HHS Public Access
Author manuscript
J Physiol. Author manuscript; available in PMC 2023 April 01.

Published in final edited form as:
J Physiol. 2022 April ; 600(8): 1825–1837. doi:10.1113/JP281061.

Hepatic Stellate Cells in Physiology and Pathology

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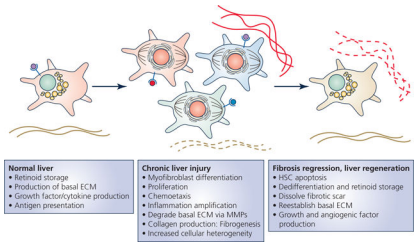
Abstract

Hepatic stellate cells (HSCs) comprise a minor cell population in the liver but serve numerous critical functions in the normal liver and in response to injury. HSCs are primarily known for their activation upon liver injury and producing the collagen-rich extracellular matrix in liver fibrosis. In the absence of liver injury, HSCs reside in a quiescent state, in which their main function appears to be storage of retinoids, or vitamin A containing metabolites. Less appreciated functions of HSCs include amplifying the hepatic inflammatory response and expressing growth factors that are critical for liver development and both the initiation and termination of liver regeneration. Recent single-cell RNA sequencing studies have corroborated earlier studies that HSC activation involves a diverse array of phenotypic alterations and identified unique HSC populations. This review serves to highlight these many functions of HSCs, and briefly describe recent genetic tools that will help to thoroughly investigate the role of HSCs in hepatic physiology and pathology.

Graphical Abstract



Hepatic stellate cells (HSCs) are best known for their production of fibrosis in response to liver injury, but they display many other phenotypes and vital functions. In the normal liver, HSCs help to orchestrate liver development, produce and maintain the basal extracellular matrix (ECM), produce growth factors and cytokines, present antigens, and serve as the body's main storage depot for retinoids or vitamin A-containing metabolites. Upon liver injury, HSCs lose the retinoid-containing lipid droplets and differentiate into myofibroblasts and proliferate. Single cell sequencing studies have uncovered that activated HSCs display a high degree of

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Author contributions
D.R.K. and K.S.M organized, wrote, and edited this manuscript. Both authors approve of the final version of this manuscript, qualify for authorship, and agree to be accountable for all aspects of the work should questions related to accuracy and integrity of any part of this review article arise.

Competing interests
The authors have no conflicts of interest to declare.

cellular heterogeneity for specific functions. Some are more proliferative, while others are more inflammatory, and others more fibrogenic. If liver injury is resolved, HSC numbers will decrease due to a large degree of apoptosis, and remaining HSCs will at least partially dedifferentiate into more quiescent HSCs. These HSCs help remove the fibrotic scar and reestablish the basal ECM scaffold. If liver regeneration is required, HSCs will coordinate this by building of the basal ECM and production of growth factors, cytokines, and angiogenic factors.

Keywords

stellate cell; liver; fibrosis

Introduction

Hepatic stellate cells (HSCs) are resident mesenchymal cells located in the perisinusoidal area between endothelial cells and hepatocytes known as the Space of Disse (Figure 1). As non-parenchymal cells, most estimates suggest HSCs comprise between 5–10% of all resident cells within the liver (Giampieri *et al.*, 1981). Recent analyses based on single-cell or single-nuclei isolation and RNA sequencing report as low as 0.3% up to ~15% of isolated liver cells or nuclei express known HSC markers (MacParland *et al.*, 2018; Massalha *et al.*, 2020; Zhu *et al.*, 2020; Andrews *et al.*, 2021; Diamanti *et al.*, 2021; Nault *et al.*, 2021; Payen *et al.*, 2021; Richter *et al.*, 2021). HSCs are predominantly viewed within the scope of producing fibrosis in response to liver injury, as HSCs are the main contributor to hepatic extracellular matrix deposition during all etiologies of liver injury (Mederacke *et al.*, 2013). The central dogma for stellate cells is that they reside in a quiescent state in the normal liver and become “activated” in response to liver injury. This activation mainly arises from oxidative stress and inflammatory signals, causing an array of phenotypic alterations related to cellular activation: enhanced proliferation, differentiation to myofibroblast-like cells, chemotaxis, fibrogenesis, and production of extracellular matrix remodeling enzymes (Friedman, 2008). However, recent evidence suggests heterogeneous populations of HSCs exist with diverse phenotypic responses to activation. Much less is known about the importance of HSCs outside of this fibrotic response to liver injury. Since HSCs comprise such a relatively minor population of cells and until recently it has been challenging to create HSC-specific genetic models, uncovering roles for HSCs in normal hepatic or systemic physiology has been challenging. This review seeks to describe the multitude of HSC functions and shine light on the current lack of understanding regarding the roles of HSCs in normal hepatic physiology.

The Quiescent Hepatic Stellate Cell

Retinoid Storage and Function

In their inactive or quiescent state, HSCs are characterized by the presence of cytoplasmic lipid droplets. These lipid droplets contain a large amount of vitamin A metabolites, or retinoids, which allowed for the original identification of these cells by Karl Wilhelm von Kupffer using gold chloride staining (Kupffer, 1876). In fact, up to 70–95% of all retinoid storage in the body resides within HSCs (Wake, 1971; Knook *et al.*, 1982; Hendriks *et al.*,

1985). These studies also described that HSC retinoid storage was increased when provided high levels of vitamin A or retinyl esters (Kupffer, 1876; Hendriks *et al.*, 1985). Due to the presence of these lipid droplets, “fat-storing cells” or “lipocytes” were early names for hepatic stellate cells. “Ito cells” was another common name for HSCs after Toshio Ito reinvigorated interest in the cells after Kupffer had concluded they may just be a subset of Kupffer macrophage cells (Reuben, 2002). The retinoids in these droplets are comprised predominantly of retinyl esters of exclusively long-chain acyl moieties (retinyl-palmitate > retinyl-stearate > retinyl-oleate > retinyl-linoleate) (Knook *et al.*, 1982; Hendriks *et al.*, 1985; Yamada *et al.*, 1987). In addition to retinyl esters, HSC lipid droplets contain triglycerides, and small amounts of cholesterol, cholesterol esters, phospholipids, and free fatty acids (Yamada *et al.*, 1987; Moriwaki *et al.*, 1988). While lipid droplet retinoid content varies based on dietary retinoid consumption, altered triglyceride consumption has little effect on HSC triglyceride content (Moriwaki *et al.*, 1988). Strangely, despite the diversity of lipid species stored in these droplets, blocking synthesis of retinyl esters completely prevents the formation of lipid droplets in HSCs (O’Byrne *et al.*, 2005). Retinyl ester synthesis in the liver is dependent on the enzyme lecithin:retinol acyltransferase (LRAT), and LRAT−/− mice completely lack HSC lipid droplets and hepatic retinyl ester storage (Batten *et al.*, 2004; O’Byrne *et al.*, 2005). In LRAT-deficient mice, dietary retinol can still be esterified by intestinal diacylglycerol acyltransferase 1 (DGAT1), and retinyl esters (and retinol) are instead stored primarily in adipose tissue (O’Byrne *et al.*, 2005). The adipose from LRAT−/− mice displayed a 3–4-fold increase in cytosolic retinol-binding protein type III (CRBPIII) (O’Byrne *et al.*, 2005), which is known to be important for retinol uptake by cells (Piantedosi *et al.*, 2005). These adipose tissue retinoid stores were still able to be mobilized when the mice were placed on a retinoid-deficient diet (O’Byrne *et al.*, 2005).

Vitamin A is essential and must be obtained from dietary sources. Vitamin A can be obtained from animal-based foods in the form of retinyl esters, or from plant-based foods in the form of carotenoids which can then be converted to retinol. Retinoids, or more appropriately, the biologically active forms which are retinaldehyde or retinoic acid, have various vital physiologic roles. In 1913, it was discovered that a certain lipid-soluble substance, later identified as vitamin A, affected growth rates independent of dietary carbohydrate, lipid, and protein (McCollum, 1913). Shortly after, it was realized that vitamin A was an important factor in detection of light by the retina (Rosenheim & Drummond, 1925; Wald, 1935), which explains why vitamin A metabolites have the name of retinoids. In addition to supporting growth and vision, retinoids support numerous immune system and reproductive system functions (reviewed in (Haaker *et al.*, 2020)). Thus, retinoid storage in HSCs likely preserves many vital functions for numerous cell types throughout the body.

Due to inexistent hepatic retinoid stores, LRAT-deficient mice were believed to be more prone to developing retinoid deficiency compared to wildtype mice. While no phenotypic symptoms were observed, after only 1 month on a retinoid-deficient diet, LRAT−/− mice had depleted their adipose tissue retinoid stores, serum retinol concentrations and retinol binding protein (RBP) were decreased, and hepatic RBP expression was elevated by 10-fold (O’Byrne *et al.*, 2005). These changes in serum/liver RBP are used as biomarkers of retinoid deficiency when performed in combination with serum vitamin A measurement.

For reasons that remain poorly understood, loss of retinoid storage in HSCs is one of the classic hallmarks of HSC activation. However, it seems this retinoid lipid droplet depletion is a consequence of activation rather than a cause. LRAT deficient mice lacking HSC retinoid stores showed no activation of HSCs compared to wildtype mice (O'Byrne *et al.*, 2005). However, chronic liver diseases resulting in cirrhotic liver failure (involving excessive HSC activation and fibrogenesis) are associated with symptomatic vitamin A deficiency (Mahmood *et al.*, 2008; Venu *et al.*, 2013; Chaves *et al.*, 2015). There is conflicting data regarding whether retinoids can directly alter stellate cell activation. Treatment with retinoic acid has been suggested to decrease HSC proliferation and collagen expression *in vitro* and *in vivo* (Davis *et al.*, 1990). However, subsequent studies with 9-*cis*-retinoic acid and 9,13-di-*cis*-retinoic acid suggest promotion of fibrosis by activation of plasminogen activator and tumor growth factor β (TGFβ) (Okuno *et al.*, 1997; Okuno *et al.*, 1999). Activated HSCs downregulate LRAT expression, greatly decreasing their ability to form and store retinyl esters (Shmarakov *et al.*, 2019). In summary, retinoid storage is the primary function of quiescent HSCs, and retinoid depletion upon HSC activation can lead to systemic vitamin A deficiency.

Transcriptional Regulation of Adipocyte-like Phenotype

Likely intricately related to the presence of these lipid droplets, quiescent HSCs express a virtually identical transcriptional program to mature adipocytes. Expression of peroxisome proliferator-activated receptor gamma (PPARγ) and sterol regulatory-element-binding protein-1 (SREBP-1c) in HSCs governs fatty acid storage and metabolism. In quiescent cells, PPARγ and SREBP-1c expression is high, but are both dramatically reduced upon HSC activation (She *et al.*, 2005; Tsukamoto, 2005). Enhancement of PPARγ and SREBP-1c expression or activity prevents or reverses HSC activation, suggesting that maintenance of the adipogenic phenotype maintains quiescence (Miyahara *et al.*, 2000; She *et al.*, 2005; Tsukamoto, 2005). Conversely, knockdown of PPARγ in cultured human HSCs significantly increases activation (Tao *et al.*, 2020). Interestingly, despite the complete loss of lipid droplets in LRAT−/− HSCs, these cells display maintained expression of PPARγ and SREBP-1c, perhaps explaining their preserved quiescent phenotype (O'Byrne *et al.*, 2005). HSCs also express the "adipokines" leptin and adiponectin, and adiponectin signaling is antifibrotic (Kamada *et al.*, 2003; Ding *et al.*, 2005). In clinical practice, treating patients with nonalcoholic steatohepatitis (NASH) with the PPARγ agonist pioglitazone significantly reduces hepatic fibrosis (Boettcher *et al.*, 2012; Bril *et al.*, 2018) which could be the result of direct PPARγ activation in the HSCs or via enhanced adiponectin signaling. Cumulatively, these data strongly suggest that maintenance of this adipogenic phenotype prevents HSC activation.

One hypothesis for why the adipogenic phenotype and lipid droplets are lost during activation is that activation requires a great deal of energy, supplied by the metabolism of fatty acids. Activated HSCs display enhanced expression of a transcriptional regulator of fatty acid oxidation enzymes peroxisome proliferator-activated receptor beta (PPARβ) (She *et al.*, 2005). Surprisingly, peroxisome proliferator-activated receptor alpha (PPARα), which also regulates gene expression for fat oxidation enzymes/transporters appears to be reduced in activated HSCs (Miyahara *et al.*, 2000). During activation, fatty acids can be

sourced from the hydrolysis of lipid droplet-stored triglycerides, or the de-esterification of the stored retinyl esters (Shmarakov *et al.*, 2019), and these processes are at least partially dependent on autophagy of the lipid droplets (Hernandez-Gea *et al.*, 2012). A number of retinyl ester hydrolases and lipases, including adipose triglyceride lipase (ATGL), patatin-like phospholipase domain-containing protein 3 (PNPLA3), hormone-sensitive lipase (HSL), and carboxyl ester lipase have been shown to be important for retinyl ester release from lipid droplets (Weng *et al.*, 1999; Mello *et al.*, 2008; Pirazzi *et al.*, 2014; Taschler *et al.*, 2015). Inhibiting fatty acid oxidation can block HSC activation (Hernandez-Gea *et al.*, 2012), suggesting that liberating fatty acid fuel from the lipid droplets may be required for HSC activation. However, HSC activation also enhances glucose and amino acid catabolism (reviewed in (Trivedi *et al.*, 2021)). This enhanced glucose utilization does increase mitochondrial oxidation, but more significantly increases glycolytic flux and lactate production, which is believed to directly contribute to activation (Chen *et al.*, 2012). Conversely, reducing mitochondrial pyruvate metabolism, which would theoretically increase intracellular lactate, has been suggested to decrease HSC activation (McCommis *et al.*, 2017).

The Activated Hepatic Stellate Cell

Extensive work in the 1970s-80s described that HSCs were found near the fibrotic collagen fibers in injured livers and were the predominant source of this extracellular matrix in injured livers (McGee & Patrick, 1972; Kent *et al.*, 1976; Okanoue *et al.*, 1983; Martinez-Hernandez, 1984; Friedman *et al.*, 1985; Martinez-Hernandez, 1985). It is now understood that activation of HSCs involves a host of phenotypic changes in addition to extracellular matrix production. Activation of HSCs includes: differentiation from adipogenic to myofibroblast phenotype with loss of lipid droplets and expression of contractile fibers, increased cell proliferation, increased HSC chemotaxis as well as signaling to attract leukocytes, and development of matured rough endoplasmic reticulum to support production of extracellular matrix fibers and matrix remodeling enzymes. Ultimately, all these cellular processes are coordinated to enhance the local accumulation of extracellular matrix capable of forming a scar in areas of liver injury. Unsurprisingly, blocking signaling pathways for protein synthesis via p70S6K knockout or inhibition decreases HSC activation and fibrogenesis (Reiter *et al.*, 2022). As HSC activation and fibrogenesis are not the primary focus of this review, we will refer to several excellent reviews of this topic (Friedman, 2000; Bataller & Brenner, 2005; Zisser *et al.*, 2021). However, we must acknowledge that hepatic fibrosis is a vitally important aspect to the pathology of all chronic liver diseases. The degree of liver fibrosis is the greatest predictor of outcomes in alcoholic liver disease (Lackner *et al.*, 2017), NASH (Hagstrom *et al.*, 2017; Taylor *et al.*, 2020), viral hepatitis (Vergniol *et al.*, 2011), and cholestatic liver injury (Saffioti *et al.*, 2021). Justifiably, there has been tremendous focus on development of anti-fibrotic therapies.

Hepatic Stellate Cell Heterogeneity

Even early studies of HSCs in normal livers described a range of HSC features, primarily based upon liver zonal location. For example, some HSCs lack expression of the diagnostic

contractile fiber desmin, while other cells lack extensive vitamin A droplets (Ballardini *et al.*, 1994; Ramm *et al.*, 1995). This heterogeneity led to the hypothesis that some HSCs are more important for retinoid storage, while others are "primed" for quick activation. Single-cell RNA sequencing of non-steatotic human liver samples from all isolated cells, identified 2 HSC populations, that could not be explained by quiescent versus activated signatures (Payen *et al.*, 2021). Instead, one population was characterized by expression of the cell surface proteoglycan *GPC3* and the neurotrophic receptor *NTRK2*, while the other population expressed the dopamine norepinephrine-converting enzyme *DBH* and the hedgehog signaling modulator *HHIP* (Payen *et al.*, 2021). Using RNA *in situ* hybridization, it was determined the *GPC3*-expressing population resided almost exclusively near the portal and central vein regions, while the *DBH*-expressing population was more diffuse in the perisinusoidal space other than the portal and central vein (Payen *et al.*, 2021). Gene ontology analyses of these two populations suggested that the $GPC3^{+}$-cells were characterized by glycosaminoglycan metabolism and elastic fiber constituents, while the DBH^{+}-cells more reminiscent of antigen-presenting cells (Payen *et al.*, 2021). Thus, these quiescent HSCs can be characterized by different spatial zonation and likely important functional differences (Figure 2).

HSCs appear to be even more heterogeneous upon activation from liver injury. A classical marker of HSC activation is the expression of the contractile fiber alpha smooth muscle actin (αSMA, gene name *ACTA2*). However, not all cells that start to express collagen 1α1 (Col1α1) also express αSMA, and vice versa, which was shown *in vitro* and *in vivo* using mice expressing a dual reporter with green fluorescent protein expressed from the Col1α1 promoter and red fluorescent protein expressed from the αSMA promoter (Magness *et al.*, 2004). Indeed, while 52% of the cells expressed both activation/fibrogenesis markers, 14% expressed only Col1α1 and 9% expressed only αSMA, and each population displayed distinct patterns of gene expression (Magness *et al.*, 2004). Our appreciation of the diversity of HSC populations has been expanded recently by single-cell RNA sequencing studies. Using a western diet-fed mouse model of NASH, a total of 9 separate HSC populations could be identified by unique gene expression patterns despite all of them expressing many of the classical HSC genes (Rosenthal *et al.*, 2021). In this study, quiescent HSCs from control mice belonged predominantly to 2 separate clusters. HSCs from the NASH livers resided in 6 of the 7 other clusters with one representing the classical activated myofibroblast, but other populations being defined by either a weak activation signature, a proliferation signature, or an inflammatory signature. A third group of mice was placed on the NASH-inducing diet but was then switched back to chow diet to provoke NASH regression. HSCs isolated from this group resided in many of the same clusters as the NASH livers, but also contained a relatively unique population that was more similar to quiescent HSCs, and contained much fewer HSCs from active NASH livers (Rosenthal *et al.*, 2021). In another study of a mouse model of fibrosis caused by the hepatotoxin carbon tetrachloride, single-cell RNA sequencing identified 8 separate HSC populations reflecting different degrees of activation or even some populations displaying enhanced expression of genes important for tissue repair and angiogenesis (Zhang *et al.*, 2021). Altogether, these studies suggest that HSCs, even in the activated state, have an array of functions independent of, or in addition to fibrogenesis.

Hepatic Stellate Cells During Fibrosis Regression

Successful treatment of various chronic liver diseases have revealed the ability to reverse hepatic fibrosis (reviewed in (Kisseleva & Brenner, 2021)). One key question has been if and how HSCs could be inactivated to reduce fibrosis. Indeed, placing culture-activated HSCs onto culture dishes containing a basement membrane-like extracellular matrix can reverse their activation (Gaca *et al.*, 2003). Likewise, treatment with an adipogenic cocktail, or exogenous PPARγ or SREBP-1c expression reverts activated HSCs to quiescent, lipid storing cells (She *et al.*, 2005). More recently, evidence of HSC reversion or inactivation *in vivo* has also been obtained. Using yellow fluorescent protein (YFP) HSC reporter mice treated with CCl_4 or intragastric ethanol but then allowed to recover to significantly resolve the fibrosis showed that many HSCs that were previously activated (YFP+) no longer expressed the activated myofibroblast marker αSMA (Kisseleva *et al.*, 2012). These inactivated cells displayed a much more quiescent gene expression phenotype, with reduced expression of collagens, αSMA, and tissue inhibitor of metalloproteinase 1, and normalized expression of PPARγ (Kisseleva *et al.*, 2012). If a similar experiment is performed in HSC-specific PPARγ knockout mice, regression of liver fibrosis is greatly slowed (Liu *et al.*, 2020), arguing the importance of returning to the adipogenic HSC phenotype to resolve hepatic fibrosis. However, these inactivated HSCs are distinct from quiescent HSCs in uninjured livers, as they appear to be primed for an enhanced fibrotic response upon TGFβ treatment *in vitro*, or after reinjury with carbon tetrachloride *in vivo* (Kisseleva *et al.*, 2012). Lastly, single-cell sequencing has also described a relatively unique population of HSCs in livers after removal of a NASH-inducing diet (Rosenthal *et al.*, 2021). Altogether, these studies suggest that activated HSCs can be inactivated during the regression of fibrosis, but these cells likely do not return to a completely quiescent phenotype.

While some activated HSCs become inactivated, it has also been well established that ~50% of activated HSCs undergo apoptosis during fibrosis regression (Iredale *et al.*, 1998; Kisseleva *et al.*, 2012). This apoptotic cell death is driven by activation of extrinsic receptor-mediated pathways such as FAS or TRAIL, enhanced caspase 3 and caspase 8 activity, and upregulation of proapoptotic proteins such as p53 and BAX as well as activation of hepatic natural killer cells (Radaeva *et al.*, 2006; Glassner *et al.*, 2012; Kisseleva *et al.*, 2012). One key characteristic of HSCs that avoid apoptosis and instead inactivate is enhanced expression of the pro-survival genes for heat-shock proteins 1a/b (Kisseleva *et al.*, 2012). Unsurprisingly, artificially inducing HSC apoptosis to deplete HSCs can significantly limit fibrosis progression or resolve hepatic fibrosis (Parsons *et al.*, 2004; Oakley *et al.*, 2005; Puche *et al.*, 2013). Thus, after removal or treatment of the pathologic etiology driving fibrosis, it is clear that hepatic fibrosis can regress by a combination of HSC apoptosis and HSC inactivation.

Roles of Hepatic Stellate Cells Not Directly Related to ECM Deposition

HSCs as Immune Regulators

The common dogma is that stellate cells become activated by sensing inflammatory signals such as TGFβ that arise from liver injury. In actuality, HSCs not only respond to inflammatory signals, but also produce many cytokines, chemokines, and

immunomodulatory signals in both normal and injured liver. HSCs produce TGFβ and the TGFβ receptor, and autocrine signaling is critically important for HSC activation (Bissell *et al.*, 1995; Purps *et al.*, 2007). The same is true for many other cytokines and growth factors. Of course, these factors can also act as paracrine signals to other liver cell types and immune cells, altering their activation.

HSCs also express toll-like receptors and can respond to bacterial infection by sensing lipopolysaccharide (Brun *et al.*, 2005). HSCs also act as antigen presenting cells, capable of activating T cells (Winau *et al.*, 2007). Activated stellate cells are also known to express programmed death ligand 1 (PD-L1)(Yu *et al.*, 2004), which can bind the programmed cell death protein 1 (PD-1) receptor on an array of immune cells including macrophages, T cells, and B cells. Overall, these signaling and antigen presenting properties of HSCs help to respond and amplify the immune response from liver injury, which can also propagate HSC activation.

HSCs in Liver Development and Regeneration

Understanding the embryonic origin of HSCs has been difficult, as HSCs express classical gene markers for all three germ layers (endoderm, mesoderm, and ectoderm). Lineage tracing suggests mesoderm origin from the septum transversum, as both Wilms tumor suppressor-expressing and mesoderm posterior 1-expressing cells develop into HSCs (Asahina *et al.*, 2009; Asahina *et al.*, 2011), and HSCs express the mesoderm-specific transcription factor FoxF1 (Kalinichenko *et al.*, 2003). Yet expression of desmin, cytokeratin-7/8, and CD34 suggests endoderm origin, while expression of glial fibrillary acidic protein (GFAP), nestin, neurotrophins, and other markers suggest ectodermal origin from the neural crest (reviewed in (Friedman, 2008)). Desmin and GFAP expression are used as defining markers for identification of HSCs, with αSMA expression considered the defining marker of activated HSCs. It is not entirely known how the pool of HSCs is maintained. It has been suggested that bone marrow-derived cells could contribute to both quiescent and activated HSCs (Baba *et al.*, 2004; Russo *et al.*, 2006), however fate tracing with LratCre and bone-marrow transplantation experiments exclude this premise (Mederacke *et al.*, 2013). Thus, it seems likely that HSCs are liver-resident cells that proliferate to maintain or expand their pool size.

Several lines of evidence suggest that HSCs play significant roles during liver development. HSCs reside in the progenitor cell niche of the liver and are known to produce cytokines and growth factors capable of stimulating various hepatic cell types, including tumor growth factor alpha, epidermal growth factor, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), stem cell factor, acidic and basic fibroblast growth factors (FGF), macrophage colony-stimulating factor, platelet activating factor, and others (reviewed in (Friedman, 2008)). These factors stimulate the stellate cells themselves in an autocrine manner, but can also stimulate hepatocytes, endothelial cells, cholangiocytes, macrophages, neutrophils, and others. Interestingly, as many as 46% of HSCs transiently express Col1α1 during the postnatal period, yet these HSCs lack αSMA expression and other myofibroblast characteristics and instead maintain quiescent HSC expression phenotypes (Kisseleva *et al.*, 2012). The purpose of this transient collagen expression is not understood.

In comparison to liver development, we have a better understanding of the impact of HSCs during liver regeneration. In most instances of liver injury, liver regeneration is primarily accomplished by re-entry into the cell cycle and proliferation of existing hepatocytes to regain normal liver mass and function (reviewed in (Michalopoulos, 2007)). HSCs produce and secrete HGF on a nearly continuous basis, which is stored in an inactive form within the basal extracellular matrix. Upon liver injury, active HGF is released and can bind its cMet receptor on hepatocytes, biliary cells, and endothelial cells. The crucial role of HSCs in liver regeneration has been demonstrated in several studies in which depletion of HSCs or inhibition of HSC activation has prevented regeneration. In one such study, depletion of stellate cells via gliotoxin injection during a model of acetaminophen-induced liver injury produced a dramatic decrease in proliferating hepatocytes, a decrease in growth factor gene expression, and significantly increased liver necrosis (Shen *et al.*, 2011). Inhibition of HSC activation with L-cysteine was also shown to reduce cell proliferation of virtually all hepatic lineages in partial hepatectomy and 2-acetylaminofluorene model of hepatic injury (Pintilie *et al.*, 2010). Lastly, genetic inhibition of HSC activation also reduces hepatocyte proliferation and worsens liver injury from various etiologies in mice (Kalinichenko *et al.*, 2003; Passino *et al.*, 2007). Altogether, these studies suggest that HSC activation after injury is vital to hepatocyte proliferation during liver regeneration.

Another important aspect to liver regeneration is the formation of new vasculature or angiogenesis. Growth factors are also vital to angiogenesis, and the positioning of HSCs and liver sinusoidal endothelial cells within the Space of Disse allows for direct contact of these cells as well as paracrine signaling. Factors produced by HSCs such as PDGF, TGFβ, FGF, vascular endothelial growth factor, and angiopoietin all enhance angiogenesis (reviewed in (Kitto & Henderson, 2021)).

It appears that HSCs also play an important role in the termination of liver regeneration. During the final phases of regeneration, HSCs rebuild the basal extracellular matrix which can then sequester the high levels of growth factors and prevent them from activating hepatocytes causing them to exit the cell cycle (Block *et al.*, 1996). Additionally, HSCs are a source of TGFβ, which is known to be an inhibitor of cell proliferation. Preventing TGFβ production from activated HSCs has been shown to enhance hepatocyte proliferation in mice after partial hepatectomy (Ebrahimkhani *et al.*, 2011). Thus, HSCs appear to be of vital importance during the early and late stages of hepatic regeneration after injury (Figure 3).

HSCs as Pleuripotent Stem Cells?

Another intriguing possibility for HSCs to be involved in response to injury is they may have the capacity to differentiate into other liver cell types, including hepatocytes. It has been suggested that up to 40% of rat HSCs express the stem cell marker CD133, as well as a host of other stem cell markers (Kordes *et al.*, 2007). In culture, these cells were able to be differentiated by cytokines known to differentiate endothelial progenitor cells into cells resembling endothelial cells, and even branched tube-like structures that expressed endothelial nitric oxide synthase and ve-cadherin (Kordes *et al.*, 2007). Additionally, these CD133+ HSCs were shown to be differentiated into hepatocyte-like cells when cultured on collagen with a medium containing specific growth factors/cytokines (Kordes *et al.*, 2007).

This *in vitro* data displays the plasticity of HSC and points to a possible flexibility of HSC to fill in the gaps of liver cells during regeneration. This stemness also appears to exist *in vivo*, as shown by fate-tracing with GFP-labeled HSCs. Intriguingly, both before and after liver injury, GFP was detected in bile duct cells and sinusoidal cells, demonstrating the differentiation capacity of HSCs (Yang *et al.*, 2008). After these reporter mice were subjected to a methionine and choline deficient diet supplemented with 0.15% ethionine to cause hepatocellular injury and activate stellate cells, GFP+ hepatocytes arose that remained even after return to normal diet and liver injury regression (Yang *et al.*, 2008). However, more recent fate tracing with HSCs genetically labelled by LratCre and ZsGreen suggested that HSCs were not a source of new hepatocytes, cholangiocytes, or endothelial cells after chemical toxin, bile-duct ligation, fatty liver injury, or partial hepatectomy (Mederacke *et al.*, 2013). Thus, while these studies highlight the potential for HSCs to differentiation into other liver cell types, this pleuripotent potential of HSCs is rather controversial.

Future Studies of HSCs in Physiology

While the fibrotic response of HSCs in response to liver injury and their importance to liver regeneration become increasingly understood, the question of how important HSCs are to normal physiology largely remains. As described above, the most widely known significance for HSCs in the normal liver is retinoid storage. Use of HSC depletion models would allow for testing of how livers devoid of HSCs are able to carry out other important processes in hepatic and systemic physiology. These types of depletion models have been previously generated, but unfortunately only studied within the context of injury and regeneration. For example, a specific model of HSC depletion using herpes simplex virus thymidine kinase expressed from the GFAP promoter and treatment with ganciclovir (Puche *et al.*, 2013), is an approach which only works when the cells are proliferating, therefore when they are activated. Additionally, GFAP is also not HSC-specific and also expressed in cholangiocytes in the liver. LratCre-mediated expression of diphtheria toxin receptor has been used to deplete a large majority of HSCs, but this approach was performed mainly to confirm that HSCs comprise the majority of myofibroblasts after liver injury (Mederacke *et al.*, 2013). The HSC-expressed diphtheria toxin receptor model would be ideal for testing HSC depletion in the normal liver. Intriguing questions to ask would be if the liver is still fully capable of performing its numerous detoxifying or biosynthetic functions. HSCs express several cytochrome P450 enzymes (Yamada *et al.*, 1997), but it is unknown how much HSCs contribute to xenobiotic detoxification in relation to hepatocytes. The liver's biosynthetic functions related to protein production (plasma proteins such as albumin and almost all critical blood clotting factors), maintenance of glycemia (via glycogenolysis and gluconeogenesis), and lipid metabolism (lipoprotein synthesis, fat oxidation, and ketogenesis) are all prominent hepatocyte functions, but could potentially be influenced by nearby HSCs. Hopefully future studies will attempt to shed light on the importance of HSCs to these aspects of normal hepatic physiology, in addition to the response to liver injury.

Conclusion

While stellate cells comprise a minor hepatic cell population by number, evidence is accumulating for their critical importance in response to liver injury. During all etiologies of

liver injury, these cells produce the fibrotic scar, respond to and amplify the inflammation signals, and also contribute to all phases of liver regeneration by the production of growth factors and cytokines. Recent single-cell or single-nuclei RNA sequencing studies have established a much greater diversity beyond quiescent versus activated HSCs. Further work is required to ascertain the importance of these numerous HSC populations in response to injury. In the absence of liver injury, the storage of retinoids and production of several growth factors are the best understood functions of HSCs. Future studies are required to truly appreciate if and how HSCs contribute to normal hepatic physiologic functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

Kyle McCommis is supported by NIH R00 HL136658.

Biographies

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Abbreviations:

αSMA	alpha smooth muscle actin
Col1α1	collagen 1 alpha 1
CRBPIII	cytosolic retinol-binding protein type III
ECM	extracellular matrix

FGF	fibroblast growth factor
HGF	hepatocyte growth factor
HSC	hepatic stellate cell
LSEC	liver sinusoidal endothelial cell
LRAT	lecithin:retinol acyltransferase
NASH	nonalcoholic steatohepatitis
PD1	programmed cell death protein 1
PD-L1	programmed death ligand 1
PDGF	platelet-derived growth factor
PPARα	peroxisome proliferator-activated receptor alpha
PPARβ	peroxisome proliferator-activated receptor beta
PPARγ	proliferator-activated receptor gamma
RBP	retinol binding protein
SREBP-1c	sterol regulatory-element-binding protein-1
TGFβ	tumor growth factor β
YFP	yellow fluorescent protein

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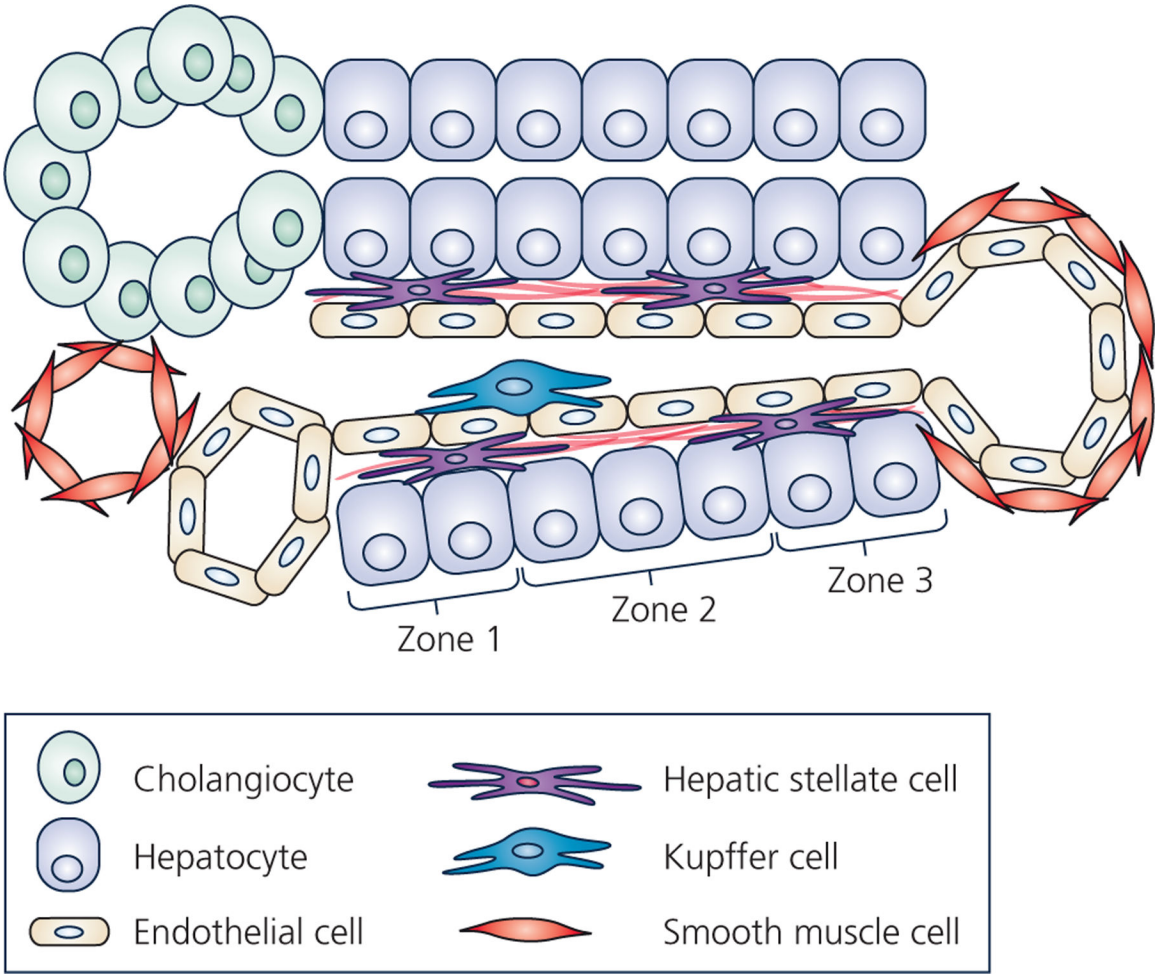

Figure 1 –. Hepatic cellular architecture.

Schematic represents a cross-section of a liver lobule, which contains the complete functional structure and main cell types of the liver. One the left, is the “portal triad” comprised of the portal vein, the hepatic artery, and the bile ductule. The bile ductule is comprised of cholangiocytes (represented as green oval-shaped cells), which collects bile produced by the hepatocytes. Bile ductules ultimately combine into the bile duct which drains bile to be stored in the gallbladder. The hepatic artery (represented as a red smooth-muscle cell-lined vessel) supplies oxygenated blood originating from the celiac artery, whereas the portal vein (represented as an endothelial cell-lined vessel) supplies nutrient- and toxin-rich blood from the stomach, pancreas, gallbladder, and spleen into the liver lobule. The portal vein and hepatic artery drain blood flow into the liver sinusoid, which is lined by liver sinusoidal endothelial cells. Ultimately, blood is collected into the central vein, lined with both endothelial cells and smooth muscle cells. Resident macrophages, known as Kupffer cells (represented as blue cell), reside in the luminal side of the sinusoid. The hepatic stellate cells (HSCs) (represented as peach-colored cells with long projections) reside in the space of Disse between hepatocytes and liver sinusoidal endothelial cells and produce the basement extracellular matrix (black lines near the HSCs in space of Disse). Regions along the length of the sinusoid are commonly referred to as zones, with zone 1

being peri-portal, zone 3 being peri-central vein, and zone 2 residing between. Hepatocytes comprise the hepatic parenchyma (the predominant cell type) and display an incredible array of functions including synthesis of serum proteins, clotting factors, lipoproteins, cholesterol and bile salts, gluconeogenesis and glycogen storage, as well as detoxification. Often these different hepatocyte functions are localized to specific zones along the sinusoid.

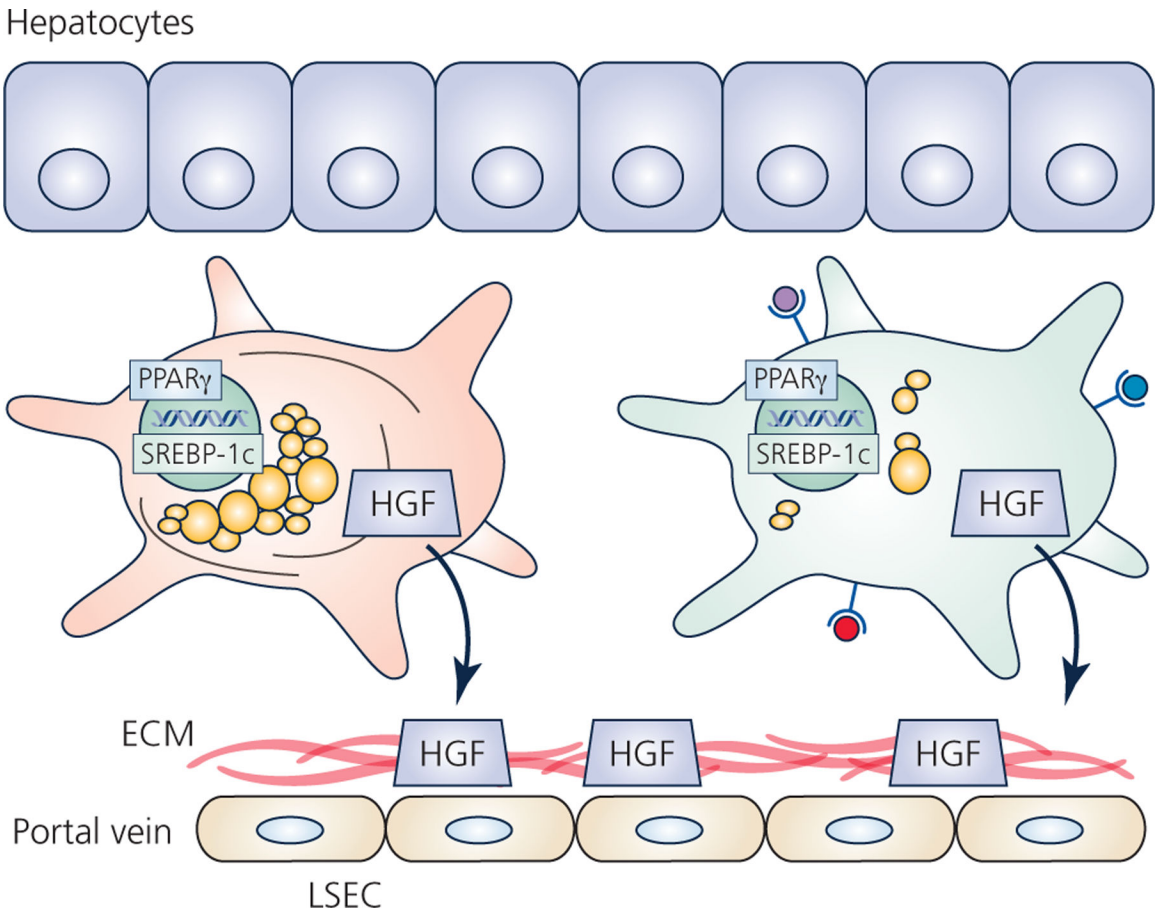

Figure 2 –. Functions and heterogeneity of quiescent HSCs.

All HSCs reside in the space of Disse between hepatocytes and liver sinusoidal endothelial cells (LSEC). Quiescence is maintained by high expression of the adipogenic transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) and sterol regulatory-element-binding protein-1c (SREBP-1c). Quiescent HSCs also produce and secrete cytokines and growth factors, such as hepatocyte growth factor (HGF) which are sequestered by the basal extracellular matrix (ECM). Physically and functionally different HSCs likely reside in different regions along the portal tract. Early studies described distinct populations of HSCs based on presence/absence of desmin fibers, or abundance of lipid droplets. In addition to these macroscopic differences, single-cell RNA sequencing studies suggest other functional differences in these distinct populations, such as altered glycosaminoglycan synthesis or increased antigen presentation.

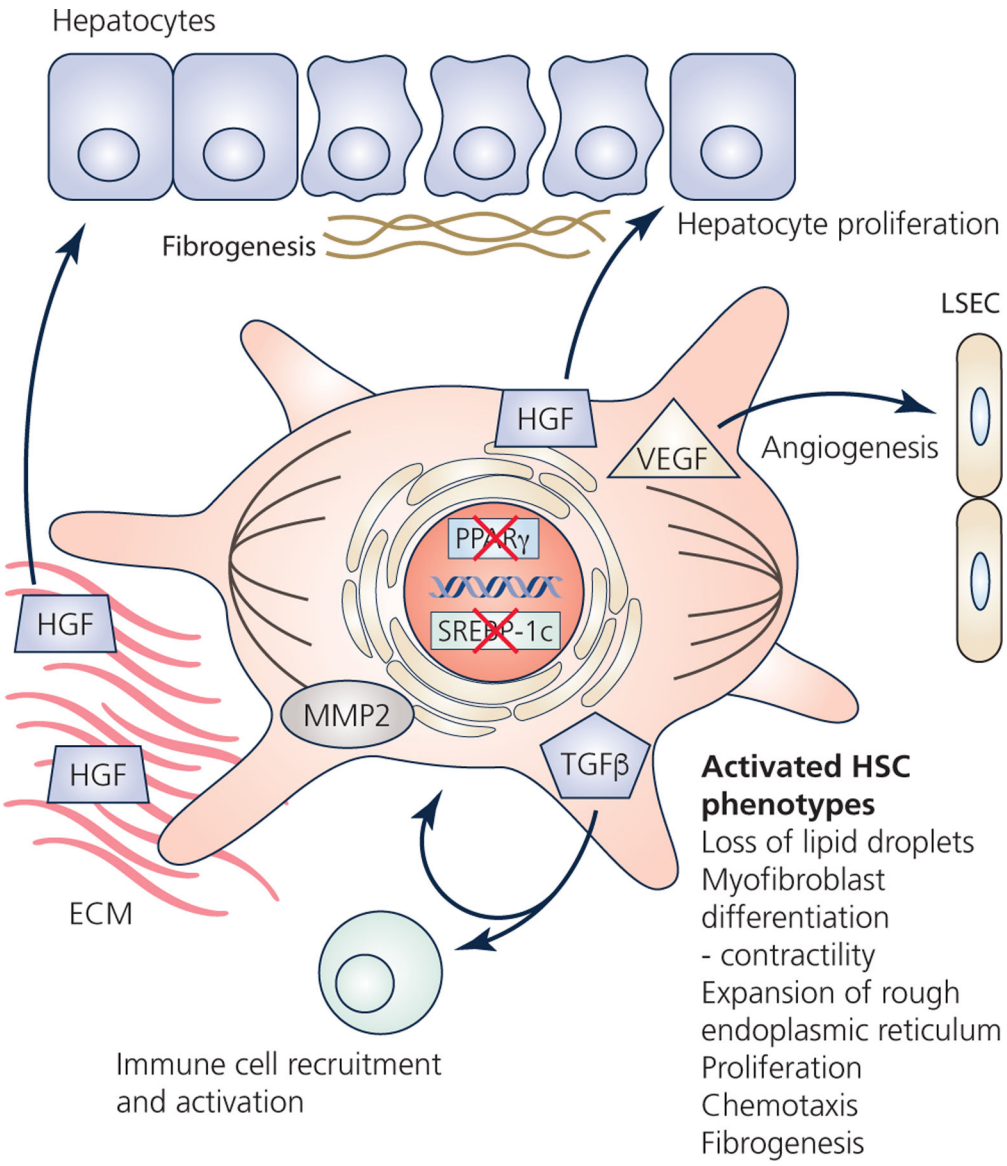

Figure 3 –. HSCs during liver injury and regeneration.

HSCs become activated by liver injury. Activation involves downregulation of the adipogenic transcription factors, and loss of the retinoid-storing lipid droplets. HSCs differentiate into contractile myofibroblasts, proliferate, and can respond to chemotactic signals. A number of secreted factors participate in the response to injury. For example, tumor growth factor beta (TGFβ) activates immune cells, but also enhances HSC activation via autocrine signaling. The HSCs continue to produce hepatocyte growth factor (HGF), but also degrade the basal extracellular matrix (ECM) by matrix metalloproteinase 2 (MMP2), which frees sequestered HGF and stimulates hepatocyte proliferation. HSCs also produce vascular endothelial growth factor (VEGF), as well as fibroblast growth factor, platelet-derived growth factor, and TGFβ which can all enhance angiogenesis. Activated HSCs also synthesize the collagen-rich fibrosis, as well as proteoglycans, glycosaminoglycans, and tissue-inhibitor of matrix metalloproteinases which all participate in the formation

of the fibrotic scar. Lastly, HSCs participate in the termination of liver regeneration by re-establishing the basal ECM which sequesters HGF. Likewise, TGFβ from HSCs can inhibit hepatocyte proliferation to help terminate liver regeneration.