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Portal myofibroblasts connect angiogenesis and fibrosis in the liver

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Abbreviations : α -SMA, alpha-smooth muscle actin; BDL, bile duct ligation; COX-2, cyclooxygenase-2; HSC, hepatic stellate cell; HSC-MF, hepatic stellate cell-derived myofibroblast; MCD, methionine-choline-deficient; MMP, matrix metalloproteinase; NAFLD, non-alcoholic fatty liver disease; PDGF-BB, platelet-derived growth factor-BB; PDGFR- β , platelet-derived growth factor receptor-beta; PIGF, placental growth factor; PMF, portal myofibroblast; TGF- β , transforming growth factor-beta ; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Abstract

In all fibrotic tissues, the extracellular matrix is produced by myofibroblasts usually characterized by *de novo* expression of alpha-smooth muscle actin (α -SMA). While in the liver, myofibroblasts mainly derive from hepatic stellate cells (HSCs), cells that are distinct from HSCs and located in the portal tract, can also give rise to myofibroblasts that we globally refer to as portal myofibroblasts (PMFs). By different mechanisms that include the formation of direct intercellular junctions with endothelial cells and the production of VEGF-A-containing microparticles, PMFs promote angiogenesis which progresses in parallel with

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3 fibrosis in response to inflammation and fibrosis, in the liver. Hepatic angiogenesis and
4 fibrosis share many signaling pathways, which can be targeted by common therapies.
5 Compared to HSC-MFs, PMFs overexpress COL15A1, which both stabilizes newly formed
6 vessels and forms a scaffold for the deposition of interstitial collagen that accumulates in the
7 fibrotic liver. Thereby, PMFs may provide a critical link between angiogenesis and fibrosis in
8 livers diseases.
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14 The incidence of chronic liver diseases keeps growing worldwide, largely due to the epidemic
15 of the metabolic syndrome and non-alcoholic fatty liver disease (NAFLD). To a great extent,
16 the prognosis of chronic liver diseases is determined by the development of liver fibrosis.
17 However, no anti-fibrotic drug that would prevent the progression of liver fibrosis towards
18 cirrhosis, is yet available and a better understanding of fibrogenesis in the liver is still needed.
19 Liver fibrogenesis is a dynamic process including quantitative and qualitative changes of the
20 extracellular matrix, of which the most prominent is the deposition of type I collagen. These
21 changes progressively disrupt normal liver architecture and result in cirrhosis formation. In
22 the fibrotic liver, like in all other fibrotic tissues, the extracellular matrix is produced by cells
23 usually characterized by *de novo* expression of alpha-smooth muscle actin (α -SMA), known
24 as myofibroblasts.
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34 *Liver myofibroblasts*

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36 Myofibroblasts form heterogeneous populations of cells, with different possible origins.
37 Current evidence indicates that in the liver, myofibroblasts mainly derive from hepatic stellate
38 cells (HSCs) (Mederacke, et al., 2013). However, cells that are distinct from HSCs and
39 located in the portal tract, can also give rise to myofibroblasts that we now globally refer to as
40 portal myofibroblasts (PMFs) (Lemoinne, et al., 2015, Lua, et al., 2016). PMFs were first
41 described in the setting of biliary-type liver fibrosis, such as that induced by bile duct ligation
42 (BDL) in rats or mice (Kinnman, et al., 2003). PMFs outnumber hepatic stellate cell-derived
43 myofibroblasts (HSC-MFs) at the onset of biliary-type liver injury (Beaussier, et al., 2007),
44 contributing for more than 70 % to liver myofibroblasts, five days after BDL (Iwaisako, et al.,
45 2014). Simultaneously in sinusoids, HSCs undergo phenotypic changes including the
46 overexpression of desmin and of platelet-derived growth factor receptor-beta (PDGFR- β) as
47 well as increased DNA synthesis, yet without fully converting into myofibroblasts, at this
48 stage (Beaussier, et al., 2007). Studies of PMFs in culture demonstrated that they required
49 transforming growth factor-beta (TGF- β) and that they were also dependent on mechanical
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3 tension, for myofibroblastic differentiation (Li, et al., 2007), a typical feature of
4 myofibroblasts (Eyden, 2008). *In vivo*, the emergence and expansion of PMFs can be
5 triggered by interactions with ductular epithelial cells, which proliferate after bile duct injury
6 in the so-called ductular reaction. Thus, a marked increase in the expression of $\alpha v\beta 6$ integrin
7 on the surface of ductular epithelial cells is induced by acute bile duct obstruction in mice,
8 which directly triggers periductal accumulation of myofibroblasts and fibrosis, through the
9 activation of TGF- β (Wang, et al., 2007). In human liver tissues, the expression of $\alpha v\beta 6$ in
10 ductular epithelial cells, was also found to be increased in acute, but not chronic, biliary-type
11 injury (Wang, et al., 2007). Reactive ductules also express profibrogenic factors such as TGF-
12 β itself or platelet-derived growth factor-BB (PDGF-BB), which also stimulates PMF
13 expansion (Kinnman, et al., 2003). Yet, the biology of PMFs remains poorly known, mainly
14 due to the lack of markers that would allow investigators to authenticate and distinguish them
15 from HSC-MFs, in the injured liver. In a majority of studies, HSC-MFs have been identified
16 on the unique basis of α -SMA expression, one of the many markers they have in common
17 with PMFs. Therefore, the contribution of PMFs may have been underestimated, and some of
18 their properties improperly allocated to HSC-MFs. By comparing the transcriptome of PMFs
19 and HSC-MFs, we identified a marker of PMFs, virtually not expressed in HSC-MFs nor in
20 any other liver cell type, *i.e.* collagen, type XV, alpha1 (COL15A1) (Lemoinne, et al., 2015),
21 and this result was subsequently confirmed by another group (Lua, et al., J Hepatol 2016). We
22 showed that both in animal models and in patients with chronic liver diseases, a marked
23 increase in the hepatic expression of COL15A1 occurred at the time of progression to
24 advanced liver fibrosis (Lemoinne, et al., 2015). This was the case not only in biliary-type
25 liver fibrosis (*i.e.* bile duct-ligated rats and patients with primary biliary cholangitis) but also
26 in post-necrotic liver fibrosis (*i.e.* carbon tetrachloride-treated rats and patients with NAFLD),
27 suggesting that PMFs or at least sub-populations of PMFs were implicated in the progression
28 of all types of liver fibrosis. Besides α -SMA expression, myofibroblasts are characterized by
29 ultrastructural features, including a prominent rough endoplasmic reticulum, a Golgi
30 apparatus producing collagen secretory granules and peripheral myofilaments (Eyden, 2008).
31 Yet another highly characteristic trait, is a cell-to-matrix junction, consisting of an aligned
32 myofilament bundle and fibronectin fibril contacting one another through a point at the cell
33 surface, termed fibronexus (Eyden, 2008). Of particular interest in this respect, we found in
34 our previous comparative analyses that PMFs compared to HSC-MFs expressed fibronectin at
35 higher levels and virtually no desmin, that both belong to the definition of myofibroblasts
36 (Eyden, 2008). Therefore, PMFs fulfill more criteria that define myofibroblasts than HSC-
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3 MFs do (Eyden, 2008).
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6 *Liver angiogenesis*

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8 Angiogenesis is a dynamic process leading to the formation of news vessels from preexisting
9 vessels. In all tissues, angiogenesis is determined by two main pathways, *i.e.* hypoxia and
10 inflammation, and comprises the following steps: i) sprouting and budding of endothelial
11 cells; ii) degradation of the extracellular matrix by proteinases and migration of endothelial
12 cells; iii) endothelial cell proliferation, tube formation and branching; iv) vessel maintenance,
13 maturation and stabilization (Elpek, 2015). Vascular endothelial growth factor (VEGF)
14 signaling is implicated in virtually all steps of angiogenesis, while the recruitment of mural
15 pericyte-like cells is required in the last step, for nascent vessels to mature and stabilize. It
16 is now well established that liver disease progression is accompanied by angiogenesis. The first
17 demonstration was provided more than thirty years ago by Rappaport *et al.*, who showed that
18 human cirrhotic livers contained more vessels than healthy livers, and that cirrhotic nodules
19 were surrounded by a dense vascular plexus (Rappaport, et al., 1983). Liver tissue hypoxia
20 occurs at early stages of liver injury and increases with disease progression, as a result of
21 structural and functional changes in the liver angioarchitecture (Corpechot, et al., 2002).
22 During liver fibrogenesis, fibrillar type I collagen progressively replaces type IV collagen in
23 the perisinusoidal space of Disse, which together with the loss of endothelial fenestrations,
24 causes sinusoidal capillarization. Therefore, fibrosis by itself can contribute to the
25 development of hypoxia, and thereby promote angiogenesis. Vascular remodeling leading to
26 capillarization of the sinusoids and generation of intrahepatic shunts characterizes hepatic
27 angiogenesis. Such changes in angioarchitecture cause a decrease in hepatocyte perfusion,
28 that aggravates hypoxia. Liver angiogenesis is also stimulated by inflammation. Chemokine-
29 dependent accumulation of monocyte-derived macrophages is an important mechanism of
30 hepatic inflammation and fibrogenesis, in human liver diseases and experimental mouse
31 models. The chemokine receptor CCR2 and its ligand CCL2 (MCP-1) promote the
32 accumulation of monocyte-derived macrophages releasing pro-inflammatory and pro-
33 angiogenic cytokines in the liver. Infiltrating CCL2-dependent inflammatory monocytes also
34 provide pro-angiogenic signals, via the production of VEGF-A and matrix metalloproteinase
35 (MMP)-9 (Ehling, et al., 2014). Three-dimensional micromorphological analyses in mouse
36 models of carbon tetrachloride- or bile duct ligation-induced liver injury, demonstrated that
37 macrophage-dependent angiogenesis during chronic liver injury was largely confined to portal
38 veins and that pharmacological inhibition of CCL2-mediated inflammatory monocyte
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3 infiltration primarily reduced angiogenic vessel sprouting in the portal vein (Ehling, et al.,
4 2014). Therefore, infiltrating bone marrow-derived inflammatory monocytes would mediate
5 the induction of hepatic angiogenesis by effects that are primarily attributable to changes in
6 the portal vein system.
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10 Angiogenesis and fibrosis progression closely correlate in experimental liver injury and
11 human liver disease. Angiogenesis and fibrogenesis are also triggered by similar pathways in
12 response to hypoxia and inflammation, so that a potential causal relationship between them
13 has been difficult to establish. In fact, dichotomous effects of angiogenesis on fibrosis have
14 now been reported, in different tissues. The administration of VEGF, while stimulating
15 angiogenesis, has been shown to reduce renal fibrosis and to stabilize renal function, in the
16 remnant kidney model of progressive renal failure (Kang, et al., 2001). Proangiogenic activity
17 induced in an adipocyte-specific VEGF-A overexpression model, has shown to suppress
18 fibrosis, inflammation and insuline resistance during the early phase of high fat diet-induced
19 adipose tissue expansion. Conversely, VEGF-A-VEGFR2 blockade had an aggravating effect
20 in this context, whereas in *ob/ob* mice with preexisting adipose tissue dysfunction, the anti-
21 angiogenic action of VEGF-A-VEGFR2 blockade caused an improvement in metabolism and
22 a decrease in inflammatory factors (Sun, et al., 2012). A large number of studies in animal
23 models of liver injury showed that the inhibition of angiogenesis led to a decrease in liver
24 fibrosis (Table 1). However, the pathways targeted in these studies, could have also promoted
25 fibrosis, directly. In addition, a few studies led to different conclusions. Thus,
26 pharmacological inhibition of the vitronectin receptor integrin alphavbeta3 which stimulates
27 endothelial cell proliferation and HSC activation, while suppressing hepatic angiogenesis,
28 aggravated liver fibrosis induced by BDL or thioacetamide (Patsenker, et al., 2009).
29 Pharmacological inhibition of the chemokine CCL2 reduced monocyte infiltration and
30 angiogenesis but not fibrosis progression in mouse models of carbon tetrachloride or BDL-
31 induced liver injury (Ehling, et al., 2014). Dichotomous effects of angiogenesis were also
32 reported in a model of fibrosis resolution generated by cholecystojejunostomy that restored
33 bile flow after BDL (Yang, et al., 2014a). In this model, VEGF-induced angiogenesis
34 promoted fibrogenesis after bile duct ligation but was also required for fibrosis resolution
35 after cholecystojejunostomy. Accordingly, VEGF-neutralizing antibodies prevented the
36 development of fibrosis but also disrupted hepatic tissue repair and fibrosis resolution (Yang,
37 et al., 2014a).
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58 *Contribution of portal myofibroblasts to liver angiogenesis*
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3 HSC-MFs and PMFs likely both contribute to liver angiogenesis, although at different stages.
4 HSCs in their quiescent state, act as pericytes that regulate the functions of sinusoidal
5 endothelial cells. Following myofibroblastic differentiation, they acquire a proangiogenic
6 phenotype and secrete proangiogenic factors such as angiopoietin-1 (Semela, et al., 2008,
7 Thabut, et al., 2011). At an early stage of liver injury, HSC-MFs promote enhanced coverage
8 of sinusoids and angiogenesis in areas of active fibrogenesis at the leading edge of developing
9 fibrotic septa (Novo, et al., 2007). At later stages, endothelial cell proliferation correlate with
10 the expansion of PMFs, which has suggested a role of PMF in liver angiogenesis (Lemoinne,
11 et al., 2015). Further evidence that this was the case, was provided by the immunostaining of
12 human cirrhotic livers showing that COL15A1-positive PMFs displayed a perivascular
13 distribution and outlined vascular capillaries within large fibrotic septa. Using a cell model of
14 PMFs that we previously described in detail, we demonstrated that PMFs were able to
15 enhance angiogenesis *in vitro* and *in vivo*, by different mechanisms including the formation of
16 direct intercellular junctions with endothelial cells and the production of VEGF-A-containing
17 microparticles (Lemoinne, et al., 2015). Compared to HSC-MFs, PMFs largely overexpress
18 COL15A1 and also COL18A1, which are the $\alpha 1$ chains of collagen XV and collagen XVIII,
19 respectively. Both collagens belong to the superfamily of multiplexins and their C-terminal
20 parts, endostatin and restin, respectively are anti-angiogenic, which could provide a negative
21 retrocontrol in PMF-induced angiogenesis. Collagen XV provides stability and resilience to
22 mechanical forces in the skeletal muscle and microvessels. It is contained in the basement
23 membrane of continuous capillaries, serving as a scaffold that anchors the basement
24 membrane to interstitial collagen, and loss of its expression results in collapsed capillaries in
25 mice. It is absent from specialized capillaries like fenestrated liver sinusoids, and the normal
26 liver contains almost no collagen XV, with the exception of trace amounts in the portal and
27 periportal area. Collagen XV forms a proangiogenic matrix for endothelial cells, and thereby
28 could contribute to the angiogenic properties of PMFs. Increased expression of collagen XV
29 has been reported in hepatocellular carcinogenesis in mice and humans. In human
30 hepatocellular carcinoma, collagen XV was identified as a prominent histopathological
31 component of intratumoral capillaries (Kimura, et al., 2016).
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53 *In summary*, PMFs appear to be critical in pathological angiogenesis, which constantly occurs
54 in advanced liver fibrosis. While it remains to be elucidated how angiogenesis and fibrosis are
55 linked to each other during the progression of liver diseases, we suggest that COL15A1-
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3 producing PMFs could provide an important link by both stabilizing newly formed vessels
4 and forming a scaffold for the deposition of interstitial collagen (Figure 1).
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30 31 **Figure legend**

32
33 **Figure 1.** Role of portal myofibroblasts in liver angiogenesis and fibrosis.

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35 **A)** In normal liver, quiescent hepatic stellate cells (HSCs) for a continuum with portal
36 mesenchymal cells, which include vascular smooth muscle cells (SMCs), portal fibroblasts
37 and few mesenchymal progenitor cells. The extracellular matrix is poorly abundant and
38 contains trace amounts of COL15A1 along mesenchymal progenitors. B) In advanced
39 fibrosis, portal myofibroblasts (PMFs) proliferate and promote angiogenesis. COL15A1
40 expressed by PMFs provides a scaffold for interstitial collagen produced in excess mostly by
41 hepatic stellate cell-derived myofibroblasts (HSC-MFs).
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Table 1. Effects of angiogenesis inhibition on liver fibrosis in experimental models

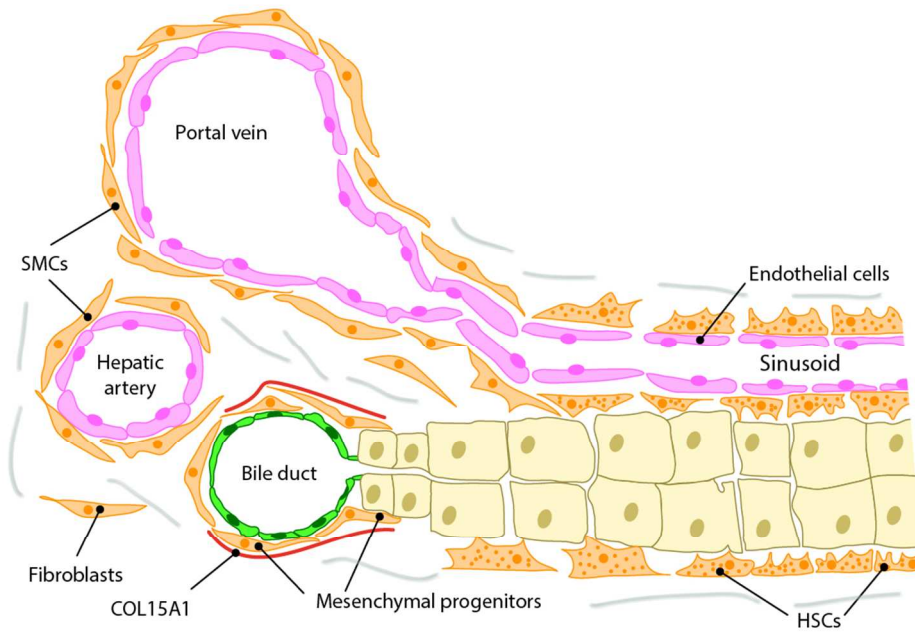
Reference	Animal model	Anti-angiogenic treatment	Assay and change in liver fibrosis
(Yoshiji, et al., 2003)	Mouse - CCl ₄	Anti-VEGFR-1 Ab Anti-VEGFR-2 Ab	Hydroxyproline (↘)
(Tugues, et al., 2007)	Rat - CCl ₄	Sunitinib (multiple TK inhibitor)	Masson trichrome staining (↘)
(Taura, et al., 2008)	Mouse - CCl ₄ , BDL	Blockade of angiopoietin signaling by Adstie2	Hydroxyproline, Sirius red staining (↘)
(Mejias, et al., 2009)	Rat - BDL	Sorafenib (multiple TK inhibitor)	Sirius red staining (↘)
(Patsenker, et al., 2009)	Rat - BDL, TAA	Cilenglinide ($\alpha\beta3/\alpha\beta5$ integrin inhibitor)	Hydroxyproline, Sirius red staining (↗)
(Thabut, et al., 2011)	Rat - BDL	Sorafenib	Magnetic resonance elastography, Sirius red staining (↘)
(Van Steenkiste, et al., 2011)	Mouse - CCl ₄	Anti-PlGF Ab	Sirius red staining (↘)
Hennenberg <i>et al.</i> 2011 (Hennenberg, et al., 2011)	Rat - BDL	Sorafenib	Sirius red staining (↘)
May <i>et al.</i> 2011 (May, et al., 2011)	Mouse	Blockade of VEGF signaling by transgenic conditional induction of a VEGF decoy receptor	Goldner staining (↗)
(Sahin, et al., 2012)	Mouse - CCl ₄	CxCl9 (angiostatic chemokine)	Hydroxyproline, Sirius red staining (↘)
(Huang, et al., 2013)	Rat - CCl ₄	Bevacizumab (anti-VEGF Ab)	Hydroxyproline, Sirius red staining (↘)
(Gao, et al., 2013)	Rat - TAA	Celecoxib (COX-2 inhibitor)	Masson trichrome staining (↘)
(Yang, et al., 2014b)	Rat - High-fat/MCD	Sorafenib, Brivanib (multiple TK inhibitors)	Hydroxyproline (↘)
(Yang, et al., 2014a)	Mouse - BDL - BDL followed by cholecystojejunostomy)) Anti-VEGF Ab)	Hydroxyproline, Sirius red staining (↘) (↗)
(Ehling, et al., 2014)	Mouse - CCl ₄ , BDL	CC12 pharmacological	Sirius red staining (→)

		inhibitor	
(Liu, et al., 2015)	Rat - Dimethylnitrosamine	Sorafenib + gadolinium chloride	Hydroxyproline, Sirius red staining (↘)
(Yan, et al., 2015)	Mouse - CCl ₄	Anti-CD147 Ab	Hydroxyproline, Sirius red staining (↘)

AdsTie2, adenovirus expressing the extracellular domain of Tie2; PlGF, placental growth factor; COX-2, cyclooxygenase-2; MCD, methionine-choline-deficient.

For Peer Review

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