A), for hepatic stellate cell markers (desmin and αSMA) and for endothelial cell markers (VEGFR2, CD31, vWF).

Table 5.2. Extracellular matrix components produced by different feeders: Immunohistochemistry (IHC) Assays

ECM Molecules	hHpSCs w/ companion cells	HUVECs	hMSCs	Human fetal liver-derived			STO
				CD31+	KDR+	Depletion of stroma	
Type III collagen	++	-	-	-	-	-	-
Type IV collagen	+	+	-	+	+	+	++
Type I collagen	-	-	-	-	-	+	++
Laminin	-	-	-	+	+	+	++
Fibronectin	-	+	+	+	+	+	++
Heparan sulfate-PG	n.d.	-	-	-	-	-	++
Chondroitin sulfate-PG	n.d.	-	+	-	-	+	n.d.
Effects of feeder on hHpScs	Self-replication		Lineage restriction to hepatoblasts				

FIGURE LEGENDS

Figure 5.1. Colony of hHpSCs and human hepatoblasts in culture:

Both hHpSC colony (A) and human hepatoblasts (B) expressed EpCAM (shown in green). NCAM (shown in red, A) was expressed near peripheral region and the center of the colony. Hepatoblasts expressed strongly alpha-fetoprotein (AFP, shown in red, B). Both cells were stained with DAPI (blue). Scale bar, 100 μm.

Figure 5.2. Cell surrounding a colony of hHpSCs are αSMA$^+$ hHpSTCs:

Cells surrounding a colony of human hepatic stem cells (hHpSCs) are αSMA$^+$ hHpSTCs. A typical human hepatic stem cell colony (A) is positive for NCAM inside the colony (B, D) and for αSMA in the companion cells at the edge of colony (C, D). Magnification, 10x.

Figure 5.3. Primary culture of hHpSCs with Angioblasts:

KDR$^+$ selected cells cultured in EGM-2 for 7 days expressed vWF (green) (A and B). CD31$^+$ selected cells cultured in EGM-2 for 4 days expressed both vWF (green) and CD31 (red) (C). Scale bar, 100 μm. Angioblasts associated with hHpSC colony in culture (D). Magnification, 10x.

Figure 5.4. Quiescent versus Activated hHpSTCs

Quiescent hHpSTCs express low levels of desmin, αSMA, CD146, type I collagen, and other matrix molecules (fibronectin, proteoglycans). Injury processes, especially exposure to serum or to certain factors (e.g. PDGF and TGF-β1), causes them to activate and to transition to myofibroblast-like stromal cells with greatly elevated production of αSMA, matrix components, and release of various growth factors such as HGF. Shown is a colony of hHpSCs encircled by companion cells expressing low levels of CD146. On the same plate, there is an adjacent colony with companion cells that have undergone activation resulting in very high levels of CD146.

Figure 5.5 Morphology and immunohistochemistry of different feeders:

A: hMSCs. B: HUVECs. C-D: human fetal liver-derived feeder cells on days 4 (C) and 7 (D). E-H: day-11 culture of magnetically immunoselected KDR^+ cells (E-F) and supernatant cells depleted of fibroblasts (G-H) in serum-free (or low-serum; data not shown) condition were positive for αSMA (F and H). Magnification, 10x.

Figure 5.6. Immunohistochemistry on fibroblast-depleted supernatant cells cultured in EGM-2 medium for 8 days:

Cells are positive for desmin (B and H), αSMA (I), laminin (C), fibronectin (F),

collagen types I (L) and IV (E), and negative for endothelial marker, vWF (K). Note that more cells express αSMA than desmin. Phase contrast image for each double staining is shown (A, D, G, and J). Bar, 50μm.

Figure 5.7. Human hHpSCs co-cultured with different feeders.

A-F: human hHpSCs cultured alone (A), co-cultured with HUVECs (B), hMSCs (C), or human fetal liver-derived feeders (D). Magnification, 10x.

Figure 5.8. Human hHpSCs co-cultured with αSMA$^+$ supernatant cells, from which fibroblasts were depleted, derived from human fetal livers.

Use of these feeders resulted in lineage restriction to hepatoblasts. Immunohistochemistry for human AFP on human hepatic stem cell colonies (A and B) and on co-culture of hHpSCs and human fetal liver-derived feeders (C and D) at day 8. Magnification, 10x.

Figure 5.9. Normalized mRNA expression of matrix molecules

Fold changes of the mRNA expression levels in each cell type were normalized to the ribosomal RNA (18S) of the same cell type.

Figure 5.10. Behavior of hHpSCs on substrata of purified matrix components:

The hHpSCs maintain their stem cell characteristic on plastic or on type III collagen (A and B). hHpSCs lineage restricted to hepatoblasts on top of type IV collagen or on laminin (C and D). hHpSCs further differentiated into mature hepatocytes when embedded in type I collagen gel. Higher magnification for D.

Figure 5.11. Summary of the changes in matrix mhemistry and matrix receptors in the hHpSCs and their mesenchymal cell partners during differentiation.

The summary is prepared from a combination of our findings (data on the hHpSCs, hepatoblasts, hHpSTCs and other human liver-derived feeders) and those in the literature (angioblasts and endothelia from other sources and mature biliary epithelia).

Figure 5.1

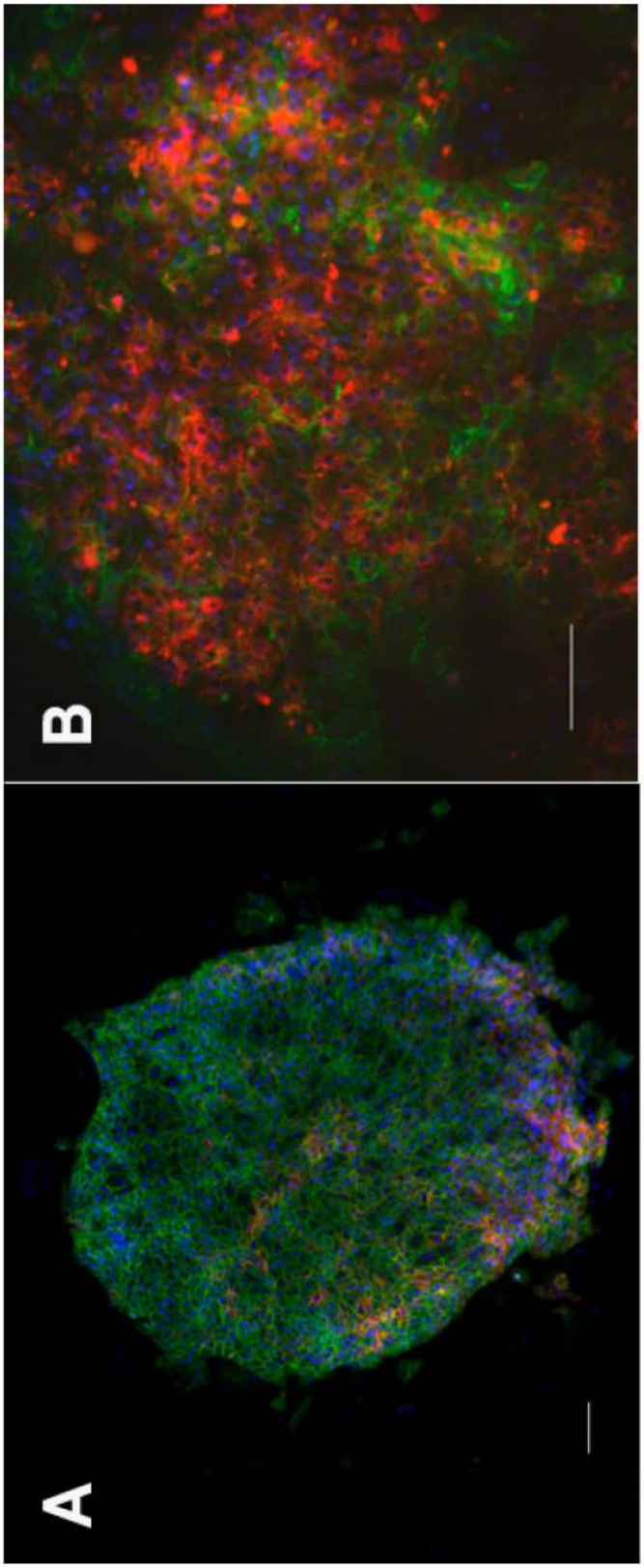


Figure 5.2

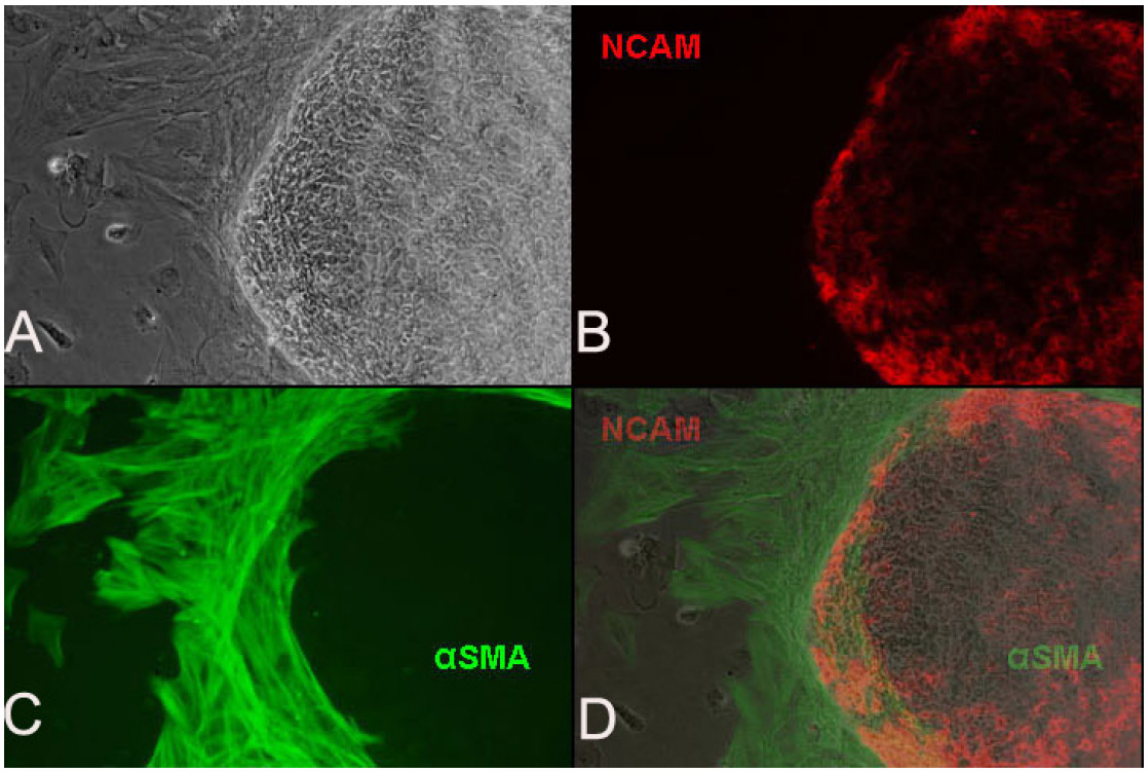


Figure 5.3

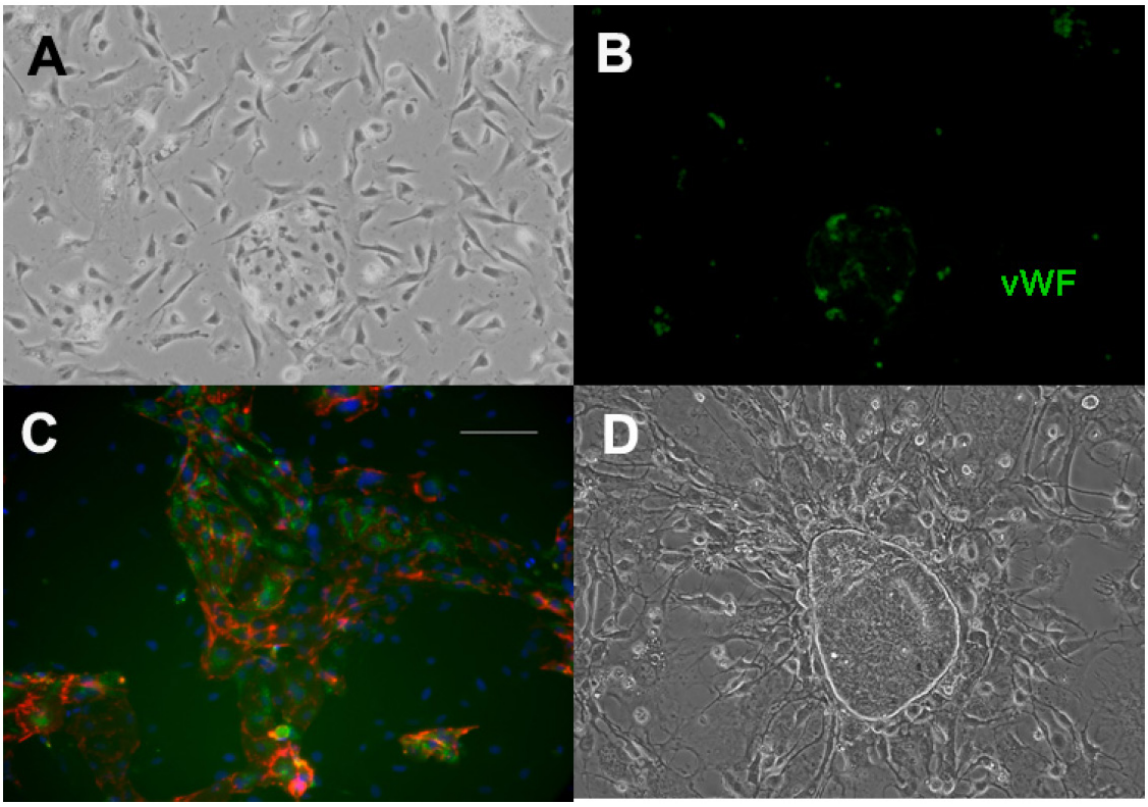


Figure 5.4

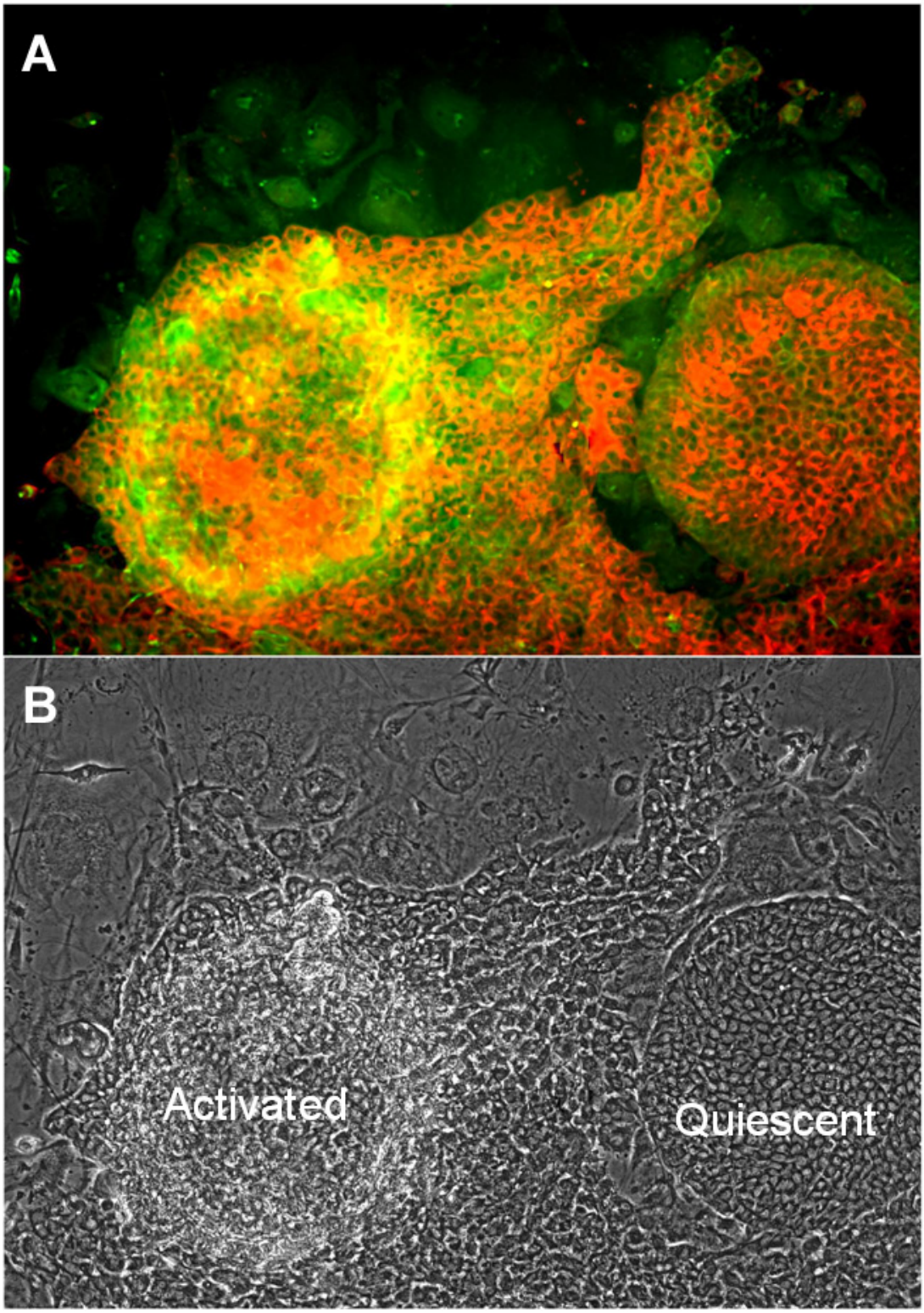


Figure 5.5

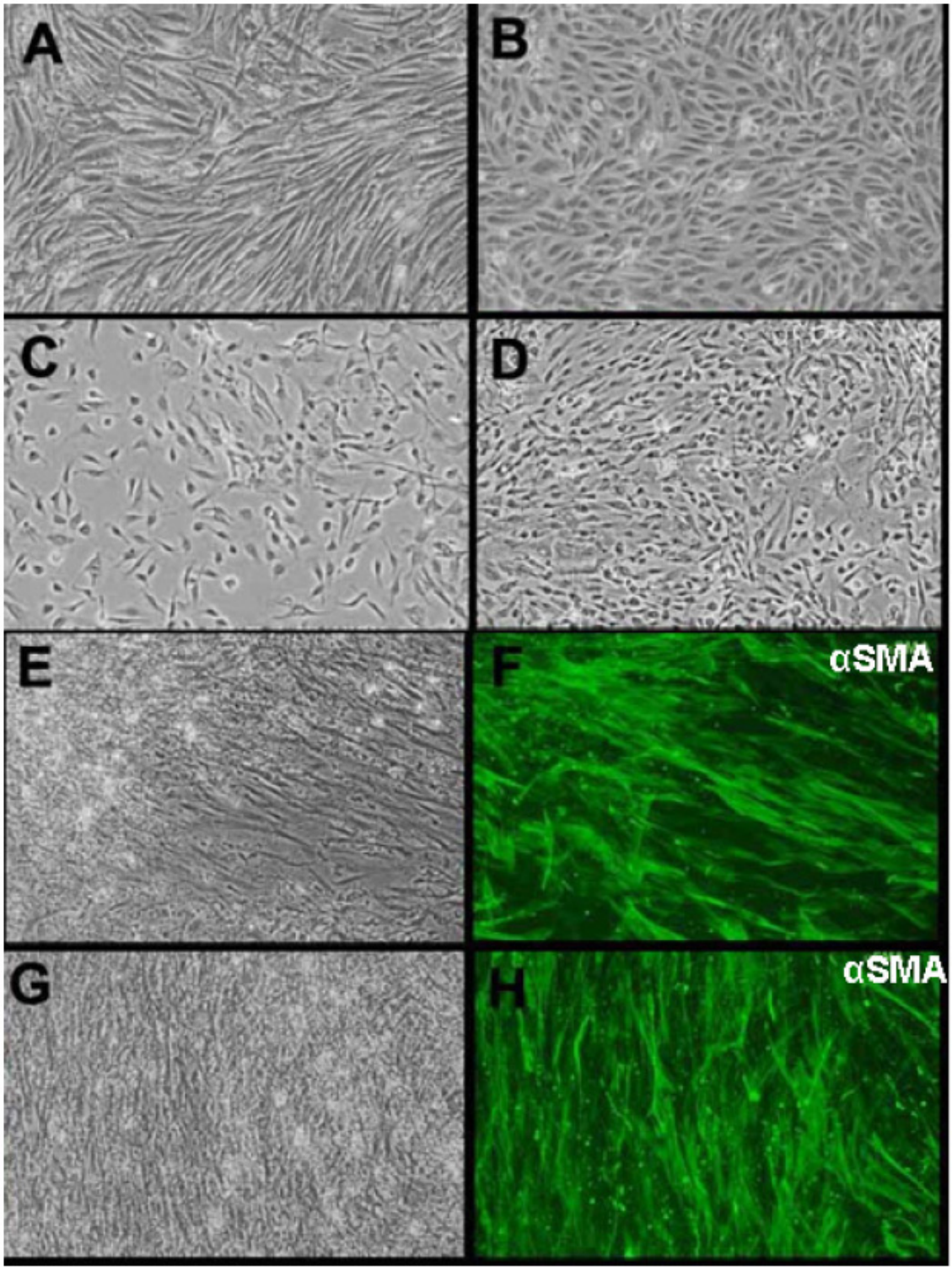


Figure 5.6

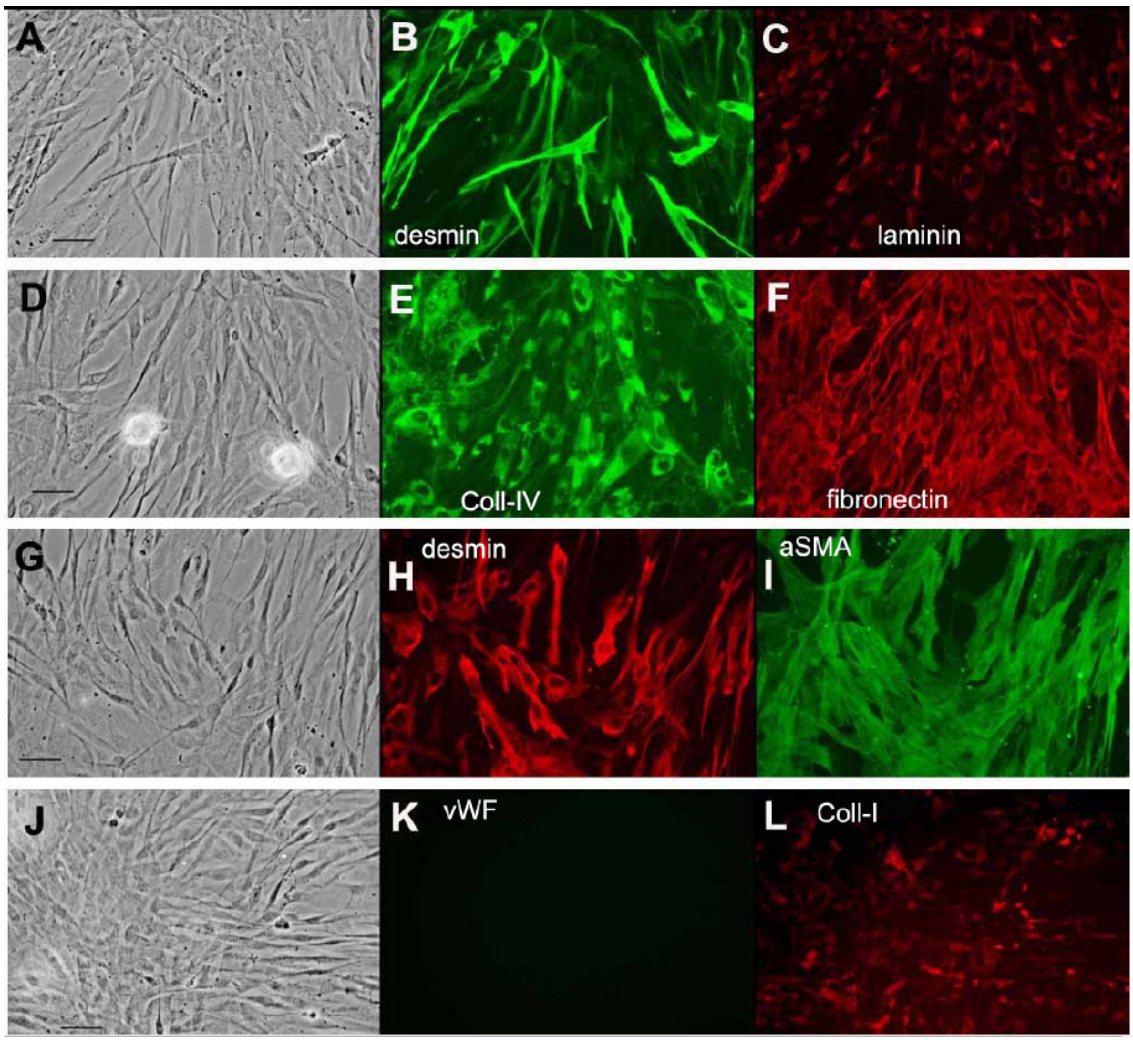

A
B
desmin
C
laminin
D
E
Coll-IV
F
fibronectin
G
desmin
H
aSMA
I
J
K
vWF
L
Coll-I

Figure 5.7

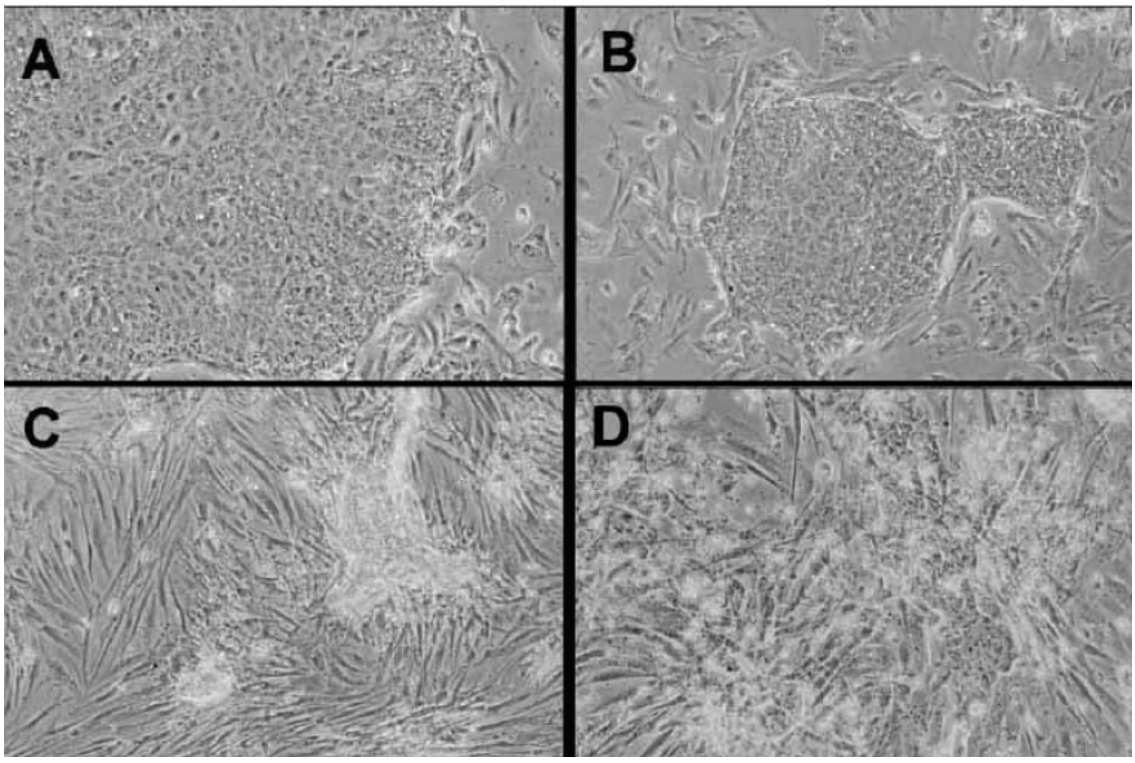


Figure 5.8

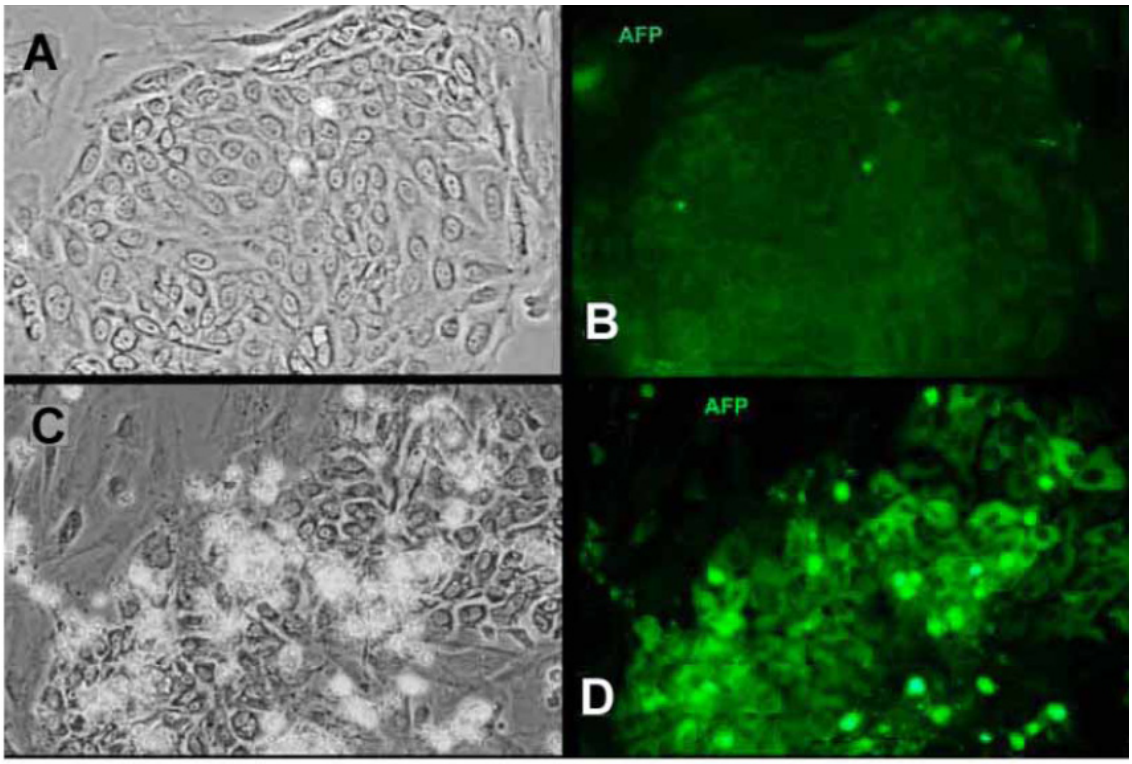


Figure 5.9

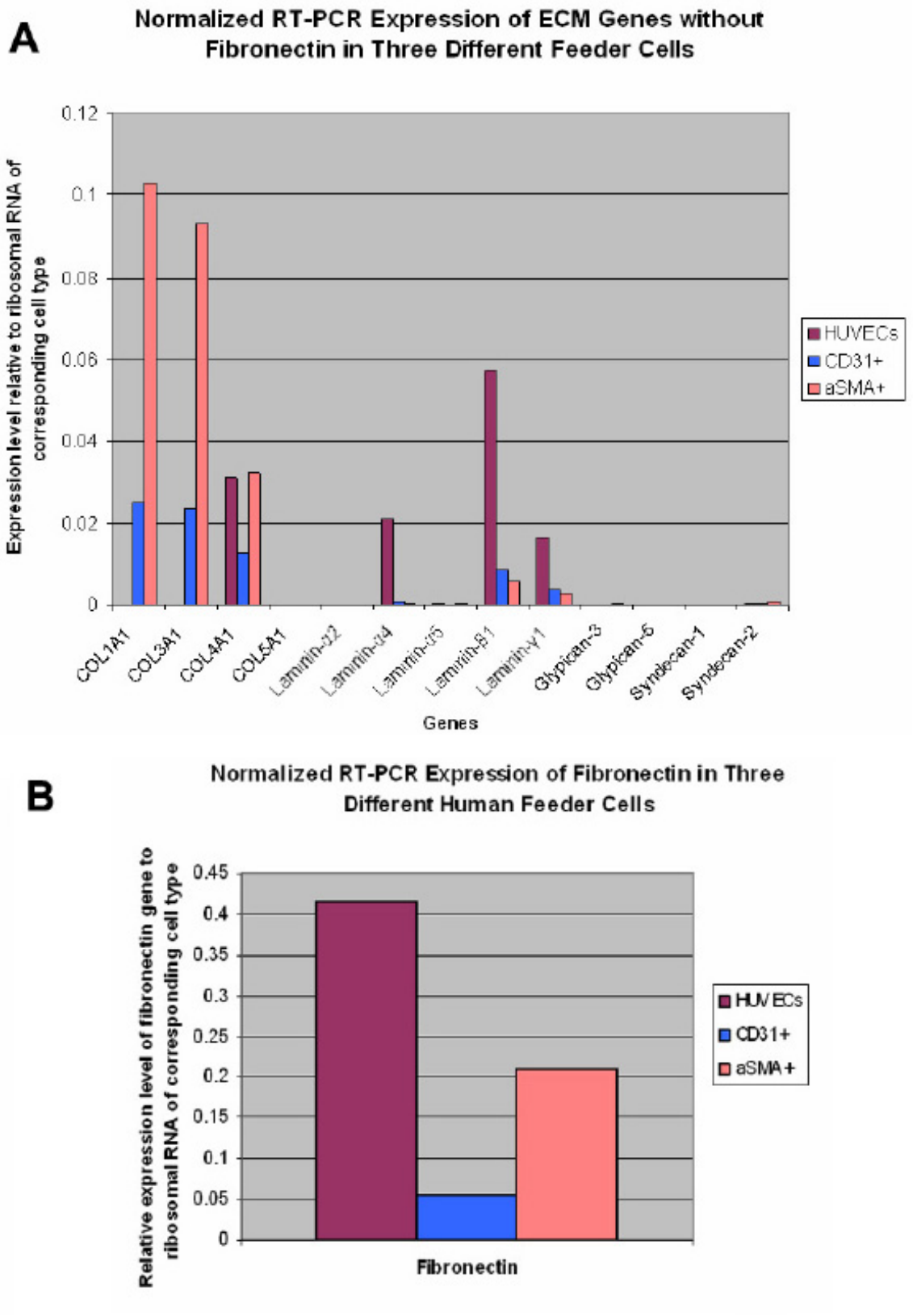

A
Normalized RT-PCR Expression of ECM Genes without Fibronectin in Three Different Feeder Cells
Expression level relative to ribosomal RNA of corresponding cell type
0.12
0.1
0.08
0.06
0.04
0.02
0
COL1A1
COL3A1
COL4A1
COL5A1
Laminin-α2
Laminin-α4
Laminin-α5
Laminin-β1
Laminin-γ1
Glypican-3
Glypican-6
Syndecan-1
Syndecan-2
Genes
HUVECs
CD31+
aSMA+
B
Normalized RT-PCR Expression of Fibronectin in Three Different Human Feeder Cells
Relative expression level of fibronectin gene to ribosomal RNA of corresponding cell type
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Fibronectin
HUVECs
CD31+
aSMA+

Figure 5.10

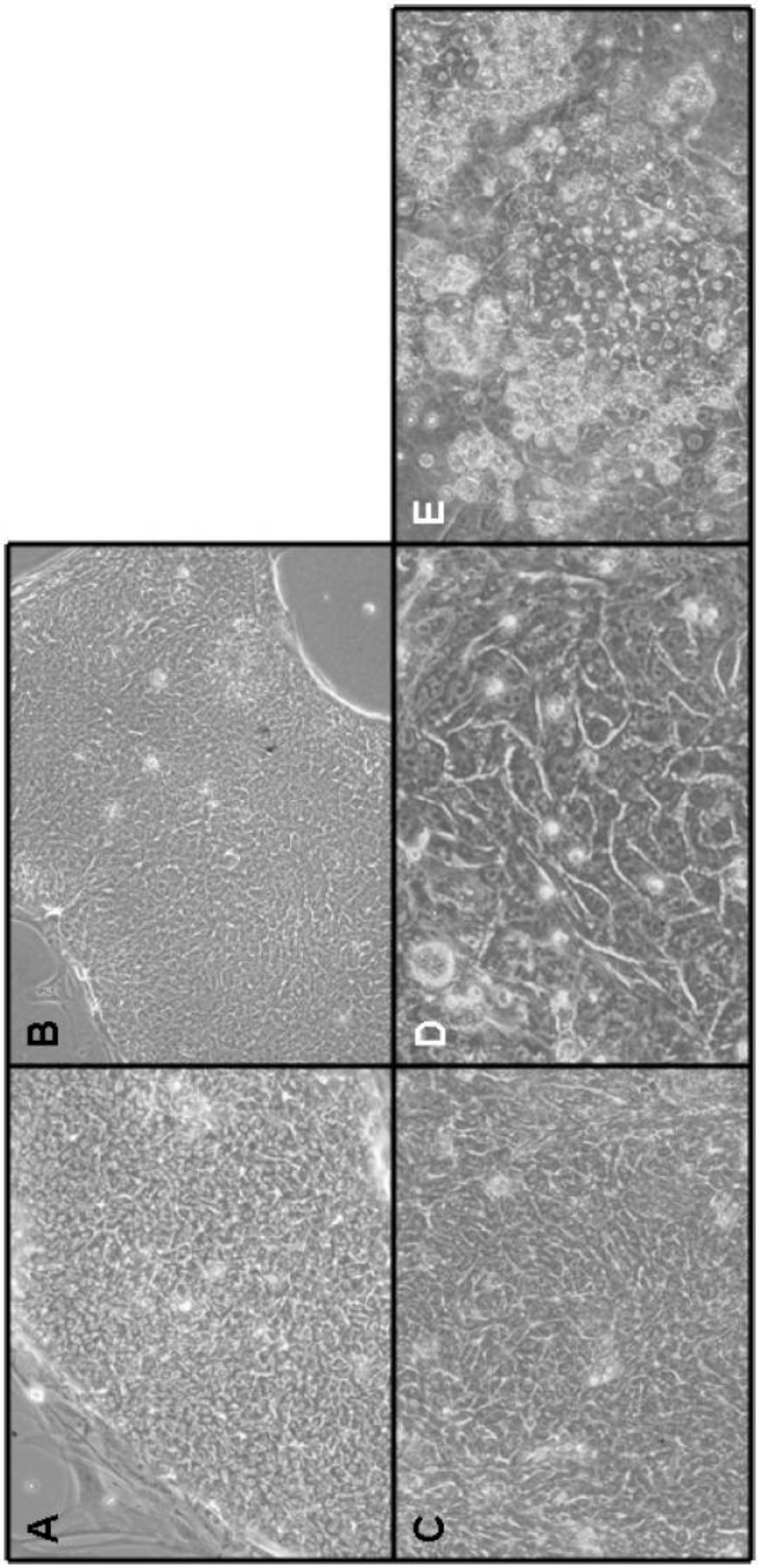
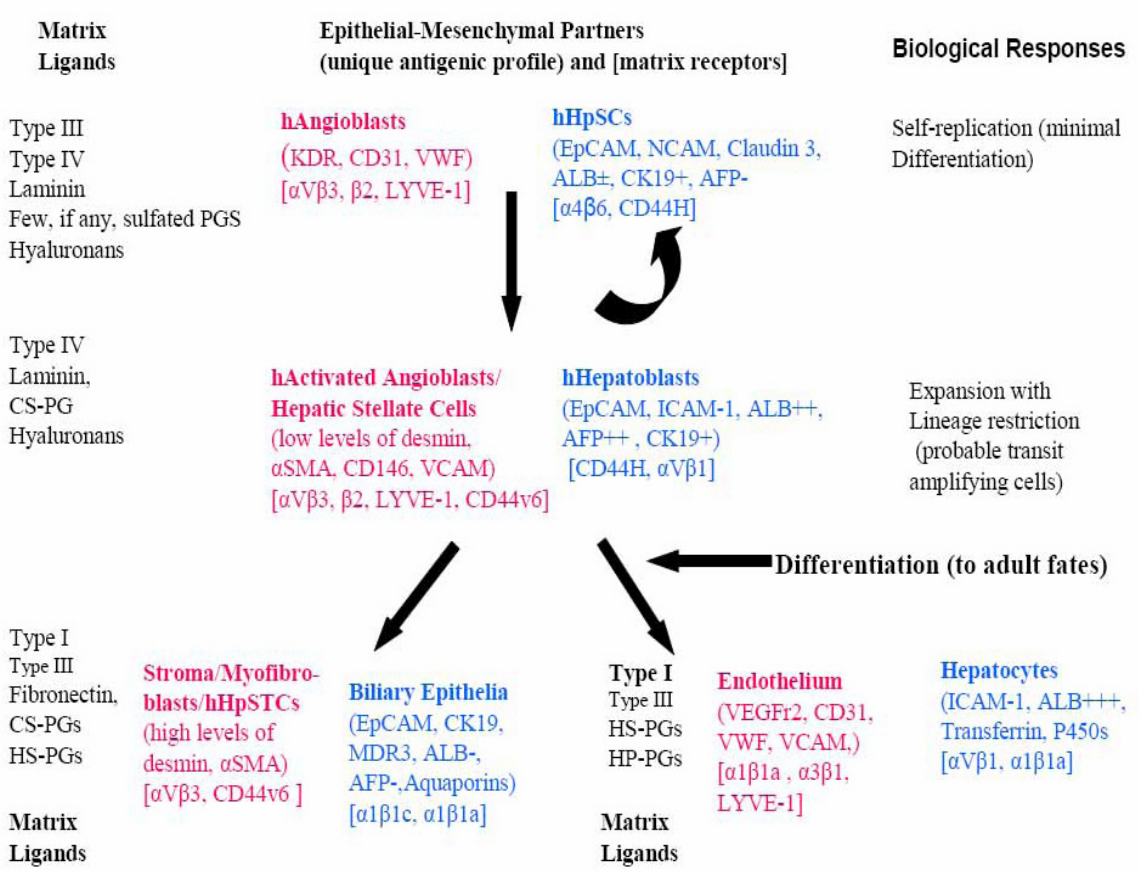


Figure 5.11


Matrix Ligands
Epithelial-Mesenchymal Partners (unique antigenic profile) and [matrix receptors]
Biological Responses
Type III
Type IV
Laminin
Few, if any, sulfated PGS
Hyaluronans
hAngioblasts
(KDR, CD31, VWF)
[αVβ3, β2, LYVE-1]
hHpSCs
(EpCAM, NCAM, Claudin 3, ALB±, CK19+, AFP-
[α4β6, CD44H]
Self-replication (minimal Differentiation)
Type IV
Laminin,
CS-PG
Hyaluronans
hActivated Angioblasts/ Hepatic Stellate Cells
(low levels of desmin, αSMA, CD146, VCAM)
[αVβ3, β2, LYVE-1, CD44v6]
hHepatoblasts
(EpCAM, ICAM-1, ALB++, AFP++ , CK19+)
[CD44H, αVβ1]
Expansion with Lineage restriction (probable transit amplifying cells)
Differentiation (to adult fates)
Type I
Type III
Fibronectin,
CS-PGs
HS-PGs
Matrix Ligands
Stroma/Myofibro-blasts/hHpSTCs
(high levels of desmin, αSMA)
[αVβ3, CD44v6]
Biliary Epithelia
(EpCAM, CK19, MDR3, ALB-, AFP-,Aquaporins)
[α1β1c, α1β1a]
Type I
Type III
HS-PGs
HP-PGs
Matrix Ligands
Endothelium
(VEGFr2, CD31, VWF, VCAM,)
[α1β1a , α3β1, LYVE-1]
Hepatocytes
(ICAM-1, ALB+++, Transferrin, P450s
[αVβ1, α1β1a]

CHAPTER VI

IMMUNOSELECTION OF CELLS BY ENDOTHELIAL MARKERS AND THE DEVELOPMENT OF THE SERUM-FREE DEFINED MEDIUM FOR ANGIOBLASTS

Endothelial cells play an important role in normal liver development and during liver regeneration. Through paracrine signaling, the endothelial cells release key cytokines and growth factors that induce hepatic progenitor cells to grow and/or differentiate during organogenesis. To study the effects of paracrine signals from endothelial cells on hepatic progenitor cells *in vitro*, establishing a model system utilizing liver-derived angioblasts or endothelial cells is critical. To achieve this requires methods for purifying the angioblasts or endothelial cells as well as wholly defined culture conditions permissive for these two categories of cells. In this chapter I describe my findings for both of these methodologies, methods that were partially successful and that should lead relatively quickly to completed ones for use with human fetal liver-derived angioblasts and endothelia. Further, the model systems being established should facilitate analyses of paracrine signaling events between liver-specific angioblasts/endothelia and hepatic parenchymal cells.

RESULTS

Isolation and expansion of endothelial progenitor cells from human fetal livers

The first attempt on isolation and expansion of endothelial progenitor cells (EPCs) from human fetal livers was done by density gradient separation method and with EndoCult Liquid Medium from StemCell Technologies, Inc. The density gradient separation methodologies have been widely used to isolate endothelial cells from human peripheral blood. The EndoCult Liquid Medium Kit is designed specifically for growth of human EPCs isolated from human peripheral blood, cord blood and bone marrow. Cells at the interface layer after density gradient separation presented as fibroblast-like cells in the EndoCult Liquid Medium on plastic and on collagen type I-coated plates. Possible contamination of the cell suspensions with fibroblastic cells could allow for their selection over that of the endothelial cells. To eliminate contamination by fibroblastic cells, anti-human fibroblast microbeads were used with the magnetically activated cell sorting system from Miltenyi Biotec to debulk or deplete these cells after density gradient separation. Yet, the fibroblast-depleted cell population cultured in EndoCult Liquid Medium on plastic and on collagen type I-coated plates still gave rise to fibroblast-like cells. Immunohistochemistry on the fibroblast-depleted population cultured on plastic showed negative staining for the endothelial marker, von Willebrand factor (vWF), but positive for α-smooth muscle actin (αSMA) and desmin, markers indicating hepatic stellate cells (HpSTCs) (Figure 6.1). Whether or not the immunoselection method worked cannot be determined, since the culture conditions strongly selected for HpSTCs or caused any angioblasts/endothelia present to transition into HpSTCs. The failure of this strategy led to attempts to immunoselect angioblasts/endothelia using endothelial marker-specific antibodies.

Cell surface markers that have been used for immunoselection of angioblasts/endothelia include CD133/1, CD34, CD105 (endoglin), KDR (VEGFR2/flk-1) and CD31 (PECAM). The percentage of each antigenic positive cell population in human fetal liver cell suspensions is shown in the Table 6.1. In primary cell cultures, $CD105^{+}$-, $CD133/1^{+}$-, and $EpCAM^{-}/KDR^{+}$-sorted cells plated onto culture plastic gave rise to fibroblast-like cells in both serum-supplemented KM and in EndoCult Liquid Medium. By contrast, $CD133/1^{+}$-sorted cells yielded parenchymal-like cells (Figure 6.2). Since CD133/1 is expressed also on HpSCs, it was necessary to learn if the immunoselection had isolated them or the angioblasts. Immunochemical staining on the 6-day primary culture of $CD133/1^{+}$ cells showed that both parenchymal-like cells and fibroblast-like cells are negative for CD146, KDR, CD133/1 and CD34, and that the parenchymal-like cells are positive for NCAM and weakly expressed EpCAM, indicating that these are HpSCs (Figure 6.2). In the passage 1 cell cultures of both $CD133/1^{+}$ and $EpCAM^{-}/KDR^{+}$ sorted cells in low-serum media, only fibroblast-like cells were observed and these grew very fast under the culture conditions used. Immunohistochemistry staining indicated that the cells were negative for endothelial markers (CD31 (PECAM), CD133/1 and KDR), negative for HpSC markers (EpCAM and NCAM), but positive for CD29f (β1 integrin), a membrane protein expressed on fibroblasts and various other cell types, but not on the endothelial cells. CD34-selected cells resulted in minimum attachment after plating.

To debulk the cell suspensions of parenchymal cells prior to immunoselection for angioblasts/endothelia, the human fetal livers were processed to permit the parenchymal cells to remain as clumps and then fractionated them into a pellet with slow-speed centrifugation spins. The supernatent was then used for immunoselection using endothelial cell markers.

KDR^+ cells selected from supernatant cells after slow-speed spin using indirect isolation method occupied 0.33% of total liver cells in the supernatent. Immunohistochemistry on paraffin-embedded human fetal liver sections using monoclonal anti-human KDR antibody showed positive expression of KDR at blood vessels including those associated with ductal plates (Figure 6.3). The KDR-selected cells cultured within EGM-2 (Endothelial Growth Medium supplemented with 2% of FBS (fetal bovine serum) from Cambrex Inc.) gave rise to cultures of endothelial cells as recognized morphologically (Figure 6.3). Immunohistochemistry showed positive expression of CD31, KDR and vWF by these cells in 7-day cultures, indicating their identity as endothelial cells (Figure 6.3). However, the culture is dominated by HpSTCs recognizable by $\alpha SMA^+/vWF^-$ non-endothelial cells. These fibroblast-like cells could be hepatic stellate cells.

$CD31^+$ cells selected from supernatant cells after slow speed-spins using direct isolation method yielded 1.6% of total liver cells. The CD31-selected cells cultured within EGM-2 on plastic gave rise to endothelial cells with the majority of cells expressing both CD31 and vWF for the first 4-5 days (Figure 6.4). After 5 days of culture, fibroblast-like cells began to appear, grew rapidly and quickly dominated the cultures within another 3 days (by 8 days of culture). Passaging of the endothelial cells at 4 days, prior to the take-over of the stroma, did not help. After 4 days of culture, fibroblast-like cells began to outgrowth the passaged endothelial cells. Clearly, immunoselection using endothelial cell markers can enrich for angioblasts/endothelial subpopulations, but they are outcompeted by stroma and HpSTCs by the culture conditions used: culture plastic and medium with any serum.

Efforts to convert to serum-free medium were not successful initially. EGM without serum was tried with the $CD31^+$ sorted cells but only after 4 days of culture in 2% FBS-

supplemented EGM (EGM-2). The endothelial population was sustained for a few days without cell growth, but then gradually died off in the culture. When changed back to EGM-2, fibroblast-like cells had survived the few days without serum and began to grow rapidly, quickly dominating the culture. The stroma/fibroblast-like cells seem to survive better than the angioblasts/endothelia under short-term serum-free conditions. As has been shown previously, it is not logical to culture for several days with serum and then convert to serum free conditions. Rather, one has to develop a serum-free, wholly defined medium into which one can plate cells immediately. To do that requires ideally that one use an endothelial cell line, already adapted to culture, as a model and develop a hormonally defined medium for it. That hormonally defined medium can be used as a starting point for establishing serum-free, wholly defined conditions for primary cultures of angioblasts/endothelia.

Partial Development of Serum-free medium for maintaining HUVECs *in vitro*

A serum-free medium has been developed for maintaining HUVECs (human umbilical vein endothelial cells) on tissue culture plastic and on fibronectin for up to three weeks. Components of this medium include RPMI 1640 basal medium (85%), Invitrogen's knock-out serum-replacement reagent (15%), L-glutamine (1mM), 2-mercaptoethanol (0.1 mM), and 10 ng/ml each of FGF-basic, LIF, VEGF, and EGF. In three test runs of using this medium for HUVEC cells, one round completely failed (no cell survival in 48 hours after changing to serum-free medium); one showed 4 days of survival but no cell growth, followed by cell death; and the third run showed 26 days of survival with similar cell growth compared with HUVECs in EGM-2 medium (Figure 6.5). The HUVECs cultured in the serum-free medium expressed vWF and CD31. Whether the cells survived or not was due, presumably,

to when the switch to the hormonally defined medium occurred after plating. This hints that matrix substrata must be identified to complete the definition of the serum-free conditions. Preliminary results testing this medium on immunoselected endothelial cells ($CD133^+$ and KDR^+) indicated failure: no endothelial cells survived in the culture. Although the results seem a failure, it is more likely that the medium as is (or with perhaps a factor or two) could still be valuable if an appropriate extracellular matrix substrata is found to use in combination with the medium. Matrix substrata are the primary determinants of attachment and survival of cells. Thus, there is considerable hope that matrix components plus the medium developed thus far will enable wholly defined conditions for angioblasts/endothelia to be established. Candidate matrix substrata, based on evidence in the literature (including the integrins expressed by angioblasts/endothelia) include at least three types of collagen (type III, type IV and type VI), laminin, and hyaluronans (or a combination of these matrix molecules).

DISCUSSION

Isolation and expansion of endothelial progenitor cells from human fetal livers

Previous studies showed that both the endothelial progenitor cells (EPCs, or angioblasts) and hemangioblasts express KDR, CD133/1, CD34, and CD117, where expression of CD34 and CD117 are also shared with hematopoietic stem cells and expression of KDR is also shared with mature endothelial cells. As EPCs differentiate into mature endothelial cells, CD133/1 expression is downregulated. Therefore, immunoselection of $CD133/1^+$ or KDR^+ cells should be able to fish out the EPCs. The results from my experiments, however, did not prove this hypothesis due, in part, to inefficiency of the magnetically activated cell sorting (MACS) selection method, and, more importantly, to the

fact that I did not have serum-free defined culture conditions that could be selective for the cells.

Any culture medium supplemented with serum results in rapid selection for stroma/fibroblasts or HpSTCs more efficiently than for the EPCs and committed/mature endothelial cells. CD133/1, prominin, has been reported to be expressed by not only the EPCs, but also variety of tissue-specific precursor cells as well as many cancer cells. Previous studies in our lab showed that hHpSCs express CD133/1. Cells selected by anti-human CD133/1 microbeads gave rise to both non-parenchymal cells and hepatic parenchymal cells, supporting the concept that CD133/1 is shared by at least two cell types within the human fetal livers: angioblasts and HpSCs.

The term, EPC, encompasses subpopulations of endothelial cells ranging from hemangioblasts to fully differentiated endothelial cells. Angioblasts are immature EPCs that express CD133/1, whereas mature endothelial cells do not express CD133/1. There also might be distinctions in the phenotype of angioblasts or endothelia from different tissues (e.g. hepatic and pancreatic associated EPCs). Thus, it is possible that angioblasts could give rise to committed EPCs that have tissue-specificity. If so, the antigenic profile of these candidate tissue-specific EPCs may not be exactly the same as the antigenic profile of angioblasts. Testing this hypothesis must await the completion of the establishment of serum-free, wholly defined conditions for angioblasts/endothelia, so that model systems of any tissue-specific forms might be established.

KDR and CD31 were found to be the most efficient markers among those tested for immunoselection of endothelial cells from human fetal livers. The $EpCAM^{-}/KDR^{+}$ sorted cells gave rise only to fibroblast-like cells in the culture, whereas KDR^{+} cells selected from

the total cell population gave rise to both endothelial cells and hHpSTCs (αSMA^+ fibroblast-like cells). Two possible reasons could be considered to explain the paradox. First, endothelial cells tend to form aggregates with parenchymal cells that are positive for EpCAM. Isolation method involving depletion of $EpCAM^+$ parenchymal cells may in fact also deplete endothelial cells. Secondly, the antibody used for selecting endothelial cells from the interface layer of density gradient separations was rabbit anti-human KDR IgG where the one used for selecting EPCs from supernatant after slow-spin was mouse monoclonal anti-human KDR IgG1. The monoclonal antibody generated from mice may be more specific than the polyclonal antibody generated from rabbit. Less specific antibody could result in lower purity of the interesting cells and higher contamination from other cell types.

The reason that fibroblast-like cells always dominated the culture after one week is most likely to be the use of serum, a problem noted by investigators for more than 50 years. Serum supplied in the culture media could either select for specific subpopulations (that is the stroma) or could lineage restrict the angioblasts/endothelia to cell types with a stromal/ fibroblastic and/or hHpSTCs phenotype. Either interpretation indicates that there is a strong need for completing the development of serum-free conditions that would select for and would sustain angioblasts and their descendents, endothelia. The lack of appropriate culture conditions is compounded by the fact that magnetic immunoselection technologies do not result in high purity of endothelial cell subpopulations. The extent of contamination of the endothelia/angioblasts by smooth muscle cells, HpSTCs, and other mesenchymal cell types in combination with culture conditions that strongly select for these contaminating cell types can obviate the efforts to establish model systems of angioblasts and endothelia.

Development of Serum-free Conditions for maintaining HUVECs *in vitro*

HUVECs were able to survive and expand in both EGM-2 and in the partially defined conditions that included a serum-free medium supplemented with four growth factors. FGF-basic and LIF have been used widely in culturing human and mouse embryonic stem (ES) cells. VEGF and EGF have been reported to promote cell growth of endothelial progenitor cells. Combination of these four growth factors enabled HUVECs to grow on plastic or on fibronectin-coated plates. However, the preliminary studies testing this serum-free medium on primary cutures of endothelial cells immunoselected from human fetal livers were not successful. EGM-2 could be used to grow immunomagnetically sorted $CD31^+$ cells on plastic, although fibroblast-like cells grow fast after 5 days in the same medium. Compared with HUVECs, a mature endothelial cell type, the human fetal liver-derived endothelial progenitor cells (EPCs) may need more key molecules in order to proliferate *in vitro*. These key molecules could be provided by serum. Components of EGM-2 that are not in the newly developed serum-free medium include IGF-1 (insulin-like growth factor-1), heparin, hydrocortisone, ascorbic acid and FBS. The concentrations of each ingredient in the EGM-2 as well as the exact components of the basal medium of EGM-2 are proprietary information of the manufacturer. Perhaps this information could also help to develop further the serum-free conditions for human fetal liver-derived EPCs.

TABLE

Table 6.1. Percentages of cells selected by magnetically activated cell sorting (MACS) method using surface marker-specific antibodies in human fetal liver cell suspension

Surface markers	Cell populations before selection	Average of 3 Experiments
CD133/1 (direct selection using anti-human CD133/1 microbeads)	Cells after slow speed spin without Ficoll	0.4%
	Interface cells after Ficoll-density gradient separation, no slow-speed spin	0.48%
	Interface cells after Ficoll-density gradient separation of supernatant cells after slow-spin	0.22%
KDR (indirect selection with rabbit anti-human KDR IgG, Assay Designs)	$EpCAM^-$ interface cells after Ficoll-density gradient separation of supernatant cells after slow-speed spin	0.5%
KDR (indirect selection with mouse anti-human KDR IgG1, Cell Sciences)	Supernatant cells after slow-speed spin	0.33%
CD31 (direct selection using anti-human CD31 microbeads)	Supernatant cells after slow-speed spin	1.6%
CD105 (direct selection using anti-human CD105 microbeads)	Fibroblast-depleted interface cells after Ficoll-density gradient separation	4%
CD34 (indirect selection)	Interface cells after Ficoll-density gradient separation	1%

FIGURE LEGENDS

Figure 6.1. Fibroblast-depleted human fetal liver-derived cell population cultured on plastic

Most cells presented as fibroblast-like cells and expressed both αSMA and desmin indicating that they are mostly HpSTCs. Scale bar, 100 μm. Phase contrast (A) and fluorescent (B) images are different regions of the cell culture.

Figure 6.2. CD133/1^{+} cells selected by magnetically activated cell sorting (MACS) from human fetal livers

Phase contrast (A and C) and fluorescent (B and D) images of the CD133^{+} human fetal liver-derived cells cultured on plastic. Some cells are positive for NCAM (A and B), one marker of human hepatic stem cells (hHpSCs). A few cells are positive for EpCAM (C and D), another marker for hHpSCs. Scale bar, 100 μm.

Figure 6.3. *In vivo* and *in vitro* identification of angioblasts/endothelial cells from human fetal livers

KDR^{+} cells are located mostly at blood vessels including that associated with ductal plate (B). Negative control (A) was done with secondary antibody only. KDR^{+} cells selected by MACS method and cultured on plastic for 7 days were positive for CD31 (PECAM) (C and D), vWF (von Willebrand factor) (E and F) and KDR (G and H), three endothelial markers. Scale bar, 100 μm.

Figure 6.4. Cells selected by anti-human CD31 microbeads from human fetal livers

$CD31^+$ cells cultured in EGM-2 on plstic for 4 days strongly expressed CD31 (red) and vWF (green). Scale bar, 100 μm. Phase contrast (A) and fluorescent (B) images are different regions of the cell culture.

Figure 6.5 HUVECs cultured in two different media on plastic for 26 days.

HUVECs cultured on tissue culture plastic and in EGM-2 (A) versus in partially developed serum-free medium (B). Magnification, 10x.

Figure 6.1

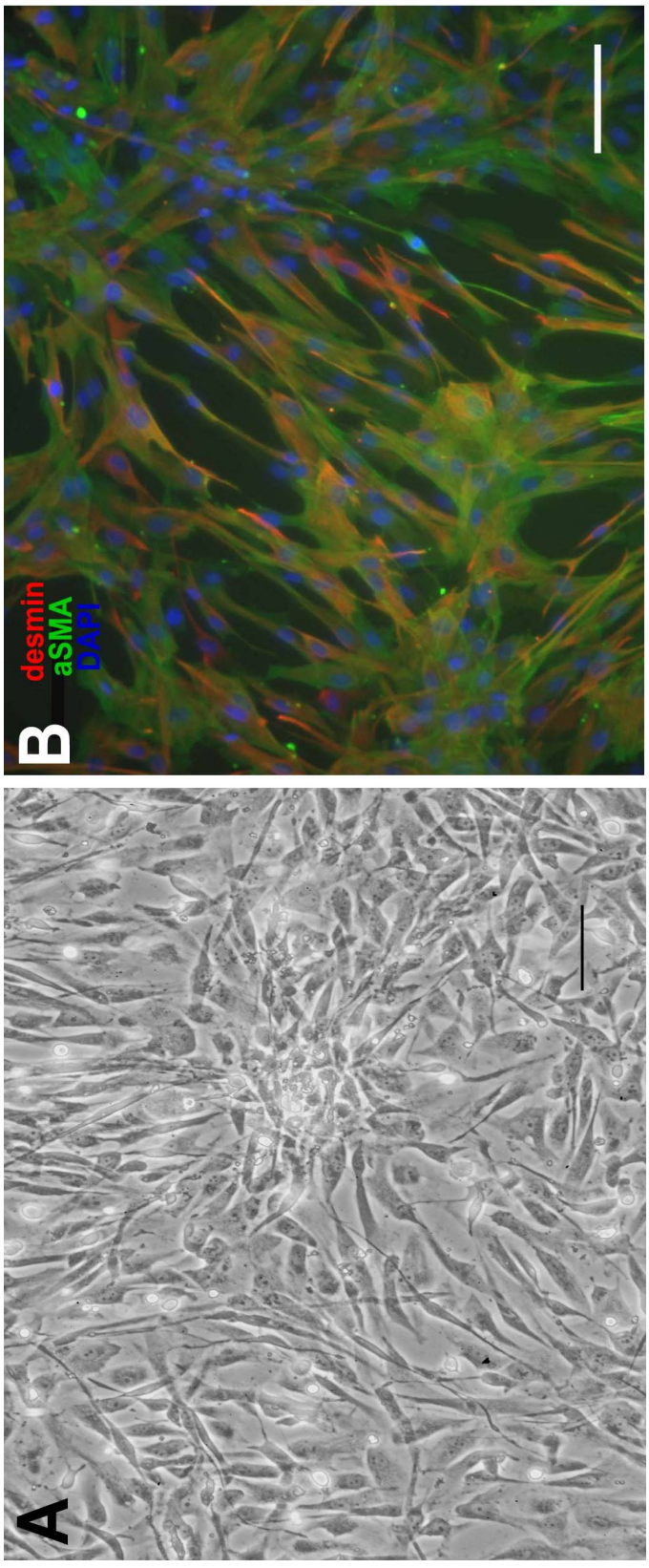


Figure 6.2

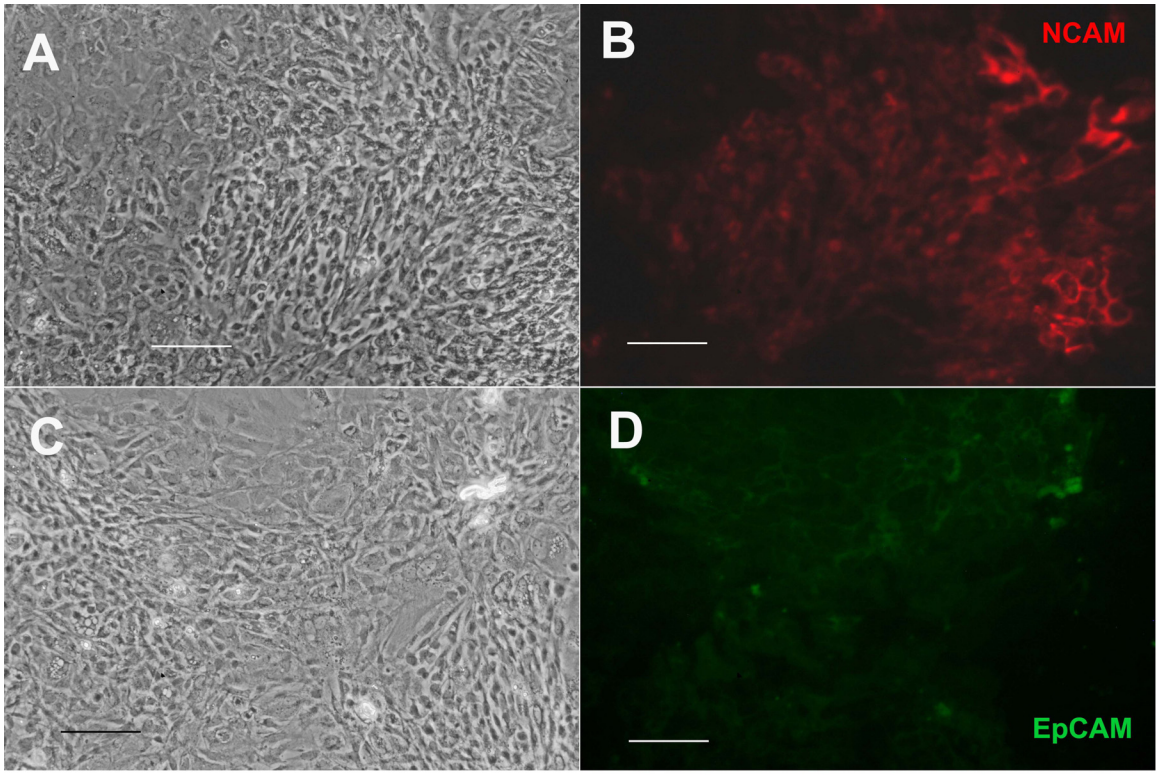


Figure 6.3

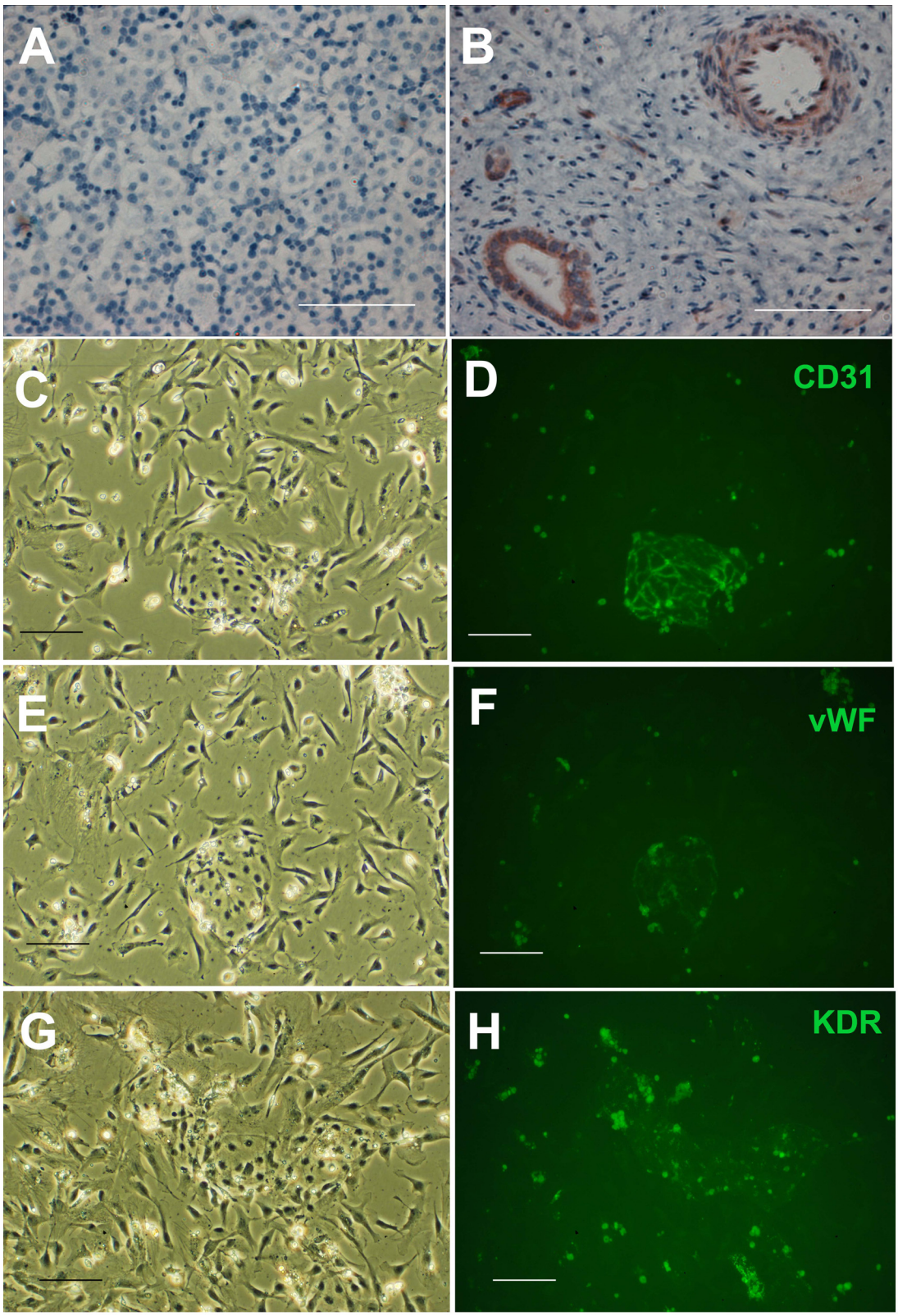


Figure 6.4

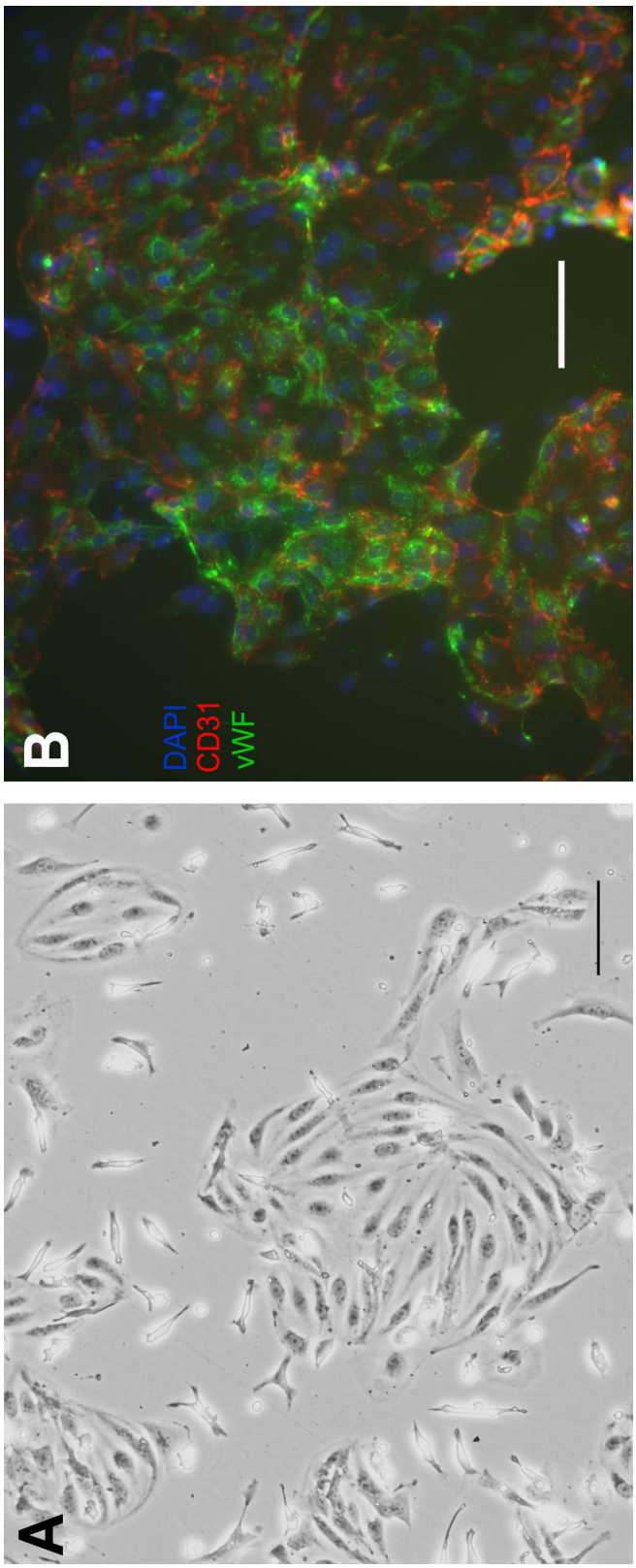
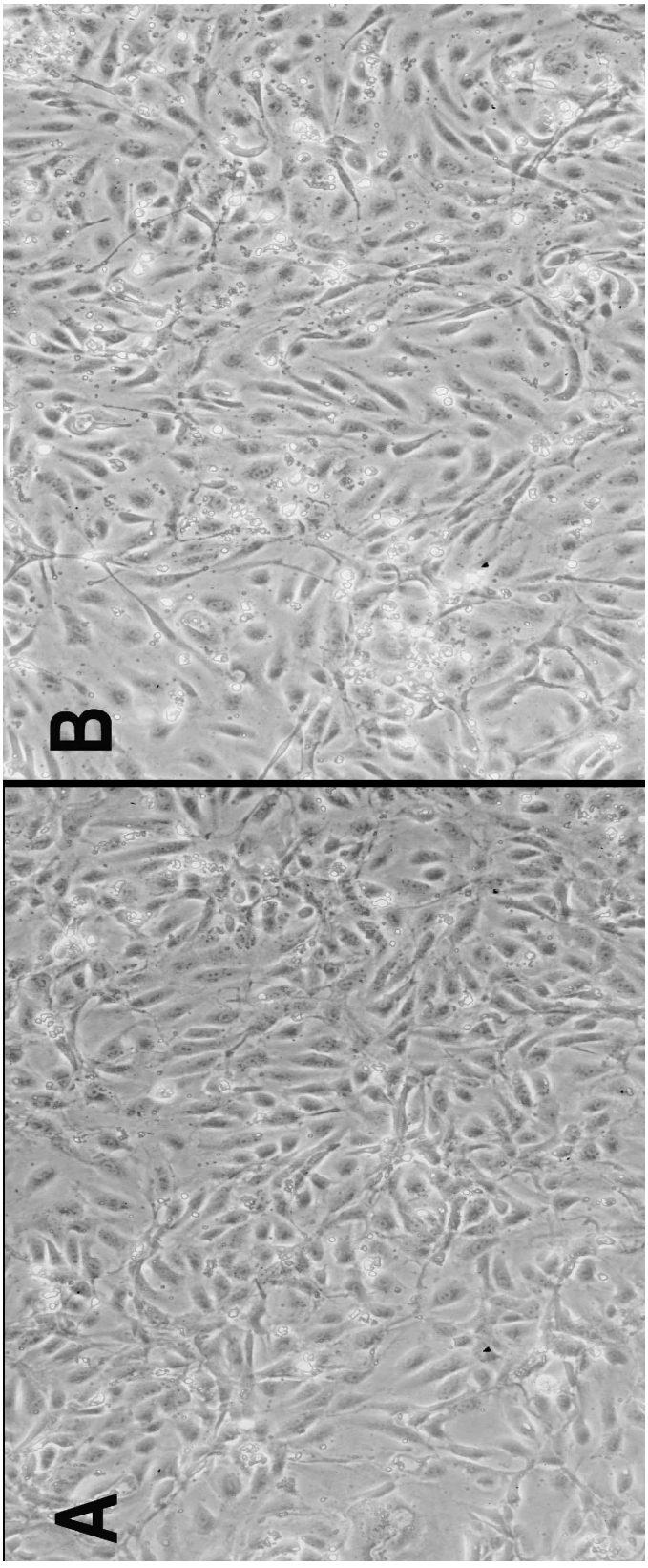


Figure 6.5



CHAPTER VII

MOUSE EMBRYONIC FIBROBLAST-SECRETED SIGNALS PROMOTE CELL GROWTH OF LIVER PROGENITORS

(Data was produced by Mr. Joshua Uronis, research analyst in the lab. Results were organized and discussion prepared by Hsin-Lei Yao.)

Mouse embryonic fibroblasts, STO cells from SIM (Sandos Inbred Mice) embryonic mouse stroma, have long been used as a feeder to support a variety of cells in culture, including embryonic stem cell (Martin and Evans, 1975), rodent hepatoblasts (Kubota and Reid, 2000), spermatogonial stem cells (Kubota et al, 2004; Kubota and Brinster, 2006); human hepatic stem cells and hepatoblasts (Schmelzer et al, 2007), and rodent HpSTCS (Kubuto et al, 2007). The molecular mechanism by which STO cells maintain the supported cell types *in vitro* is still understood though facets of the paracrine signaling are described below. Here we showed that a serum-free hormonally defined medium conditioned by STO cells was able to promote expansion of two hepatoblast-like, cloned cell lines, one from fetal rat livers, RTER6, and the other from a well differentiated human hepatoma, HepG2. Precise combinations of different STO cell-secreted matrix components (embryonic collagens, laminin, certain proteoglycans) and soluble signals, such as leukemia inhibitory factor (LIF), interleukin-6 or -11 (IL-6 or -11) and transforming growth factor-β1 (TGF-β1), may be crticial factors in regulation of the associated hepatic progenitor cells.

RESULTS

Effects of Mesenchymal Cells Conditioned Media on Colony Formation of Rat Hepatic Progenitor (rter6) Cells and Human Hepatoblastoma (HepG2) Cells

Rat hepatic progenitor cells (rter6) are unable to generate colonies efficiently on inert substrata such as plastic or on extracellular matrix-coated plates but will produce colonies if on STO feeders (Kubota and Reid, 2000). In this experiment rter6 cells were cultured on top of either mouse embryonic fibroblast cell line (STO cells) or human fetal lung fibroblast cell line (MRC5). The stocks of feeders were grown in serum supplemented medium until confluence, were rinsed thoroughly, and then switched to serum-free KM for 48 hours to generate a serum-free, conditioned medium. The colony numbers of rter6 cells co-cultured for 10 days with STO feeder cells in serum-free medim supplemented with STO conditioned medium increased 2.39-fold compared with KM (Table 7.1). Colony number of rter6 cells co-cultured for 10 days with MRC5 feeder cells in serum-free mouse embryonic fibroblast (STO) conditioned medium increased 1.57-fold compared with in hormonally-defined medium (HDM) (Table 7.1). In serum-free HDM, MRC5 cells seemed to promote more colony formation of rter6 cells compared with STO feeder. In STO conditioned medium, rter6 cells on both feeders had the similar colony formation ability.

Human hepatoblastoma (HepG2) cells can form colony on uncoated tissue culture plastic. Serum-free conditioned media from four different feeder cell types were used to test the colony formation of HepG2 cells. These four feeder cells include (1) mouse embryonic fibroblast cell line (STO cells); (2) human fetal lung fibroblast cell line (MRC5 cells); (3) immortalized adult human hepatic stellate cell line (h-tert-HSC); and (4) primary human fetal liver-derived stromal cells. Compared with hormonally-defined medium (HDM), the serum-

free STO conditioned medium increased colony formation of HepG2 cells, while the serum-free media conditioned by MRC5 cells, h-tert-HSC or primary human fetal liver-derived stromal cells had inhibitory effects on HepG2 cell colony formation.

Enzyme-Linked Immunoabsorbent Assay (ELISA) on Conditioned Media from STO Cells, Human Fetal Liver Cells, and Co-culture of Both

Concentration of 23 human cytokines, 17 mouse cytokines and 2 non-species specific cytokines were tested on cultured media conditioned by STO feeder, human fetal liver-derived progenitor cells and co-culture of both. For human cytokines, increased concentration of soluble interleukin-1 receptorα (IL-1Rα), interleukin-1α (IL-1α), IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, macrophage chemoattractant protein-2 (MCP-2), eotaxin, soluble tumor necrosis factor receptor2 (sTNF-R2), and regulated on activation, normal T-cell expressed, and presumably secreted (RANTES) was observed in co-culture compare with human fetal liver-derived progenitor cells cultured alone (Figure 7.1, Table 7.2). Those with decreased concentration in co-culture include interleukin-11 (IL-11), granulocye macrophage-colony stimulating factor (GM-CSF), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, soluble TNF receptor1 (sTNF-R1) and hepatocyte growth factor (HGF) (Figure 7.2).

For mouse cytokines, increased concentration of interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-10, IL-11, IL-12, granulocye macrophage-colony stimulating factor (GM-CSF), eotaxin, granulocyte-colony stimulating factor (G-CSF), macrophage inflammatory protein-1α (MIP-1α), interferon-γ (IFN-γ) and lipopolysaccharide-induced CXC chemokine (LIX) were observed in co-culture compared with STO cells cultured alone (Figure 7.2, Table 7.2). There

was no significant decrease of concentration of any mouse cytokines tested in co-culture. For non-species specific cytokines, transforming growth factor-β1 (TGF-β1) was increased in co-culture (532.3 pg/ml) compared with STO cells cultured alone (10.2 pg/ml) and with human fetal liver-derived progenitor cells cultured alone (22.3 pg/ml).

Human hepatic stem cells have been found to differentiate into hepatoblasts when co-cultured with STO feeder cells (Schmelzer et al, 2007 JEM). Combined concentration of human and mouse cytokines in co-culture revealed that interleukin-4 (IL-4), IL-5, IL-10, CXC chemokine (mouse keratinocyte-derived chemokines (KC) and human IL-8), eotaxin, macrophage chemoattractant protein-1 (MCP-1) and regulated on activation, normal T-cell expressed, and presumably secreted (RANTES) had dramatic increase (≥ 5-fold and > 50 pg/ml) of protein concentration compared with human fetal liver cells cultured alone (Table 7.2).

Effects of Soluble Cytokines on Colony Formation of Rat Hepatic Progenitor (rter6) Cells and Human Hepatocarcinoma (HepG2) Cells

Nine cytokines have been added individually into serum-free, hormonally-defined medium (HDM) used for 10 days to test their effect on colony formation of rat hepatic progenitor cells (rter6 cells) grown on STO feeder layers. Leukemia inhibitory factor (LIF, 0.5 ng/ml), interleukin-11 (IL-11, 10 ng/ml), and transforming growth factor-β1 (TGF-β1, 0.05 ng/ml) increased the colony number and colony area of rter6 cells compared with controls (Figure 7.3). Interleukin-6 (IL-6), interleukin-13 (IL-13), hepatocyte growth factor (HGF), growth related oncogene-α (GRO-α), macrophage inflammatory protein-1α (MIP-1α) and tumor necrosis factor-α (TNF-α) had no effect on colony formation of rter6 cells.

Several cytokines and candidate stimulatory molecules were added individually into HDM and STO conditioned medium to test their effects on colony formation of HepG2 cells. Hydrocortisone increased colony formation 25% in both HDM and STO conditioned medium compared with control. Insulin-like growth factor-II (IGF-II), interleukin-6 (IL-6), interleukin-11 (IL-11), interleukin-13 (IL-13), tumor necrosis factor-α (TNF-α), growth related oncogene (GRO; CXC chemokine), human growth hormones, and high density lipoproteins (HDL) had no effects on HepG2 cell colony formation. Transforming growth factor-β1 (TGF-β1) had inhibitory effect on HepG2 cell growth as well as survival. Epidermal growth factor (EGF) significantly decreased HepG2 colony formation and caused the cells to migrate away from colonies. Soon after separated from colonies, the dispersed cells died quickly.

DISCUSSION

Mouse embryonic fibroblast (STO) conditioned medium increased colony formation of both rat hepatic progenitor (rter6) cells and a well differentiated human hepatoma (HepG2) cells when compared with non-conditioned medium. This finding suggests soluble signals secreted by STO cells could promote proliferation of hepatic progenitors derived from two mammalian species *in vitro*. However, our previous study showed that primary human hepatic stem cells (hHpSCs) derived from human fetal livers cultured on top of STO feeder demonstrated slower cell growth and differentiated into hepatoblasts (Schmelzer et al., 2007 JEM). Two possible reasons could explain these conflictive results. HepG2 cells are derived from adult liver-derived hepatoma, thus representing an abnormal human cell type that is different than freshly isolated human fetal liver-derived HpSCs. Rter6 cells are derived from

fetal rat liver and are partially transformed to be able to establish them as a clonogenic cell line; they are also a different species than human. Some signals produced by STO cells that induce differentiation and reduce cell growth of primary human hepatic stem cells may have no effect on rter6 and HepG2 cells.

Interleukin-11 (IL-11) and leukemia inhibitory factor (LIF) promoted colony formation of rat hepatic progenitor (rter6) cells on top of STO feeder. IL-11 and LIF are two members of IL-6 cytokine superfamily. LIF has been shown to support mouse ES cells sufficiently *in vitro* in feeder-free condition (Smith et al., 1988; Williams et al., 1988). Our finding supports the positive role of IL-6 cytokine family in promoting growth of progenitor cells *in vitro*. The inhibitory effect of EGF on HepG2 cells compliments our previous study that EGF reduced colony formation of rat hepatoblasts but increased colony formation of diploid adult rat hepatocytes (Kubota and Reid, 2000).

TGF-beta1 increased colony number and area of rter6 cells on top of STO feeder, but inhibited cell growth of HepG2 cells on plastic. It seems that TGF-beta1 has biphasic effects on hepatic progenitor cells derived from two different species. However, the concentration used for stimulating HepG2 cells (5 ng/ml) was 100-fold higher than that for rter6 cells (0.05 ng/ml). It is possible that cytokine effect from TGF-beta1 is dosage dependent where limited amount of TGF-beta1 could promote proliferation of hepatic progenitor cells but too much dosage would have inhibitory effect on cell growth. There might be a threshold concentration of TGF-beta1 below which thehepatic progenitor cells could benefit from it.

This is the first report of large screening of protein concentration of over forty human and mouse cytokines produced by mouse embryonic fibroblast (STO) cell line and human hepatic stem cells *in vitro* using emzyme-linked immunoabsorbent assay (ELISA). Co-

culture of human hepatic stem cells (hHpSCs) and STO feeder cells induced higher expression of several human and mouse cytokines including in majority the inflammatory signals and some factors known to be hepatic growth stimulating. Interleukin-4 (IL-4) is one of the inflammatory cytokines that elevated dramatically in the co-culture. The biological activities of interleukin-4 (IL-4) and IL-13 overlap as they bind the same receptor, IL-13 receptor (IL-13R), a heterodimer consisting of IL-13Rα1 chain and IL-4 receptor α chain (IL-4α) (Mentink-Kane and Wynn, 2004; Umeshita-Suyama et al., 2000). Both IL-4 and IL-13 utilize IL-4α/Stat6 signaling pathway and mediate inflammation of the liver. Liver fibrosis is significantly reduced in the combined absence of IL-4 and IL-13 (Chiaramonte et al. 1999). Interleukin-5 (IL-5) and eotaxin are two inflammatory cytokines triggering liver fibrosis. Expression of IL-5 and eotaxin is induced by IL-4/Stat6 signaling pathway (Duran et al., 2004). Stat6 deficient mice had reduced serum IL-5 and eotaxin level (Jaruga et al., 2003).

Mouse keratinocyte-derived chemokines (KC) and human IL-8 are two CXC chemokines related to liver cirrhosis and inflammation process. Injection of recombinant adenoviruses carrying KC gene resulted in activation of proinflammatory genes in the liver such as IL-1α and IL-1β and stimulation of profibrotic genes including type I collagen, leading to massive liver necrosis (Stefanovic and Stefanovic, 2006). Overexpression of KC gene also upregulated TGF-β1 and alpha smooth muscle actin (αSMA) genes, indicating activated hepatic stellate cells. Macrophage chemoattractant protein-1 (MCP-1) is a cytokine involved in the attraction of monocytes and activated T lymphocytes. Upregulation of MCP-1 is associated with neutrophil infiltration in the lung and liver after trauma hemorrhage. Neutrophil infiltration is a critical step in the development of organ damage.

IL-11 is one of IL-6 cytokine family and has been shown to promote colony formation of rat hepatic progenitor cells co-cultured with STO feeder. Significant reduction of IL-11 concentration in the culture without enough compensation from other IL-6 family cytokines may have detrimental effects on hepatic progenitor cell growth. Elevation of these inflammatory cytokines and reduction of proliferative cytokine in the STO-hHpSCs co-culture medium may retard proliferation of hHpSCs and lineage restrict hHpSCs into more committed parenchymal cells.

In addition to the increase of inflammatory cytokines, anti-inflammatory cytokines and those proven to be therapeutic after hepatic injury were also elevated in STO-hHpSCs co-culture by STO cells. These include mouse interleukin-10 (IL-10), mouse interferon-γ (IFN-γ), mouse macrophage inflammatory protein-2 (MIP-2) and lipopolysaccharide-induced CXC chemokine (LIX). IL-10 is an anti-inflammatory cytokine (Kim et al., 2007). IFN-γ can diminish IL-13 signaling by rapidly mobilizing IL-13Rα2 to cell surface as non-signaling a decoy receptor for IL-13 (Daines and Hershey, 2002). LIX is a mouse homologue of human epithelial neutrophil activating protein (ENA-78), a CXC chemokine. ENA-78 and another CXC chemokine, MIP-2, are important for hepatic regeneration after liver injury (Ren et al., 2003). Co-elevation of inflammatory and therapeutic cytokines in the co-culture raises interesting questions: do those inflammatory cytokines trigger the expression of the therapeutic cytokines by STO to rescue hHpSCs from detrimental effects caused by the inflammatory cytokines? Or do hHpSCs after exposing to the inflammatory cytokines secret some messenger molecules to signal STO producing “rescuing” cytokines? The answers for above questions could provide helpful information for developing therapeutic methods for liver fibrosis and for improving bioreactor design in liver tissue engineering.

TABLES

Table 7.1. Effect of STO conditioned medium on colony formation of rter6 cells (rat cloned hepatoblast cell line) on STO or MRC5 feeder

On STO feeder (murine embryonic stroma):

Rter6 seeding density (cells/cm^2)		100	200	400	Average fold increase of colony no.
Average colony no.	HDM	7.25	15.25	27.25	2.39
	+ STO-CM	18	34.5	66	
Fold increase		2.49	2.26	2.42	
P-value		**0.0065	***0.0009	***0.0003	

On MRC5 feeder (human fetal lung stromal cell line):

Rter6 seeding density (cells/cm^2)		100	200	400	Average fold increase of colony no.
Average colony no.	HDM	14.75	23.5	42.5	1.57
	+ STO-CM	23	37.25	66.5	
Fold increase		1.56	1.59	1.56	
P-value not significant					

Table 7.2 ELISA analyses of conditioned medium from STO cells and from human fetal liver cells

Mouse cytokines	Concentration (pg/ml)		Human Cytokines	Concentration (pg/ml)		
					STO/hFLC co-culture	
	STO	STO/hFLC co-culture		hFLCs	Human cytokine	Human and mouse
TNF-α	0	1.4	TNF-α	0	0.5	1.9
IL-1β	0	3	IFN-γ	0.8	2.5	23.4
IL-12	0	11.3	IL-10	1	4	62.8
IL-2	7.7	12	IL-1β	3	4.8	7.8
IFN-γ	0	20.9	IL-5	0	7.5	133.7
GM-CSF	5.8	35.3	IL-12	1	8	19.3
IL-11	0	46.2	IL-2	0.8	10	22
IL-10	0	58.8	GM-CSF	73	11	46.3
Eotaxin	33.96	68.3	IL-13	0	16	n/a
G-CSF	2.6	118.7	MCP-2	10	18	n/a
IL-5	3.8	126.2	IL-1α	13.8	24.1	n/a
MIP-2	0	182	IL-4	5.2	34.3	317.8
IL-6	28.2	192.3	Eotaxin	15.5	34.4	102.7
MIP-1α	86.7	283.17	IL-1Rα	24	34.7	n/a
IL-4	2.3	283.5	IL-11	1221	43.1	89.3
LIX	299	499	MIP-1β	292	111	n/a
KC	800	794	sTNFR2	66	166	n/a
MCP-1	2095	2094	IL-8	172	177	971
			HGF	399.9	253.8	n/a
			sTNFR1	687	283	n/a
			TGF-β1	136.3	n/a	287.8
			IL-6	327	290	482.3

	RANTES	67	320	n/a
	MIP-1α	981	360	643.17
	MCP-1	539	531	2625

FIGURE LEGENDS

Figure 7.1. Comparison of human cytokines produced in co-culture and in human fetal liver cell culture.

Concentration (pg/ml) of human cytokines produced in human fetal liver cell single culture and in co-culture of STO feeder cells and human fetal liver cells at low (top) and high (bottom) levels.

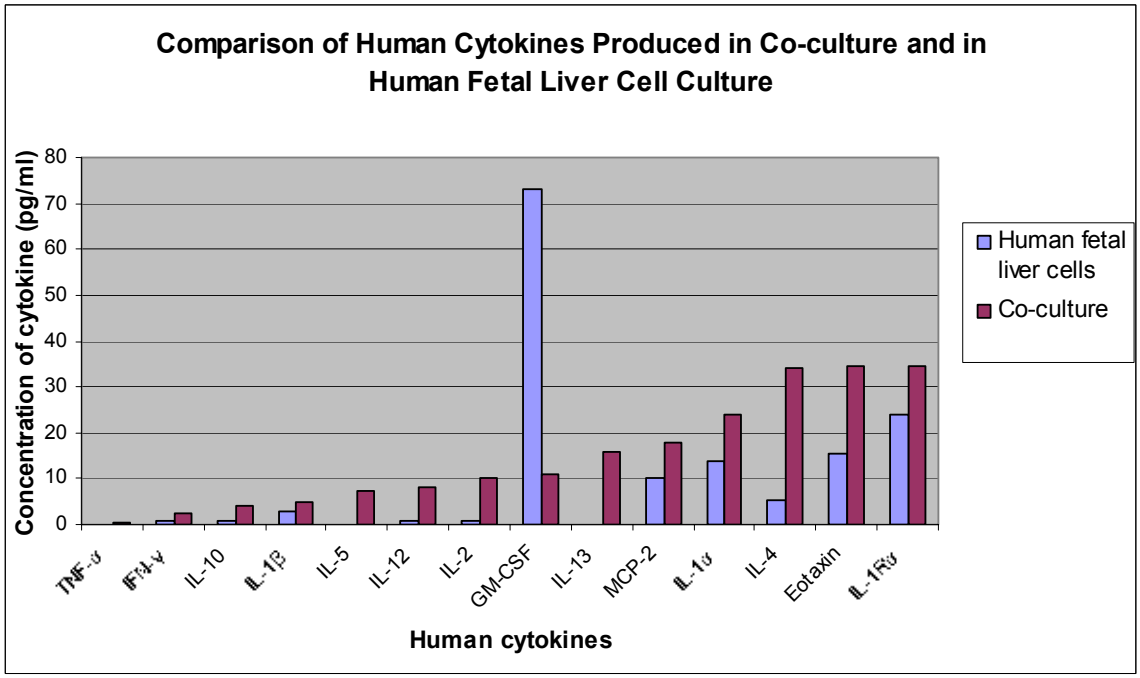
Figure 7.2. Comparison of mouse cytokines produced in co-culture and in STO feeder cell culture.

Concentration (pg/ml) of mouse cytokines produced in STO feeder cell single culture and in co-culture of STO feeder cells and human fetal liver cells at low (top) and high (bottom) levels.

Figure 7.3. Cytokine effects on colony formation of rter6 cells

Colony number (top) and area (in pixels; bottom) of rat hepatic progenitor (rter6) cells in hormonally defined medium (HDM) with or without cytokine are shown.

Figure 7.1


Comparison of Human Cytokines Produced in Co-culture and in Human Fetal Liver Cell Culture
Concentration of cytokine (pg/ml)
0
10
20
30
40
50
60
70
80
IL-10
IL-5
IL-12
IL-2
GM-CSF
IL-13
MCP-2
IL-4
Eotaxin
Human cytokines
Human fetal liver cells
Co-culture

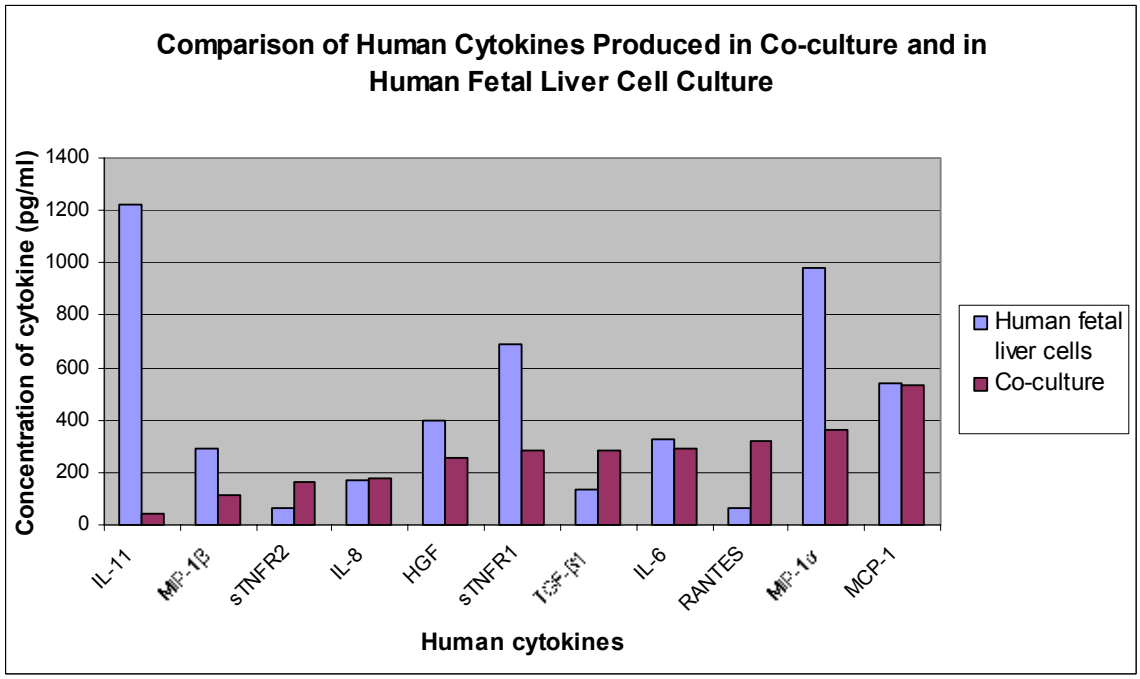
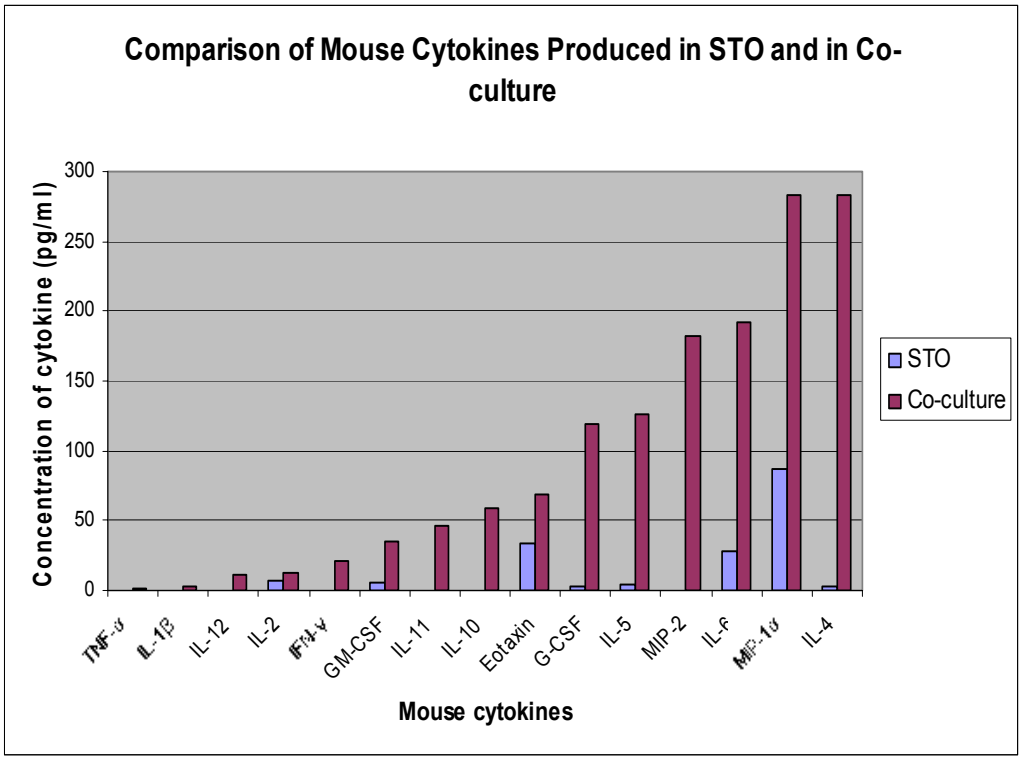

Comparison of Human Cytokines Produced in Co-culture and in Human Fetal Liver Cell Culture
Concentration of cytokine (pg/ml)
0
200
400
600
800
1000
1200
1400
IL-11
sTNFR2
IL-8
HGF
sTNFR1
IL-6
RANTES
MCP-1
Human cytokines
Human fetal liver cells
Co-culture

Figure 7.2


Comparison of Mouse Cytokines Produced in STO and in Co-culture
Concentration of cytokine (pg/ml)
300
250
200
150
100
50
0
GM-CSF
IL-11
IL-10
Eotaxin
G-CSF
IL-5
MIP-2
IL-4
Mouse cytokines
STO
Co-culture

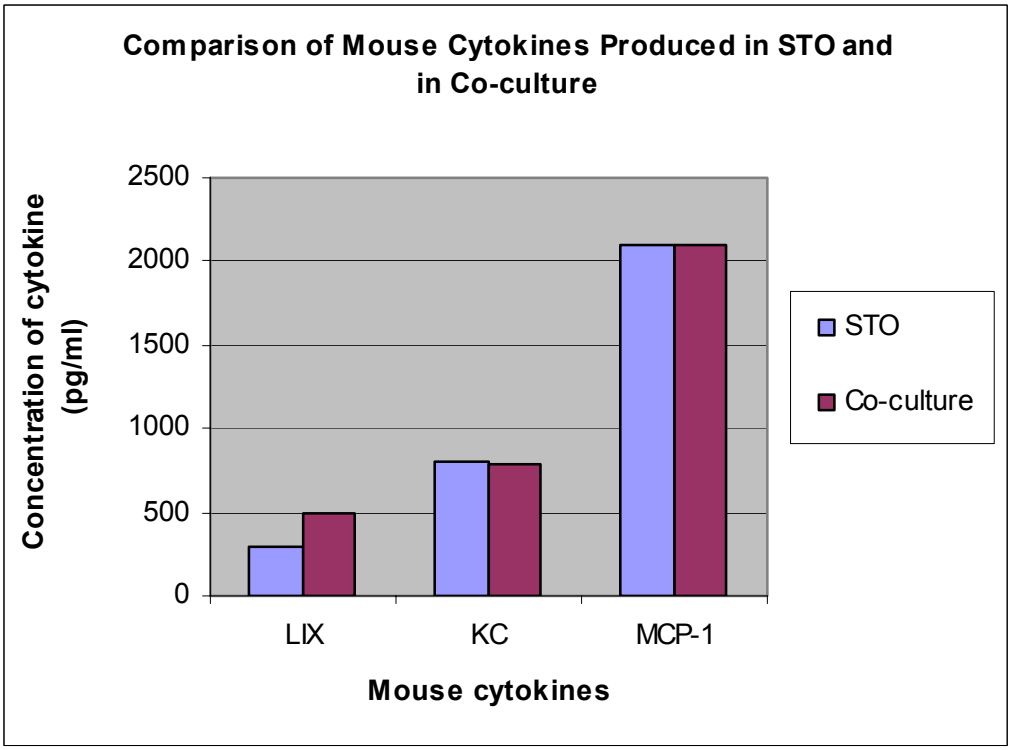
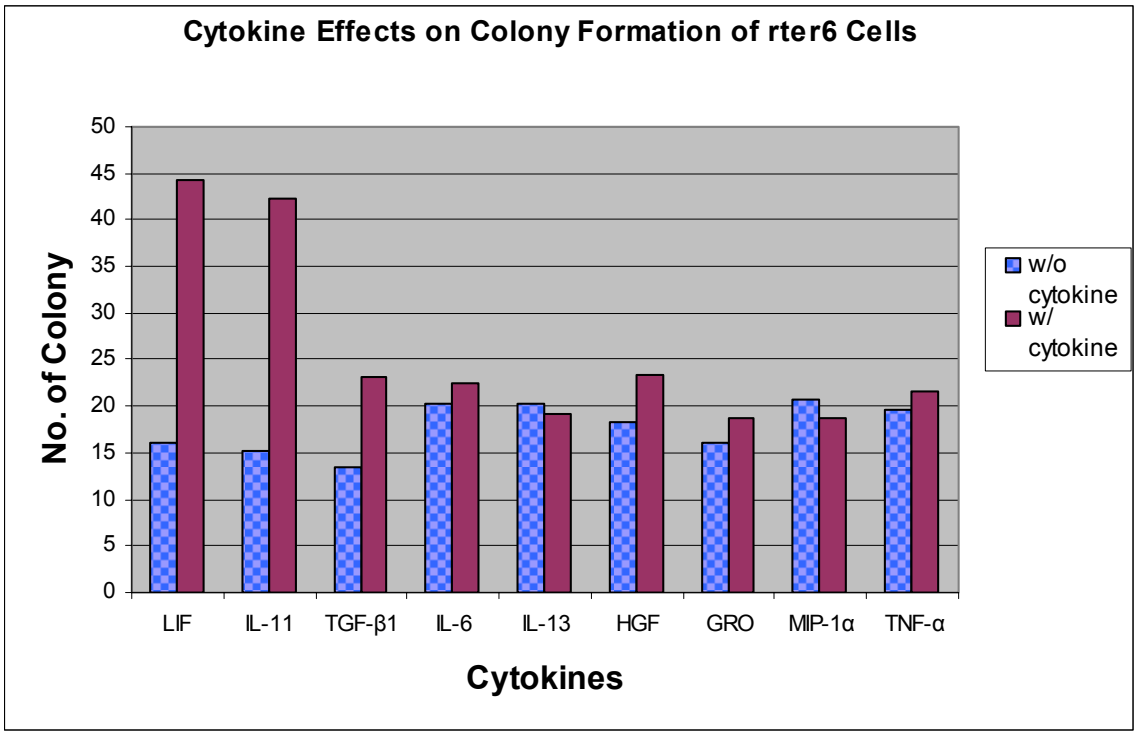
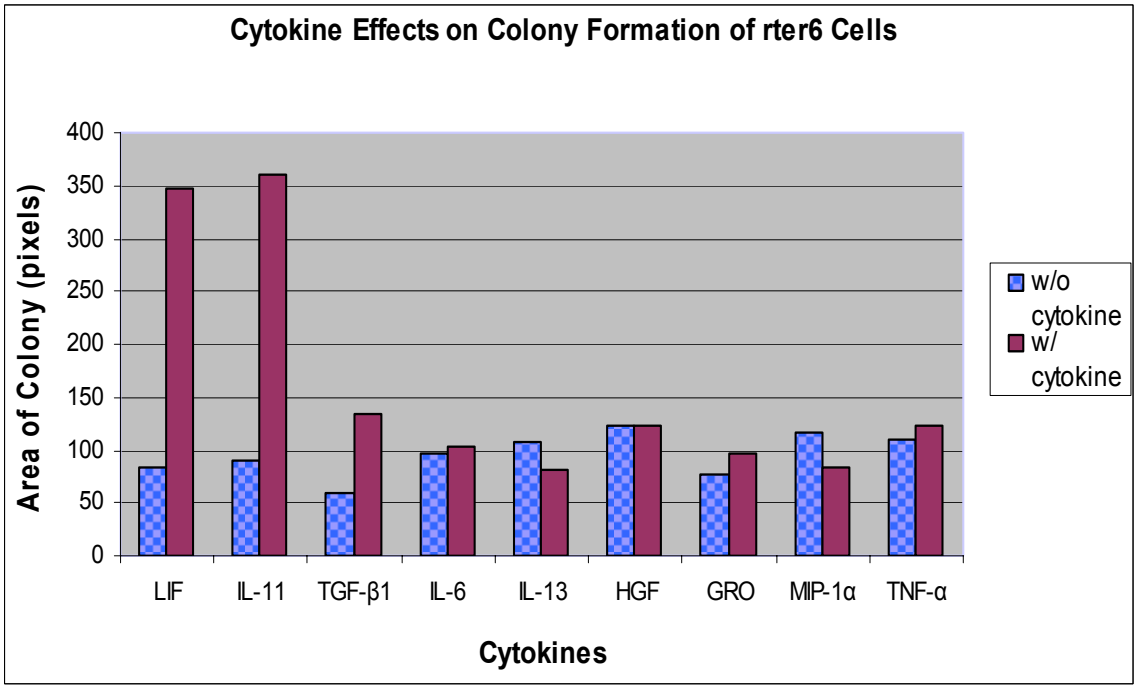

Comparison of Mouse Cytokines Produced in STO and in Co-culture
Concentration of cytokine (pg/ml)
2500
2000
1500
1000
500
0
LIX
KC
MCP-1
Mouse cytokines
STO
Co-culture

Figure 7.3


Cytokine Effects on Colony Formation of rter6 Cells
No. of Colony
50
45
40
35
30
25
20
15
10
5
0
LIF
IL-11
TGF-β1
IL-6
IL-13
HGF
GRO
MIP-1α
TNF-α
Cytokines
w/o cytokine
w/ cytokine


Cytokine Effects on Colony Formation of rter6 Cells
Area of Colony (pixels)
400
350
300
250
200
150
100
50
0
LIF
IL-11
TGF-β1
IL-6
IL-13
HGF
GRO
MIP-1α
TNF-α
Cytokines
w/o cytokine
w/ cytokine

CHAPTER VIII

FUTURE DIRECTIONS

Human hepatic stem cells (hHpSCs) differentiated into hepatoblasts when co-cultured with activated hepatic stellate cells (HpSTCs) from human fetal livers or with bone marrow-derived human mesenchymal stem cells (hMSCs) that contained HpSTCs. These feeder cells were assumed to release matrix and soluble signals that induce hHpSCs to differentiate. A high-throughput screening of different cytokines/growth factors present in the medium of co-culture and the medium of hHpSCs cultured alone using proteomic approaches, such as enzyme-linked immunoabsorbent assay (ELISA), were done on STO feeder cells and now could be done on feeders of angioblasts/endothelia versus HpSTC feeders to test which cytokines/growth factors are upregulated in the co-culture compared with single culture.

Primary cultures of HpSTCs isolated from human fetal liver were activated in serum-containing medium and grew rapidly in cultures. However, when cells were on culture plastic and in Kubota's Medium, the αSMA^+/$desmin^+$ cells surrounding hHpSCs colonies did not grow fast, and had minimal or no effect on differentiation of hHpSCs, thus indicating that they are quiescent hHpSCs. Activated hHpSTCs are known to be critical components associated with liver fibrosis. Understanding the activation mechanism by which the quiescent cells convert to cells releasing key matrix and soluble signals could help to develop a method to suppress the activation process in these non-parenchymal cells, leading to new therapeutic approaches in clinical treatment of liver fibrosis. It would be interesting to isolate

these hHpSTCs in quiescent form and to compare their gene expression profile with that of activated hHpSTCs. The cDNA microarray could be a useful tool to achieve this goal. Transcription factors involved in several inflammatory signaling pathways during liver fibrosis would be important target genes to be examined in the microarray analyses.

Primary cultures of HpSTCs derived from adult human or rodent livers did not support survival and growth of hHpSCs *in vitro* whereas quiescent hHpSTCs from fetal livers did. This raises a question: do fetal liver-derived hHpSTCs have different properties than adult liver-derived ones? Our paper on HpSTC precursors from E13 rat embryos (Kubota et al., 2007 in press) versus papers on HpSTCs from adult rodent livers indicate some critical distinctions that, presumably, are paralleled in distinctions to be found from HpSTCS from fetal versus adult human livers. Comparison of gene expression profiles from these model systems could provide answers to these questions.

Proinflammatory cytokines such as interleukin-4 (IL-4), IL-5 and eotaxin were upregulated by both hHpSCs and STO cells in co-culture and could be factors in the phenomenon we describe in which hHpSCs differentiate into hepatoblasts when co-cultured with STO feeders. Understanding the downstream signaling pathways of these cytokines inside the hHpSCs would help to unfold the differentiation mechanism by which hepatic stem cells (HpSCs) utilize. STO cells derived from mutant mouse embryos deficient in target cytokine/growth factor gene(s) may be used to see if hHpSCs grown on top of the genetically modified STO feeder could maintain their stemness in serum-free medium.

Serum supplementation in the first twenty four hours of plating could be a factor affecting the hHpSCs given that serum contains variety of growth factors and cytokines, and it varies from batch to batch in lots provided commercially. It would be interesting to test if

some of the proinflammatory cytokines in serum are factors in differentiation of hHpSCs either by direct assays of the factors on the cells or by blocking the effects of the factors to learn what happens in their absence. Antibodies or antiserum specific against particular cytokines could be used to quench binding efficiency of the target cytokines to their corresponding receptors on the hHpSCs, resulting in reduction of their signaling pathway.

Primary cultures of hHpSCs can be maintained for several months on culture plastic and in serum-free condition. Addition of the proinflammatory cytokines individually or in different combination into the serum-free medium and test of the differentiation state of the hHpSCs could be done to know if the target cytokine(s) can lineage restrict the hHpSCs to more committed cells. Alternatively transfection of the hHpSCs using adenoviruses carrying target cytokine gene(s) can overexpress the target cytokine(s) constitutively as autocrine signals, thus is a way to test the differentiation effects of particular cytokines.

My minimal success in isolation and long-term expansion of endothelial progenitor cells, angioblasts, from human fetal livers means that more robust purification methods and more highly defined, serum-free culture conditions need to be developed. Magnetically activated cell sorting (MACS) method can be used to isolate particular surface marker-specific cells, but without high purity and with other cell types contaminated in the selected cell population. Flow cytometry-activated cell sorting (FACS) method is the most accurate purification technology in today's biomedical research and could be used in the future to sort out the endothelial progenitor cells from human fetal livers. Our previous study showed that shear stress introduced by flow cytometry is detrimental to the survival of hepatic epithelial cells. However, unlike parenchymal cells, endothelial cells are constantly exposed to hemodynamic, thus are potentially capable of bearing shear stress. HUVECs grown on the

micropatterned strips of fibronectin and subjected to a pulsatile shear stress (12 ± 4 dyn/cm^2) parallel to micropatterned strips for 12 hours had significantly reduced apoptosis induced by micropatterned anisotropic substrata (Wu et al., 2007).

Any serum, even low concentrations of serum, that was used in my study is the probable could cause of overgrowth of fibroblastic cells in human fetal liver-derived endothelial cell cultures. The partially developed serum-free medium, developed using HUVECs as a model system, needs to be further developed so that it can be used for primary cultures of liver-derived endothelial cells. This would be helpful to reduce overgrowth of fibroblastic cells and to select the endothelial cells for long-term culture.

REFERENCES

Abelev, G.I. Alpha-fetoprotein in ontogenesis and its association with malignant tumors. Adv. Cancer Res. **14**, 295, 1971.

Abergel, A., Sapin, V., Dif, N., Chassard, C., Darcha, C., Marcand-Sauvant, J., Gaillard-Martinie, B., Rock, E., Dechelotte, P., and Sauvant, P. Growth arrest and decreasae of alpha-SMA and type I collagen expressionby palmitic acid in the rat hepatic stellate cell line PAV-1. Dig. Dis. Sci. **51**, 986, 2006.

Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature **439**, 599, 2006

Agelli, M., Dello Sbarba, P., Halay, E.D., Faris, R.A., Hixson, D.E., and Reid, L.M. Putative liver progenitor cells: conditions for long-term survival in culture. Histochem. J. **29**, 205, 1997.

Alison, M.R., Golding, M., Sarraf, C.E., Edwards, R.J., and Lalani, E.N. Liver damage in the rat induces hepatocyte stem cells from biliary epithelial cells. Gastroenterology **110**, 1182, 1996.

Alison, M.R., Vig, P., Russo, F., Bigger, B.W., Amofah, E., Themis, M., and Forbes, S. Hepatic stem cells: from inside and outside the liver? Cell Prolif. **37**, 1, 2004

Alvarez-Buylla, A., and Lim, D.A. For the long run: maintaining germinal niches in the adult brain. Neuron **41**, 683, 2004

Amit, M., and Itskovitz-Eldor, J. Feeder-free culture of human embryonic stem cells. Methods Enzymol. **420**, 37, 2006.

Amit, M., Margulets, V., Segev, H., Shariki, K., Laevsky, I., Coleman, R., and Itskovitz-Eldor, J. Human feeder layers for human embryonic stem cells. Biol. Reprod. **68**, 2150, 2003.

Anatskaya, O.V., Vinogradov, A.E., and Kudryavtsev, B.N. Hepatocyte polyploidy and metabolism/life-history traits: hypotheses testing. J. Theor. Biol. **168**, 191, 1994.

Ankoma-Sey, V., and Friedman, S.L. Hepatic stellate cells. In: Strain, A., and Diehl, A.M. eds. Liver Growth and Repair. London, England: Chapman & Hall, 1998, pp.512-537.

Anneren, C., Cowan, C.A., and Melton, D.A. The Src family of tyrosine kinases is important for embryonic stem cell self-renewal. J. Biol. Chem. **279**, 31590, 2004.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito ,K., Koh, G.Y., and Suda, T. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell **118**, 149, 2004.

Aruffo, A., Stamenkovic, I., Melnick, M. et al. CD44 is the principal cell surface receptor for hyaluronate. Cell. **61**, 1303, 1990.

Ashery-Padan, R., and Gruss, P. Pax6 lights-up the way for eye development. Curr. Opin. Cell Biol. **13**, 706, 2001.

Aubin, J.E. Autofluorescence of viable cultured mammalian cells. J. Histochem. Cytochem. **27**, 36, 1979.

Auerbach, R., Huang, H., and Lu, L. Hematopoietic stem cells in the mouse embryonic yolk sac. Stem Cells **14**, 269, 1996.

Auerbach, R., Wang, S.J., Yu, D., Gilligan, B., and Lu, L.S. Role of endothelium in the control of mouse yolk sac stem cell differentiation. Dev. Comp. Immunol. **22**, 333, 1998.

Austin, L., and Burgess, A.W. Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. J. Neurol. Sci. **101**, 193, 1991.

Baba, S., Fujii, H., Hirose, T. et al. Commitment of bone marrow cells to hepatic stellate cells in mouse. J. Hepatol. **40**, 255, 2004.

Bachem, M.G., Meyer, D., Melchior, R., Sell, K.M., and Gressner, A.M. Activation of rat liver perisinusoidal lipocytes by transforming growth factors derived from myofibroblastlike cells. A potential mechanism of self perpetuation in liver fibrogenesis. J. Clin. Invest. **89**, 19, 1992.

Bagley, R.G., Walter-Yohrling, J., Cao, X., Weber, W., Simons, B., Cook, B.P., Chartrand, S.D., Wang, C., Madden, S.L., and Teicher, B.A. Endothelial precursor cells as a model of tumor endothelium: characterization and comparison with mature endothelial cells. Cancer Res. **63**, 5866, 2003.

Balzar, M., Bakker, H.A., Briaire-de-Bruijn, I.H., Fleuren, G.J., Warnaar, S.O., and Litvinov, S.V. Cytoplasmic tail regulates the intercellular adhesion function of the epithelial cell adhesion molecule. Mol. Cell Biol. **18**, 4833, 1998.

Balzar, M., Winter, M.J., de Boer, C.J., and Litvinov, S.V. The biology of the 17-1A antigen (Ep-CAM). J. Mol. Med. **77**, 699, 1999.

Bardin, N., Anfosso, F., Masse, J.M., Cramer, E., Sabatier, F., Le Bivic, A., Sampol, J., and Dignat-George, F. Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion. Blood **98**, 3677, 2001.

Baudin, B., Bruneel, A., Bosselut, N., and Vaubourdolle, M. A protocol for isolation and culture of human umbilical vein endothelial cells. Nat. Protoc. 2, 481, 2007.

Benveniste, H., Einstein, G., Kim, K.R., Hulette, C., and Johnson, G.A. Detection of neuritic plaques in Alzheimer's disease by magnetic resonance microscopy. Proc. Natl. Acad. Sci. USA **96**, 14079, 1999.

Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M.T., Spring, J., Gallo, R.L., and Lose, E.J. Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. Annu. Rev. Cell Biol. **8**, 365, 1992.

Bhatia, M. AC133 expression in human stem cells. Leukemia **15**, 1685, 2001.

Bhushan, A., Itoh, N., Kato, A., Thiery, J.P., Czernichow, P., Bellusci, S., and Scharfmann, R. Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development **128**, 5109, 2001.

Blacker, K.L., Williams, M.L., and Goldyne, M. Mitomycin C-treated 3T3 fibroblasts used as feeder layers for human keratinocyte culture retain the capacity to generate eicosanoids. J. Invest. Dermatol. **89**, 536, 1987.

Blaner, W.S. Retinoid (Vitamin A) Metabolism and the liver. In: Arias, I.M., Boyer, J.L., Fausto, N. et al., eds. The liver: biology and pathology. New York: Raven Press. 1994

Blomhoff, R., Green, M.H., Berg, T. et al. Transport and storage of vitamin A. Science. **250**, 399, 1990.

Bongso, A., Fong, C.Y., Ng, S.C., and Ratnam, S. Isolation and culture of inner cell mass cells from human blastocysts. Hum. Reprod. **9**, 2110, 1994.

Boulton, R.A., Alison, M.R., Golding, M., Selden, C., and Hodgson, H.J. Augmentation of the early phase of liver regeneration after 70% partial hepatectomy in rats following selective Kupffer cell depletion. J. Hepatol. **29**, 271, 1998.

Braun, L., Mead, J.E., Panzica, M., Mikumo, R., Bell, G.I., and Fausto, N. Transforming growth factor beta mRNA increases during liver regeneration: a possible paracrine mechanism of growth regulation. Proc. Natl. Acad. Sci. U S A **85**, 1539, 1988.

Breuhahn, K., Longerich, T., and Schirmacher, P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. Oncogene **25**, 3787, 2006.

Brill, S., Holst, P., Sigal, S., Zvibel, I., Fiorino, A., Ochs, A., Somasundaran, U., and Reid, L.M. Hepatic progenitor populations in embryonic, neonatal, and adult liver. Proc. Soc. Exp. Biol. Med. **204**, 261, 1993.

Brill, S., Zvibel, I., and Reid, L.M. Maturation-dependent changes in the regulation of liver-specific gene expression in embryonal versus adult primary liver cultures. Differentiation **59**, 95, 1995.

Brooks, B., Bundred, N.J., Howell, A., Lang, S.H., and Testa, N.G. Investigation of mammary epithelial cell-bone marrow stroma interactions using primary human cell culture as a model of metastasis. Int. J. Cancer **73**, 690, 1997.

Burdon, T., Smith, A., and Savatier, P. Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol. **12**, 432, 2002.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A., Kronenberg, H.M., and Scadden, D.T. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature **425**, 841, 2003.

Cassiman, D., Barlow, A., Vander Borght, S. et al. Hepatic stellate cells do not derive from the neural crest. J. Hepatol. **44**, 1098, 2006.

Charlotte, F., Win, K.M., Preaux, A.M., Mavier, P., Dhumeaux, D., Zafrani, E.S., and Rosenbaum, J. Immunolocalization of heparin-binding growth factors (HBGF) types 1 and 2 in rat liver. Selective hyperexpression of HBGF-2 in carbon tetrachloride-induced fibrosis. J. Pathol. **169**, 471, 1993.

Chen, D., and McKearin, D. Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. Curr. Biol. **13**, 1786, 2003.

Cheng, L., Hammond, H., Ye, Z., Zhan, X., and Dravid, G. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells **21**, 131, 2003.

Chessebeuf, M., and Padieu, P. Rat liver epithelial cell cultures in a serum-free medium: primary cultures and derived cell lines expressing differentiated functions. In Vitro **20**, 780, 1984.

Chiaramonte, M.G., Donaldson, D.D., Cheever, A.W., and Wynn, T.A. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. J. Clin. Invest. **104**, 777, 1999.

Choo, A.B., Padmanabhan, J., Chin, A.C., and Oh, S.K. Expansion of pluripotent human embryonic stem cells on human feeders. Biotechnol. Bioeng. **88**, 321, 2004.

Christensen, J.L., Wright, D.E., Wagers, A.J. et al. Circulation and chemotaxis of fetal hematopoietic stem cells. PLoS Biol. **2**, E75, 2004.

Christiano, A.M. Epithelial stem cells: stepping out of their niche. Cell **118**, 530, 2004.

Corda, S., Samuel, J.L., and Rappaport, L. Extracellular matrix and growth factors during heart growth. Heart Fail. Rev. **5**, 119, 2000.

Couvelard, A., Bringuier, A.F., Dauge, M.C., Nejjari, M., Darai, E., Benifla, J.L., Feldmann, G., Henin, D., and Scoazec, J.Y. Expression of integrins during liver organogenesis in humans. Hepatology **27**, 839, 1998.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D., et al. Derivation of embryonic stem-cell lines from human blastocysts. N. Eng. J. Med. **350**, 1353, 2004.

Craig, C.E., Quaglia, A., Selden, C., Lowdell, M., Hodgson, H., and Dhillon, A.P. The histopathology of regeneration in massive hepatic necrosis. Semin. Liver Dis. **24**, 49, 2004.

Crawford, J.M. Development of the intrahepatic biliary tree. Semin. Liver Dis. **22**, 213, 2002.

Cressman, D.E., Greenbaum, L.E., DeAngelis, R.A., Ciliberto, G., Furth, E.E., Poli, V., and Taub, R. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science **274**, 1379, 1996.

Crosby, H.A., Nijjar, S., de Ville de Goyet, J., Kelly, D.A., and Strain, A.J. Progenitor Cells of the Biliary Epithelial Cell Lineage. Semin. Cell Dev. Biol. **13**, 397, 2002.

Cunha, G.R., Young, P., Christov, K., Guzman, R., Nandi, S., Talamantes, F., and Thordarson, G. Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. Acta Anat. **152**, 195, 1995.

Cunningham, B.A., Hemperly, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R., and Edelman, G.M.. Neural cell adhesion molecule:structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science **236**, 799, 1987.

Cvekl, A., and Tamm, E.R. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. BioEssays **26**, 374, 2004.

Daines, M.O., and Hershey, G.K.K. A novel mechanism by which interferon-γ can regulate interleukin (IL)-13 responses. J. Biol. Chem. **277**, 10387, 2002.

D'Amour, K.A., Agulnick, A.D., Eliazer, S., Kelly, O.G., Kroon, E., and Baetge, E.E. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat. Biotechnol. **23**, 1534, 2005.

Dan, Y.Y., Riehle, K.J., Lazaro, C., Teoh, N., Haque, J., Campbell, J.S., and Fausto, N. Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. Proc. Natl. Acad. Sci. USA **103**, 9912, 2006.

Date, M., Matsuzaki, K., Matsushita, M., Tahashi, Y., Sakitani, K., and Inoue, K. Differential regulation of activin A for hepatocyte growth and fibronectin synthesis in rat liver injury. J. Hepatol. **32**, 251, 2000.

Davis, G..E.., and Senger, D.R. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ. Res. **97**, 1093, 2005.

De Felici, M., and Dolci, S. Leukemia inhibitory factor sustains the survival of mouse primordial germ cells cultured on TM4 feeder layers. Dev. Boil. **147**, 281, 1991.

De Felici, M., and McLaren, A. In vitro culture of mouse primordial germ cells. Exp. Cell Res. **144**, 417, 1983.

Doetsch, F. A niche for adult neural stem cells. Curr. Opin. Genet. Dev. **13**, 543, 2003.

Dong, P.D., Munson, C.A., Norton, W., Crosnier, C., Pan, X., Gong, Z., Neumann, C.J., and Stainier, D.Y. Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. Nat. Genet. **39**, 397, 2007.

Donovan, P.J., Stott, D., Cairns, L.A., Heasman, J., and Wylie, C.C. Migratory and postmigratory mouse primordial germ cells behave differently in culture. Cell **44**, 831, 1986.

Dou, C.L., and Levine, J.M. Differential effects of glycosaminoglycans on neurite growth on laminin and L1 substrates. J. Neurosci. **15**, 8053, 1995.

Draper, J.S., Moore, H.D., Ruban, L.N., Gokhale, P.J., and Andrews, P.W. Culture and characterization of human embryonic stem cells. Stem Cells Dev. **13**, 325, 2004.

Draper, J.S., Smith, K., Gokhale, P., Moore, H.D., Maltby, E., Johnson, J., Meisner, L., Zwaka, T.P., Thomson, J.A., and Andrews, P.W. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat. Biotechnol. **22**, 53, 2004.

Duncan, S.A. Mechanisms controlling early development of the liver. Mech. Dev. **120**, 19, 2003.

Duran, A., Rodriguez, A., Martin, P., Serrano, M., Flores, J.M., Leitges, M., Diaz-Meco, M.T., and Moscat, J. Crosstalk between PKCζ and the IL4/Stat6 pathway during T-cell-mediated hepatitis. EMBO J **23**, 4595, 2004.

Dvorak, P., Dvorakova, D., and Hampl, A. Fibroblast growth factor signaling in embryonic and cancer stem cells. FEBS letters **580**, 2869, 2006.

Dzierzak, E., and Medvinsky, A. Mouse embryonic hematopoiesis. Trends Genet. **11**, 359, 1995.

Ehring, B., Biber, K., Upton, T.M., Plosky, D., Pykett, M., and Rosenzweig, M. Expansion of HPCs from cord blood in a novel 3D matrix. Cytotherapy **5**, 490, 2003.

Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C., and Le Douarin, N.M. Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. Proc. Natl. Acad. Sci. U S A **94**, 5141, 1997.

Enzan, H., Himeno, H., Hiroi, M. et al. Development of hepatic sinusoidal structure with special reference to the Ito cells. Microsc. Res. Tech. **39**, 336, 1997.

Eswarakumar, V.P., Lax, I., and Schlessinger, J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. **16**, 139, 2005.

Fabris, L., Strazzabosco, M., Crosby, H.A., Ballardini, G., Hubscher, S.G., Kelly, D.A., Neuberger, J.M., Strain, A.J., and Joplin, R. Characterization and isolation of ductular cells coexpressing neural cell adhesion molecule and Bcl-2 from primary cholangiopathies and ductal plate malformations. Am. J. Pathol. **156**, 1599, 2000.

Factor, V.M., Radaeva, S.A., and Thorgeirsson, S.S. Origin and fate of oval cells in Dipin-induced hepatocarcinogenesis in the mouse. Am. J. Pathol. **145**, 409, 1994.

Fair, J.H., Cairns, B.A., Lapaglia, M., Wang, J., Meyer, A.A., Kim, H., Hatada, S., Smithies, O., and Pevny, L. Induction of hepatic differentiation in embryonic stem cells by co-culture with embryonic cardiac mesoderm. Surgery **134**, 189, 2003.

Farini, D., Scaldaferri, M.L., Iona, S., La Sala, G., and De Felici, M. Growth factors sustain primordial germ cell survival, proliferation and entering into meiosis in the absence of somatic cells. Dev. Biol. **285**, 49, 2005.

Fausto, N., Lemire, J.M., and Shiojiri, N. Cell lineages in hepatic development and the identification of progenitor cells in normal and injured liver. Proc. Soc. Exp. Biol. Med. **204**, 237, 1993.

Fisher, H.W., and Puck, T.T. On the Functions of X-Irradiated "Feeder" Cells in Supporting Growth of Single Mammalian Cells. Proc. Natl. Acad. Sci. USA **42**, 900, 1956.

Forbes, A.J., Lin, H., Ingham, P.W., and Spradling, A.C. Hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in Drosophila. Development **122**, 1125, 1996.

Forbes, S., Vig, P., Poulsom, R., Thomas, H., and Alison, M. Hepatic stem cells. J. Pathol. **197**, 510, 2002.

Fornaro, M., Manzotti, M., Tallini, T., Slear, A.E., Bosari, S., Ruoslahti, E., and Languino, L.R. Beta1C integrin in epithelial cells correlates with a nonproliferative phenotype: forced expression of beta1C integrin inhibits prostate epithelial cell prololiferation. Am. J. Pathol. **153**, 1079, 1998.

Fuccillo, M., Joyner, A.L., and Fishell, G. Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development. Nat. Rev. Neurosci. **7**, 772, 2006.

Fuchs, E., Tumbar, T., and Guasch, G. Socializing with the neighbors: stem cells and their niche. Cell **116**, 769, 2004.

Fujimoto, I., Bruses, J.L., and Rutishauser, U. Regulation of cell adhesion by polysialic acid. Effects on cadherin, immunoglobulin cell adhesion molecule, and integrin function and independence from neural cell adhesion molecule binding or signaling activity. J. Biol. Chem. **276**, 31745, 2001.

Fujio, K., Evarts, R.P., Hu, Z., Marsden, E.R., and Thorgeirsson, S.S. Expression of stem cell factor and its receptor, c-kit, during liver regeneration from putative stem cells in adult rat. Lab. Invest. **70**, 511, 1994.

Fujiwara, T., Dehart, D.B., Sulik, K.K., and Hogan, B.L. Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. Development **129**, 4685, 2002.

Fukuda, K., and Yasugi, S. Versatile roles for sonic hedgehog in gut development. J. Gastroenterol. **37**, 239, 2002.

Fukuda, K., and Yasugi, S. The molecular mechanisms of stomach development in vertebrates. Dev. Growth Differ. **47**, 375, 2005.

Garcion, E., Halilagic, A., Faissner, A., and French-Constant, C. Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. Development **131**, 3423, 2004.

Gard, A.L., White, F.P., and Dutton, G.R. Extra-neural glial fibrillary acidic protein (GFAP) immunoreactivity in perisinusoidal stellate cells of rat liver. J. Neuroimmunol. **8**, 359, 1985.

Geerts, A., De Bleser, P., Hautekeete, M.L. et al. Fat-storing (Ito) cell biology. In: Arias, I.M., Boyer, J.L., Fausto, N. et al., eds. The liver: biology and pathology. New York: Raven Press. 1994.

Geerts, A., Niki, T., Hellemans, K. et al. Purification of rat hepatic stellate cells by side scatter-activated cell sorting. Hepatology. **27**, 590, 1998.

Geerts, A. On the origin of stellate cells: mesodermal, endodermal or neuro-ectodermal? J. Hepatol. **40**, 331, 2004.

Geerts, A. History, heterogeneity, developmental biology and functions of quiescent hepatic stellate cells. Semin. Liver Dis. 21, 311, 2001.

Geerts, A., Geuze, H.J., Slot, J.W., Voss, B., Schuppan, D., Schellinck, P., and Wisse, E. Immunogold localization of procollagen III, fibronectin and heparan sulfate proteoglycan on ultrathin frozen sections of the normal rat liver. Histochemistry **84**, 355, 1986.

Gehring, W.J., and Ikeo, K. Pax6: mastering eye morphogenesis and eye evolution. Trends Genet. **15**, 371, 1999.

Genbacev, O., Krtolica, A., Zdravkovic, T., Brunette, E., Powell, S., Nath, A., Caceres, E., McMaster, M., McDonagh, S., Li, Y., et al. Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders. Fertil. Steril. **83**, 1517, 2005.

Gerlach, J. Development of a Hybrid Liver Support System: A Review. Int. J. Artif. Organs

19, 645, 1996.

Gittes, G.K., Galante, P.E., Hanahan, D., Rutter, W.J., and Debase, H.T. Lineage-specific morphogenesis in the developing pancreas: Role of mesenchymal factors. Development **122**, 439, 1996.

Gomez, R.A., and Norwood, V.F. Recent advances in renal development. Curr. Opin. Pediatr. **11**, 135, 1999.

Gonzalez, A.M., Hill, D.J., Logan, A., Maher, P., and Baird, A. Complimentary distribution of fibroblast growth factor-2 (FGF-2) and FGF receptor-1 FGFR1 messenger RNA expression and protein presence in the mid-trimester human fetus. Ped. Res. **39**, 375, 1996.

Goridis, C., and J.F. Brunet. Neural Cell Adhesion Molecule (NCAM): structural diversity, function and regulation of expression. Semin. Cell Biol. **3**, 189, 1992.

Goss, J.A., Mangino, M.J., Callery, M.P., and Flye, M.W. Prostaglandin E2 downregulates Kupffer cell production of IL-1 and IL-6 during hepatic regeneration. Am. J. Physiol. **264**, G601, 1993.

Gouon-Evans, V., Boussemart, L., Gadue, P., Nierhoff, D., Koehler, C.I., Kubo, A., Shafritz, D.A., and Keller, G. BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. Nat. Biotechnol. **24**, 1402, 2006.

Greenwel, P., Rubin, J., Schwartz, M., Hertzberg, E.L., and Rojkind, M. Liver fat-storing cell clones obtained from a CCl4-cirrhotic rat are heterogeneous with regard to proliferation, expression of extracellular matrix components, interleukin-6, and connexin 43. Lab. Invest. **69**, 210, 1993.

Grisham, J.W., and Thorgeirsson, S.S. Liver stem cells. In: Potter, C.S. eds. Stem Cells. London, England: Academic Press. 1997, pp.233.

Gronthos, S., Franklin, D.M., Leddy, H.A., Robey, P.G., Storms, R.W., and Gimble, J.M. Surface protein characterization of human adipose tissue-derived stromal cells. J. Cell Physiol. **189**, 54, 2001.

Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R., and Zaret, K.S. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev. **10**, 1670, 1996.

Gupta, S., Gorla, G.R., and Irani, A.N. Hepatocyte transplantation: emerging insights into mechanisms of liver repopulation and their relevance to potential therapies. J. Hepatol. **30**, 162, 1999.

Hansen, D., Wilson-Berry, L., Dang, T., and Schedl, T. Control of the proliferation versus meiotic development decision in the C. elegans germline through regulation of GLD-1 protein accumulation. Development **131**, 93, 2004.

Haruna, Y., Saito, K., Spaulding, S., Nalesnik, M.A., and Gerber, M.A. Identification of bipotential progenitor cells in human liver development. Hepatology **23**, 476, 1996.

Hata, K., Kagami, H., Ueda, M., Torii, S., and Matsuyama, M. The characteristics of cultured mucosal cell sheet as a material for grafting; comparison with cultured epidermal cell sheet. Ann. Plast. Surg. **34**, 530, 1995.

Hayashi, K., Yasugi, S., and Mizuno, T. Pepsinogen gene transcription induced in heterologous epithelial-mesenchymal recombinations of chicken endoderms and glandular stomach mesenchymes. Development **103**, 725, 1998.

Hebrok, M., Kim, S.K., and Melton, D.A. Notochord repression of endodermal Sonic hedgehog permits pancreas development. Genes Dev. **12**, 1705, 1998.

Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G., and Schaper, F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem. J. **374**, 1, 2003.

Hellerbrand, Wang, S.C., Tsukamoto, H., Brenner, D.A., and Rippe, R.A. Expression of intracellular adhesion molecule 1 by activated hepatic stellate cells. Hepatology **24**, 670, 1996.

Hentsch, B., Lyons, I., Li, R. et al. Hlx homeo box gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut. Genes Dev. **10**, 70, 1996.

Hermann, A., Maisel, M., Liebau, S., Gerlach, M., Kleger, A., Schwarz, J., Kim, K.S., Antoniadis, G., Lerche, H., and Storch, A. Mesodermal cell types induce neurogenesis from adult human hippocampal progenitor cells. J. Neurochem. **98**, 629, 2006.

Hidalgo, A., Sanz-Rodriguez, F., Rodriguez-Fernandez, J.L. et al. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-dependent adhesion to fibronectin and VCAM-1 on bone marrow hematopoietic progenitor cells. Exp. Hematol. **29**, 345, 2001.

Hill, D.J. Development of the endocrine pancreas. Rev. Endocr. Metab. Dis. **6**, 229, 2005.

Hitraya, E.G., Tan, E.M., Rudnicka, L., and Jimenez, S.A. Expression of extracellular matrix genes in adult human dermal microvascular endothelial cells and their regulation by heparin and endothelial cell mitogens. Lab. Invest. **73**, 393, 1995.

Hombauer, H., and Minguell, J.J. Selective interactions between epithelial tumour cells and bone marrow mesenchymal stem cells. Br. J Cancer **82**, 1290, 2000.

Hovatta, O., Mikkola, M., Gertow, K., Stromberg, A.M., Inzunza, J., Hreinsson, J., Rozell, B., Blennow, E., Andang, M., and Ahrlund-Richter, L. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum. Reprod. **18**, 1404, 2003.

Hu, Z., Evarts, R.P., Fujio, K., Marsden, E.R., and Thorgeirsson, S.S. Expression of hepatocyte growth factor and c-met genes during hepatic differentiation and liver development in the rat. Am. J. Pathol. **142**, 1823, 1993.

Huang, H., and Auerbach, R. (1993). Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. Proc. Natl. Acad. Sci. USA **90**, 10110, 1993.

Infanger, M., Ulbrich, C., Baatout, S., Wehland, M., Kreutz, R., Bauer, J., Grosse, J., Vadrucci, S., Cogoli, A., Derradji, H., et al. Modeled gravitational unlodaing induced downregulation of endothelin-1 in human endothelial cells. J. Cell Biochem. [Epub ahead of print], 2007 Mar 5.

Jarnagin, W.R., Rockey, D.C., Koteliansky, V.E., Wang, S.S., and Bissell, D.M. Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. J. Cell Biol. **127**, 2037, 1994.

Jaruga, B., Hong, F., Sun, R., Radaeva, S., and Gao, B. Crucial role of IL-4/Stat6 in T cell-mediated hepatitis: up-regulating eotaxins and IL-5 and recruiting leukocytes. J. Immunol. **171**, 3233, 2003.

Jensen, U.B., Lowell, S., and Watt, F.M. The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis. Development **126**, 2409, 1999.

Jessberger, S., Clemenson Jr, G.D., and Gage, F.H. Spontaneous Fusion and Non-clonal Growth of Adult Neural Stem Cells. Stem Cells **25**, 871, 2006.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., and Verfaillie, C.M. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature **418**, 41, 2002.

Johnson, J.P. Cell adhesion molecules in the development and progression of malignant melanoma. Cancer Metastasis Rev. **18**, 345, 1999.

Jung, J., Zheng, M., Goldfarb, M., and Zaret, K.S. Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science **284**, 1998, 1999.

Kai, T., and Spradling, A. An empty Drosophila stem cell niche reactivates the proliferation of ectopic cells. Proc. Natl. Acad. Sci. USA **100**, 4633, 2003.

Kallunki, P., and Tryggvason, K. Human basement membrane heparan sulfate proteoglycan core protein: a 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. J. Cell Biol. **116**, 559, 1992.

Kanatsu-Shinohara, M., Miki, H., Inoue, K., Ogonuki, N., Toyokuni, S., Ogura, A., and Shinohara, T. Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol. Reprod. **72**, 985, 2005.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., and Shinohara, T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol. Reprod. **69**, 612, 2003.

Kannangai, R., Diehl, A.M., Sicklick, J., Rojkind, M., Thomas, D., and Torbenson, M. Hepatic angiomyolipoma and hepatic stellate cells share a similar gene expression profile. Hum. Pathol. **36**, 341, 2005.

Kawashima, H., Atarashi, K., Hirose, M., Hirose, J., Yamada, S., Sugahara, K., and Miyasaka, M. Oversulfated chondroitin/dermatan sulfates containing GlcAbeta1/IdoAalpha1-3GalNAc(4,6-O-disulfate) interact with L- and P-selectin and chemokines. J. Biol. Chem. **277**, 12921, 2002.

Kedinger, M., Simon-Assmann, P., Bouziges, F., and Haffen, K. Epithelial-mesenchymal interactions in intestinal epithelial differentiation. Scand. J. Gastroenterol. **Suppl 151**, 62, 1988.

Kedinger, M., Simon-Assmann, P.M., Lacroix, B., Marxer, A., Hauri, H.P., and Haffen, K. Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. Dev. Biol. **113**, 474, 1986.

Kemp, D.M., Thomas, M.K., and Habener, J.F. Developmental aspects of the endocrine pancreas. Rev. Endocr. Metab. Dis. **4**, 5, 2003.

Kiel, M.J., and Morrison, S.J. Maintaining hematopoietic stem cells in the vascular niche. Immunity **25**, 862, 2006.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell **121**, 1109, 2005.

Kiger, A.A., Jones, D.L., Schulz, C., Rogers, M.B., and Fuller, M.T. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science **294**, 2542, 2001.

Kikkawa, Y., Mochizuki, Y., Miner, J.H., and Mitaka, T. Transient expression of laminin alpha1 chain in regenerating murine liver: restricted localization of laminin chains and nidogen-1. Exp. Cell Res. **305**, 99, 2005.

Kikuchi, S., Griffin, C.T., Wang, S.S., and Bissell, D.M. Role of CD44 in epithelial wound repair: Migration of rat hepatic stellate cells utilizes hyaluronic acid and CD44v6. J. Biol. Chem. **280**, 15398, 2005.

Kim, C.W., Goldberger, O.A., Gallo, R.L., and Bernfield, M. Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue-, and development-specific patterns. Mol. Biol. Cell **5**, 797, 1994.

Kim, J.K., Chon, C.Y., Kim, J.H., Kim, Y.J., Cho, J.H., Bang, S.M., Ahn, S.H., Han, K., and Moon, Y.M. Changes in serum and ascetic monocyte chemotactic protein-1 (MCP-1) and IL-10 levels in cirrhotic patients with spontaneous bacterial peritonitis. J. Interferon Cytokine Res. **27**, 227, 2007.

Kim, S.K., and Melton, D.A. Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor. Proc. Natl. Acad. Sci. USA **95**, 13036, 1998.

King, F.J., Szakmary, A., Cox, D.N., and Lin, H. Yb modulates the divisions of both germline and somatic stem cells through piwi- and hh-mediated mechanisms in the Drosophila ovary. Mol. Cell **7**, 497, 2001.

Kishigami, S., and Mishina, Y. BMP signaling and early embryonic patterning. Cytokine & Growth Factor Rev. **16**, 265, 2005.

Kisseleva, T., and Brenner, D.A. Hepatic stellate cells and the reversal of fibrosis. J. Gastroenterol. Hepatol. **21**, S84, 2006.

Klein, R.S., Rubin, J.B., Gibson, H.D., DeHaan, E.N., Alvarez-Hernandez, X., Segal, R.A., and Luster, A.D. SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. Development **128**, 1971, 2001.

Knittel, T., Kobold, D., Saile, B. et al. Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. Gastroenterology. **117**, 1205, 1999.

Knittel, T., Fellmer, P., Muller, L., and Ramadori, G. Bone morphogenetic protein-6 is expressed in nonparenchymal liver cells and upregulated by transforming growth factor-beta 1. Exp. Cell Res. **232**, 263, 1997.

Kobayashi, C., Kagami, H., Kito, K., Ishikawa, K., Ebisawa, K., Ueda, M., and Terasaki, H. Selective and efficient culturing of retinal pigment epithelial cells using a feeder layer. Cytotherapy **7**, 427, 2005.

Kollet, O., Dar, A., Shivtiel, S., Kalinkovich, A., Lapid, K., Sztainberg, Y., Tesio, M., Samstein, R.M., Goichberg, P., Spiegel, A., Elson, A., and Lapidot, T. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat. Med. **12**, 657, 2006.

Kollet, O., Shivtiel, S., Chen, Y.Q. et al. HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. J. Clin. Invest. **112**, 160, 2003.

Kristensen, D.M., Kalisz, M., and Nielsen, J.H. Cytokine signalling in embryonic stem cells. APMIS **113**, 756, 2005.

Kubota, H., and Brinster, R.L. Technology insight: In vitro culture of spermatogonial stem cells and their potential therapeutic uses. Nat. Clin. Pract. Endocrinol. Metab. **2**, 99, 2006.

Kubota, H., and Reid, L.M. Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. Proc. Natl. Acad. Sci. U S A **97**, 12132, 2000.

Kubota, H., Avarbock, M.R., and Brinster, R.L. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol. Reprod. **71**, 722, 2004.

Kubota, H., Yao, H., and Reid, L.M. Identification and characterization of vitamin A-storing cells in fetal liver: Implications for functional importance of hepatic stellate cells in liver development and hematopoiesis. Stem Cells [Epub ahead of print], 2007 Jun 21.

Kubota, H., Storms, R.W., and Reid, L.M. Variant forms of alpha-fetoprotein transcripts expressed in human hematopoietic progenitors. Implications for their developmental potential towards endoderm. J. Biol. Chem. **277**, 27629, 2002.

Kulig, K.M., and Vacanti, J.P. Hepatic tissue engineering. Transpl. Immunol. **12**, 303, 2004

Kurpinski, K., Chu, J., Hashi, C., and Li, S. Anisotropic mechanosensing by mesenchymal stem cells. Proc. Natl. Acad. Sci. USA **103**, 16095, 2006.

Kutteh, W.H., Rainey, W.E., and Carr, B.R. Regulation of interleukin-6 production in human fetal Kupffer cells. Scand. J. Immunol. **33**, 607, 1991.

Kutteh, W.H., Rainey, W.E., Beutler, B., and Carr, B.R. Tumor necrosis factor-alpha and interleukin-1 beta production by human fetal Kupffer cells. Am. J. Obstet. Gynecol. **165**, 112, 1991.

Labbe, E., Letamendia, A., and Attisano, L. Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. Proc. Natl. Acad. Sci. USA **97**, 8358, 2000.

Lammert, E., Cleaver, O., and Melton, D. Induction of pancreatic differentiation by signals from blood vessels. Science **294**, 564, 2001.

Lammert, E., Cleaver, O., and Melton, D. Role of endothelial cells in early pancreas and liver development. Mech. Dev. **120**, 59, 2003.

Lapidot, T. Mechanism of Human Stem Cell Migration and Repopulation of NOD/SCID and B2mnull NOD/SCID Mice: The Role of SDF-1/CXCR4 Interactions. Ann. N. Y. Acad. Sci. **938**, 83, 2001.

Lee, J.M., Dedhar, S., Kalluri, R., and Thompson, E.W. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J. Cell Biol. **172**, 973, 2006.

Lee, C.K., and Piedrahita, J.A. Effects of growth factors and feeder cells on porcine primordial germ cells in vitro. Cloning **2**, 197, 2000.

Lee, J.B., Lee, J.E., Park, J.H., Kim, S.J., Kim, M.K., Roh, S.I., and Yoon, H.S. Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. Biol. Reprod. **72**, 42, 2005.

Lee, J.B., Song, J.M., Lee, J.E., Park, J.H., Kim, S.J., Kang, S.M., Kwon, J.N., Kim, M.K., Roh, S.I., and Yoon, H.S. Available human feeder cells for the maintenance of human embryonic stem cells. Reproduction **128**, 727, 2004.

Leith, J.T., Padfield, G., Faulkner, L.E., Quinn, P., and Michelson, S. Effects of feeder cells on the X-ray sensitivity of human colon cancer cells. Radiother. Oncol. **21**, 53, 1991.

Lemaigre, F., and Zaret, K.S. Liver development update: new embryo models, cell lineage control, and morphogenesis. Curr. Opin. Genet. Dev. **14**, 582, 2004.

Levy, V., Lindon, C., Harfe, B.D., and Morgan, B.A. Distinct stem cell populations regenerate the follicle and interfollicular epidermis. Dev. Cell **9**, 855, 2005.

Libbrecht, L., Cassiman, D., Desmet, V., and Roskams, T. Expression of neural cell adhesion molecule in human liver development and in congenital and acquired liver diseases. Histochem. Cell Biol. **116**, 233, 2001.

Lilja, H., Arkadopoulos, N., Blanc, P., Eguchi, S., Middleton, Y., Meurling, S., Demetriou, A.A., and Rozga, J. Fetal rat hepatocytes: isolation, characterization, and transplantation in the Nagase analbuminemic rats. Transplantation 64, 1240, 1997.

Lilja, H., Blanc, P., Demetriou, A.A., and Rozga, J. Response of cultured fetal and adult rat hepatocytes to growth factors and cyclosporine. Cell Transplant. **7**, 257, 1998.

Lim, D.A., Tramontin, A.D., Trevejo, J.M., Herrera, D.G., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron **28**, 713, 2000.

Lin, H. The stem-cell niche theory: lessons from flies. Nat. Rev. Genet. **3**, 931, 2002.

Lints, T.J., Hartley, L., Parsons, L.M. et al. Mesoderm-specific expression of the divergent homeobox gene Hlx during murine embryogenesis. Dev. Dyn. **205**, 457, 1996.

Lin, Y., Weisdorf, D.J., Solovey, A., and Hebbel, R.P. Origins of circulating endothelial cells and endothelial outgrowth from blood. J. Clin. Invest. **105**, 71, 2000.

Livak, K.J., and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delata Deelta C (T)) Method. Methods **25**, 402, 2001.

Lough, J., and Sugi, Y. Endoderm and heart development. Dev. Dyn. **217**, 327, 2000.

Lu, M., Grove, E.A., and Miller, R.J. Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. Proc. Natl. Acad. Sci. USA **99**, 7090, 2002.

Lu, L.S., Wang, S.J., and Auerbach, R. In vitro and in vivo differentiation into B cells, T cells, and myeloid cells of primitive yolk sac hematopoietic precursor cells expanded > 100-fold by coculture with a clonal yolk sac endothelial cell line. Proc. Natl. Acad. Sci. USA **93**, 14782, 1996.

Ludwig, T.E., Bergendahl, V., Levenstein, M.E., Yu, J., Probasco, M.D., and Thomson, J.A. Feeder-independent culture of human embryonic stem cells. Nat. Methods **3**, 637, 2006.

Macdonald, J.M., Xu, A.S.L., Kubota, H., LeCluyse, E., Hamillton, G., Liu, H., Rong, Y.W., Moss, N., Lodestro, A., Luntz, T., Wolfe, S.P., and Reid, L.M. Ex Vivo Maintenance of Cells from the Liver Lineage. In: Lanza, W.L., Langer, R., and Vacanti, J. eds. Methods of Tissue Engineering. San Diego, CA: Academic Press, 2002, pp.151-201.

Madison, B.B., Braunstein, K., Kuizon, E., Portman, K., Qiao, X.T., and Gumucio, D.L. Epithelial hedgehog signals pattern the intestinal crypt-villus axis. Development **132**, 279, 2005.

Maher, J.J. Cell-specific expression of hepatocyte growth factor in liver. Upregulation in sinusoidal endothelial cells after carbon tetrachloride. J. Clin. Invest. **9**1, 2244, 1993.

Maher, J.J. Rat hepatocytes and Kupffer cells interact to produce interleukin-8 (CINC) in the setting of ethanol. Am. J. Physiol. **269**, G518, 1995.

Maher, J.J., and McGuire, R.F. Extracellular matrix gene expression increases preferentially in rat lipocytes and sinusoidal endothelial cells during hepatic fibrosis in vivo. J. Clin. Invest. **86**, 1641, 1990.

Malik, R., Selden, C., and Hodgson, H. The role of non-parenchymal cells in liver growth. Semin. Cell Dev. Biol. **13**, 425, 2002.

Marquardt, H., and Heidelberger, C. Influence of "feeder cells" and inducers and inhibition of microsomal mixed-function oxidases on hydrocarbon-induced malignant transformation of cells derived from C3H mouse prostate. Cancer Res. **32**, 721, 1972.

Marra, F., Valente, A.J., Pinzani, M., and Abboud, H.E. Cultured human liver fat-storing cells produce monocyte chemotactic protein-1. Regulation by proinflammatory cytokines. J. Clin. Invest. **92**, 1674, 1993.

Marsden, E.R., Hu, Z., Fujio, K., Nakatsukasa, H., Thorgeirsson, S.S., and Evarts, R.P. Expression of acidic fibroblast growth factor in regenerating liver and during hepatic differentiation. Lab. Invest. **67**, 427, 1992.

Martin, M.J., Muotri, A., Gage, F., and Varki, A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat. Med. **11**, 228, 2005.

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokota, T. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. EMBO J. **18**, 4261, 1999.

Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K.S. Liver organogenesis promoted by endothelial cells prior to vascular function. Science **294**, 559, 2001.

Maxwell, P.H., Ferguson, D.J., Osmond, M.K., Pugh, C.W., Heryet, A., Doe, B.G., Johnson, M.H., and Ratcliffe, P.J. Expression of a homologously recombined erythopoietin-SV40 T antigen fusion gene in mouse liver: evidence for erythropoietin production by Ito cells. Blood **84**, 1823, 1994.

McClelland, R.E., Wauthier, E., Schmelzer, E., and Reid, L. Type III collagen substratum permissive for self-replication ex vivo of Human Hepatic Stem Cells. Submitted, 2007.

Mentink-Kane, M.M., and Wynn, T.A. Opposing roles for IL-13 and IL-13 receptor α2 in health and disease. Immunol. Rev. **202**, 191, 2004.

Michalopoulos, G.K., and DeFrances, M.C. Liver regeneration. Science **276**, 60, 1997.

Miyake, K., Medina, K., Ishihara, K. et al. A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. J. Cell Biol. **114**, 557, 1991.

Miyazono, K., Maeda, S., and Imamura, T. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev. **16**, 251, 2005.

Moore, K.A., and Lemischka, I.R. Stem cells and their niches. Science **311**, 1880, 2006.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M. et al. The purification and characterization of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. USA. **92**, 10302, 1995.

Morrison, S.J., Uchida, N., Weissman, I.L. The biology of hematopoietic stem cells. Annu. Rev. Cell Dev. Biol. **11**, 35, 1995.

Mullhaupt, B., Feren, A., Fodor, E., and Jones, A. Liver expression of epidermal growth factor RNA. Rapid increases in immediate-early phase of liver regeneration. J. Biol. Chem. 269, 19667, 1994.

Naito, M., Hasegawa, G., and Takahashi, K. Development, differentiation, and maturation of Kupffer cells. Microsc. Res. Tech. **39**, 350, 1997.

Narita, T., Saitoh, K., Kameda, T. et al. BMPs are necessary for stomach gland formation in the chicken embryo: a study using virally induced BMP-2 and Noggin expression. Development **127**, 981, 2000.

Naveiras, O., and Daley, G.Q. Stem cells and their niche: a matter of fate. Cell Mol. Life Sci. **63**, 760, 2006.

Neubauer, K., Knittel, T., Aurisch, S., Fellmer, P., and Ramadori, G. Glial fibrillary acidic protein--a cell type specific marker for Ito cells in vivo and in vitro. J. Hepatol. **24**, 719, 1996.

Niki, T., Pekny, M., Hellemans, K. et al. Class VI intermediate filament protein nestin is induced during activation of rat hepatic stellate cells. Hepatology. **29**, 520, 1999.

Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J., Denhardt, D.T., Bertoncello, I., Bendall, L.J., Simmons, P.J., and Haylock, D.N. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood **106**, 1232, 2005.

Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., and Kamalati, T. HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. Development 121, 2897, 1995.

Nohe, A., Keating, E., Knaus, P., and Petersen, N.O. Signal transduction of bone morphogenetic protein receptors. Cell. Signal. **16**, 291, 2004.

Nokubo, M., Nagy, I., Kitani, K. et al. Characterization of the autofluorescence of rat liver plasma membranes. Biochim. Biophys. Acta. **939**, 441, 1988.

Oh, S.H., Hatch, H.M., and Petersen, B.E. Hepatic oval 'stem' cell in liver regeneration. Semin. Cell Dev. Biol. 13, 405, 2002.

Ohlstein, B., Kai, T., Decotto, E., and Spradling, A. The stem cell niche: theme and variations. Curr. Opin. Cell Biol. **16**, 693, 2004.

Ohta, M., Sakai, T., Saga, Y., Aizawa, S., and Saito, M. Suppression of hematopoietic activity in tenascin-C-deficient mice. Blood **91**, 4074, 1998.

Osborn, L., Hession, C., Tizard, R. et al. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell **59**, 1203, 1989.

Overturf, K., Al-Dhalimy, M., Finegold, M., and Grompe, M. The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion. Am. J. Pathol. **155**, 2135, 1999.

Paik, Y.H., Lee, K.S., Lee, H.J., Yang, K., Lee, S.J., Lee, D.K., Han, K.H., Chon, C.Y., Lee, S.I., Moon, Y.M., et al. Hepatic stellate cells primed with cytokines upregulate inflammaation in response to peptidoglycan or lipoteichoic acid. Lab. Invest. **86**, 676, 2006.

Palmer, T.D., Willhoite, A.R., and Gage, F.H. Vascular niche for adult hippocampal neurogenesis. J. Comp. Neurol. **425**, 479, 2000.

Papayannopoulou, T., Craddock, C., Nakamoto, B. et al. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. Proc. Natl. Acad. Sci. USA. **92**, 9647, 1995.

Peichev, M., Naiyer, A.J., Pereira, D., Zhu, Z., Lane, W.J., Williams, M., Oz, M.C., Hicklin, D.J., Witte, L., Moore, M.A., et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood **95**, 952, 2000.

Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D., and Lapidot, T. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science **283**, 845, 1999.

Pellegrini, G., Traverso, C.E., Franzi, A.T., Zingirian, M., Cancedda, R., and De Luca, M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet **349**, 990, 1997.

Perez-Moreno, M., Jamora, C., and Fuchs, E. Sticky business: orchestrating cellular signals at adherens junctions. Cell **112**, 535, 2003.

Perez-Pomares, J.M., and Munoz-Chapuli, R. Epithelial-mesenchymal transitions: a mesodermal cell strategy for evolutive innovation in Metazoans. Anat. Rec. **268**, 343, 2002.

Pesce, M., Farrace, M.G., Piacentini, M., Dolci, S., and De Felici, M. Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). Development **118**, 1089, 1993.

Petersen, B.E., Goff, J.P., Greenberger, J.S. et al. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. Hepatology. **27**, 433, 1998.

Petersen, B.E., Grossbard, B., Hatch, H., Pi, L., Deng, J., and Scott, E.W. Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. Hepatology **37**, 632, 2003.

Pierce, A., Lyon, M., Hampson, I.N., Cowling, G.J., and Gallagher, J.T. Molecular cloning of the major cell surface heparan sulfate proteoglycan from rat liver. J. Biol. Chem. **267**, 3894, 1992.

Pinzani, M., Abboud, H.E., and Aron, D.C. Secretion of insulin-like growth factor-I and binding proteins by rat liver fat-storing cells: regulatory role of platelet-derived growth factor. Endocrinology **127**, 2343, 1990.

Pinzani, M., Abboud, H.E., Gesualdo, L., and Abboud, S.L. Regulation of macrophage colony-stimulating factor in liver fat-storing cells by peptide growth factors. Am. J. Physiol. **262**, C876, 1992.

Plaschke-Schlutter, A., Behrens, J., Gherardi, E., and Birchmeier, W. Characterization of the scatter factor/hepatocyte growth factor gene promoter. J. Biol. Chem. **270**, 830, 1995.

Potgens, A.J., Schmitz, U., Kaufmann, P.M., and Frank, H.G. Monoclonal antibody CD133-2 (AC141) against hematopoietic stem cell antigen CD133 shows crossreactivity with cytokeratin 18. J. Histochem. Cytochem. **50**, 1131, 2002.

Powell, T.M., Paul, J.D., Hill, J.M., Thompson, M., Benjamin, M., Rodrigo, M., McCoy, J.P., Read, E.J., Khuu, H.M., Leitman, S.F., et al. Granulocyte colony-stimulating factor mobilizes functional endothelial progenitor cells in patients with coronary artery disease. Arterioscler. Thromb. Vasc. Biol. **25**, 296, 2005.

Puck, T.T., and Marcus, P.I. A Rapid Method for Viable Cell Titration and Clone Production with Hela Cells in Tissue Culture: the Use of X-Irradiated Cells to Supply Conditioning Factors. Proc. Natl. Acad. Sci. USA **41**, 432, 1955.

Puck, T.T., Marcus, P.I., and Cieciura, S.J. Clonal growth of mammalian cells in vitro; growth characteristics of colonies from single HeLa cells with and without a feeder layer. J. Exp. Med. **103**, 273, 1956.

Quesenberry, P.J., Becker, P.S. Stem cell homing: Rolling, crawling, and nesting. Proc. Natl. Acad. Sci. USA **95**, 15155, 1998.

Rai, R.M., Loffreda, S., Karp, C.L., Yang, S.Q., Lin, H.Z., and Diehl, A.M. Kupffer cell depletion abolishes induction of interleukin-10 and permits sustained overexpression of tumor necrosis factor alpha messenger RNA in the regenerating rat liver. Hepatology **25**, 889, 1997.

Ray, J., and Gage, F.H. Differential properties of adult rat and mouse brain-derived neural stem/progenitor cells. Mol. Cell. Neurosci. **31**, 560, 2006.

Reber, L., Da Silva, C.A., and Frossard, N. Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. Eur. J. Pharmacol. **533**, 327, 2006.

Reid, L.M., Fiorino, A.S., Sigal, S.H., Brill, S., and Holst, P.A. Extracellular matrix gradients in the space of Disse: relevance to liver biology. Hepatology **15**, 1198, 1992.

Reid, L.M., Agelli, M., and Ochs, A. Method of expanding hepatic precursor cells. US Patent # 5,576,207 Date of application: August 7, 1991.

Ren, X., Carpenter, A., Hogaboam, C., and Colletti, L. Mitogenic properties of endogenous and pharmacological doses of macrophage inflammatory protein-2 after 70% hepatectomy in the mouse. Am. J. Pathol. **163**, 563, 2003.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat. Biotechnol. **18**, 399, 2000.

Reyes, M., Dudek, A., Jahagirdar, B., Koodie, L., Marker, P.H., and Verfaillie, C.M. Origin of endothelial progenitors in human postnatal bone marrow. J. Clin. Invest. **109**, 337, 2002.

Rhee, H., Polak, L., Fuchs, E. Lhx2 Maintains Stem Cell Character in Hair Follicles. Science **312**, 1946, 206.

Rheinwald, J.G. Serial cultivation of normal human epidermal keratinocytes. Methods Cell Biol. **21A**, 229, 1980.

Rheinwald, J.G., and Green, H. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell **6**, 317, 1975.

Richards, M., Fong, C.Y., Chan, W.K., Wong, P.C., and Bongso, A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat. Biotechnol. **20**, 933, 2002.

Richards, M., Tan, S., Fong, C.Y., Biswas, A., Chan, W.K., and Bongso, A. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem cells **21**, 546, 2003.

Rizvi, A.Z., and Wong, M.H. Epithelial stem cells and their niche: there's no place like home. Stem Cells **23**, 150, 2005.

Roberts, D.J., Johnson, R.L., Burke, A.C., Nelson, C.E., Morgan, B.A., and Tabin, C. Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. Development **121**, 3163, 1995.

Roberts, D.M., Kearney, J.B., Johnson, J.H., Rosenberg, M.P., Kumar, R., and Bautch, V.L. The VEGF receptor flt-1 (VEGFR-1) modulates flk-1 (VEGFR-2) signaling during blood vessel formation. Am. J. Pathol. **164**, 1531, 2004.

Robinson, G.W., and Hennighausen, L. Inhibins and activins regulate mammary epithelial cell differentiation through mesenchymal-epithelial interaction. Development **124**, 2701, 1997.

Robinson, G.W., Karpf, A.B.C., and Kratochwil, K. Regulation of mammary gland development by tissue interaction. J. Mammary Gland Biol. Neoplasia. **4**, 9, 1999.

Rose-John, S. GP130 stimulation and the maintenance of stem cells. Trends Biotechnol. **20**, 417, 2002.

Rosen, G.D., Sanes, J.R., LaChance, R. et al. Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. Cell. **69**, 1107, 1992.

Rosenzweig, M., Pykett, M., Marks, D.F., and Johnson, R.P. Enhanced maintenance and retroviral transduction of primitive hematopoietic progenitor cells using a novel three-dimensional culture system. Gene therapy **4**, 928, 1997.

Roskams, T., Moshage, H., De Vos, R., Guido, D., Yap, P., and Desmet, V. Heparan sulfate proteoglycan expression in normal human liver. Hepatology **21**, 950, 1995.

Roskams, T., Yang, S.Q., Koteish, A., Durnez, A., DeVos, R., Huang, X., Achten, R., Verslype, C., and Diehl, A.M. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. Am. J. Pathol. **163**, 1301, 2003.

Roskams, T.A., Theise, N.D., Balabaud, C., Bhagat, G., Bhathal, P.S., Bioulac-Sage, P., et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology **39**, 1739, 2004.

Roskams, T., De Vos, R., Van Eyken, P., Myazaki, H., Van Damme, B., and Desmet, V. Hepatic OV-6 expression in human liver disease and rat experiments: evidence for hepatic progenitor cells in man. J. Hepatol. **29**, 455, 1998.

Roskams, T.A., Theise, N.D., Balabaud, C., Bhagat, G., Bhathal, P.S., Bioulac-Sage, P., Brunt, E.M., Craqford, J.M., Crosby, H.A., Desmet, V., et al. Nomenclature of the Finer Branches of the biliary Tree: Canals, Ductules, and Ductular Reactions in Human Livers. Hepatology **39**, 1739, 2004.

Roskoski, R., Jr. Structure and regulation of Kit protein-tyrosine kinase--the stem cell factor receptor. Biochem. Biophys. Res. Commun. **338**, 1307, 2005.

Rossi, J.M., Dunn, N.R., Hogan, B.L., and Zaret, K.S. Distinct mesodermal signals, including BMPs form the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev. **15**, 1998, 2001.

Rubin, L.L., and de Sauvage, F.J. Targeting the Hedgehog pathway in cancer. Nat. Rev. Drug Discov. **5**, 1026, 2006.

Ruoslahti, E., and Yamaguchi, Y. Proteoglycans as modulators of growth factor activities. Cell **64**, 867, 1991.

Ryu, B.Y., Kubota, H., Avarbock, M.R., and Brinster, R.L. Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc. Natl. Acad. Sci. USA **102**, 14302, 2006.

Sakakur, T., Kusano, I., Kusakabe, M., Inaguma, Y., and Nishizuka, Y. Biology of mammary fat pad in fetal mouse: capacity to support development of various fetal epithelia in vivo. Development **100**, 421, 1987.

Sakakur, T., Sakagami, Y., and Nishizuka, Y. Persistence of responsiveness of adult mouse mammary gland to induction by embryonic mesenchyme. Dev. Biol. **72**, 201, 1979.

Sakurai, H., and Nigam, S.K. In vitro branching tubulogenesis: Implications for developmental and cystic disorders, nephron number, renal repair, and nephron engineering. Kidney Int. **54**, 14, 1998.

Sales, V.L., Engelmayr, G.C.J., Mettler, B.A., Johnson, J.A.J., Sacks, M.S., and Mayer, J.E.J. Transforming growth factor-beta 1 modulates extracellular matrix production, proliferation, and apoptosis of endothelial progenitor cells in tissue-engineering scaffolds. Circ. Res. **114**, 1193, 2006.

Santhanam, A.V., Smith, L.A., He, T., Nath, K.A., and Katusic, Z.S. Endothelial Progenitor Cells Stimulate Cerebrovascular Production of Prostacyclin By Paracrine Activation of Cyclooxygenase-2. Circ. Res. **100**, 1379, 2007.

Sato, N., Sanjuan, I.M., Heke, M., Uchida, M., Naef, F., and Brivanlou, A.H. Molecular signature of human embryonic stem cells and its comparison with the mouse. Dev. Biol. **260**, 404, 2003.

Saxena, R., and Theise, N. Canals of Hering: recent insights and current knowledge. Semin. Liver Dis. **24**, 43, 2004.

Scadden, D.T. The stem-cell niche as an entity of action. Nature **441**, 1075, 2006.

Schirmacher, P., Geerts, A., Pietrangelo, A. et al. Hepatocyte growth factor/hepatopoietin A is expressed in fat-storing cells from rat liver but not myofibroblast-like cells derived from fat-storing cells. Hepatology **15**, 5, 1992.

Schlaf, G., Schmitz, M., Heine, I., Demberg, T., Schieferdecker, H.L., and Gotze, O. Upregulation of fibronectin but not of entactin, collagen IV and smooth muscle actin by anaphylatoxin C5a in rat hepatic stellate cells. Histol. Histopathol. **19**, 1165, 2004.

Schmelzer, E., McClelland, R.E., Melhem, A., Zhang, L., Yao, H., Wauthier, E., Turner, W.S., Furth, M.E., Gerber, D., Gupta, S., and Reid, L.M. Hepatic Stem Cells and the Liver's Maturational Lineages: Implications for Liver Biology, Gene Expression, and Cell Therapies. In: Potten, C.S., Clarke, R., Wilson, J., and Renehan, A. eds. Tissue Stem Cells. New York: Taylor & Francis. 2006, pp.161-214.

Schmelzer, E., Wauthier, E., and Reid, L.M. The phenotypes of pluripotent human hepatic progenitors. Stem Cells **24**, 1852, 2006.

Schmelzer, E., Zhang, L., Bruce, A., Wauthier, E., Ludlow, J., Yao, H., Moss, N., Melhem, A., McClelland, R.l., Turner, W., et al. Human hepatc stem cells from fetal and postnatal donors. J. Exp. Med. (in press) 2007.

Schmidt, C., Bladt, F., Goedecke, S. et al. Scatter factor/hepatocyte growth factor is essential for liver development. Nature **373**, 699, 1995.

Schnabl, B., Bradham, C.A., Bennett, B.L., Manning, A.M., Stefanovic, B., and Brenner, D.A. TAK1/JNK and p38 have opposite effects on rat hepatic stellate cells. Hepatology **34**, 953, 2001.

Schwabe, R.F., Bataller, R., and Brenner, D.A. Human hepatic stellate cells express CC5 and RANTES to induce prolireration and migration. Am. J. Physiol. Gastrointest. Liver Physiol. **285**, G949, 2003.

Seftalioglu, A., and L. Karakoc. Expression of CD146 adhesion molecules (MUC18 or MCAM) in the thymic microenvironment. Acta Histochem. **102**, 69, 2000.

Selden, C., and Hodgson, H. Cellular therapies for liver replacement. Transpl. Immunol. **12**, 273, 2004.

Selden, C., Jones, M., Wade, D., and Hodgson, H. Hepatotropin mRNA expression in human foetal liver development and in liver regeneration. FEBS Lett. **270**, 81, 1990.

Serls, A.E., Doherty, S., Parvatiyar, P., Wells, J.M., and Deutsch, G.H. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. Development **132**, 35, 2005.

Shalaby, F., Ho, J., Stanford, W.L., Fischer, K.D., Schuh, A.C., Schwartz, L., Bernstein, A., and Rossant, J. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell **89**, 981, 1997.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature **376**, 62, 1995.

Shi, Y.F., Zhang, Q., Cheung, P.Y., Shi, L., Fong, C.C., Zhang, Y., Tzang, C.H., Chan, B.P., Fong, W.F., Chun, J., et al. Effects of rhDecorin on TGF-beta1 induced human hepatic stellate cell LX-2 activation. Biochim. Biophys. Acta **1760**, 1587, 2006.

Shin, M., Watanuki, K., and Yasugi, S. Expression of Fgf10 and Fgf receptors during development of the embryonic chicken stomach. Gene Expr. Patterns **5**, 511, 2005.

Shiojiri, N., Lemire, J.M., and Fausto, N. Cell lineages and oval cell progenitors in rat liver development. Cancer Res. **51**, 2611, 1991.

Shiojiri, N. Development and differentiation of bile ducts in the mammalian liver. Microsc. Res. Tech. **39**, 328, 1997.

Sicklick, J.K., Li, Y.X., Choi, S.S., Qi, Y., Chen, W., Bustamante, M., Huang, J., Zdanowicz, M., Camp, T., Torbenson, M.S., et al. Role for hedgehog signaling in hepatic stellate cell activation and viability. Lab. Invest. **85**, 1368, 2005.

Sicklick, J.K., Li, Y.X., Melhem, A., Schmelzer, E., Zdanowicz, M., Huang, J., Caballero, M., Fair, J.H., Ludlow, J.W., McClelland, R.E., Reid, L.M., and Diehl, A.M. Hedgehog

signaling maintains resident hepatic progenitors throughout life. Am. J. Physiol. Gastrointest. Liver Physiol. **290**, G859, 2006.

Sigal, S.H., Brill, S., Reid, L.M., Zvibel, I., Gupta, S., Hixson, D., Faris, R., and Holst, P.A. Characterization and enrichment of fetal rat hepatoblasts by immunoadsorption ("panning") and fluorescence-activated cell sorting. Hepatology **19**, 999, 1994.

Sigal, S.H., Gupta, S., Gebhard, D.F., Jr., Holst, P., Neufeld, D., and Reid, L.M. Evidence for a terminal differentiation process in the rat liver. Differentiation **59**, 35, 1995.

Sigal, S.H., Rajvanshi, P., Gorla, G.R., Sokhi, R.P., Saxena, R., Febhard Jr., D.R., Reid, L.M., and Gupta, S. Partial Hepatectomy-Induced Polyploidy Attenuates Hepatocyte Replication and Activates Cell Aging Events. Am. J. Physiol. **276**, G1260, 1999.

Sigal, S.H., Rajvanshi, P., Reid, L.M., and Gupta, S. Demonstration of differentiation in hepatocyte progenitor cells using dipeptidyl peptidase IV deficient mutant rats. Cell Mol. Biol. Res. **41**, 39, 1995.

Sigal, S.H., Brill, S., Fiorino, A.S., and Reid, L.M. The liver as a stem cell and lineage system. Am. J. Physiol. **263**, G139, 1992.

Singh, A.B., and Harris, R.C. Autocrine, paracrine and juxtacrine signaling by EGF-R ligands. Cell. Signal. **17**, 1183, 2005.

Skottman, H., and Hovatta, O. Culture conditions for human embryonic stem cells. Reproduction **132**, 691, 2006.

Smith, A.G., Nichols, J., Robertson, M. et al. Differentiation inhibiting activity (DIA/LIF) and mouse development. Dev. Biol. **151**, 339, 1992.

Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M. and Rogers, D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature **336**, 688, 1988.

Song, X., Wong, M.D., Kawase, E., Xi, R., Ding, B.C., McCarthy, J.J., and Xie, T. Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development **131**, 1353, 2004.

Sonnenberg, E., Meyer, D., Weidner, K.M., and Birchmeier, W. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. J. Cell Biol. **123**, 223, 1993.

Soria, B. In vitro differentiation of pancreatic β-cells. Differentiation **68**, 205, 2001.

Sosa-Pineda, B., Wigle, J.T., and Oliver, G. Hepatocyte migration during liver development requires Prox1. Nat. Genet. **25**, 254, 2000.

Stacey, G.N., Cobo, F., Nieto, A., Talavera, P., Healy, L., and Concha, A. The development of 'feeder' cells for the preparation of clinical grade hES cell lines: challenges and solutions. J. Biotechnol. **125**, 583, 2006.

Stefanovic, L., and Stefanovic, B. Mechanism of direct hepatotoxic effect of KC chemokine: sequential activation of gene expression and progression from inflammation to necrosis. J. Interferon Cytokine Res. **26**, 760, 2006.

Stewart, F. Roles of mesenchymal-epithelial interactions and hepatocyte growth factor-scatter factor (HGF-SF) in placental development. Rev. Reprod. **1**, 144, 1996.

Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., Cheng, T., Dombkowski, D., Calvi, L.M., Rittling, S.R., and Scadden, D.T. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J. Exp. Med. **201**, 1781, 2005.

Stoker, M.G., and Sussman, M. Studies on the Action of Feeder Layers in Cell Culture. Exp. Cell Res. **38**, 645, 1965.

Strobl, H., Riedl, E., Scheinecker, C., Bello-Fernandez, C., Pickl, W.F., Rappersberger, K., Majdic, O., and Knapp, W. TGF-beta 1 promotes in vitro development of dendritic cells from CD34+ hemopoietic progenitors. J. Immunol. **157**, 1499, 1996.

Strom, S., Fisher, R., Thompson, M., Sanyal, A., Cole, P.E., Ham, J.M., and Posner, M.P. Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. Transplantation **63**, 559, 1997.

Stuart, K.A., Riordan, S.M., Lidder, S., Crostella, L., Williams, R., and Skouteris, G.G. Hepatocyte growth factor/scatter factor-induced intracellular signalling. Int. J. Exp. Pathol. **81**, 17, 2000.

Stumm, R.K., Rummel, J., Junker, V., Culmsee, C., Pfeiffer, M., Krieglstein, J., Hollt, V., and Schulz, S. A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia. J. Neurosci. **22**, 5865, 2002.

Sumanasinghe, R.D., Bernacki, S.H., and Loboa, E.G. Osteogenic Differentiation of Human Mesenchymal Stem Cells in Collagen Matrices: Effect of Uniaxial Cyclic Tensile Strain on Bone Morphogenetic Protein (BMP-2) mRNA Expression. Tissue Eng. **12**, 3459, 2006.

Sun, H., Lesche, R., Li, D.M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X., and Wu, H. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc. Natl. Acad. Sci. USA **96**, 6199, 1999.

Susick, R., Moss, N., Kubota, H., Lecluyse, E., Hamilton, G., Luntz, T., Ludlow, J., Fair, J.,

Gerber, D., Bergstrand, K., et al. Hepatic progenitors and strategies for liver cell therapies. Ann. N. Y. Acad. Sci. **944**, 398, 2001.

Suzuki, A., Zheng, Y.W., Kaneko, S. et al. Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. J. Cell Biol. **156**, 173, 2002.

Takiguchi, K., Yasugi, S., and Mizuno, T. Gizzard epithelium of chick embryos can express embryonic pepsinogen antigen, a marker protein of proventriculus. Roux's Arch. Dev. Biol. **195**, 475, 1986.

Tanimizu, N., Nishikawa, M., Saito, H., Tsujimura, T., and Miyajima, A. Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J. Cell Sci. **116**, 1775, 2003.

Tenchini, M.L., Ranzati, C., and Malcovati, M. Culture techniques for human keratinocytes. Burns **18 Suppl 1**, S11, 1992.

Theise, N.D., Saxena, R., Portmann, B.C., Thung, S.N., Yee, H., Chiriboga, L., Kumar, A., and Crawford, J.M. The canals of Hering and hepatic stem cells in humans. Hepatology **30**, 1425, 1999.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. Embryonic stem cell lines derived from human blastocysts. Science **282**, 1145, 1998.

Thorgeirsson, S.S., and Grisham, J.W. Overview of recent experimental studies on liver stem cells. Semin. Liver Dis. **23**, 303, 2003.

Thornton, A.J., Ham, J., and Kunkel, S.L. Kupffer cell-derived cytokines induce the synthesis of a leukocyte chemotactic peptide, interleukin-8, in human hepatoma and primary hepatocyte cultures. Hepatology **14**, 1112, 1991.

Tiedemann, K., Olander, B., Eklund, E., Todorova, L., Bengtsson, M., Maccarana, M., Westergren-Thorsson, G., and Malmstrom, A. Regulation of the chondroitin/dermatan fine structure by transforming growth factor-beta1 through effects on polymer-modifying enzymes. Glycobiology **15**, 1277, 2005.

Tulina, N., and Matunis, E. Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science **294**, 2546, 2001.

Turner, W.S., Schmelzer, E., McClleland, R., Wauthier, E., Chen, W., and Reid, L.M. (2007). Human hepatoblast phenotype maintained by gyaluronan hydrogels. J. Biomed. Mater. Res. B Appl. Biomater. **82**, 156, 2007.

Uchida, N., Buck, D.W., He, D., Reitsma, M.J., Masek, M., Phan, T.V., Tsukamoto, A.S., Gage, F.H., and Weissman, I.L. Direct isolation of human central nervous system stem cells. Proc. Natl. Acad. Sci. USA **97**, 14720, 2000.

Uemura, M., Swenson, E.S., Gaca, M.D., Giordano, F.J., Reiss, M., and Wells, R.G. Smad2

and Smad3 play different roles in rat hepatic stellate cell function and alpha-smooth muscle actin organization. Mol. Biol. Cell **16**, 4214, 2005.

Umeshita-Suyama, R., Sugimoto, R., Akaiwa, M., Arima ,K., Yu, B., Wada, M., Kuwano, M., Nakajima, K., Hamasaki, N., and Izuhara, K. Characterization of IL-4 and IL-13 signals dependent on the human IL-13 receptor α chain 1: redundancy of requirement of tyrosine residue for STAT3 activation. Int. Immunol. **12**, 1499, 2000.

Urase, K., and Yasugi, S. Induction and inhibition of epithelial differentiation by the mixture cell aggregates of the mesenchymes from the chicken embryonic digestive tract. Dev. Growth Differ. 35, 33, 1993.

Urase, K., Fukuda, K., Ishii, Y., Sakamoto, N., and Yasugi, S. Analysis of mesenchymal influence on the pepsinogen gene expression in the epithelium of chicken embryonic digestive tract. Roux's Arch. Dev. Biol. **205**, 382, 1996.

Van Den Heuvel, M.C., Slooff, M.J., Visser, L., Muller, M., De Jong, K.P., Poppema, S., and Gouw, A.S. Expression of anti-OV6 antibody and anti-N-CAM antibody along the biliary line of normal and diseased human livers. Hepatology **33**, 1387, 2001.

van Oosten, M., van de, B.E., de Vries, H.E. et al. Vascular adhesion molecule-1 and intercellular adhesion molecule-1 expression on rat liver cells after lipopolysaccharide administration in vivo. Hepatology **22**, 1538, 1995.

Varga, A.C., and Wrana, J.L. The disparate role of BMP in stem cell biology. Oncogene **24**, 5713, 2005.

Vassy, J., Rigaut, J.P., Briane, D. et al. Confocal microscopy immunofluorescence localization of desmin and other intermediate filament proteins in fetal rat livers. Hepatology **17**, 293, 1993.

Vermeulen, M., Le Pesteur, F., Gagnerault, M.C. et al. Role of Adhesion Molecules in the Homing and Mobilization of Murine Hematopoietic Stem and Progenitor Cells. Blood. **92**, 894, 1998.

Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J., and Aguila, H.L. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood **103**, 3258, 2004.

Vongchan, P., Warda, M., Toyoda, H., Toida, T., Marks, R.M., and Linhardt, R.J. Structural characterization of human liver heparan sulfate. Biochim. Biophys. Acta **1721**, 1, 2005.

Wake, K. Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. Int. Rev. Cytol. **66**, 303, 1980.

Wandzioch, E., Kolterud, A., Jacobsson, M. et al. Lhx2-/- mice develop liver fibrosis. Proc. Natl. Acad. Sci. U S A. **101**, 16549, 2004.

Wang, R,, Yashpal, N,, Bacchus, F., and Li, J. Hepatocyte growth factor regulates proliferation and differentiation of epithelial monolayers derived from islets of postnatal rat pancreas. J. Endocrinol. **183**, 163, 2004.

Wang, S.C., Ohata, M., Schrum, L., Rippe, R.A., and Tsukamoto, H. Expression of interleukin-10 by in vitro and in vivo activated hepatic stellate cells. J. Biol. Chem. **273**, 302, 1998.

Wang, S.J., Greer, P., and Auerbach, R. Isolation and propagation of yolk-sac-derived endothelial cells from a hypervascular transgenic mouse expressing a gain-of-function fps/fes proto-oncogene. In vitro Cell Dev. Biol. **32**, 292, 1996.

Wang, X., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., and Grompe, M. The origin an dliver repopulating capacity of murine oval cells. Proc. Natl. Acad. Sci. USA. **100 Suppl 1**, 11881, 2003.

Wells, M.J., Hatton, M.W., Hewlett, B., Podor, T.J., Sheffield, W.P., and Blajchman, M.A. Cytokeratin 18 is expressed on the hepatocyte plasma membrane surface and interacts with thrombin-antithrombin complexes. J. Biol. Chem. **272**, 28574, 1997.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature **336**, 684, 1988.

Winwood, P.J., and Arthur, M.J.P. (1998). Kupffer cells and endothelial cells. In: Strain, A., and Diehl, A.M. eds. Liver Growth and Repair. London: Chapman & Hall. 1998, pp.512-537.

Winwood, P.J., Schuppan, D., Iredale, J.P., Kawser, C.A., Docherty, A.J., and Arthur, M.J. Kupffer cell-derived 95-kd type IV collagenase/gelatinase B: characterization and expression in cultured cells. Hepatology **22**, 304, 1995.

Wong, C.E., Paratore, C., Dours-Zimmermann, M.T., Rochat, A., Pietri, T., Suter, U., Zimmermann, D.R., Dufour, S., Thiery, J.P., Meijer, D., et al. Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. J. Cell Biol. **175**, 1005, 2006.

Wright, D.E., Bowman, E.P., Wagers, A.J. et al. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J. Exp. Med. **195**, 1145, 2002.

Wu, C.C., Li, Y.S., Haga, J.H., Kaunas, R., Chiu, J.J., Su, F.C., Usami, S., and Chien, S. Directional shear flow and Rho activation prevent the endothelial cell apoptosis induced by micropatterned anisotropic geometry. Proc. Natl. Acad. Sci. USA **104**, 1254, 2007.

Xie, T., and Spradling, A.C. Decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. Cell **94**, 251, 1998.

Xu, A., Luntz, T., Macdonald, J., Kubota, H., Hsu, E., London, R., and Reid, L.M. In: Lanza, R., Langer, R., and Vacanti, J. eds. Principles of Tissue Engineering. 2nd ed. San Diego: Academic Press. 2000.

Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., and Carpenter, M.K. Feeder-free growth of undifferentiated human embryonic stem cells. Nat. Biotechnol. **19**, 971, 2001.

Xu, R.H., Chen, X., Li, D.S., Li, R., Addicks, G.C., Glennon, C., Zwaka, T.P., and Thomson, J.A. BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat. Biotechnol. **20**, 1261, 2002.

Xu, R.H., Peck, R.M., Li, D.S., Feng, X., Ludwig, T., and Thomson, J.A. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat. Methods **2**, 185, 2005.

Yang, Z.J., and Wechsler-Reya, R.J. Hit 'em where they live: targeting the cancer stem cell niche. Cancer Cell **11**, 3, 2007.

Yasunaga, M., Tada, S., Torikai-Nishikawa, S., Nakano, Y., Okada, M., Jakt, L.M., Nishikawa, S., Chiba, T., Era, T., and Nishikawa, S. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. Nat. Biotechnol. **23**, 1542, 2005.

Ye, F., Duvillie, B., and Scharfmann, R. Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells. Diabetologia **48**, 277, 2005.

Ying, Q.L., Nichols, J., Chambers, I., and Smith, A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell **115**, 281, 2003.

Yokoi, Y., Namihisa, T., Kuroda, H. et al. Immunocytochemical detection of desmin in fat-storing cells (Ito cells). Hepatology. **4**, 709, 1984.

Yoshitomi, H., and Zaret, K.S. Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development **131**, 807, 2004.

Yu, Y., Flint, A., Dvorin, E. L., and Bischoff, J. AC133-2, a novel isoform of human AC133 stem cell antigen. J. Biol. Chem. **277**, 20711, 2002.

Zaret, K. Early liver differentiation: genetic potentiation and multilevel growth control. Curr. Opin. Genet. Dev. **8**, 526, 1998.

Zaret, K.S. Regulatory phases of early liver development: paradigms of organogenesis. Nat. Rev. Genet. **3**, 499, 2002.

Zern, M.A., and Reid, L.M. Extracellular Matrix Chemistry and Biology. New York: Academic Press. 1993.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y., and Li, L. Identification of the haematopoietic stem cell niche and control of the niche size. Nature **425**, 836, 2003.

Zhang, H., and Bradley, A. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development **122**, 2977, 1996.

Zhang, L., Theise, N., and Reid, L.M. Human Liver Stem Cells and their Maturational Lineages in Normal versus Disease States: Symmetries between Liver Development and Liver Regeneration. submitted. 2007

Zhao, R., and Duncan, S.A. Embryonic development of the liver. Hepatology **41**, 956, 2005.

Zhou, X., Murphy, F.R., Gehdu, N. et al. Engagement of {alpha}v{beta}3 Integrin Regulates Proliferation and Apoptosis of Hepatic Stellate Cells. J. Biol. Chem. **279**, 23996, 2004.

Zindy, F., Lamas, E., Schmidt, S., Kirn, A., and Brechot, C. Expression of insulin-like growth factor II (IGF-II) and IGF-II, IGF-I and insulin receptors mRNAs in isolated non-parenchymal rat liver cells. J. Hepatol. **14**, 30, 1992.