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EDITORIAL

Can a fibrotic liver afford epithelial-mesenchymal transition?

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Abstract

The question whether epithelial-mesenchymal transition (EMT) occurs during liver fibrogenesis is a controversial issue. *In vitro* studies confirm that hepatocytes or cholangiocytes undergo EMT upon transforming growth factor β (TGF-β) stimulation, whereas *in vivo* experiments based on genetic fate mapping of specific cell populations suggest that EMT does not occur in fibrotic animal models. In this review we present current data supporting or opposing EMT in chronic liver disease and discuss conditions for the occurrence of EMT in patients. Based on the available data and our clinical observations we hypothesize that EMT-like alterations in liver cirrhosis are a side effect of high levels of TGF-β and other pro-fibrotic mediators rather than a biological process converting functional parenchyma, *i.e.*, hepatocytes, into myofibroblasts at a time when essential liver functions are deteriorating.

Key words: Epithelial-mesenchymal transition; Liver fibrosis; Liver cirrhosis; Transforming growth factor-β

Core tip: This review provides a personal notion about

whether a complete epithelial-mesenchymal transition (EMT) occurs in human fibrotic livers. We consider three aspects that might determine the occurrence of EMT: (1) capacity of parenchymal cells; (2) potential benefit for the liver and the whole body; and (3) microenvironment within a fibrotic liver. Clinical evidence suggests that in humans, EMT-like alterations occur mainly in advanced chronic liver disease, *i.e.*, cirrhosis. In such a severe disease state, the most urgent mission for a liver is to maintain a maximum number of functional hepatocytes, while hepatic stellate cells and portal fibroblasts provide an ample supply of myofibroblasts. It appears that there is no need for additional sources of myofibroblasts in a cirrhotic liver. EMT-like alterations in parenchymal cells are most likely a side effect of high levels of EMT-promoting factors such as TGF-β.

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INTRODUCTION

The progression of liver fibrosis is a dynamic process characterized by excessive deposition of extracellular matrix (ECM). Myofibroblasts (MFB) are the major ECM-producing cells[1,2]. MFB are derived from different cell types with sinusoidal hepatic stellate cells (HSC), portal fibroblasts and bone marrow-derived fibrocytes being the most prominent sources[3]. Whether hepatocytes and/or cholangiocytes differentiate into MFB by way of epithelial-to-mesenchymal transition (EMT) is still controversial[4-10]. In this review, we discuss actual data supporting or opposing the occurrence of EMT during liver fibrogenesis.

Why does EMT occur during embryogenesis?

A hypothetical biological process requires three preconditions: (1) the process has to provide a benefit to either the local organ or the system; (2) the cells must be capable of performing the process; and (3) the process must be supported by the surrounding microenvironment. EMT is classified into three subtypes[11]: Type 1 EMT, which is associated with implantation, embryo formation, and organ development; type 2 EMT, which is a repair-associated function that generates fibroblasts and other related cells in order to reconstruct tissues following trauma and inflammatory injury; and type 3 EMT in neoplastic cells that have previously undergone genetic and epigenetic changes, particularly in genes that favor clonal outgrowth and the dissemination of tumors. So far, type 1 EMT is the best-characterized subclass, occuring in the embryo at gastrulation[12,13]. A subset of cells from the epiblast moves to the midline to form the primitive streak. These cells undergo EMT and internalize to generate mesoderm and endoderm, while those remaining in the epiblast become ectoderm[12,13]. EMT and MET between endoderm and mesoderm are critical mechanisms for organogenesis, for example in the kidney[14-16]. However, EMT does not play an important role during liver organogenesis because hepatoblasts, from which hepatocytes and BEC are subsequently derived, arise from endoderm rather than mesoderm[17].

EVIDENCE SUPPORTING AND OPPOSING EMT IN LIVER FIBROSIS

According to a brief definition of EMT, that is, "epithelial cells changing their phenotype and acquiring mesenchymal properties" [11], two types of adult liver cells can undergo EMT under experimental conditions: hepatocytes and cholangiocytes[18]. Given that HSC are mesenchymal cell in the first place, regardless if quiescent or activated, the conversion of HSC into MFB is not considered EMT. Thus the term EMT refers to the process of hepatocytes or cholangiocytes obtaining phenotypes of mesenchymal cells and differentiating into MFB.

Parenchymal cells express mesenchymal markers in patients with advanced chronic liver disease

Evidence supporting the occurrence of EMT during liver fibrogenesis is based on immunohistochemistry and co-staining studies. Expression of multiple mesenchymal markers, including vimentin, S100A4 [fibroblast-specific protein (FSP-1)], heat shock protein 47 (HSP47), snail, and α-smooth muscle actin (α-SMA), has been reported in parenchymal cells of patients with different chronic liver disease[17,19,20]. Diehl AM's group showed that S100A4 is expressed in reactive ducts of patients with primary biliary cholangitis (PBC) and of cirrhotic patients with non-alcoholic steatohepatitis (NASH)[19,21]. Díaz *et al*[17] found that in pediatric patients with biliary atresia and adult patients with primary sclerosing cholangitis (PSC)/PBC, cholangiocytes and reactive ducts express FSP-1, the collagen chaperone HSP47, the intermediate filament protein vimentin, and the transcription factor snail. Dooley *et al*[22] showed that a portion of hepatocytes in patients with HBV-associated cirrhosis expressed Snail. These results suggest that parenchymal cells do indeed express mesenchymal markers in chronic liver disease. It should be noted that parenchymal cells expressing mesenchymal markers have only been found in patients with advanced chronic liver disease, *e.g.*, cirrhosis so far. There is no data showing that parenchymal cells of patients express mesenchymal markers at early stages of liver fibrosis.

In vitro studies confirm the occurrence of EMT in liver cells

Further evidence supporting the occurrence of EMT of liver parenchymal cells comes from *in vitro* studies.

Fetal rat hepatocytes treated with transforming growth factor β (TGF-β) underwent an EMT, presenting high levels of vimentin and Snail and lack of cytokeratin 18 and E-cadherin[23]. Murine primary hepatocytes cultured on monolayers of dry collagen undergo dedifferentiation and lose polarity and liver function within 3 d[24]. Changing culture conditions by seeding hepatocytes within a sandwich of two soft collagen gel layers preserves an epithelial phenotype for extended periods[24]. Upon TGF-β stimulation, primary hepatocytes on both dry collagen monolayer and soft collagen gel sandwich quickly exhibit myofibroblast-like morphological changes, lose tight junction proteins (*e.g.,* Occludin and E-cadherin), and express mesenchymal markers (vimentin, connective tissue growth factor, S100A4, *et al*)[4,24,25]. In contrast to hepatocytes of untreated mouse livers, hepatocytes derived from carbon tetrachloride (CCl_4)-induced cirrhotic mice express vimentin, a mesenchymal marker, *in vitro* and *in vivo*[25].

TGF-β induces hepatocytes' EMT through regulating the expression of transcription factors, in particular Snail, the master gene of EMT, and hepatocyte nuclear factor 4α (HNF4α), the master gene of hepatocyte differentiation[26,27]. The Snail family induces EMT in different epithelial cells, including hepatocytes. In fetal liver, TGF-β induces apoptosis of hepatocytes. Snail confers hepatocytes resistance to TGF-β-induced cell death[26,27]. In addition, Snail expression is sufficient to induce EMT in adult hepatocytes. HNF4α is an essential transcription factor maintaining the epithelial phenotype of hepatocytes[28]. During EMT of hepatocytes, expression of HNF4α is largely inhibited by TGF-β administration[27]. The inhibitory effects are performed by upregulating Snail, which represses transcription of the *HNF4α* gene through direct binding to its promoter[27]. The balance between these two transcription factors plays a pivotal role in regulating EMT/MET dynamics in hepatocytes[29].

Besides hepatocytes, primary cholangiocytes isolated from rats following one week of bile duct ligation (BDL) express S100A4 while showing reduced expression levels of epithelial markers such as cytokeratin 19 and 7[21]. When an immature cholangiocyte line was treated with conditioned medium from myofibroblastic HSC, these cholangiocytes underwent complete EMT[21]. Consistent with the findings in rat cholangiocytes, Rygiel *et al*[30] reported that administration of TGF-β induced expression of mesenchymal markers in cultured primary human cholangiocytes. These results show that (1) *in vitro* cell culture conditions (*e.g.,* putting cells on monolayer gel) induce hepatocytes' loss of epithelial feature; and (2) pro-EMT factors in cultured medium, such as TGF-β, induce rapid EMT of liver parenchymal cells.

Current fibrotic animal models deny the occurrence of EMT during liver fibrogenesis

Although a study of the CCl_4-induced fibrotic mouse model stated the occurrence of EMT during liver fibrosis[31], later studies based on genetic cell fate mapping provided convincing evidence that in contrast to liver parenchymal cells in primary culture, EMT does not occur in fibrotic animal models induced by BDL, CCl_4, and 3,5-diethoxycarbonyl-1,4-dihydrocollidine[4,5,10]. This issue has been discussed intensively[3,6,8].

TGF-β: BETWEEN FIBROSIS AND EMT

As mentioned above, one key finding supporting the occurrence of EMT in damaged liver is that parenchymal cells express mesenchymal markers. Why would they do that? One explanation might be that there are high levels of growth factors such as TGF-β surrounding these cells.

TGF-β is not only the most important pro-fibrotic cytokine[32], but also the most efficient growth factor promoting EMT[33]. It has been confirmed that liver parenchymal cells undergo EMT in culture medium with TGF-β stimulation[4,24,25]. During chronic liver diseases, TGF-β is produced by multiple systemic and local cells, including macrophages, monocytes, activated HSC and reactive ducts[34,35]. In addition, TGF-β treatment also induces BMOL cells, a murine liver progenitor cells (LPC) line, to undergo EMT-like phenotype change *in vitro* (unpublished data). There is a close correlation between phosphorylated Smad2 levels and fibrotic stages in HBV- and steatosis-associated chronic liver disease[36]. This means that parenchymal cells in cirrhotic livers often reside in an environment teeming with high levels of TGF-β. It is quite likely that such a microenvironment can force the expression of mesenchymal markers in parenchymal cells.

However, the occurrence of EMT should not be defined merely by parenchymal cells expressing mesenchymal markers. To accomplish a complete EMT in the liver, hepatocytes or cholangiocytes are required to finish at least the following steps: (1) expression of mesenchymal markers; (2) loss of anchoring proteins such as E-cadherin and Occludin; and (3) release from adjoined hepatocytes/cholangiocytes and conversion into an isolated MFB. To date, there is no conclusive evidence that hepatocytes or cholangiocytes expressing mesenchymal markers undergo the latter two steps and become real MFB.

It should be re-emphasized that parenchymal cells expressing mesenchymal markers are found only in advanced stages of chronic liver disease, particularly in cirrhosis. At this stage, survival of parenchymal cells for the maintenance of liver function is of prime importance. Therefore we surmise that the most likely scenario is that expression of mesenchymal markers by parenchymal cells represents a response to high levels of TGF-β rather than evidence for EMT in a liver with severely impaired functions.

CAN LIVERS EVEN AFFORD EMT IN CIRRHOTIC PATIENTS?

Based on current data, it is too early to conclude

that EMT of liver parenchymal cells contributes to the MFB pool *in vivo*. Given the vast difference between tissue culture and the human liver, observations of EMT following TGF-β incubation *in vitro* by no means provide convincing evidence that the same phenotypic alterations occur during progression of chronic liver disease *in vivo*. On the other hand, the fact that EMT does not occur in fibrotic animal models does not rule out the possibility of EMT in patients with chronic liver disease. The currently used fibrotic models have a maximum observation period of several months, whereas the history of a patient progressing to liver cirrhosis spans years and decades[37]. The fact that patients with chronic liver disease have such a long natural history bears witness to the huge capacity of the human liver for self-repair, even under continuous attack.

The liver is the largest gland in the body, and it supports nearly every other organ in some aspect. The majority of physiological functions of the liver are performed by hepatocytes, including metabolism of carbohydrates, proteins, amino acids, lipids and some important hormones, the production and excretion of bile, metabolism and excretion of toxic substances, and synthesis of coagulation factors[38]. In order to implement these copious complex physiological functions, the liver owns special blood systems and anatomic architecture. A hepatocyte has three boundaries: the sinusoidal, lateral and canalicular membranes[28]. The cell is highly polarized with transport directed from its sinusoidal surface to the canalicular surface[28]. The canalicular domains between two adjacent hepatocytes constitute the smallest bile lumen (diameter: 1 μm)[39]. The adjoining apical membranes of a bile lumen are sealed by tight junctions (zonula occludens), representing the only physical barrier between the blood and the canalicular lumen. These tight junctions determine "paracellular permeability" between blood and bile[39]. In normal liver, hepatocytes are arranged in one-cell thick cords[40]. Such arrangement makes hepatocyte-produced bile delivery easy. If a complete EMT should occur in these hepatocytes, one key issue would be that the loss of hepatocytes from these one-cell thick cords must not alter primary liver architecture. In a patient with chronic liver disease, the organ is under continuous insult and yet manages to maintain a normal function to support the body's physiological requirements for several decades. To achieve this feat, the liver has to avoid any response that is likely to disturb the above-mentioned hepatocyte arrangement.

Deposition of ECM by MFB is a key process in liver repair. In acute liver injury, particularly during acute liver failure, the severely damaged organ recruits enormous numbers of ECM-producing MFB in order to maintain a relatively intact liver architecture[41]. Furthermore, MFB and the ECM they produce are providing a niche for the activation of LPC, a major cell source for liver regeneration in acute liver failure[42,43]. Under these conditions, most mature hepatocytes have gone extinct[44,45]. Still these copious amounts of MFB do not cause fibrosis: Once the damaging etiology is removed, the damaged liver can recover its function and restore its architecture completely, although fibrotic septa produced by MFB persist for several months or years. This process is summarized as "wound healing".

In chronic liver disease, enduring damage induces excessive ECM deposition beyond the liver's capacity for degradation[46]. Such excessive ECM deposition combined with local hepatocyte death and regeneration finally results in distortion of the hepatic architecture and vascular structures[47]. The process is described as liver fibrosis and its end stage cirrhosis. Actually, the line between "wound healing" and "fibrosis" is a blurred one. Defining the two processes only according to disease time, for example acute or chronic, is artificial. It is impossible to claim that ECM deposition in the liver during chronic disease is completely "fibrogenesis", rather than "wound healing". During several decades of chronic liver disease progression, the human body is constantly trying to repair and restore the damaged liver. Before decompensated liver cirrhosis is established, withdrawal of etiology can still reverse liver fibrosis and even cirrhosis to some degree[48,49]. Regeneration and repair represent two aspects of host defence. When we discuss whether EMT occurs in chronic liver damage or not, it is important to consider whether there is actually any requirement for hepatocytes to transdifferentiate into MFB through EMT. In our view, it is highly doubtful if MFB derived from other cell sources, *e.g.,* HSC, should be insufficient to produce the amount of ECM required for healing and repair.

Morphologically, at least five fibrotic septa patterns are demonstrated in patients with liver fibrosis: portal to portal, portal to central, central to central, chicken-wire and portal pipestem[40,50]. Etiology, the topographic localization and nature of injury, and disease stage are critical factors that determine the pattern of liver fibrosis.

Patients with alcoholic steatohepatitis (ASH) or NASH usually have pericellular fibrosis, *i.e.,* the deposition of fibrillar matrix is concentrated around the sinusoids and groups of hepatocytes and displays a chicken-wire like shape[40,50]. It is well recognized that this "chicken-wire fibrosis" is dependent on sinusoidal HSC activation. Do hepatocytes undergo EMT and transdifferentiate into MFB in these circumstances? Most likely not: In patients with ASH or NASH, hepatocytes manifest with steatosis, ballooning degeneration, and containing Mallory-Denk bodies. These cells usually do not have an intact liver function. Severe ASH or NASH leads to lytic necrosis and apoptosis of hepatocytes. In the end-stage of these diseases, particularly in ASH, there may be large amounts of parenchymal extinction, suggesting secondary vascular events[40]. Under these circumstances, the most important mission

for surviving hepatocytes is maintaining liver function. It is difficult to fathom that such a liver would induce EMT in functionally impaired, or even in some of the few remaining functional hepatocytes. On the other hand, no data indicate that there might be insufficient HSC-derived MFBs to produce the amount of ECM required for tissue repair and/or fibrogenesis.

In contrast to ASH and NASH, ECM deposition in biliary disease is dependent on portal fibroblasts. In cholestatic diseases such as PBC and PSC, fibrosis initiates from portal tracts, induced by obstruction, loss, or inflammation of bile ducts[51]. Geographically, peribiliary fibroblast-derived MFB are primarily responsible for the deposition of portal tract collagen[52]. The biliary fibrosis due to activation of peribiliary and portal fibroblasts explains the lack of subdivision with parenchymal fibrotic septa until late stages of the disease[40]. Morphologically, the MFB of bridging septa in cholestatic livers strongly resemble the MFB of the portal field[53]. These cells can be distinguished from HSC-derived MFB using combined staining for fibrillin-1 and elastin[52]. Activated HSC generate fibrillin-1-positive but elastin-negative ECM, whereas MFB inside the portal tracts produce both fibrillin-1- and elastin-positive ECM[54]. In addition, activated portal tract fibroblasts express some different protein markers such as cellular retinol-binding protein-1[55].

Besides activation of portal fibroblasts, ductular reaction (DR), which is defined as "ductules accompanied by an inflammatory infiltrate and by fibrosis", is a critical histological feature in most cholestatic liver diseases[51,56-58]. It is mainly reactive ducts that have previously been reported to express mesenchymal markers[17,21]. Will these reactive ducts expressing mesenchymal markers differentiate into MFB? To date there is no evidence supporting this hypothesis. DR in cholestatic liver disease has several cell sources, including the small intralobular bile ducts, ductules, canals of Hering and from "ductular metaplasia" of periportal hepatocytes[58]. Cholestatic pathogenesis is initiated by bile leakage due to obstruction of extrahepatic bile ducts and loss of small intrahepatic bile ducts. DR accompanied by inflammation and fibrosis constitutes a protective response to the destruction of interlobular bile ducts. These reactive ducts provide abortive bypass mechanisms for the drainage of bile in the diseased liver, and thus protect hepatocytes from the deleterious effect of bile acid overload[59]. It has been well recognized that LPC residing in canals of Hering are the major source of DR in cholestatic diseases[58]. In advanced stages of PSC, severe destruction of small ductules including canals of Hering reduces the number and size of DR[60]. Thus, it is clear that DR is a key process of the liver in order to restore the architecture of a damaged biliary tree. LPC are activated and undergo differentiation to cholangiocytes to recover ruined bile ducts. On the other hand, DR and the accompanying inflammatory response indeed play an important role in portal and periportal fibrosis by producing and secreting a variety of biologically active fibrosis-associated mediators, including TGF-β2, connective tissue growth factor, platelet derived growth factor, tumor necrosis factor-α, interleukin (IL)-6, IL-8, monocyte chemotactic protein-1 and nitric oxide[61]. Thus, these data suggest that DR contributes to biliary fibrosis through producing critical pro-fibrogenesis factors rather than differentiating into mesenchymal cells.

HYPOTHESIS: HEPATOCYTES ARE NOT ALLOWED TO PERFORM EMT IN A CIRRHOTIC LIVER

Human liver cirrhosis develops over years or decades. Histologically it is characterized by diffuse nodular regeneration surrounded by dense fibrotic septa with subsequent parenchymal extinction and collapse of liver structures, causing pronounced distortion of hepatic vascular architecture[40,47,62,63]. Of all these histological features, parenchymal extinction is rarely found in animal models[46]. Parenchymal extinction denotes the loss of contiguous hepatocytes, producing lesions that remodel into septa that vary from 0.05 mm to several millimeters in thickness[60,62]. Only recently, an elegant study from Stueck and Wanless showed that repopulation of parenchymal extinction lesions in cirrhotic human liver is dependent on LPC activation[60]. This result suggests that without LPC-derived hepatocytes, the remaining mature hepatocytes in a cirrhotic liver are not sufficient to ensure liver function. The most urgent mission of a cirrhotic liver is to maintain a maximum number of functional mature hepatocytes, either by proliferation of the remaining hepatocytes, or from LPC. Proliferating cells cannot perform EMT in breast cancer[64,65]. Consistent with breast cancer cells, TGF-β administration or over-expression of Snail induce EMT as well as cell cycle arrest, which favors survival signals in hepatocytes[27]. Thus, a cirrhotic liver is unlikely to support or induce a biological process like EMT in the surviving parenchymal cells. On the other hand, the decision if EMT is required for hepatic fibrogenesis and tissue repair might also depend on whether MFB derived from other cell sources provide sufficient ECM. At the present time, there are no studies indicating that the activated HSC, portal fibroblasts and fibrocytes provide insufficient MFB.

CONCLUSION

It may be too early yet to exclude the occurrence of type 2 EMT in patients with chronic liver damage. However, current evidence indicates that EMT only occurs in the advanced stages of chronic liver disease. In this phase, *i.e.,* during cirrhosis, mature hepatocytes performing vital functions are decreasing in numbers. LPCs are activated to replenish hepatocytes in order

Table 1 Selected evidence supporting or opposing epithelial-mesenchymal transition during liver fibrogenesis

	Ref.
Supporting evidence	
In vitro	
Primary cultured hepatocytes or cholangiocytes with TGF-β stimulation undergo complete EMT	[4,21,23-27,29,30]
Patients	
Liver parenchymal cells in patients with advanced chronic liver disease express mesenchymal markers	[17,19-22,30]
Opposing evidence	
Techniques based on genetic cell fate mapping of specific cell populations provided convincing evidence that EMT does not occur in fibrotic animal models	[4,5,10]
Clinical observation	
There is no data showing that parenchymal cells of patients express mesenchymal markers during early stages of fibrosis	
Parenchymal cells expressing mesenchymal markers are found only in patients with advanced chronic liver disease, *e.g.*, cirrhosis.	
A cirrhotic liver is not likely to drive remaining parenchymal cells towards a non-essential biological process like EMT	
No studies indicate that activation of HSC, portal fibroblasts and fibrocytes produce insufficient MFB	
In the cirrhotic liver, parenchymal cells expressing mesenchymal markers might be caused by high levels of surrounding pro-EMT factors, *e.g.*, TGF-β	

TGF-β: Transforming growth factor β; EMT: Epithelial-mesenchymal transition; HSC: Hepatic stellate cells; MFB: Myofibroblasts.

to maintain crucial liver functions[60]. Under these conditions, it seems rather counterproductive for a severely damaged liver to induce conversion of hepatocytes into MFB. There are multiple alternative sources of MFB, including HSC, portal fibroblasts and fibrocytes. To date, there is no evidence suggesting that these cell sources produce insufficient MFB for liver repair or fibrogenesis. We propose that in the cirrhotic liver, parenchymal cells express mesenchymal markers in response to high levels of surrounding pro-EMT factors, *e.g.*, TGF-β.

The notions discussed in this paper are based on our observations only, and at present lack supporting experimental evidence. We hope that future studies and observations will provide clinical evidence to confirm, correct or refute our hypothesis. Table 1 summarizes current evidence supporting or opposing EMT during liver fibrogenesis.

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