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REVIEW

Hepatic Stellate Cells: Unique Characteristics in Cell Biology and Phenotype

Mitsuru Sato*, Shinsuke Suzuki, and Haruki Senoo

Department of Anatomy, Akita University School of Medicine, Akita 010-8543, Japan

ABSTRACT. **Hepatic stellate cells (HSCs), a mesenchymal cell type in hepatic parenchyma, have unique features with respect to their cellular origin, morphology, and function. Normal, quiescent HSCs function as major vitamin A-storing cells containing over 80% of total vitamin A in the body to maintain vitamin A homeostasis. HSCs are located between parenchymal cell plates and sinusoidal endothelial cells, and extend well-developed, long processes surrounding sinusoids *in vivo* as pericytes. However, HSCs are known to be 'activated' or 'transdifferentiated' to myofibroblast-like phenotype lacking cytoplasmic lipid droplets and long processes in pathological conditions such as liver fibrosis and cirrhosis, as well as merely during cell culture after isolation. HSCs are the predominant cell type producing extracellular matrix (ECM) components as well as ECM degrading metalloproteases in hepatic parenchyma, indicating that they play a pivotal role in ECM remodeling in both normal and pathological conditions. Recent findings have suggested that HSCs have a neural crest origin from their gene expression pattern similar to neural cell type and/or smooth muscle cells and myofibroblasts. The morphology and function of HSCs are regulated by ECM components as well as by cytokines and growth factors *in vivo* and *in vitro*. Liver regeneration after partial hepatectomy might be an invaluable model to clarify the HSC function in elaborate organization of liver tissue by cell-cell and cell-ECM interaction and by growth factor and cytokine regulation.**

Key words: hepatic stellate cell/extracellular matrix/gene expression/cytoskeleton/hepatic sinusoid/liver regeneration

Introduction

Hepatic stellate cells (HSCs), referred to also as Ito cells, vitamin A-storing cells, lipocytes, or fat-storing cells, are located between parenchymal cell plates and endothelial linings (Fig. 1), and have several important functions: 1) retinoid storage and homeostasis; 2) remodeling of extracellular matrix (ECM) by production of both ECM components and matrix metalloproteinases (MMPs); 3) production of growth factors and cytokines; 4) contraction and dilation of the sinusoidal lumen in response to endothelin, angiotensin, thromboxane, or prostaglandins (Wake, 1980; Blaner *et al.*, 1985; Clément *et al.*, 1984; Milani *et al.*, 1989; Friedman, 1990; Blomhoff and Wake, 1991; Loreal *et al.*, 1993: Geerts *et al.*, 1994; Senoo *et al.*, 1998). It has been reported that HSCs are involved in development and regeneration of liver tissue, reorganization of hepatic ECM, development of hepatic fibrosis, or cancer cell invasiveness (Clément *et al.*, 1984; Milani *et al.*, 1989; Loreal *et al.*, 1993: Friedman, 2000). HSCs are major mesenchymal cell type producing ECM components including type I, III, IV, and VI collagen, fibronectin, laminin, and proteoglycans.

HSCs exhibit mainly two phenotypes, 'quiescent' state and 'activated' or 'transdifferentiated' state (Friedman, 2000; Friedman and Arthur, 2002). Cytoplasmic lipid droplets containing retinyl esters and long cytoplasmic processes with fine branching are characteristics of quiescent HSCs, as seen in normal liver tissues. In activated state, HSCs lack both lipid droplets (Friedman *et al.*, 1993; Tsukamoto *et al.*, 1996) and long processes, and display proliferative and fibrogenic myofibroblast-like phenotype (Bachem *et al.*, 1992; Matsuoka and Tsukamoto, 1990). The 'activation' or 'transdifferentiation' process of HSCs is regulated by paracrine and autocrine loops of growth factors (Friedman

*To whom correspondence should be addressed: Mitsuru Sato, Department of Anatomy, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan.
Tel: +81–18–884–6057, Fax: +81–18–834–7808
E-mail: mitsuru@med.akita-u.ac.jp

Abbreviations: HSC, hepatic stellate cell; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; GFAP, glial fibrillary acidic protein; N-CAM, neural cell adhesion molecule; DDR, discoidin domain receptor; TGF-β1, transforming growth factor β1; PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; HGF, hepatocyte growth factor; IL-10, interleukin-10; RAR, retinoic acid receptor; RXR, retinoid-X receptor; PPAR, peroxisome proliferator-activated receptor.

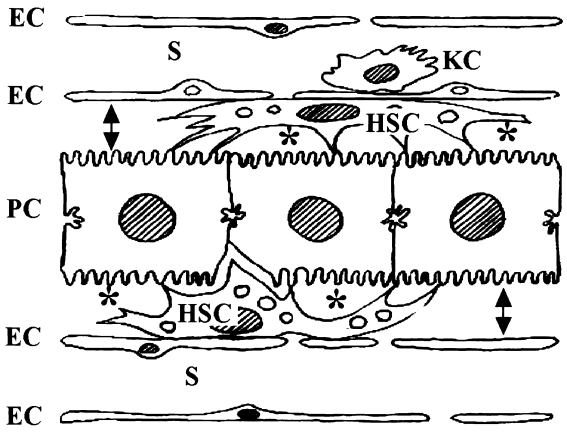

Fig. 1. A schematic drawing depicting the localization of HSCs between parenchymal cell and endothelial cell. Arrows indicate a classical definition of the perisinusoidal space of Disse between parenchymal cell and endothelial cell, whereas asterisks indicate a new concept of the perisinusoidal space of Disse between parenchymal cell and the complex of HSC and endothelial cell. EC, endothelial cells; HSC, hepatic stellate cells; KC, Kupffer cells; PC, parenchymal cells; S, sinusoids.

and Arthur, 1989; Friedman and Arthur, 2002; Matsuoka and Tsukamoto, 1990; Bachem *et al.*, 1992) in association with fibrosis in the pathological conditions such as liver injury and cirrhosis. HSC activation is accompanied by changes in gene expression and phenotype, and finally leads to enhanced cytokine expression and responsiveness, and accelerated production of ECM components, MMPs, and tissue inhibitors of metalloproteinase (TIMP) to promote ECM remodeling (Maher and McGuire, 1990; Nakatsukasa *et al.*, 1990; Iredale *et al.*, 1995).

It is well known that after isolation and primary culture using ordinary polystyrene culture dishes the quiescent phenotype of HSCs is changed to myofibroblast-like phenotype and to proliferate and secrete a large amounts of ECM components, resembling 'activated' HSCs seen *in vivo* after liver injury. We have shown that subcultured HSCs can restore at least morphologically a quiescent phenotype by culturing using interstitial collagen gel as a substratum (Miura *et al.*, 1997; Sato *et al.*, 1998). When cultured in or on extracellular type I collagen gel, HSCs alter their morphology accompanied by the elongation of long processes, as well as changes in collagen production and growth rate (Senoo *et al.*, 1996; Senoo and Hata, 1994). In this review, we introduce unique features of HSCs in cell biology and gene expression, and describe a phenotypic change in HSCs by 'activation' or 'transdifferentiation' *in vivo* and *in vitro*. We also discuss the regulatory role of ECM components in HSC morphology and function in culture, and finally a role of HSCs in liver regeneration following partial hepatectomy.

Characteristics of gene expression including cytoskeleton proteins in HSCs

HSCs consisting of approximately 15% of the total cell number in the liver are the major cells storing vitamin A (retinoids) (Li and Friedman, 2001). All HSCs in hepatic parenchyma have a similar function but are heterogeneous in desmin expression, vitamin A storage, proliferative activity, cytokine production, and ECM production (Blomhoff and Wake, 1991; Greenwel *et al.*, 1993; Zou *et al.*, 1998; Higashi and Senoo, 2003). The activated HSCs in culture show myofibroblast-like cell shapes with well-developed stress fibers of actin cytoskeleton. However, when cultured using interstitial collagen gel, HSCs are induced to elongate long cytoplasmic processes by reorganization of microtubules (Miura *et al.*, 1997; Sato *et al.*, 1998). In addition to actin and microtubule cytoskeleton, HSCs express several types of intermediate filaments including glial fibrillary acidic protein (GFAP) (Neubauer *et al.*, 1996; Niki *et al.*, 1996; Niki *et al.*, 1999), vimentin, and desmin (Burt *et al.*, 1986), and their expression is changed by the activation process.

Recent evidence has suggested that HSCs are derived from the neural crest since HSCs express GFAP and nestin (Niki *et al.*, 1999), and that neural crest stem cells can differentiate into myofibroblasts expressing α-smooth muscle actin which is a marker of activated HSCs. It is well known that HSCs are reactive to the Golgi staining (Wake, 1980) which is usually used for neurofilament staining. HSCs also display unique characteristics of gene expression similar to that of neural cell types; e.g. expression of neural cell adhesion molecule (N-CAM) (Knittel *et al.*, 1996), synaptophysin (Cassiman *et al.*, 1999), nestin, neurotrophins and neurotrophin receptors (Cassiman *et al.*, 2001), GFAP, or microtubule associated protein 2 (Sato *et al.*, 2001). These findings also suggest the neural crest origin of HSCs. Activated HSC, a major ECM-producing cell type in liver fibrogenesis, shows common phenotypic features to smooth muscle cells and myofibroblasts, both of which are originated from the neural crest and express vimentin, desmin, and α-smooth muscle actin.

Morphology and ultrastructure of HSCs

The liver parenchyma is composed of several cell types; 1) hepatic parenchymal cells, an epithelial component; 2) sinusoidal endothelial cells characterized by well-developed fenestrae or pores; 3) hepatic stellate cells (HSCs), perisinusoidal mesenchymal cells; 4) Kupffer cells, tissue macrophages; 5) other cell types, for instance, pit cells and dendritic cells. The cellular elements of liver parenchyma are organized within and around the sinusoids, and the perisinusoidal space of Disse separates the epithelium (parenchymal cells) from the complex of HSCs and sinusoidal endothelium (Fig. 1). It has been regarded that HSCs are localized in the perisinusoidal space of Disse, which is defined as

the space between parenchymal cell plate and sinusoidal wall composing of endothelial cells. However, HSCs have close contact with endothelial cells through incomplete basement membrane components (Fig. 1), and interstitial collagen fibers and nerve fibers run through the space between HSCs and parenchymal cells, and hence it is reasonable to define the perisusoidal space of Disse as the space between parenchymal cell plates and the complex of HSC and endothelial cell (Wake, 1995). The localization and long cytoplasmic processes of HSCs favor to promote their interactions with neighboring cell types. HSCs have direct contact with endothelial cells, and interact with parenchymal cells *via* microspines extending from cytoplasmic processes.

Interaction of cells with ECM components affects diverse cellular functions including cell differentiation, migration, proliferation, and survival (Hynes, 1992), and signals from ECM can regulate embryonic growth processes and differentiation, and tissue remodelling and repair (Davis *et al.*, 1987; Jones *et al.*, 1993; Senoo *et al.*, 1996). In normal liver the space between parenchymal cell and the complex of HSC and endothelial cell contains fibrillar ECM components such as type I and type III collagen and the basement membrane components which have usually no electron-dense structure as seen in the typical basement membrane of other tissues. Therefore, neither hepatic epithelial cells (parenchymal cells) nor sinusoidal endothelial cells have a typical basement membrane structure, despite the presence of basement membrane components under the endothelial cells (between HSC and endothelial cell).

The normal ECM components are essential to maintain the differentiated function and morphology of resident liver cells, namely parenchymal cells, endothelial cells, and HSCs. In liver fibrosis, the hepatic ECM composition is both quantitatively and qualitatively altered; the total amounts of collagenous and noncollagenous ECM components increase, and the ECM components between endothelial cells and HSCs are changed from the normal basement membrane-like matrices to fibrous collagen-rich matrices (Friedman, 2000). In addition to HSCs, extrahepatic stellate cells have also been found in several organs including lung, kidney, and intestine (Wake, 1980; Nagy *et al.*, 1997), and suggested to be involved in fibrous changes.

HSCs have been found to express several types of integrin receptors including $\alpha1\beta1$, $\alpha2\beta1$, $\alpha V\beta1$ and $\alpha6\beta4$ (Pinzani *et al.*, 1998). In addition to the classical ECM receptors, a non-integrin receptor, discoidin domain receptor-2 (DDR-2), has been found to be expressed in HSCs (Ankoma-Sey *et al.*, 1998). The morphology and function of HSCs might be regulated by interaction with interstitial type I and III collagen and other ECM components *via* integrin and non-integrin receptors.

In addition to the ECM production, HSCs have been found to produce several types of MMPs. Matrix proteases including MMPs have a pivotal role in remodeling of the ECM during liver injury and regeneration. MMPs including MMP-1, MMP-2, MMP-3, MMP-9, and MMP-11 are expressed in HSCs or hepatic parenchymal cells (Milani *et al.*, 1994; Takahara *et al.*, 1997; Geisler *et al.*, 1997), whereas tissue inhibitor of metalloproteinase-1 and -2 (TIMP-1 and -2) are expressed only in HSCs (Iredale *et al.*, 1992; Herbst *et al.*, 1997). HSCs produce virtually all the key MMPs including MMP-2 and MMP-3, which degrade the normal ECM components in the space between parenchymal cells and endothelial cells, whereas HSCs produce also TIMP-1 and -2, and hence can inhibit the interstitial collagenase activities (Knittel *et al.*, 1999; Friedman and Arthur, 2002). Taken together, HSCs are a predominant type of cells contributing to the hepatic ECM remodeling in both normal and pathological conditions.

ECM components regulate cell morphology in cultured HSCs

Cultured HSCs have been found to exhibit mainly three types of morphology according to a substratum: 1) myofibroblast-like cell shape when cultured on polystyrene surface, lysine- or aminoalkylsilane-coated surface, type I or type IV collagen-coated surface, 2) asteroid shape when cultured in or on interstitial type I or type III collagen gel, or 3) rounded shape when cultured in or on Engelbreth-Holm-Swarm murine tumor extracellular matrix (Matrigel) containing the basement membrane components (Miura *et al.*, 1997; Sato *et al.*, 1998; Kojima *et al.*, 1998). HSCs cultured on polystyrene surface show a flattened, myofibroblast-like shape with well-developed stress fibers (Fig. 2A). On the other hand, during culture using type I or type III collagen gel as a substratum HSCs display a drastic change in morphology accompanying the elongation of long cytoplasmic processes (Fig. 2B). Alternatively, HSCs cultured using Matrigel show neither cell spreading nor process elongation

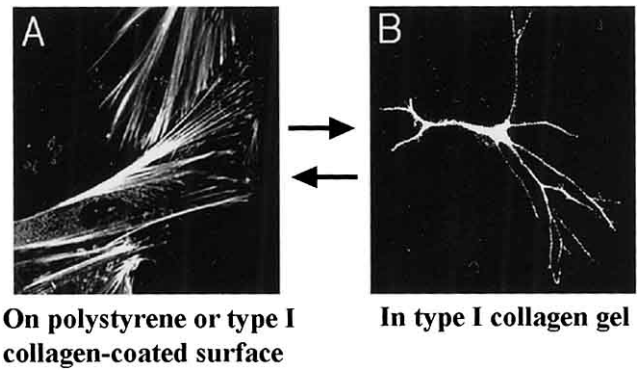

Fig. 2. Reversible morphological change in cultured HSCs by extracellular type I collagen fibrils used as a substratum. HSCs, cultured on polystyrene surface, as well as on type I collagen-coated surface, and stained with FITC-phalloidin, show well-developed stress fibers (A). HSCs, cultured in type I collagen gel and stained for microtubules, show well-developed cytoplasmic processes (B). Magnification, 630×.

and keep a round shape. Such morphological alteration in cultured HSCs is reversible (Senoo *et al.*, 1996). Differential response of HSCs to collagen types may be functionally important, since HSCs extend their processes *in vivo* along the perisinusoidal space containing fibrillar type I and type III collagen, whereas HSCs display smooth cell surface in contact with the endothelial cell membrane and/or the imperfect basal membrane components (Wake, 1980).

Dual fluorescence staining of microtubule and F-actin in HSCs cultured on type I collagen gel has revealed that the core of the elongated processes consists of microtubules, while the periphery contains F-actin, and that the elongating front of processes displays a similar structure to that of growth cone of neurites (Miura *et al.*, 1997; Sato *et al.*, 1998). The process elongation in cultured HSCs depends on integrin-binding to extracellular collagen fibrils, although the involvement of non-integrin collagen receptor such as DDR cannot be ruled out. Since no process elongation occurs in the culture on type I or type III collagen-coated surface (monomeric collagen molecules), two- or three-dimensional structure of native, fibrillar form of interstitial collagen is prerequisite for process elongation, as reported for response to native form of collagen fibrils but not to monomeric collagen in other cell types (Grab *et al.*, 1996; Mercier *et al.*, 1996; Vogel *et al.*, 1997). The process elongation of HSCs also depends on intracellular signaling such as protein phosphorylation and dephosphorylation, phosphatidylinositol-3 kinase, and small G proteins. The reorganization of F-actin is triggered by integrin-binding to ECM components, followed by signal transduction mechanisms including protein phosphorylation. In HSC culture, interstitial collagen signals appear to finally result in the reorganization of microtubules (Miura *et al.*, 1997; Sato *et al.*, 1998). HSCs can neither spread out nor elongate processes in or on Matrigel, but display rather normal *in vivo* functions when cultured on the basement membrane components (Friedman *et al.*, 1989). In addition to the induction of process elongation by extracellular, interstitial collagen fibrils, it has been also reported that interstitial type I collagen gel used as a substratum alters the HSC function to that of the quiescent, normal *in vivo* phenotype, such as lower proliferation activity and suppressed ECM production (Senoo *et al.*, 1996). Therefore, HSCs require the basement membrane components for normal, unactivated function, whereas they need interstitial collagen fibers for the process elongation and part of the function. Such interpretation is consistent with *in vivo* ECM status around HSCs in hepatic parenchyma (Wake, 1980).

Production of and regulation by growth factors and cytokines in HSCs

Transforming growth factor β1 (TGF-β1) and platelet-derived growth factor (PDGF) are the best characterized fibrogenic and proliferative cytokines, respectively, for HSCs. TGF-β1 has been found to be increased in experimental and human hepatic fibrosis, and TGF-β1 induces ECM production by HSCs (Friedman, 2000). Although there are many sources of this cytokine, autocrine expression in HSCs is the most important (Gressner, 1995).

In TGF-β1 knockout mice, collagen accumulation in response to liver injury is markedly reduced as compared to that in normal mice (Hellerbrand *et al.*, 1999). TGF-β1 gene expression is up-regulated in activated HSCs (Kim *et al.*, 1998). It has been demonstrated that TGF-β1 activity is increased by proteolytic activation of latent form of TGF-β1 by urokinase-type plasminogen activator in activated HSCs. TGF-β1 has been described to mediate up-regulation of collagen synthesis by HSCs (Brenner *et al.*, 1993; Inagaki *et al.*, 1995). In addition to the transcriptional regulation, the half-life of collagen α1(I) mRNA is 20-fold longer in activated HSCs compared to that of quiescent HSCs (Stefanovic *et al.*, 1999).

Hepatic injury is associated with both increased autocrine platelet-derived growth factor (PDGF) and up-regulation of PDGF receptor (Pinzani *et al.*, 1998). Activation of PDGF receptor leads to Ras recruitment and activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAP kinase) pathway. Studies using hepatocyte growth factor (HGF)-deficient or HGF receptor-deficient mice have revealed that both HGF and its receptor are essential for liver development (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). On the other hands, IL-10 down-regulates inflammation and increases interstitial collagenase activity (Wang *et al.*, 1998; Thompson *et al.*, 1998). In addition to the effects of soluble cytokines, reversion of HSC activation may be possible by reconstituted, perisinusoidal ECM components.

Nuclear receptors and HSCs

Of retinoid metabolites retinoic acids regulate cell growth and differentiation *via* nuclear receptors, retinoic acid receptors (RARs) and retinoid-X receptors (RXRs). It has been found that HSCs express RARs and RXRs, which levels are reduced during activation process (Ohta *et al.*, 1997). Retinoic acid signaling is greatly suppressed in HSCs during experimental cholestatic liver fibrosis and correlated with a decrease in the mRNA levels for RXR and retinoic acid receptors (Ohta *et al.*, 1997). Furthermore, retinoic acids have been reported to prevent phenotypic change and proliferation in activated HSCs (Davis *et al.*, 1988; Davis *et al.*, 1990; Pinzani *et al.*, 1992). Although intracellular lipid droplets containing vitamin A disappear by HSC activation, whether or not retinoid loss is directly related to HSC activation is still unclear.

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-dependent transcription factors. PPARγ is expressed mainly in adipose tissue and has a key role in adipogenesis,

although it is also expressed in other tissues at much lower levels. Ligand-activated PPARγ acts as heterodimer with 9-cis retinoic acid receptor (RXR) to alter the target gene expression by binding to PPAR responsive elements, consisting of a hexameric direct repeat (TGACCT) separated by a single nucleotide, and has been shown to have a key role in adipogenesis (Kliewer *et al.*, 1992; Tontonoz *et al.*, 1995; Forman *et al.*, 1995). HSCs have been found to express PPARγ, expression and transcriptional activity of which are reduced in transdifferentiated HSCs in culture (Galli *et al.*, 2000). Moreover, PDGF, the most potent mitogen for activated HSCs, suppresses PPARγ activity in HSCs through MAP kinase. Decline in PPARγ expression and transcriptional activity is associated with HSC proliferation, and the ligand-mediated activation of PPARγ exerts an inhibition of PDGF-induced proliferation of activated HSCs as well as interference with activated phenotype of HSCs and suppression of α1(I) procollagen promoter activity and mRNA expression. These results suggest the blocking of transdifferentiation process of HSCs *via* RXR/PPARγ signaling pathway, and hence the involvement of PPARγ in phenotypic change in HSCs and in liver fibrogenesis.

Liver regeneration

The liver has an extraordinary capacity to regenerate and restore from damaged tissue after chemical or mechanical injury. Rat 70% partial hepatectomy has been used as a liver regeneration model (Higgins and Anderson, 1934), providing insights into alteration in gene expression of transcription factors, growth factors, and cytokines, and in reorganization of ECM or hepatic sinusoid architecture. Since hepatic resident cells proliferate differentially among distinct cell types constituting hepatic parenchyma; in rat after 70% partial hepatectomy DNA synthesis in parenchymal cells peaks at 24 hours and terminates by 72 hours, whereas in sinusoidal endothelial cells it starts at 48 to 72 hours and peaks at 4 day (Grisham, 1962; Widmann and Fahimi, 1975; Martinez-Hernandez *et al.*, 1991). Growth factors such as epidermal growth factor, transforming growth factor α (TGFα), and hepatocyte growth factor (HGF) have a potent mitogenic activity for hepatic parenchymal cells (Fausto *et al.*, 1995). The difference in proliferation between parenchymal cells and endothelial cells results in the formation of avascular islands of 12–15 parenchymal cells in the liver lobule (Martinez-Hernandez and Amenta, 1995). At this time, many sinusoidal endothelial cells are enveloped by HSCs having a peak of DNA synthesis at 48 hours after partial hepatectomy. Following proliferation, HSCs and endothelial cells migrate into parenchymal cell clusters, as followed by the formation of new vascular branches and perisinusoidal spaces.

The fenestrated sinusoidal endothelial cells, lacking the typical basement membrane structure, regulate uptake of chylomicrons and lipoproteins, growth factors, and hormones between the blood circulation and hepatic parenchymal cells through the perisinusoidal space of Disse (Wisse *et al.*, 1985). During liver regeneration, ECM synthesis might play an important role in restoring the quiescent and differentiated phenotype of hepatic parenchymal cells, and ECM remodeling largely mediated by activated MMPs might be a prerequisite for parenchymal cell proliferation after partial hepatectomy (Galli *et al.*, 2000). Plasmin is responsible for activating the latent form of MMPs, and then active MMPs digest the ECM in the early stages of liver regeneration (Kim *et al.*, 1997). Since HSCs are a major type of cells producing growth factors and cytokines, ECM components, and MMPs, HSCs have a pivotal role in liver regeneration including both parenchymal cell proliferation and ECM remodeling.

Conclusions

HSCs display diverse phenotypes with respect to the morphology, function, and gene expression in hepatic parenchyma, and have invaluable roles in vitamin A-storage, ECM remodeling, and liver development and regeneration. Gene expression in HSCs is regulated by cell-cell and cell-ECM interaction, or cytokines and growth factors. Several recent reports have suggested that HSCs have a neural crest origin from similar findings on gene expression pattern between HSCs and neural cells, and from the resemblance in their phenotype to that of smooth muscle cells and myofibroblasts, both of which are known to be derived from the neural crest. HSCs showing a quiescent phenotype with cytoplasmic lipid droplets containing vitamin A and well-developed cytoplasmic processes are activated or transdifferentiated to myofibroblast-like phenotype lacking lipid droplets and cytoplasmic processes and displaying a remarkable fibrogenic nature. Whether or not activated HSCs can revert to the quiescent phenotype is a controversial issue (Friedman and Arthur, 2002). It has been suggested that the activated HSCs can at least in part retrace the activation process, for example, by signaling from retinoids or PPARγ activation, or interaction with ECM components such as native, fibrillar type I collagen or the basement membrane components.

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