

Epithelial-mesenchymal transition in liver fibrosis (Review)

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Abstract. Liver fibrosis is the result of a sustained wound healing response to sustained chronic liver injury, which includes viral, alcoholic and autoimmune hepatitis. Hepatic regeneration is the dominant outcome of liver damage. The outcomes of successful repair are the replacement of dead epithelial cells with healthy epithelial cells, and reconstruction of the normal hepatic structure and function. Prevention of the development of epithelial-mesenchymal transition (EMT) may control and even reverse liver fibrosis. EMT is a critical process for an epithelial cell to undergo a conversion to a mesenchymal phenotype, and is believed to be an inflammation-induced response, which may have a central role in liver fibrosis. The origin of fibrogenic cells in liver fibrosis remains controversial. Numerous studies have investigated the origin of all fibrogenic cells within the liver and the mechanism of the signaling pathways that lead to the activation of EMT programs during numerous chronic liver diseases. The present study aimed to summarize the evidence to explain the possible role of EMT in liver fibrosis.

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1. Introduction

Liver fibrosis is a complex pathophysiological process of numerous chronic liver diseases, which are characterized by the deposition of the extracellular matrix (ECM). Hepatic stellate cells (HSCs), fibroblasts and myofibroblasts participate in the process via different mechanisms. The inevitable consequence of sustainable liver fibrosis is liver cirrhosis and hepatic cancer, and therefore, preventing liver fibrosis is the primary measure. At present, studies focus on the mechanisms that potentially delay the process of liver fibrosis and even reverse it. Accumulating evidence has shown that mesenchymal cells have an important role in hepatic fibrogenesis. The epithelial-mesenchymal transition (EMT) is suggested as one of the important origins of mesenchymal cells.

During the EMT, epithelial cells lose their epithelial characteristics and gradually obtain a mesenchymal phenotype. The source of the mesenchymal cells participating in tissue repair and regeneration remains to be elucidated. Bi *et al* (1) reported that alendronate sodium significantly arrested the progression of liver fibrosis. Deng *et al* (2) observed that biliary epithelial cells (BECs) undergoing EMT may contribute to fibrogenesis in biliary atresia by detecting the antigen for cytokeratin-7 (CK-7), heat-shock protein 1 (HSP1), HSP47 and α -smooth muscle actin (α -SMA) in liver sections from patients with biliary atresia. This progress involves the switch of cadherin from E-cadherin to N-cadherin, the dissolution of intracellular connections, the upregulation of matrix remodeling factors and the rearrangement of the cellular cytoskeleton.

2. Different cell types in liver fibrosis

Liver fibrogenic cells. Liver fibrogenic cells are a heterogenous cell group, which includes the α -SMA⁺ myofibroblasts (MFs). Liver fibrogenic cells may have a major role in liver fibrosis according to recent studies, and the origin of these cells remains to be elucidated. HSCs are considered the major source as they are the main ECM-producing cells in the injured liver. Hepatic MFs may also originate from bone marrow-derived mesenchymal cells and cells from EMT and endothelial-mesenchymal transition (EnMT).

HSCs. Activation of HSCs is a central event in liver fibrosis. Recently, a number of studies have demonstrated that HSCs are derived from mesodermal-derived multipotent

mesenchymal progenitor cells. HSCs are significant in producing the ECM, particularly collagen type 1, which is regulated by complex stimuli and pathways. Transforming growth factor- β (TGF- β) is prominent among these stimuli. TGF- β has 3 major isoforms: TGF- β 1, TGF- β 2 and TGF- β 3. Generally, TGF- β 1 is stored in an inactivated state, and once activated, it will enhance the transcription of the target gene via its receptors to Smad proteins. As it responds to Smad, the further matrix production in HSCs differs between acute and chronic injury (3). In addition to TGF- β , there are numerous other factors that exhibit profibrogenic effects on HSCs, such as retinoids and angiotensin II (4-6). During liver fibrosis, parenchymal injury and sustained inflammation generate a large panel of signals that induce the activation of quiescent HSCs. HSC activation is associated with the activation of nuclear factor κ B (NF- κ B) and activator protein 1, which are activated following the stimulation of intracellular signaling cascades. Platelet-derived growth factor has been shown to activate mitogen-activated protein kinase (MAPK) signaling, specifically c-Jun N-terminal protein kinase, extracellular signal-regulated kinase (ERK) and p38, and finally, regulate HSCs proliferation. Following the activation of HSCs, a variety of changes in gene transcription occur. The target genes include, but are not limited to, the following: α -SMA, type 1 collagen, MMP-2, TGF- β 1, TGF- β receptors, TIMPs 1 and 2 (7,8). Persistent activation leads to changes in HSC behavior, such as proliferation, chemotaxis, fibrogenesis and cytokine release, and all these changes are discrete (9). Liver MFs originating from activated HSCs exhibit high proliferative capacity, upregulate the expression of typical mesenchymal cell markers, such as α -SMA, type 1 collagen, fibronectin, desmin and vimentin intermediate filaments (10).

Portal fibroblasts (PFs). The PFs are spindle shaped and exhibit biological similarities with activated HSCs; however, they have different genetic profiles and signaling responses (11,12). They are of mesenchymal origins that undergo myofibroblastic differentiation. PFs do not express α -SMA, glial fibrillary acidic protein filaments and desmin, cluster of differentiation 146 (CD146) and cellular retinol-binding protein-1 proteins (13,14), nor store retinoids, which is different from HSCs. In response to tissue injury in liver fibrosis, PFs undergo myofibroblastic activation. Proliferation of the MFs originated from PFs primarily occurs in disease associated with ductular reaction and/or cholestasis, in which the initial injury site is the portal area (15,16).

Fibrocytes. Fibrocytes originated from hematopoietic stem cells are capable to differentiate into MFs. Once tissue is damaged, fibrocytes are recruited to the injured organ and secrete growth factors. The migration of fibrocytes is regulated by C-C chemokine receptor type 2 (CCR2) and CCR1. Studies have shown that the extent of differentiation into MFs depends on different organs and the type of injury (17,18).

Bone marrow-derived MFs. Certain hepatic MFs can also originate from the bone marrow-derived mesenchymal stem cells (MSCs), which most likely represent a population that is different from hematopoietic-derived fibrocytes (9,19,20).

Other cells. Studies have shown that MFs may also be derived from hepatocytes or cholangiocytes through EMT in the liver (21). Zeisberg *et al* (22) were the first to report the evidence for hepatocyte EMT *in vivo*. They demonstrated that in the transgenic mice challenged with CCL4, in which hepatocyte-derived cells are permanently labeled by β -galactosidase (β -gal), 45% of the cells expressing the fibroblast-specific protein 1 (FSP1) were also positive for β -gal expression. Furthermore, the CCL4-induced liver fibrosis can be limited by the inhibition of the TGF- β 1 pathway. As a summary, the results demonstrated that hepatocyte EMT was triggered by TGF- β 1 and had a role in liver fibrosis. Cholangiocytes symbolize a unique epithelial cell compartment in the diseased liver. The biliary epithelial cells cannot be ruled out of the assumption that liver epithelial cells undergo EMT in liver fibrosis. Upon liver injury, cholangiocytes proliferate and switch from a quiescent to a 'reactive' state. Reactive cholangiocytes are known to express a variety of cytokines and pro-fibrogenic growth factors. They are likely to contribute to fibrosis and inflammation by promoting activation, proliferation and collagen synthesis in the surrounding pro-fibrogenic cells (23,24). However, Omenetti *et al* (25) showed a complete EMT in an immature cholangiocyte cell line *in vitro*, suggesting the possibility of direct contribution of cholangiocytes to fibrosis via EMT. In biliary atresia, biliary epithelial cells expressed FSP-1 and vimentin, while hepatocytes did not. Furthermore, the study showed that the expression of mesenchymal markers in biliary epithelial cells was observed in all liver disease with a ductular proliferation component. In mice exposed to common bile duct ligation (BDL), which is an experimental liver fibrosis model that induces strong ductular reaction, biliary epithelial cells underwent EMT, as shown by type I collagen and α -SMA expression (26).

3. Basic concept of EMT

EMT allows the epithelial cells to lose their polarity, to undergo complex biochemical changes and to assume multiple mesenchymal cell phenotypes, which includes a significantly increased production of ECM components, migratory capacity, invasiveness and elevated resistance to apoptosis. The progress was first described by Hay in 1995 in a chick model of primitive streak formation (27). In 2003, it was agreed at the first meeting of The EMT International Association, that epithelial-mesenchymal transformation and epithelial-mesenchymal transdifferentiation would be termed EMT. In March 2008, EMT was classified into three different subtypes at an EMT meeting at Cold Spring Laboratory based on the different biological contexts in which they occur (28,29). i) The type 1 EMTs are associated with implantation, embryo formation and organ development, neither cause organ fibrosis nor induce invasive phenotype. ii) The type 2 EMTs, in contrast to type 1, are connected to wound healing, tissue regeneration and organ fibrosis, and involve secondary epithelial or endothelial cells transitioning to resident tissue fibroblasts. As is observed during wound healing and tissue regeneration, the type 2 EMTs are positively correlated with inflammation and cease once inflammation is attenuated. iii) The type 3 EMTs are part of the metastatic process, and occur in neoplastic cells that have previously undergone genetic and epigenetic changes.

4. Type 2 EMTs

Type 2 EMTs are associated with organ fibrosis and regeneration occurring in the liver, lung, kidney and intestine. FSP1, α -SMA and collagen 1 are the characterized markers of the mesenchymal products generated by the EMTs during the development of organ fibrosis (29-31). The aforementioned markers, along with vimentin, desmin and discoidin domain receptor 2 (DDR2), have been used to distinguish the epithelial cells that are undergoing EMTs in response to ongoing inflammation. With the development of EMTs, these cells continue to exhibit epithelial-specific morphology and molecular markers, such as E-cadherin and cytokeratin, but showed concomitant expression of FSP1 and α -SMA. When the epithelial cell markers continue to be expressed, but the mesenchymal cells markers have been already obtained, such cells possibly represent the intermediate stage of EMT, or namely a partial EMT. Eventually these cells ultimately shed all their epithelial markers (including E-cadherin and zonula occludens-1) and acquire a fully fibroblastic phenotype (31) (vimentin, α -SMA, FSP1 and β -catenin), and the cells have undergone complete EMT. In the lineage studies, during the formation of fibroblasts in liver tissues, renal and other organs including lung and heart, this transition was strongly demonstrated (32-34). Studies have demonstrated that endothelial cells can also be devoted to the formation of mesenchymal cells via a process known as EnMT (35). Li *et al* (36) studied mouse models with cell lineage analysis and demonstrated that mesothelial cells (MCs) expressing Wilms tumor 1 produce HSCs and MFs during liver fibrogenesis. The results suggest that MCs participate in liver injury via differentiation to HSCs and MFs and are able to undergo mesothelial-mesenchymal transition.

An EMT can be identified in rat fetal liver cells in response to growth factors (epithelial growth factor and TGF- β) and dimethyl sulfoxide (37,38). HSCs cultured *in vitro* were shown to coexpress epithelial and mesenchymal markers, which provided indirect evidence of EMT (39,40). Increasing evidence has shown that TGF- β can induce an EMT in mice hepatocytes *in vitro*. The mechanism demonstrated that TGF- β induced EMT via a MAPK-dependent pathway and a Smad2/3-dependent pathway. Studies have shown that hepatic growth factors can decrease the level of TGF- β , restore E-cadherin, and decrease the amount of active matrix metalloproteinase 9 (MMP-9) (41) potentially. Other studies have demonstrated that the E-cadherin/ β -catenin signaling axis also has an important role for EMT involving epithelial cells. Bone morphogenetic protein 7 (BMP-7) has been used in the mouse model of liver, kidney and lung fibrosis, and the results demonstrated that BMP-7 functions as an endogenous inhibitor of TGF- β induced EMT (31). TGF- β 1 is recognized as a major cytokine in organ fibrosis and is an inducer of collagen production and HSC proliferation (42).

5. Biomarkers of EMT

To demonstrate the EMT, a variety of biomarkers have been used. Among these markers, some are acquired and some are attenuated during the process of transition. The following are a few of the commonly used markers and mechanisms.

A change or switch of E-cadherin during the EMT in tissue fibrosis, cancer and embryonic development is the prototypical epithelial cell marker. During the transition, the expression of E-cadherin is decreased, and in addition, EMT is promoted by the loss of E-cadherin function (43,44). The switches from E-cadherin to N/OB-cadherin have been increasingly used in recent years to monitor the progress of EMT during embryonic development, fibrosis and cancer progression. Integrins are other EMT markers, which in general have limited utility, as various integrins are expressed on mesenchymal and epithelial cells. DDR2 upregulates MMP1 and cell motility upon binding to type 1 or type X collagen, and is associated with types 2 and 3 EMT. FSP-1 (also known as S100A4 and MTS-1), is a member of the Ca²⁺-binding S100 proteins. In tissue fibrosis, FSP-1 is expressed by epithelial cells undergoing type 2 EMT transition to mesenchymal cells, and it has been used as a prototypical marker for detecting EMT in fibrosis and cancer. Vimentin, another marker of EMT, is expressed in various cells including fibroblasts and endothelial cells, and it should not be treated as a typical marker of type 2 EMT as adult epithelial cells express vimentin in response to different insults (45). Fibronectin serves as a scaffold for the ECM, which has been used as an indicator of type 1 EMT. The increased fibronectin expression is associated with type 2 and type 3 EMT *in vitro*.

6. EMT in liver fibrosis: TGF- β /Smad and non-Smad signaling pathway

TGF- β is believed to be a potent inducer of EMT and a key mediator of wound healing, fibrosis (46) and cancer. TGF- β 1 is a well-established cytokine that induces the profibrogenic pathway and fibrosis in liver (47). Furthermore, TGF- β 1 expression is also associated with morphological alterations, such as EMT in hepatocytes and changes in survival signaling pathways (48). In the TGF- β signaling pathway, active TGF- β 1 ligands initiate signaling by binding to TGF- β receptor type I (T β RI) and T β RII serine/threonine kinases. T β RI phosphorylates Smad2 and Smad3, which form a complex with Smad4 and translocate to the nucleus. Smad proteins convey signals from TGF- β to nucleus. Once in the nucleus, the complex of Smads can regulate the transcription of target genes. Activation of several Smad independent pathways have been identified as crucial for EMT induction by TGF- β , which includes phosphoinositide 3-kinase (PI3K)-Akt (49), focal adhesion kinase (50), p38 MAPK (51), and ERK (52). Recent studies have implicated Krüppel-like factor-8 (53), hyaluronan synthase 2 (54) and microRNA miR-203 (55) as critical regulators of EMT. Kim *et al* (56) demonstrated that the NF- κ B decoy oligodeoxynucleotide inhibited the EMT process in fibrotic liver *in vivo*. The overexpression of TGF- β 1 is associated with liver fibrosis in diverse animal models and in patients with chronic liver disease. TGF- β 1 crucially controls the expression of ECM network components, such as fibrillar collagens and fibronectin, ECM-degrading protease inhibitors plasminogen activator inhibitor-1 and TIMPs and finally regulates ECM deposition. The activity of TGF- β 1 is strongly induced during chronic liver injury with links between connective tissue growth factor and TGF- β 1 in the HSC activation process (57), which acquire myofibroblastic features and produce ECM proteins in turn. TGF- β 1 initiates and maintains

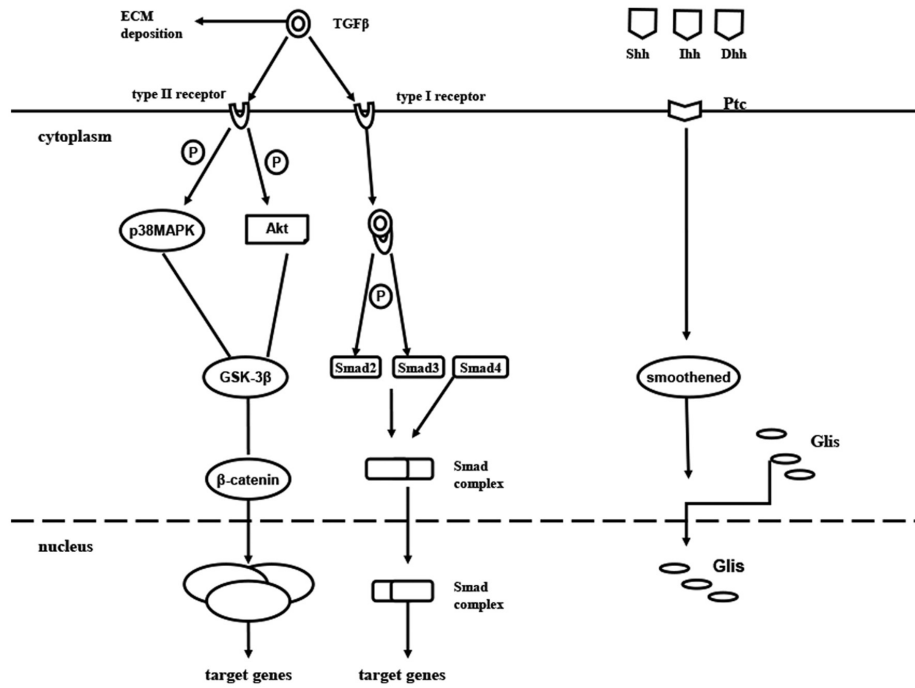


Figure 1. Cellular signaling pathways of EMT in liver fibrosis. Active TGFβ1 ligands initiate signaling by binding to TβRI and TβRII serine/threonine kinases. TβRI phosphorylates Smad2 and Smad3, which form a complex with Smad4 and translocate to the nucleus. Smad proteins convey signals from TGF-β to the nucleus. Once in the nucleus, the complex of Smad can regulate the transcription of target genes. EMT, epithelial-mesenchymal transition; TGF, transforming growth factor; TβRI, TGFβ receptor type I; P, phosphorylate; MAPK, mitogen-activated protein kinase; GSK-3β, glycogen synthase kinase-3β; ILK, integrin-linked kinase; TCF/LEF-1 complex, T-cell factor/lymphoid enhancer-binding factor-1 complex; Hh, hedgehog; Shh, Sonic Hh; Ihh, Indian Hh; Dhh, Desert Hh; Ptc, patch; ECM, extracellular matrix; Gli3, glioblastoma family.

the EMT in a variety of biological systems by activating major signaling pathways and transcriptional regulators integrated in extensive cellular networks. It has been suggested that the loss of E-cadherin expression in MDCKII cells exposed to TGF-β1 occurs through a Smad-independent mechanism, which includes the MAPK and PI3K pathways with expression of Snail. However, a complete transition to the mesenchymal phenotype additionally requires Smad signaling. A previous study reported that TGF-β1 participates in the regulation of the Notch signaling pathway (58). A series of the previously mentioned genes and others described to be involved in EMT (includes *Notch2* and *Snail*) were identified as TGF-β1 target genes. Park *et al* (59) reported that geniposide suppresses EMT, which leads to liver fibrosis by inhibiting multiple TGF-β1-mediated molecular mediators involved in hepatic injury. Lee *et al* (60) demonstrated that apamin suppressed the TGF-β1-induced hepatocyte EMT *in vitro* and CCl4-injected fibrosis *in vivo*.

The hedgehog (Hh) pathway has been identified as an essential morphogene for tissue remodeling in adult tissue. Hh ligands, Sonic Hh (Shh), Indian Hh (Ihh), Desert Hh (Dhh), bind to the patch (Ptc), releasing smoothened (Smo) into the cytosol (61). The aforementioned released Smo promotes the translocation of the cytoplasmic glioblastoma family (Gli3: Gli1, Gli2 and Gli3) into the nucleus, which acts as a transcriptional factor, activating Hh signaling (62-64). Evidence has shown that Hh signaling is activated in damaged liver, where it regulates tissue reconstruction. The level of Hh expression was suggested to be parallel to the degree of fibrosis (65). Furthermore, Hh signaling has been

demonstrated to activate quiescent hepatic stellate cells into MF-HSCs (66) (Fig. 1).

7. Controversy

Hepatocyte EMT. EMT was first demonstrated to occur in the fibrosis tissue in the kidney, *in vitro* (30). Following this, a mice model of renal fibrosis induced by unilateral ureteral obstruction lost the epithelial marker E-cadherin and gained mesenchymal cells markers (such as α-SMA). As the origin of fibroblastic cells remains under debate, it is appealing that the liver epithelial cells may have the possibility participate to fibrosis via EMT. Hepatocyte EMT was observed when cells were incubated with TGF-β1 (67), which was characterized by a decrease in epithelial marker E-cadherin expression and concomitant acquisition of mesenchymal markers (type I collagen and vimentin). While substantial experimental evidence supports that EMT makes a contribution to embryonic development and tumor metastasis, and renal fibrosis, the role of EMT in liver fibrosis remains under debate. Taura *et al* (68) bred the triple transgenic mice expressing *ROSA26 stop β-gal*, Albumin Cre and collagen α1 (1) green fluorescent protein and induced fibrosis by CCl4 injections. The study examined the expression of four different mesenchymal markers, which were FSP-1, α-SMA, vimentin and desmin. In these studies, the lack of expression of yellow fluorescent protein (YFP) supports the conclusion that EMT does not contribute to fibrosis in these models. Furthermore, the complete absence of its colocalization with YFP suggests that liver epithelial cells do not transition to either mesenchymal cells or MFs via EMT in the mouse models examined (69).

Cholangiocyte EMT. Cholangiocyte EMT was recently challenged with lineage-tracing methodology. Scholten *et al* (70) studied several strains expressing *Cre* under cholangiocyte-, HSC- or FSP-1-specific promoters in two liver fibrosis models (chronic CCl₄ intoxication and common BDL) with *Cre-Lox* technology for lineage tracing. Following permanent genetic *Cre*-mediated labeling of cholangiocytes, the fundamental experiment traced the fate of cells expressing *K19* in this case. The study concluded that EMT of cholangiocytes identified by genetic labeling does not contribute to liver fibrosis in mice.

8. Conclusion

There have been considerable advances in the understanding of the mechanisms of the EMT. The possibility that EMT makes a contribution to liver fibrogenesis reinforced that not only HSCs, but bone marrow-derived cells and circulating fibrocytes, could contribute to this process. The research of EMT in the next few years holds a significant potential as a viable therapeutic target. Future research probes into the molecular similarities and differences among the EMT programs. Furthermore, the identification of the signaling pathways that lead to activation of EMT programs during liver fibrosis is providing novel insights into the plasticity of cellular phenotypes and possible therapeutic interventions.

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