Cellular and Molecular Mechanisms of Novel Therapies to Ameliorate Liver Sinusoidal Dysfunction in Cirrhotic Portal Hypertension

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CELLULAR AND MOLECULAR MECHANISMS OF NOVEL THERAPIES TO AMELIORATE LIVER SINUSOIDAL DYSFUNCTION IN CIRRHOTIC PORTAL HYPERTENSION

Ph.D. thesis presented by

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For the degree of Doctor

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Alla mia famiglia

“Every great advance in science has issued from a new audacity of imagination”
John Dewey

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Directors' report (Informe de los directores de tesis)

Barcelona, 7 de Maig

Jordi Gracia-Sancho, Investigador Ramón y Cajal a IDIBAPS, i Jaime Bosch Genover, catedràtic de la Facultat de Medicina de la Universitat de Barcelona i Consultor Sènior del Servei d'Hepatologia de l'Hospital Clínic de Barcelona,

CERTIFIQUEN:

Que la tesi doctoral CELLULAR AND MOLECULAR MECHANISMS OF NOVEL THERAPIES TO AMELIORATE LIVER SINUSOIDAL DYSFUNCTION IN CIRRHOTIC PORTAL HYPERTENSION, presentada per Giusi Marrone per a optar al títol de Doctor per la Universitat de Barcelona s'ha realitzat sota la nostra direcció i compleix tots els requisits necessaris per ser defensada davant el Tribunal d'avaluació corresponent.

Jordi Gracia-Sancho

Jaime Bosch Genover

Abbreviations

α-SMA	alpha-smooth muscle actin
5-LO	5-Lipoxygenase
Ad-CTRL	Adenovirus control
Ad-KLF2	Adenovirus KLF2
ADMA	Asymmetric dimethylarginine
AKT	Protein kinase B (PKB)
ARE	Antioxidant response elements
BH_4	Tetrahydrobioterin
CBDL	Common bile duct ligation
CCl_4	Carbon tetrachloride
cGMP	cyclic guanosine monophosphate
COX	Cyclooxygenase
CysLTs	Cysteinyl leukotrienes
ECM	Extracellular matrix
eNOS	endothelial nitric oxide synthase
ERK5	Extracellular signal regulated kinase 5
ET-1	Endothelin-1
FPP	Farnesyl-pyrophosphate
GGPP	Geranylgeranyl-pyrophosphate
GRK2	G protein-coupled receptor kinase 2
H_2O_2	Hydrogen peroxide
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HMG-CoA	Hydroxyl-methyl-glutaryl-coenzyme A
HO-1	Heme oxygenase
HPVG	Hepatic venous pressure gradient

HSC	Hepatic stellate cells
Hsp90	Heat shock protein 90
ICAM-1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IHVR	Intrahepatic vascular resistance
iNOS	inducible nitric oxide synthase
IP3R	Inositol 1,4,5-trisphosphate receptor
JAK	Janus kinase inhibitor
KC	Kupffer cells
Keap1	Kelch-like ECH-associated protein 1
KLF2	Krüppel-like Factor 2
KLF4	Krüppel-like Factor 4
KLF6	Krüppel-like Factor 6
LSEC	Liver sinusoidal endothelial cells
MEF2	Myocyte-enhancer factor-2 pathway
MEK5	Mitogen-activated protein kinase 5
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
NOS	nitric oxide synthase
NOSIP	Nitric oxide synthase interacting protein
NOSTRIN	Nitric oxide synthase trafficking
NQO1	NADPH dehydrogenase quinone 1
Nrf2	Nuclear related factor 2
O_2^-	Superoxide anion

ObR	Leptin receptor
ObR-Ab	Leptin receptor antibody
$ONOO^-$	Peroxynitrite
PDGF	Platelet-derived growth factor
p-eNOS	Phosphorylated endothelial nitric oxide synthase
PGH_2	Prostaglandin H2
PI3K	Phosphoinositide 3-kinase
PKG	Protein kinase G
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
STAT	Signal Transducer and Activator of Transcription
SVR	Systemic vascular resistance
TGF-β	Transforming growth factor beta
TM	Thrombomodulin
TXA_2	Thromboxane A2
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGR-1	Vascular endothelial growth factor receptor 1
XO	Xanthine oxidase

Introduction

Introduction

Liver microcirculation plays an essential role in the progression and aggravation of chronic liver diseases. The maintenance of a global liver function and a correct specific phenotype of hepatic cells are related to the sinusoidal environment. However, acute or continuous liver injury significantly de-regulates the protective phenotype of liver cells, leading to parenchymal and non-parenchymal dysfunction. In cirrhosis and portal hypertension, the damaged hepatic sinusoid loses the appropriate communication among liver cells and cannot regulate the hepatic vascular tone, homeostasis and metabolism. Studying the sinusoidal milieu opens the possibility to develop new therapeutic strategies to ameliorate liver microcirculation and viability.

I. Liver microcirculation and hepatic sinusoid

Liver microcirculation is unique among vascular beds (1). The high pressure and oxygenated arterial blood mixes with the low-pressure, de-oxygenated but nutrient-rich portal venous blood within the hepatic sinusoids, specialized capillaries with contractile properties and unique in their phenotype (2). It is due to this particular structure that hepatic cells, mainly composed by hepatocytes (parenchymal cells), liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC), and Kupffer cells (KC), tightly interact each other, and it is in these segments of the microcirculation where the exchange of substances occurs (3). The space between the hepatocytes and the sinusoidal endothelial cells is called the space of Disse (Fig. 1), where molecules of blood as large as albumin enter before making contact with the microvilli of the hepatocytes. Blood flow passes through the sinusoids from the hepatic artery and the portal vein of the periportal field (zone 1) to the central vein (zone 3) of each lobule, thus supplying the liver with oxygen and nutrients. This hepatic blood flows through sinusoids at low shear stress, rather than at high shear stress associated with flow through post-capillary venules (4).

Sinusoids become dysfunctional when its cellular components change their phenotype for the worst, as explained later on in this Ph.D. thesis.

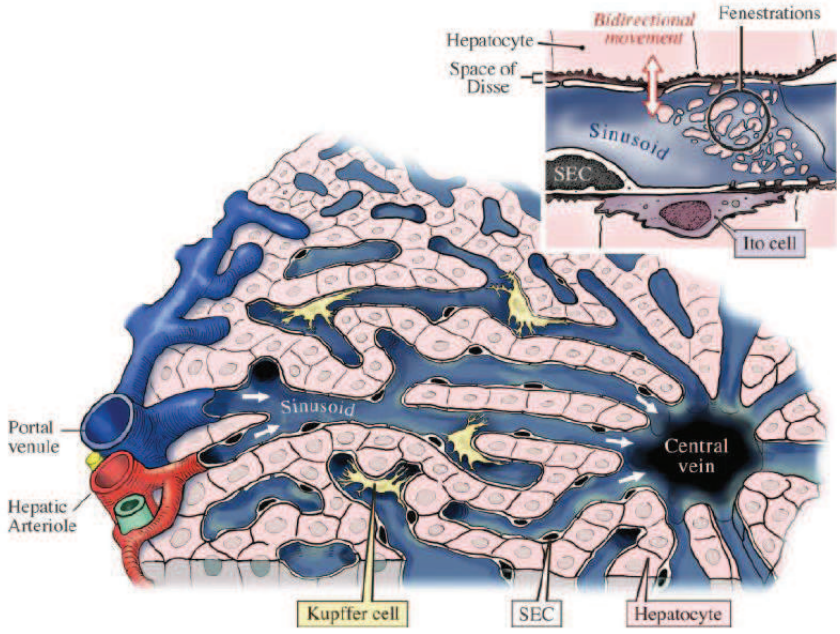


Figure 1. The hepatic sinusoids. Every hepatocyte is in close contact with the blood due to the marked porosity of the walls of the sinusoids. Adapted from WC Aird, Circ Res. 2007b.

I.1. Liver sinusoidal endothelial cells

Representing the facade of the sinusoids, liver sinusoidal endothelial cells (LSEC) were firstly recognized as highly differentiated cells in 1970-72, when Prof. E. Wisse examined the hepatic sinusoid by perfusion fixation and electron microscopy (5, 6). These cells have similar functions of endothelial cells lying the vascular wall, they participate to all aspects of the vascular homeostasis and regulation of the vascular tone, but also to physiological or pathological processes like thrombosis, inflammation, or vascular wall remodeling, been implicated in coagulation and fibrinolysis.

Although they share similar functions, LSEC are dissimilar from other endothelial cells because the lack of an organized basal membrane and the presence of open fenestrae of less than 200 nm in diameter and organized in clusters termed sieve plates (2 - 20% of the cytoplasm surface). Hence, liver endothelial wall is discontinuous and LSEC the most permeable of all mammalian endothelial cells (7). Fenestrae are the peculiarity of LSEC: dynamic structures able to contract or dilate, change in size and in porosity (number of fenestrae per μm^2) (8).

LSEC provide a microcirculation and represent a porous barrier that facilitates oxygenation of hepatocytes and enhance hepatocyte exposure to macromolecules in the portal circulation. In addition, together with Kupffer cells, LSEC are part of the reticulo-endothelial system (diffuse

mononuclear phagocyte system) and play an important role in uptake and processing of circulating factors, including pathogens (9), thus clear colloids and macromolecules from the circulation. Moreover, LSEC act as a gatekeeper against hepatic stellate cell activation since maintenance of LSEC protective phenotype avoids the activation of HSC in response to an injury (10, 11). These cells are the main source of the endothelium-derived nitric oxide (NO), an important modulator of vascular tone (12), produced by the constitutive endothelial nitric oxide synthase (eNOS). The phenotype of these cells is maintained by paracrine secretion of vascular endothelial growth factor (VEGF) by hepatocytes and HSCs (13, 14) and a downstream autocrine loop of VEGF-stimulated NO production by eNOS in the LSEC (13).

Subsequent to a damage, LSEC capillarize (lose fenestrae), promote fibrosis and inflammation, and lose their filter properties because the loss of sieve function (3) (Figure 2).

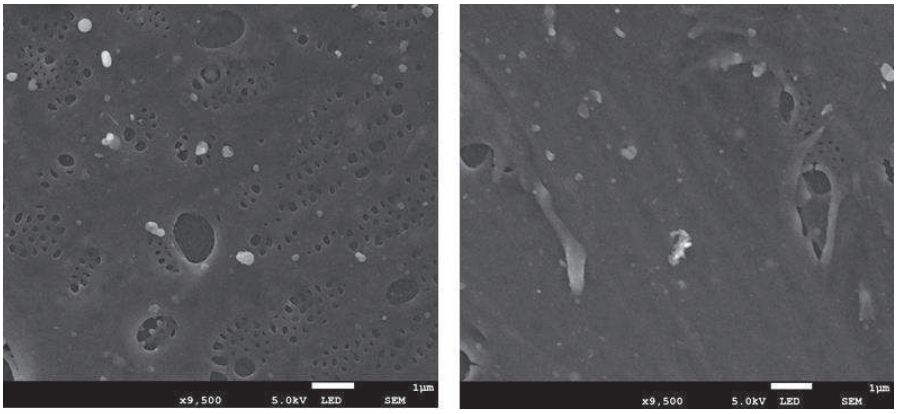


Figure 2. Fenestrae in LSEC. LSEC isolated from normal (left) and cirrhotic (right) rats.

I.2. Hepatic stellate cells

It is well known that hepatic stellate cells (HSC), localized in the space of Disse in a quiescent state, are the main collagen-synthesis cells of the liver (15, 16). The functions of these cells, in normal condition, are to regulate retinoid metabolism (17, 18), modulate blood flow (19), and are implicated in growth and metabolic activities of other cells either by direct cell–cell interaction (20) or by the release of cytokines and growth factors (21). A single stellate cell can wrap up to 4 sinusoids, thus regulate sinusoidal blood flow by contraction, and can adhere each other through adherent junctions (22). Indeed, from a histological point of view, stellate cells resemble tissue pericytes, a cell type with smooth muscle features that is thought to regulate blood flow via pericapillary constriction (23). After an injury, perisinusoidal HSC change morphologically and functionally becoming “activated”: they trans-differentiate into a myofibroblast-like cells, proliferate, migrate and contract even more, increasing dynamically the hepatic vascular

resistance (24). Moreover, activated HSC produce large amounts of extracellular matrix components that results in increased structural resistance to liver perfusion (25).

I.3. Sinusoidal phenotype modulation by shear stress

The shear stress is the force per unit area generated when a tangential force of blood flow acts on the endothelial monolayer. It is a product of fluid viscosity and the velocity gradient between adjacent layers of the flowing fluid. The endothelium (LSEC) is sensitive to the hemodynamic forces generated by the blood flow and translates them into biochemical responses, activating signal transduction and endothelium-dependent gene and protein expression that modulate endothelial cell phenotype (26). Shear stress is indispensable in the long-term maintenance of blood vessel tone and structure (26), since it modulates endothelial function and the contractility of vascular smooth muscle cells (or HSC). Blood flow pattern may change in different vascular regions from laminar and unidirectional to low, turbulent/oscillatory/disturbed and multidirectional (range of 5-15 dynes/cm^2) (Figure 3). At branch points and curved vessels, the flow is turbulent and increases the permeability of the vessels augmenting the expression of adhesion molecules, recruiting leukocyte and accumulating lipoprotein (27, 28). Moreover, in the regions under the oscillatory flow, cells modify their shape and their nuclei alignment (27, 29).

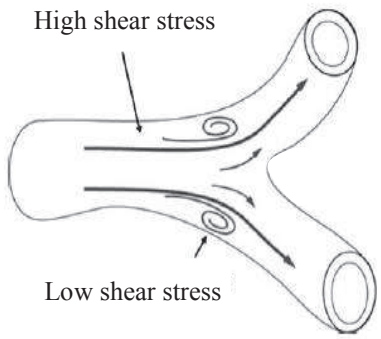


Figure 2. Flow pattern changes depending on the geometry of blood vessels. In ''straight'' regions of vasculature, endothelial cells experience ordered laminar shear stress, while at or near branch points and vascular bifurcations, endothelial cells experience low or oscillatory shear stress. Adapted from Pan S., Antioxid Redox Signal. 2009.

Several studies performed using macrovascular endothelial cells focused on the genes regulated by acute or chronic shear stress exposure: adhesion molecules such as vascular cell adhesion protein-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (30), growth factors such as platelet-derived growth factor (PDGF-A and B) (31), protein related to oxidative stress like superoxide dismutase (SOD), nuclear factor erythroid-derived 2-like 2 (Nrf2) (32, 33), cyclo-oxygenase-2 (COX-2) and eNOS (34), activation of transcription factors as c- fos (35), Egr -1 (36) and NFκB (37). Acute shear stress *in vitro* elicits similarities with endothelial responses to inflammatory cytokines (38). In contrast, after chronic shear stress, endothelial cells respond with structural remodeling and flattening to adapt shear stress (38).

The effects of different flow patterns and associated shear stresses on endothelial and vascular biology are reported in the table below (Table 1).

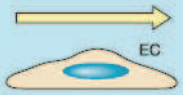
	Laminar flow / high shear stress 	Disturbed flow / low or reciprocating shear stress 
Vasoactivity	Vasodilation	Vasoconstriction
Turnover rate	Low	High
Macromolecular permeability & LDL uptake	Low	High
DNA synthesis	Low	High
Morphology	Elongated & aligned	Polygonal
Expression of adhesion molecules, inflammatory & chemokine genes	Low	High
Expression of antioxidant genes	High	Low
WBC adhesion and platelet aggregation	Inhibition	Promotion
Oxidative stress/ROS	Low	High (Sustained)
VSMC activation	Low	High
Wound repair: Endothelization	Promotion	Retardation
Heterogeneity	Low	High
Fibronectin/fibrinogen deposition	Low	High
Atherosclerosis & thrombosis	Prevention	Promotion

Table 1. Summary of effects of different flow patterns on endothelial cells. From Jiann and Chien, Physiol Rev 2011.

Dekker and colleagues firstly showed the importance of the transcription factor Kruppel-like factor 2 (KLF2) in the regulation of the flow-dependent endothelial phenotype, observing that the knockdown of this transcription factor prevented the induction of eNOS and the reduction of endothelin-1 mediated by the flow (39, 40). Later on, Parmar and colleagues discovered that over 15% of the genes regulated by flow were KLF2-dependent (41). Our group demonstrated for the first time that KLF2 is also induced by shear stress in the microvascular environment of the liver (42).

I.4. Kruppel like factor 2

The KLFs are a subclass of zinc finger transcription factor that regulate cell growth and tissue development thanks to their ability to bind to the "CACCC" sequences ("GC" boxes) in the promoters of their target genes, thus regulating their expression (43). The main distinguishing features of the KLF family is the presence of three highly conserved Cysteine2/Histidine2 zinc fingers located at the C-terminus of the protein and joined by a conserved 7 amino acid sequence, TGEKP(Y/F)X, that allow the binding to DNA and its nuclear localization (Figure 4). Although the zinc finger domains are very similar, the non–DNA-binding domains (activation and repression domains) in the N-terminus are highly divergent and mediate the transcriptional

regulation by KLFs. Indeed, the N-terminal regions allow KLFs to bind different co-activators, co-repressors and modifiers, resulting in functional diversity and specificity. KLFs regulate gene transcription recruiting chromatin modifiers and transcription machinery to promoters of specific genes. One of the best known interacting proteins is the cAMP response element binding protein (CBP), p300, p300/CBP-associated factor (P/CAF), C-terminal-binding protein (CtBP) and Sin3A (44).

Figure 4. Structure of KLF2. The transactivation and transrepression domains are at the N-terminus. The C-terminus is the Cys2/His2 zinc finger for the DNA-binding. Adapted from Atkins and Jain, Circ. Res. 2008

KLF nomenclature is based on the homology to the DNA-binding domain of the Drosophila Kruppel protein, a member of the "gap" class of segmentation gene products that regulates body segmentation in the thorax and anterior abdomen of the Drosophila embryo (45). Kruppel is the German word for "cripple." The protein is indeed appropriately named since Drosophila embryos homozygous for the protein Kruppel died because of altered anterior abdominal and thoracic segments (46).

One member of this family, KLF2, first cloned by Lingrel and colleagues (47), is a 354-aa protein that, owing to its high expression in lung tissues, was initially termed lung Kruppel-like factor (LKLF). It is now known that this transcription factor is expressed primarily in the endothelium and it is necessary for proper vessel development and for its correct functions (48-50), since its endothelial expression begins as early as embryonic day 9.5. KLF2 has been largely studied in the cardiovascular system, endothelial biology and pathobiology (i.e. atherosclerosis). It is consider a key "molecular switch" that regulates important aspects of vascular function and disease such as leukocyte adhesion to the endothelium, endothelial thrombotic function, endothelial proliferation, migration, and angiogenesis, and expression of factors implicated in regulating vasoreactivity and vascular tone. Indeed, it has been reported that KLF2 confers an anti-inflammatory and anti-thrombotic phenotype to the endothelium since it decreases the expression of adhesion molecules such as E-selectin and VCAM-1 (51), and induces the expression of eNOS (51) and thrombomodulin (TM) (52) (Figure 5), a cell surface factor essential in generating activated protein C via interactions with thrombin, leading to potent inhibition of coagulation.

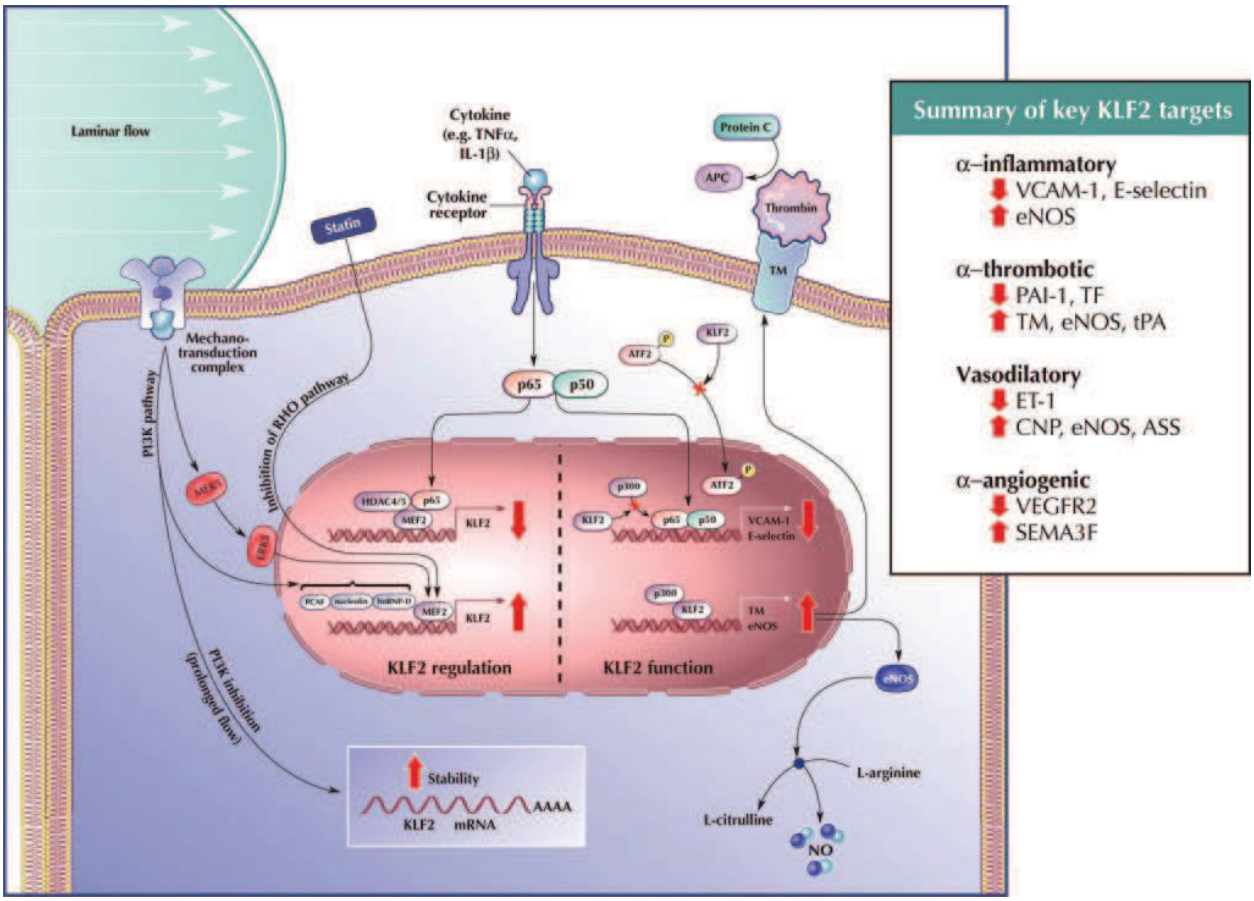


Figure 5. Schematic diagram of the regulation and function of KLF2 in endothelial cells. From Atkins and Jain, Circ. Res. 2008

The expression of this transcription factor is flow dependent (39): high levels in vascular regions exposed to laminar shear stress (which confers resistance to atherosclerosis), low levels in athero-susceptible regions exposed to a turbulent shear stress (40). Consequences of flow-induced KLF2 expression in endothelial cells include activated expression of eNOS and repressed expression of angiotensin converting enzyme, endothelin-1, and adrenomedullin, all of which are involved in the control of vascular tone in response to flow (41). Moreover, it has been demonstrated that small molecules like statins (53-55) or resveratrol (56) can also induce the expression of KLF2, and that shear stress sustains atheroprotective endothelial KLF2 expression through mRNA stabilization (57).

KLF2 induction depends on the phosphorylation/activation of the mitogen-activated protein kinase 5/extracellular signal regulated kinase 5/myocyte-enhancer factor-2 pathway (MEK5/ERK5/MEF2), since MEF2 is able to bind KLF2 promoter and induce its transcription (41) (Figure 6). In addition, prolonged flow is able to stabilize KLF2 through the pathway of the phosphoinositide 3-kinase (PI3K) (58) and the recruitment of the protein nucleolin (59), organizing a positive complex on the KLF2 promoter.

Contrary to up-regulation of KLF2 by shear stress and statins, expression of KLF2 in endothelial cells is suppressed by pro-inflammatory cytokines such as TNF-α, interleukin 1β (IL-1β), and the NFκB pathways interacting with histone de-acetylase (60).

Our group was the first demonstrating that KLF2 expression within the liver responds to flow stimulation (42). However, the exact mechanism of *hepatic KLF2* activation has not been investigated.

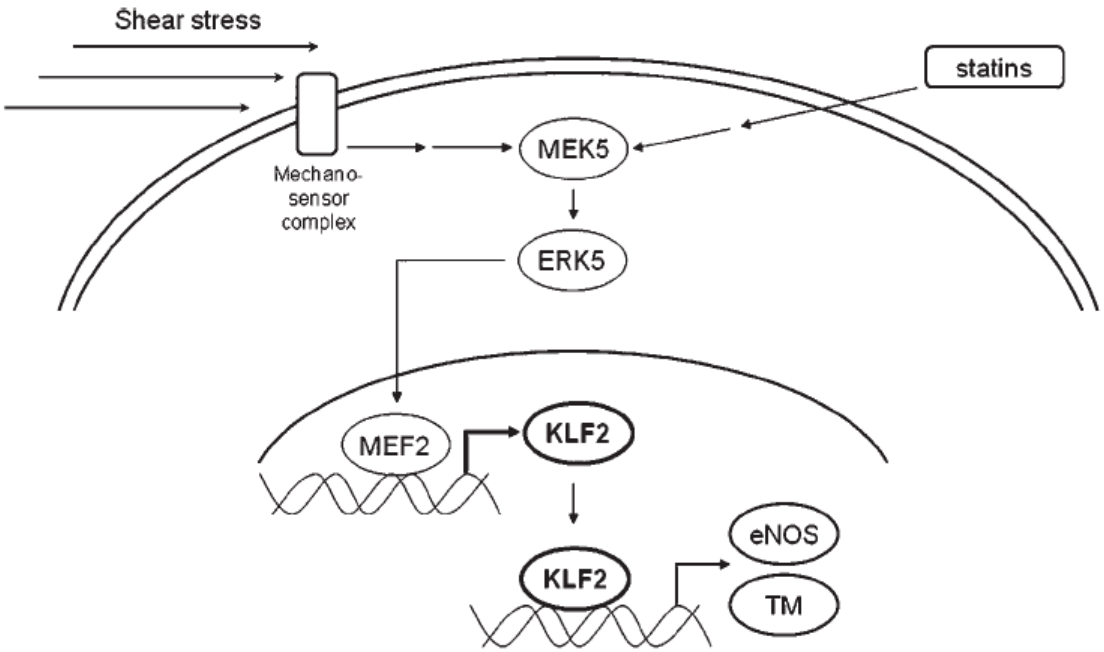


Figure 6. Scheme of KLF2 induction in endothelial cells in response to shear stress and statins. KLF2 activates vasodilatory and anti-thrombotic genes preserving a healthy endothelium. Adapted from Gracia-Sancho J et al., Gut 2011

II. Cirrhosis and portal hypertension

Portal hypertension is a clinical syndrome defined as an elevation of the hepatic venous pressure gradient (HVPG) above 5mmHg. It is well established that the primary and necessary factor for the development of portal hypertension is an increased resistance to portal blood flow (61). Normally, a healthy liver has no active role in regulating portal inflow, a function provided by resistance vessels at the splanchnic arteriolar level, thereby it may be conceived as a big vascular network with very low resistance (62). However, in portal hypertension situation, increased resistance to blood flow exists.

Depending on the level of impediment to portal flow, portal hypertension is classified as either *pre-hepatic* (i.e. portal vein thrombosis or congenital atresia), *intra-hepatic* (i.e. liver cirrhosis, hepatic fibrosis, or non-cirrhotic causes such as schistosomiasis, massive fatty change and diffuse granulomatous diseases) or *post-hepatic* (i.e. hepatic vein thrombosis, inferior vena cava thrombosis, inferior vena cava congenital malformation, and constrictive pericarditis). Among these, the most frequent cause of portal hypertension in Western Countries is liver cirrhosis, with either alcoholic in origin or chronic HBV or HVC infection, affecting over 90% of patients with portal hypertension in Europe and USA (63).

Cirrhosis is as a diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules (64). After the injury, the liver triggers a dynamic inflammatory response in order to repair the damaged tissue but, if the insult persists and tissue responds inadequately, the fibrotic process begins as a consequence of cytokines release (i.e. tumor necrosis factor alpha, TNF-α, and tissue growth factor beta, TGF-β or oxidative stress) from inflammatory cells. This leads to HSC activation with increased extracellular matrix deposition, fibrogenesis and cirrhosis (Figure 7).

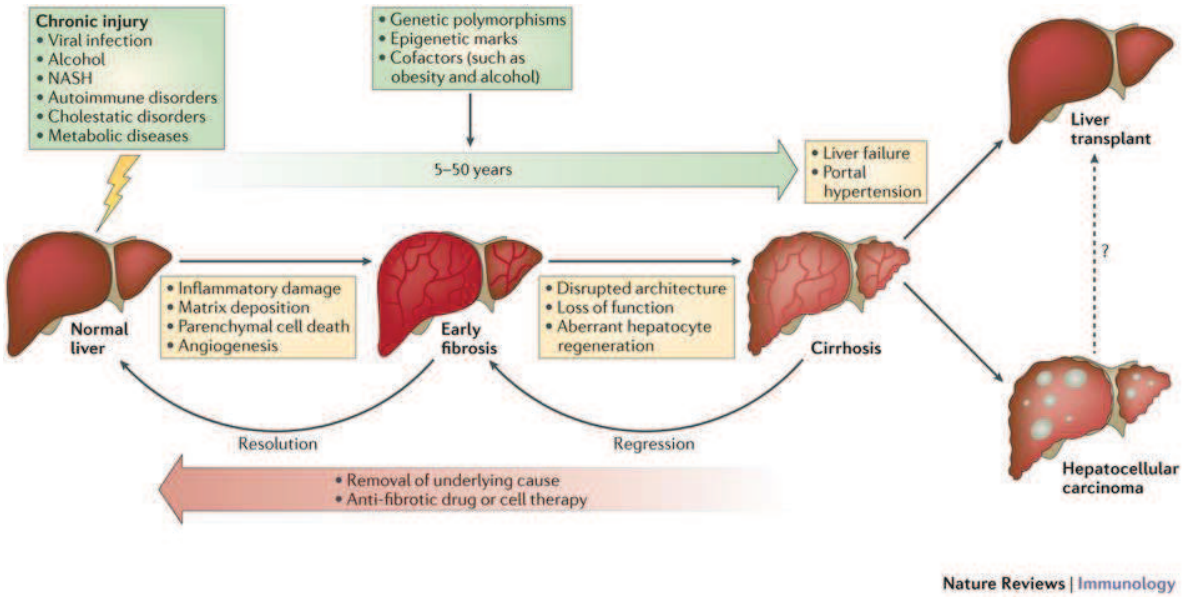


Figure 7. Natural history of chronic liver disease. From Pellicoro et al., Nat Rev Imm 2014

Thus, cirrhosis is a chronic and progressive condition that results in liver cell dysfunction and portal hypertension. These are the major responsible of cirrhosis complications: variceal bleeding, portal-systemic encephalopathy, accumulation of fluid in the peritoneal cavity (ascites), hepatorenal syndrome, portopulmonary hypertension.

A prognostic clinical sub-classification of cirrhosis with four distinct stages has been proposed with substantially differing likelihoods of mortality: stage 1 (compensated with no esophageal varices) has an estimated mortality of 1% per year, and stages 2 (compensated with varices), 3 (decompensated with ascites), and 4 (decompensated with gastrointestinal bleeding) have annual mortality rates of 3-4%, 20%, and 57%, respectively (65, 66). Infections and renal failure have been considered as stage 5, with 67% of 1-year mortality (67).

II.1. Clinical pathophysiology of portal hypertension

The portal pressure gradient is determined by the product of portal blood flow and the vascular resistance that opposes to the flow. Ohm's law defines this relationship in the equation:

$$\Delta P = Q \times R$$

in which ΔP is the portal pressure gradient, Q is the flow within the portal venous system, and R is the vascular resistance of the portal venous system, which represents the sum of the resistance of the portal vein, the hepatic vascular bed, and of the portosystemic collaterals (68).
The resistance that opposes blood flow is determined by the Poiseuille law:

$$R = 8\ n\ L / \pi r^4$$

where n is the coefficient of blood viscosity, L the length of the vessel and r its radius. Since the length of the vessel and its viscosity are relatively constant, the determining factor of resistance is the radius of the vessel. A decrease in the radius of the vessel can cause a significant increase in vascular resistance and, therefore, in the pressure gradient.

Portal pressure may be increased by an increment in portal blood flow or in vascular resistance, or a combination of both. However, it is well established that in cirrhosis, the primary factor leading to portal hypertension is an *increased resistance to portal blood flow*, associated with the development of *sinusoidal endothelial dysfunction* (69). Later on, an *increase in portal venous inflow* will help to maintain and aggravate portal hypertension (66, 69).

II.1.1. Increased intrahepatic vascular resistance to portal blood flow

Increased resistance to portal blood flow may occur at any site within the portal venous system (70). Although it is known that in cirrhosis the increase in intrahepatic resistance is due to architectural abnormalities caused by the fibrotic process (71), it is now clear that on top of these alterations there is a dynamic and reversible component representing up to 40% of the total increased intrahepatic vascular resistance (69, 72). This dynamic component is composed by

contractile elements located at sinusoidal as well as extra-sinusoidal levels, as explained in the chapter related to the hepatic sinusoid. An increased production of vasoconstrictors (69) (mainly thromboxane A2 and endothelin-1) and an exaggerated response of the hepatic vascular bed to them, as well as an insufficient availability and response to vasodilators (mainly NO) are the mechanisms that have been implicated in the pathogenesis of the dynamic component of the increased intrahepatic resistance of the cirrhotic liver (69, 73) (Figure 8). These mechanisms are explained in the chapter related to the modulation of the vascular tone.

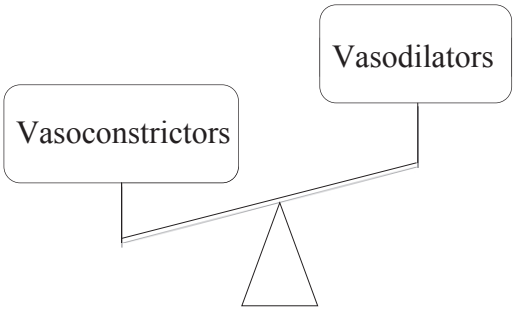


Figure 8. The balance of vasoactive factors involved in regulating the dynamic component of the intrahepatic vascular resistance is altered in cirrhosis.

The second factor contributing to aggravate the portal hypertension syndrome is an increased portal venous flow. It derives from a marked splanchnic arteriolar vasodilation that produces an increase in the volume of blood that reaches the portal vein. This situation leads to a splanchnic hyperdynamic circulation, with several neurogenic, humoral and local mechanisms (74, 75). Specifically, it has been reported an overproduction and increased response to circulating vasodilators (mainly NO) and a low response to vasoconstrictors (68, 76), as explained in the chapter II.1.3.

II.1.2. Sinusoidal endothelial dysfunction

In response to physical and chemical signals, a healthy endothelium produces a wide range of factors that regulate vascular tone, cellular adhesion, thrombo-resistance, smooth muscle cell proliferation and vessel wall inflammation (77-79). It is also able to keep the balance between tissue oxygen supply and metabolic demand. Thus, in response to increased blood volume, blood pressure or vasoconstrictor agents, which is what happens in cirrhotic portal hypertension, the hepatic endothelium should maintain its functions and find a way to accommodate the changes in order to prevent or attenuate the concomitant increase in pressure. However, in cirrhosis, liver sinusoids are not able to do that because they become dysfunctional as soon as the insult begins.

As a matter of fact, the hepatic vascular bed of cirrhotic livers exhibits impairment in the endothelium-dependent vasodilatation, named as "endothelial dysfunction" (80), because it cannot adapt the increased portal blood flow caused by the postprandial hyperemia, which determines an abrupt postprandial increase in portal pressure (81, 82).

Endothelial dysfunction is considered one of the main pathological mechanisms involved in the increased vascular tone observed in several vascular disorders such as arterial hypertension (83), diabetes (84) and hypercholesterolemia (85), and has been attributed to a diminished NO bioavailability (80, 86) and to an increased production of vasoconstrictors such as prostaglandin H2/thromboxane A_2 (PGH_2/TXA_2) (87) and endothelin (88). Gupta and colleagues published the first study that investigated the functional role of the endothelium on the increased vascular tone in the cirrhotic intrahepatic microcirculation evaluating the response of cirrhotic livers to the endothelium-dependent vasodilator acetylcholine (89). Nevertheless it has been reported that sinusoidal endothelial dysfunction is due not only to low NO bioavailability but also to increased COX-1 derived vasoconstrictor prostanoids (90-92), and that these two pathways are tightly related since inhibition of the COX-TXA_2 pathway results in the improvement in the phenotype of LSEC and HSC (93). In addition, our group recently observed that liver endothelial dysfunction occurs before the development of fibrosis or inflammation in a model of NAFLD (94) (Figure 9).

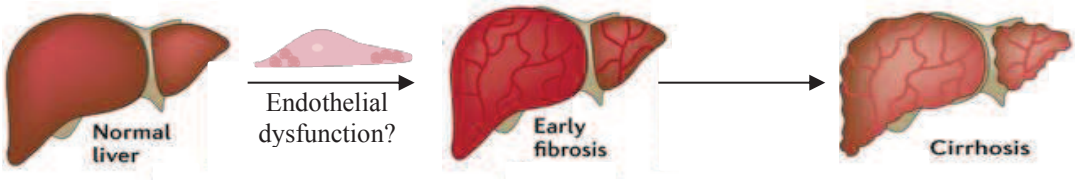


Figure 9. Does endothelial dysfunction proceed inflammation and fibrosis?

II.1.3. The hyperdynamic circulation

Systemic vascular resistance (SVR) represents the resistance of the body vascular bed against which the left heart is pumping. If SVR decreases, the body compensates it by pumping out hormones (i.e. epinephrine and norepinephrine) that cause muscle cells surrounding blood vessels to constrict, thus leading to decreased radius and thereby increased resistance. Vascular resistance is important because it is one determinant of blood pressure, and therefore organ perfusion. In cirrhotic portal hypertensive patients there is a marked reduction in the total SVR, associated with peripheral vasodilatation (95-97). This is due to the effects of increased endogenous systemic circulating vasodilator substances (mainly NO) that lead to the rise of the

hyperdynamic circulation (98). Indeed, development of a hyperdynamic splanchnic circulatory state is a major component of portal hypertension (75, 99-101). The increase in blood flow in splanchnic organs draining into the portal vein, and the subsequent increase in portal venous inflow, aggravates and perpetuates the portal hypertensive syndrome.

The role of NO in SVR has been evidenced in experimental studies where specific NO inhibitors were used, causing splanchnic vasoconstriction (102, 103). Moreover, the finding of increased serum and urinary concentrations of the products of NO oxidation, nitrite and nitrate, in patients with cirrhosis, also supports a role for NO in the genesis of the systemic circulatory disturbances of portal hypertension (104). The increased production of NO is due both to an increased expression and to an increased activity of eNOS (105, 106). Shear stress (107, 108), circulating vasoactive factors (109-111) and the angiogenic factor vascular endothelial growth factor (VEGF) (112) contribute to increase eNOS expression.

In portal hypertensive animals, NO overproduction by eNOS in the splanchnic circulation precedes the development of the hyperdynamic circulation (113). The post-translational regulation of eNOS in portal hypertension has been further evidenced by recent studies in the partial portal vein ligated model of portal hypertension, showing that up-regulation of eNOS catalytic activity, rather than eNOS overexpression, is the initial event that induces NO overproduction in the splanchnic circulation. Indeed, eNOS phosphorylation by AKT seems to be the mechanism of the initial up-regulation of eNOS activity and NO-mediated hypo-responsiveness to vasoconstrictors (106). Later on, other mechanisms for an increased production of NO become important, including an enhanced signaling of the molecular chaperone heat shock protein 90 (Hsp90) (114).

In advanced cirrhosis, the hyperdynamic circulation, together with portal hypertension, has a major role in the pathogenesis of ascites and hepatorenal syndrome, hepatopulmonary syndrome and arterial hypoxemia, variceal bleeding and portalhypertensive gastropathy. In addition, the shunting of portal blood to the systemic circulation through the portosystemic collaterals is a major determinant of hepatic encephalopathy, of decreased first-pass effect of orally administered drugs, and of decreased reticulo-endothelial system function (66, 115).

Finally, recent studies have demonstrated that the development of the hyperdynamic circulation, and its derived complications, is also associated with an increased neovascularization in splanchnic organs, through a VEGF-dependent angiogenic process. Indeed VEGF signaling blockade markedly attenuates the increase in splanchnic blood flow, as well as the increased

splanchnic vascularization observed in portal hypertensive animals (116-118). Therefore, modulation of angiogenesis may represent a potential target in the treatment of portal hypertension.

II.2. Cellular pathophysiology of cirrhotic portal hypertension

In a healthy condition, the liver is capable to maintain its functions thanks to an appropriated communication among its cellular components, guaranteed by the status of the liver sinusoidal microcirculation. Below are reported the main functions of the liver sinusoid:

- ✓ Regulation of macromolecular permeability
- ✓ Maintenance of liver homeostasis and normal coagulation
- ✓ Participation in the natural and acquired immunity
- ✓ Leukocyte traffic control
- ✓ Regulation of the hepatic vascular tone (*)
- ✓ Regulation of the oxidative stress (*)
- ✓ Sinusoidal remodeling
- ✓ Regulation and modulation of liver regeneration
- ✓ Support of lipid metabolism
- ✓ Regulation of cellular proliferation and death
- ✓ Preservation of the hepatic microcirculation

Functions tightly related with this Ph.D. thesis are described below with a particular emphasis on the cellular changes and responses occurring during the development of cirrhosis and portal hypertension (*).

II.2.1. Regulation of the hepatic vascular tone.

The hepatic vascular tone is determined by the balance between vasoconstrictors and vasodilators acting on the liver sinusoid. These vasoactive stimuli can be extrinsic (i.e. circulating angiotensin II, atrial natriuretic peptide) or intrinsic (i.e. nitric oxide, endothelin, local hormones, hypoxia), depending on where they come from (outside/inside of the liver). The primary function of extrinsic factors is to regulate arterial blood pressure modulating systemic vascular resistance, whereas the intrinsic factors regulate local blood flow within the liver and modulate intrahepatic vascular resistance thanks to their action on hepatic sinusoidal cells.

- *Nitric oxide and its bioavailability in cirrhosis. Role of liver sinusoidal endothelial cells.*

Nitric oxide is a gaseous molecule with a half-life of 3-5 seconds and with a very small size (order of picometers). It regulates vascular tone and homeostasis, and it is implicated in biological functions such as vasodilation, inhibition of platelet aggregation, insulin secretion, angiogenesis, neural development, coagulation and leukocyte adhesion to the endothelium (119, 120). The enzyme responsible for the formation of this important cellular signaling molecule is the protein nitric oxide synthase (NOS), which catalyze the production of nitric oxide from L-arginine (Figure 10). There are three main isoforms of this enzyme: the neuronal NOS (nNOS), the inducible (iNOS) and the endothelial (eNOS). eNOS is a calcium-calmodulin controlled cytosolic enzyme expressed constitutively in endothelial cells, where it produces small amounts of NO in response to mechanical/physiological stimuli such as shear stress or estrogen, vascular endothelial growth factor, acetylcholine, bradykinin and other agonists of G protein-coupled receptors (121). The formation and the activity of this enzyme is a complex process that involves both post-transcriptional and post-translational modifications. Among them, phosphorylation on serine, threonine and tyrosine, protein-protein interactions and intracellular localization play an important role. NO derived from eNOS diffuses to the smooth muscle cell (or HSC), thus modulates their contraction through the activation of its natural ligand, the soluble guanylate cyclase (sGC), an enzyme that promotes the synthesis of the second messenger cyclic guanosine monophosphate (cGMP) (Figure 10). The main target of cGMP is a cGMP-dependent protein kinase called protein kinase G (PKG), which phosphorylates numerous proteins involved in calcium homeostasis, like the inositol 1,4,5-trisphosphate receptor (IP3R). This phosphorylation leads to a decrease in the concentration of intracellular calcium levels and finally to the relaxation of smooth muscle cells and vasodilation. Moreover, protein kinase activates myosin light chain phosphatase, the enzyme that dephosphorylates myosin light chains, which also leads to smooth muscle relaxation. Thus, NO production is essential to maintain the adequate vascular tone and an anti-atherogenic endothelium (122).

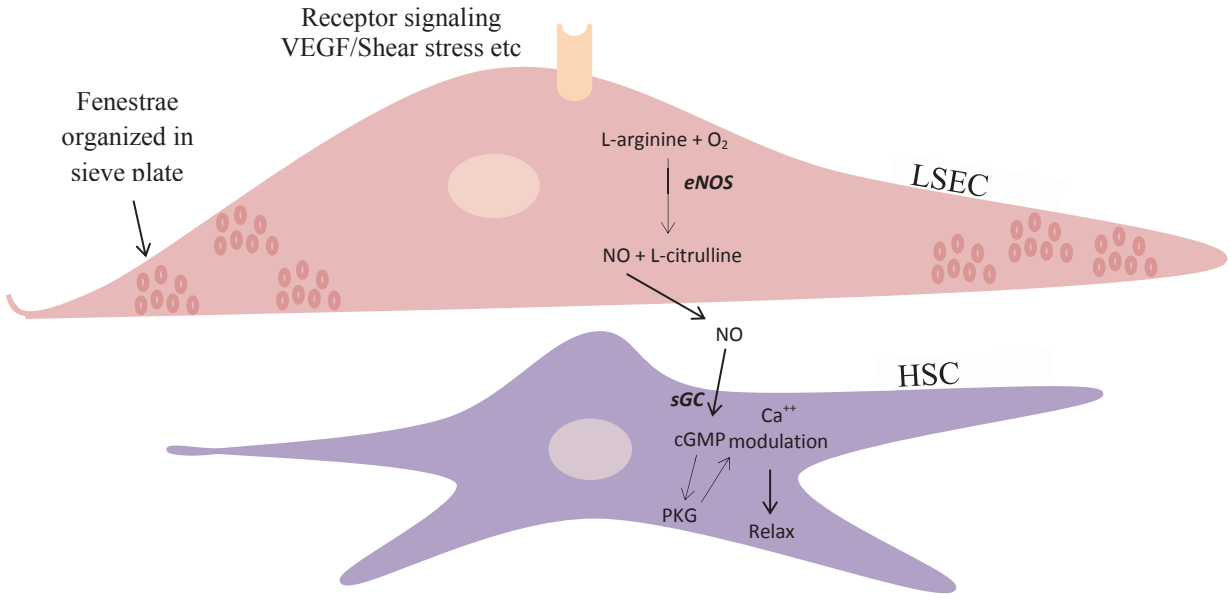


Figure 10. Nitric oxide regulation in the hepatic sinusoid. NO generated from the conversion of L-arginine to L-citrulline stimulates the HSC relaxation through the activation of the protein kinase G.

The level of expression and subcellular localization of eNOS in LSEC and the effects of its regulatory mechanisms were described by Dr. Shah and colleagues in 1996 (123). LSEC express eNOS protein in abundant quantities, but normally they release NO at low levels. After a stimulus (i.e. shear stress), LSEC increase NO release. However, the cirrhotic liver has a deficit in the production of this vasodilator, thus NO is defectively released from LSEC and this influences HSC contraction and relaxation, leading to increased intrahepatic vascular resistance. The decrease in NO bioavailability is mainly caused by a reduced activity of eNOS, without changes in its basal expression, due to post-translational modifications (124): decreased bioavailability of the eNOS cofactor tetrahydrobiopterin (BH_4) (125) or increased interaction with the inhibitory proteins caveolin -1 (126-128), nitric oxide synthase interacting protein (NOSIP) and nitric oxide synthase trafficking (NOSTRIN) (129). In addition, the reduced activity of eNOS in cirrhosis has been associated with decreased Akt-dependent eNOS phosphorylation (130) or decreased Akt activity due to its interaction with the G protein-coupled receptor inhibitor GRK-2 (131). Another important endogenous nitric oxide synthase inhibitor, the asymmetric dimethylarginine (ADMA), is also involved in the reduction of the enzymatic activity of eNOS in cirrhotic livers (132). In addition, NO bioavailability within liver microcirculation is further reduced due to its scavenging by elevated levels of superoxide anion (O_2^-), as explained in II.2.2.

As mentioned above, the decreased production of the vasodilator NO contributes to the development and progression of portal hypertension in cirrhosis. Therefore, there is a rational basis to make strategies in order to increase hepatic NO levels. In this sense, pioneering studies confirmed that the administration of NO donors reduces portal pressure in the perfused cirrhotic

liver (133, 134). Moreover, several studies investigated how to increase hepatic NO bioavailability without producing deleterious systemic effects. The cofactor BH_4 increased intrahepatic NO bioavailability, reducing intrahepatic resistance and ultimately reducing portal pressure without changes in systemic hemodynamic parameters (135). In addition, statins also restore eNOS activity increasing NO bioavailability in cirrhotic animals (136, 137), and other studies using adenovirus codifying for nNOS or eNOS achieved similar results (138, 139).

- *Hyperresponsiveness to vasoconstrictors.*

The cirrhotic liver has an increased response to certain vasoconstrictors, compared to a normal liver (92, 140-142). This increased response to vasoconstrictors is related to several changes: a) increase in the amount of contractile tissue due to the proliferation of the HSC; b) deficit of vasodilators; c) increase in vasoconstrictor receptor density or in its response.

Endothelin 1 (ET-1) is one of the most studied vasoconstrictor in the intrahepatic circulation. There are evidences that both circulating levels of ET-1 and its intrahepatic production are increased in cirrhosis (143, 144), and that ET-1 can augment the intrahepatic resistance in the cirrhotic liver (145).

The cysteinyl leukotrienes (CysLTs) are a group of biologically highly potent vasoactive substances derived from the metabolism of arachidonic acid due to the action of the 5-lipoxygenase (5-LO) which production, as well as 5-LO expression, is increased in cirrhotic livers (92, 146). The importance of the arachidonic acid pathway regarding the regulation of the hepatic vascular tone in cirrhotic livers has been mostly investigated by our laboratory. In 2003 we observed that the hyperresponse to the alpha adrenergic agonist methoxamine of cirrhotic livers disappeared when cyclooxygenase (COX) was inhibited with indomethacin but not after NO inhibition (91, 142). More specifically, the COX-derived prostanoid thromboxane A_2 is the one that regulates the response of cirrhotic livers to methoxamine (142). Later on we demonstrated that LSEC play a fundamental role in the production of these vasoconstrictors (147). Furthermore, the abnormal response of the cirrhotic liver to the endothelium-dependent vasodilator acetylcholine is also a consequence of an increased production of TXA_2 (91). Thus, the sinusoidal endothelial dysfunction, characteristic of the cirrhotic liver, is not only characterized by a reduced NO bioavailability, but also by an increament in prostanoids production and an exaggerated response to them.

- *Extracellular matrix deposition and activation of hepatic stellate cells*

Hepatic stellate cells have been considered to play a role in the regulation of sinusoidal vascular tone in the pathogenesis of intrahepatic portal hypertension because of their strategic perisinusoidal orientation within the sinusoid (25). Their contraction contributes to increase the intrahepatic sinusoidal resistance.

The extracellular matrix (ECM) is the structural framework needed to provide support for the surrounding cells, regulate intercellular communication and store cellular growth factors. It is essential for processes like growth, wound healing and fibrosis, especially when there has been a significant loss of tissue due to liver injury. ECM plays an important role in cirrhosis since sub-endothelial matrix accumulation in the space of Disse can lead to sinusoidal dysfunction or capillarization (148). After an injury HSC activate and produce large amounts of ECM components such as proteoglycan, collagen, and glycoproteins (149). They change to a myofibroblast-like phenotype and during the trans-differentiation process HSC lose their retinoid droplets, and express de novo smooth muscle proteins, including α smooth muscle actin (150). In addition, HSC respond to the elevated quantities of endothelin-1 (a prominent regulator of stellate cell contractility) (151-153) presented in the sub-endothelial space of Disse, which together with other vasocontrisctors and low nitric oxide bioavailability (Fig. 11), will further contribute to increase intrahepatic vascular resistance, aggravating portal hypertension. Compounds such as substance P, angiotensin II, norepinephrine and thrombin have been also shown to have significant, but variable, effects on stellate cell contractility (154).

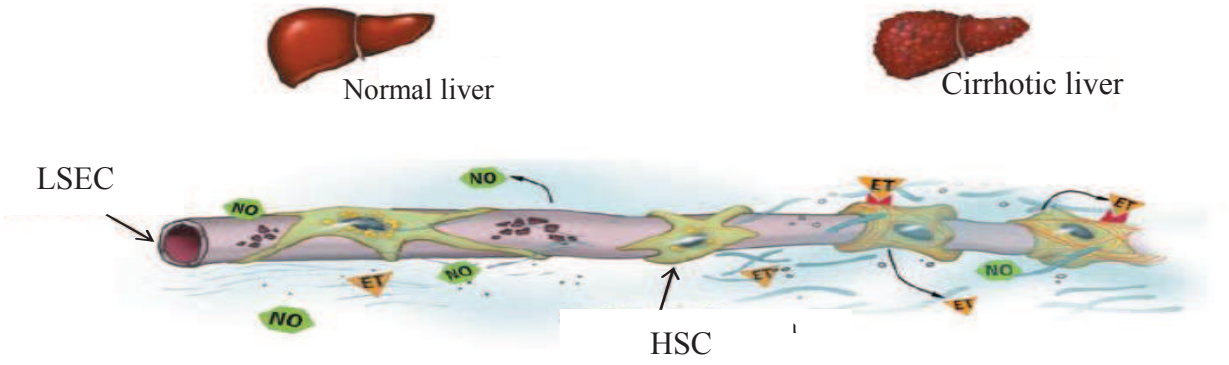


Figure 11. Activation of HSC in the cirrhotic liver. Factors regulating the contractility of the HSC. ET: endothelin. NO: nitric oxide. Adapted from Rockey DC, Hepatology 2003.

- *Cross-talk between LSEC and HSC*

Cells communicate by sending and receiving signals that may come from other cells. Sometimes the signal itself can cross the cell membrane, other times can interact with the receptor outside or inside the cell, thus making the signal specific for that receptor so for the cell. Hormones, neurotransmitters, cytokines, lipids, phospholipids, aminoacids, monoamines, proteins, glycoproteins or gases: all can be potent signal molecules involved in a specific cellular response. The first study demonstrating that liver cells modulate the phenotype of the hepatic microvascular sinusoid was published in Laboratory Investigation in 1991 by Módis et al. They found that since hepatocytes and endothelium do not establish direct cell contacts, the modulation of liver microcirculation was exerted either by secreted soluble cytokines or by the extracellular matrix (155). Several years later, Dr. DeLeve and colleagues showed that LSEC phenotype is maintained by paracrine and autocrine regulation (via VEGF) (13) and that healthy LSEC prevent HSC activation and promote reversion to quiescence through VEGF-NO-dependent and independent mechanisms (10, 11). This Ph.D. thesis is also focused on the cross-talk among these hepatic cells.

II.2.2. Regulation of the oxidative stress

Reactive oxygen species (ROS) are unstable and highly reactive molecules produced as natural byproducts of the oxygen metabolism of the cell, and have important roles in cell signaling and homeostasis (156, 157). They can be generated from endogenous but also exogenous sources. Once formed, ROS can bind any kind of molecule (proteins, lipids, DNA) and damage cell structures or alter their functions. The most well-known ROS are superoxide anion (O_2^-), hydroxyl anion (OH^-), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (158, 159). They derive from the reaction of several enzymes such as the nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase (160), the xanthine oxidase (XO) (161), uncoupled eNOS (162), COX (163), cytochrome P_{450} and proteins involved in the mitochondrial respiratory chain (164). Under physiological conditions, intracellular ROS levels are regulated by the antioxidant defense mechanisms of the organism (vitamin C and E, glutathione, catalase, glutathione peroxidase and superoxide dismutase) that keep a balance between their production and the antioxidant capacity of the cell (165-167). When cells produce more ROS than they can eliminate, the pathophysiological situation known as *oxidative stress* begins and may cause necrosis, apoptosis and inflammation, altogether contributing to increase the vascular tone (168, 169). Indeed the anion O_2^- reduces NO bioavailability leading to the formation of $ONOO^-$ that bind to the tyrosine residues of proteins in a process called protein nitrotyrosination with consequent modification in

the protein function (170-172). In cirrhosis, our group demonstrated that elevated levels of O_2^- scavenge NO, thus reducing its bioavailability (173). O_2^- was originated from different cellular sources including COX and XO, but not from eNOS or NADPH oxidase (174). In addition, intracellular ROS in HSC lead to their phenotype modification, making them pro-contractile and proliferative (175, 176).

- *Leptin*

Leptin is a cytokine-type hormone of 16kDa codified by the Ob gene (177), implicated in the regulation of weight, appetite and body thermogenesis through actions on the central nervous system (178). Different isoforms of leptin receptors (ObRs), generated by alternative splicing, have been identified: long, short and soluble forms, different because of the intracellular portion. The only receptor which presents full signaling capabilities is the long domain of the ObRb, which results in the activation of the Jak family of non-receptor tyrosine kinases and the STAT (signal transducer and activator of transcription) group of transcription factors (179), leading to the transcription of inflammatory (i.e. NFκB) and fibrogenic (i.e. pro-collagen I) genes (180, 182). Although mainly produced by adipocytes, hepatic sinusoidal cells have been shown to produce leptin end express its receptors (182, 184). Indeed, leptin modulates the biology of different cell types participating in the response to liver injury, such as KC, HSC and LSEC. In this last one, leptin stimulated their proliferation and production of ROS (184).

Elevated serum leptin levels have been found in experimental models of fibrosis and cirrhosis and in cirrhotic patients (185-188). In addition, leptin is able to decrease NO bioavailability due to its oxidative properties (189, 190), leading to endothelial dysfunction and impairing vascular tone. Its exogenous administration can potentiate the progression of liver fibrosis (191-193) . On the other hand, absence of leptin or leptin receptor signaling results in a significant reduction of fibrosis as demonstrated using experimental models of liver injury such as thioacetamide intoxication, chronic CCl_4 administration or NASH (194-196). Leptin also plays a role in the diminished degradation of fibrotic ECM occurring in the fibrogenic process (179). Although the adverse effects of leptin on liver fibrosis/cirrhosis have been extensively investigated, its role in portal hypertension remains unknown.

- *The Nrf2-mediated pathway*

One of the best known antioxidant mechanisms that is activated in various diseases as a defense response is the Nrf2-mediated pathway (197). Nrf2 is a member of the cap'n'collar family of

bZIP transcription factors and has been shown to regulate the expression of a network of cyto-protective enzymes resulting in protection against toxicity induced by exposure to electrophilic and oxidative chemicals (198). Under basal conditions, Nrf2 is retained in the cytoplasm bound to Keap1 that promotes its proteasomal degradation (199). However, upon stimulation Nrf2 is released and translocates to the nucleus where it binds to the antioxidant responsive elements (ARE) of cyto-protective genes such as glutathione, NADPH dehydrogenase quinone 1 (NQO1) and heme oxygenase (HO-1), promoting their transcription (200, 201). Nrf2 activation has been observed in hepatic stellate cells and Kupffer cells as well as in parenchymal hepatocytes where it plays complex roles in hepatic inflammation, fibrosis, hepato-carcinogenesis, and regeneration via its target gene induction (202, 203). The protective roles of Nrf2 activation in the pathogenesis of liver diseases have been extensively investigated (Figure 12). Shimozono and colleagues observed that activation of the Nrf2-mediated pathway attenuates the progression of hepatic fibrosis in a rat model of nonalcoholic steatohepatitis (NASH) (204) and Xu W. et al observed that Nrf2 activation may be a novel strategy to prevent or ameliorate toxin-induced liver injury and fibrosis (205). It is important to notice that this transcription factor increases in response to shear stress (206, 207) and becomes activated in endothelial cells over-expressing KLF2 (208, 209).

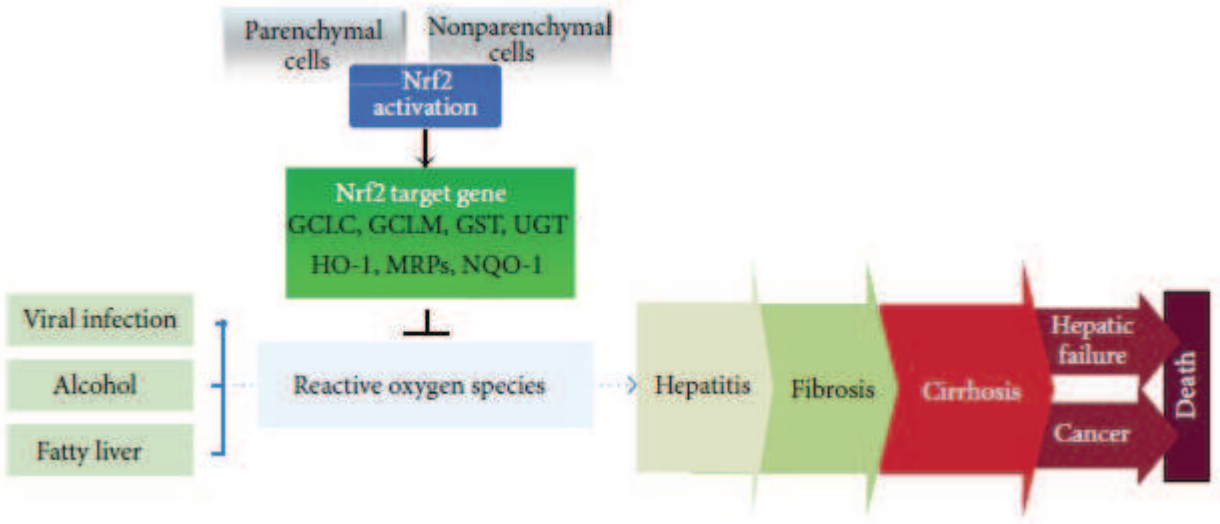


Figure 12. Scheme of the protective role of Nrf2 in liver diseases. The activation of the Nrf2-mediated pathway in hepatic cells may prevent the progression of liver disease through the inhibition of ROS production. From Shin SM, Oxid Med Cell Longev. 2013.

III. Current treatments and future perspectives

The current treatments available for portal hypertension are related to its complications. The HVPG is a good surrogate marker of portal hypertension and has robust prognostic power (210). Portal hypertension is considered clinically significant when the HVPG is above 10 mmHg since patients with HVPG of less than 10 mmHg had a 90% probability of not progressing to decompensation during median follow-up of 4 years (211). Below (Figure 13) are reported the prevention and treatment of portal hypertension and varices at various degrees of severity (66).

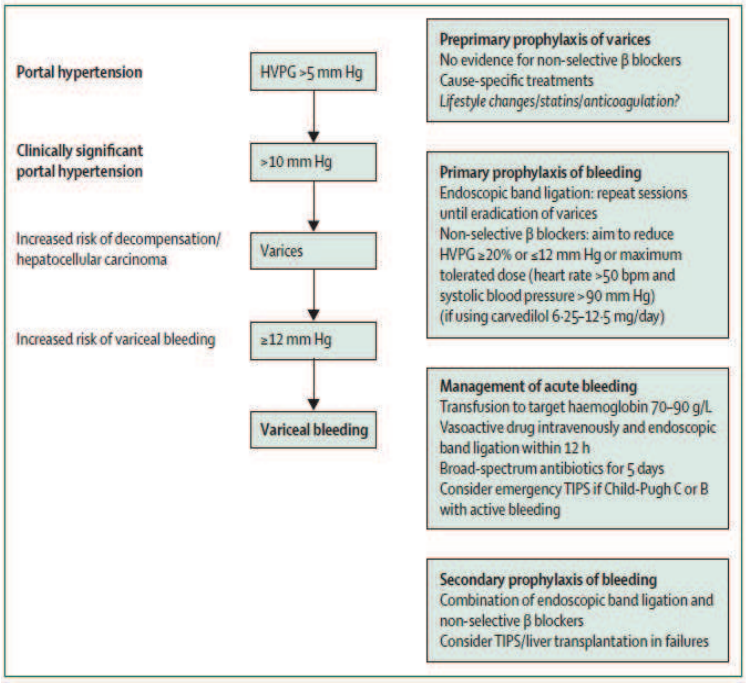


Figure 13. Prevention and treatment of portal hypertension and varices at various degrees of severity.
HVPG= hepatic-vein pressure gradient.
BPM=beats per minute.
TIPS=transjugular intrahepatic porto-systemic shunt.
From Emmanuel A Tsochatzis, Jaime Bosch, Andrew K Burroughs. Lancet 2014.

III.1. Future perspective: statins

Statins are a group of drugs that inhibit the action of the hydroxyl-methyl-glutaryl-coenzyme A (HMG-CoA) reductase (Figure 14), the limiting enzyme for the synthesis of cholesterol, depleting the cells of mevalonate and its derived products (i.e. isoprenoids) (212). Indeed, statins were firstly design to lower lipid levels, but various studies reported that much of their beneficial and pleiotropic effects were independent of cholesterol lowering (213, 214). They may be beneficial in situations of septicemia (215-217), stroke (218), rheumatoid arthritis (219) and colon carcinoma (220).

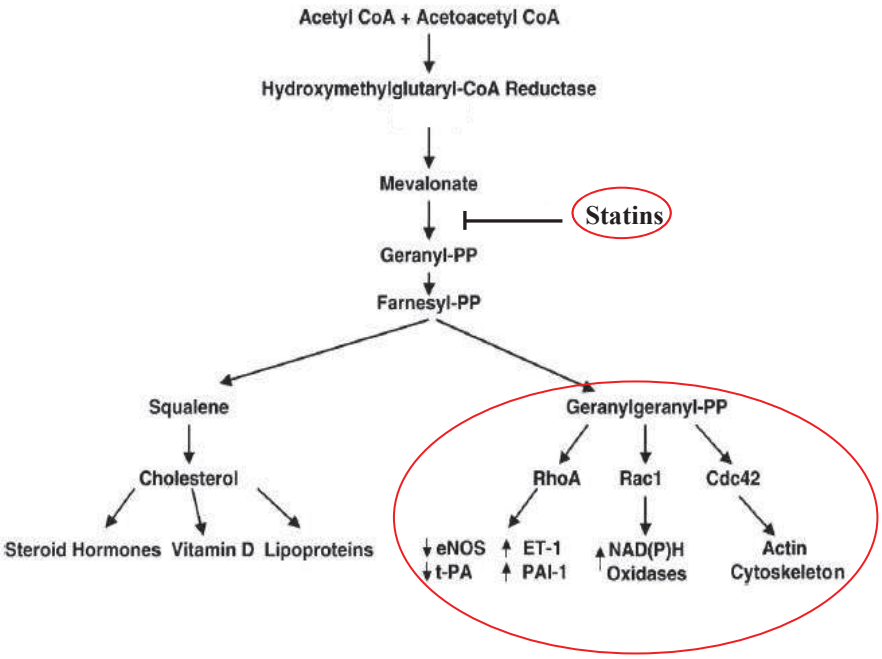


Figure 14: Metabolism and biological actions of the mevalonate pathway. The "no lipid lowering" effects of statins relate to the lack of production of the isoprenoid intermediate geranyl-geranyl-PP (GGPP) (adapted from Beckman JA and Creager MA, TCM 2006).

Under physiological conditions, mevalonate-derived products regulate cellular processes like oxidative stress, glycoproteins synthesis for the maintenance of cell membrane structures, cholesterol and steroid hormones (221). Inhibition of the mevalonate pathway prevents the formation of farnesyl-pyrophosphate (FPP), necessary for the synthesis of squalene and cholesterol, and geranylgeranyl-pyrophosphate (GGPP), which regulates the activity of the small GTPases Rho, Rac1 and Ras (222). These GTPases are involved in the regulation of cellular membrane transport and motility and can modulate the endothelial function regulating NO synthesis, endothelin, oxidative stress and the expression of inflammatory and thrombotic factors (223) (Table 2).

Anti-atherogenic	***Pro-atherogenic***
↑ KLF2	↓ NFκB
↑ NO	↓ ET-1
↑ PGI_2	↓ Ang II
↑ EDHF	↓ O^-_2
↑ TM	↓ PAI-1
↑ tPA	↓ TNFα, IL-6
	↓ P-, E-selectin
	↓ ICAM-1, VCAM-1

Table 2: The effects of statins on endothelial regulators of vascular function. Adapted from Beckman JA and Creager MA, TCM 2006.

Our group was the pioneer demonstrating the importance of statins as treatment for cirrhosis and portal hypertension (136, 224, 225). In fact, in 2004 we observed that administration of simvastatin to cirrhotic patients with portal hypertension reduced hepatic sinusoidal resistance (224) and 3 years later, following this line, that simvastatin administration to portal hypertensive rats improved endothelial function increasing NO bioavailability selectively in the liver (136). Nevertheless, the underlying mechanisms of statins improving portal hypertension still remain unknown. The effects of statins on vascular function could be due in part to the increased KLF2 expression (42). Indeed, with this Ph.D. thesis I mainly focus on the importance of KLF2 and statins in cirrhosis and portal hypertension.

III.2. Future perspective: antioxidant therapies

Additional approaches to improve sinusoidal phenotype in cirrhosis included the reduction in hepatic oxidative stress using a variety of antioxidant strategies, including an exogenous recombinant formulation of superoxide dismutase (226), N-acetylcysteine (227), vitamins (132, 228) and resveratrol (229). All these therapeutic approaches resulted in intrahepatic vascular resistance reduction associated with marked increment in hepatic NO bioavailability. Importantly, oxidative stress reduction and hepatic circulation improvement using antioxidants has been also demonstrated in patients with cirrhosis (228, 230).

Hypothesis and aims

Hypothesis and aims

Liver cirrhosis is an end-stage disease that habitually leads to death, unless liver transplantation is done, and represents the 4th cause of death in central Europe in adults (231) and the 3rd in men between 45 and 64 years of age in Spain (232).

Pathophysiologically, hepatic microvascular changes together with the formation of intrahepatic shunts and sinusoidal endothelial dysfunction lead to the development of cirrhotic portal hypertension, and later on to its complications.

The increment in the intrahepatic vascular resistance to portal blood flow is the first event occurring in portal hypertension, further aggravated by the subsequent increment in blood inflow. Thus any therapy able to decrease intrahepatic resistance or portal blood flow could have beneficial effects on the portal hypertension syndrome. In cirrhotic patients, a decrease in HVPG of at least 20% or to less than 12 mmHg is associated with a significant reduction in the development of portal hypertension complications (233).

Nowadays, cirrhosis treatments are directed to eliminate the insult (66) (i.e. venesection for haemochromatosis, antiviral therapy for viral-cirrhosis, lifestyle change for alcoholic cirrhosis) but, in decompensated cirrhosis with portal hypertension, treatments options only include non-selective β blockers (i.e. propranolol), which decrease cardiac output and cause splanchnic vasoconstriction thereby reducing portal inflow (68), and endoscopic band ligation for the prevention of bleeding and reduction of mortality (234) (propranolol has the same effect).

As explained in the introduction of this Ph.D. thesis, find a way to improve liver sinusoidal microcirculation, to ameliorate endothelial dysfunction and to decrease intrahepatic resistance could lead to fibrosis resolution, or attenuate the cirrhotic progression, together with an improvement in portal hypertension. Thus, studying the mechanisms and the “actors” involved in the regulation of the hepatic vascular tone, in the hepatic oxidative stress or in the modulation of homeostasis and hepatic metabolism is essential to know which drug could be useful in the treatment of liver diseases: a drug that may have a positive effect on those “actors”.

The works presented herein focus on the response of cirrhotic portal hypertensive livers to the blockade of the leptin signaling (using an antibody against the leptin receptor, ObR) and to the up-regulation on the intrahepatic KLF2 pathways (using adenoviral and pharmacologically approaches), as well as to further understand the underlying molecular and cellular mechanisms that modulate the hepatic vascular tone, the oxidative stress and the fibrotic process. In

particular, the leptin study proposes a new pharmacological approach, and the studies on KLF2 are related to the pleiotropic effects of statins, standing out the importance of the use of these drugs in chronic liver diseases and pointing out the effects of cellular cross-talk among LSEC and HSC.

- **Study 1. Leptin receptor blockade reduces intrahepatic vascular resistance and portal pressure in an experimental model of rat liver cirrhosis.**

The dangerous role of leptin in chronic liver diseases has been exhaustively investigated. This hormone has pro-oxidative and pro-fibrogenic properties, and it is a modulator of the vascular tone. Moreover, it has been reported how the inhibition of its signaling has beneficial effects not only in the liver, but also in different damaged tissues. In human cirrhosis, leptin serum levels as well as its signaling (derived from the activation of the leptin receptor ObR) have been found increased (186, 187). With this background the hypothesis of the study was to investigate if the blockade of the ObR could ameliorate cirrhosis and portal hypertension. For that we aimed at evaluating the hemodynamic parameters and the mechanisms regulating the hepatic vascular tone in cirrhotic animals in which the leptin signaling has been inhibited.

- **Study 2. The transcription factor KLF2 mediates hepatic endothelial protection and paracrine endothelial–stellate cell deactivation induced by statins.**

KLF2 confers vasoprotective properties to the endothelium (235). Recently, we observed that its expression is increased in the cirrhotic liver probably to help the endothelium to adapt itself to the liver injury occurring during the progression of the disease (42).

KLF2 is induced by statins in different cellular types as well as its hepatic expression in simvastatin treated cirrhotic animals (42), but its modulation by statins in LSEC has not been investigated. Moreover, it was recently reported that LSEC and HSC paracrinally regulate their phenotype (10, 11, 13), and that simvastatin attenuates the development of liver fibrosis (236). Therefore, our hypothesis was that statins could induce the expression of KLF2 also in a microvascular environment like the sinusoidal endothelium (LSEC), which may ameliorate neighboring cells (HSC), and our aim was to investigate if KLF2 could be the mediator of statins-derived hepatic vasoprotection.

- **Study 3. KLF2 exerts anti-fibrotic and vasoprotective effects in cirrhotic rat livers: behind the molecular mechanisms of statins.**

As reported in the introduction, KLF2 represents an endothelial compensatory mechanism trying to be beneficial during its marriage with the endothelium, in sickness and in health, just because its expression increases during the progression of the disease (42). So we thought "what may happen if we try to help this marriage, if we give to cirrhotic animals more KLF2?". Thus, the hypothesis of this study was that the up-regulation of KLF2 in cirrhotic animals could lead to an amelioration of the microvascular environment. Indeed, although collectively the observations regarding KLF2 are intriguing, much of our understanding (and of others) has been based on *in vitro* observations. For that, our aim was to explore the effects and the underlying mechanisms of KLF2 up-regulation in *in vitro* and *in vivo* models of liver cirrhosis, specially focusing on the effects on the HSC phenotype and liver hemodynamics.

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Study 1:

LEPTIN RECEPTOR BLOCKADE REDUCES INTRAHEPATIC VASCULAR RESISTANCE AND PORTAL PRESSURE IN AN EXPERIMENTAL MODEL OF RAT LIVER CIRRHOSIS.

María Gabriela Delgado,* Jordi Gracia-Sancho,* **Giusi Marrone**, Aina Rodríguez-Vilarrupla, Ramon Deulofeu, Juan G. Abraldes, Jaume Bosch, and Juan Carlos García-Pagán.

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Leptin receptor blockade reduces intrahepatic vascular resistance and portal pressure in an experimental model of rat liver cirrhosis

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Barcelona Hepatic Hemodynamic Laboratory, Liver Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), University of Barcelona, Spain

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Delgado MG, Gracia-Sancho J, Marrone G, Rodríguez-Vilarrupla A, Deulofeu R, Abraldes JG, Bosch J, García-Pagán JC. Leptin receptor blockade reduces intrahepatic vascular resistance and portal pressure in an experimental model of rat liver cirrhosis. *Am J Physiol Gastrointest Liver Physiol* 305: G496–G502, 2013. First published July 25, 2013; doi:10.1152/ajpgi.00336.2012.—Increased hepatic vascular resistance mainly due to elevated vascular tone and to fibrosis is the primary factor in the development of portal hypertension in cirrhosis. Leptin, a hormone associated with reduction in nitric oxide bioavailability, vascular dysfunction, and liver fibrosis, is increased in patients with cirrhosis. We aimed at evaluating whether leptin influences the increased hepatic resistance in portal hypertension. CCl_4-cirrhotic rats received the leptin receptor-blocker ObR antibody, or its vehicle, every other day for 1 wk. Hepatic and systemic hemodynamics were measured in both groups. Hepatic nitric oxide production and bioavailability, together with oxidative stress, nitrotyrosinated proteins, and liver fibrosis, were evaluated. In cirrhotic rats, leptin-receptor blockade significantly reduced portal pressure without modifying portal blood flow, suggesting a reduction in the intrahepatic resistance. Portal pressure reduction was associated with increased nitric oxide bioavailability and with decreased O_2^- levels and nitrotyrosinated proteins. No changes in systemic hemodynamics and liver fibrosis were observed. In conclusion, the present study shows that blockade of the leptin signaling pathway in cirrhosis significantly reduces portal pressure. This effect is probably due to a nitric oxide-mediated reduction in the hepatic vascular tone.

portal hypertension; nitric oxide; fibrosis; endothelium; ObR

PORTAL HYPERTENSION IS THE main complication of cirrhosis of the liver. It is mainly due to increased intrahepatic resistance, which results from structural changes inherent to progressive fibrosis and dynamic changes due to increased hepatic vascular tone (11). Deficient nitric oxide (NO) bioavailability within the liver circulation, derived both from reduced NO synthesis by endothelial NO synthase (eNOS) (17, 27, 34) and increased NO scavenging by elevated levels of superoxide anion (O_2^-) (14), is considered a main pathogenic factor increasing hepatic vascular tone in cirrhosis. This concept has been reinforced by two recently published studies showing that decreasing hepatic O_2^- levels by enhancing superoxide dismutase activity improves the intrahepatic vascular tone and reduces portal pressure in cirrhotic rats (10, 16).

Leptin, a hormone of 16 kDa expressed from the *ob* gen (9), regulates weight, appetite, and body thermogenesis and prevents fat storage in nonadipose tissue (35). Although leptin is produced primarily by adipocytes, expression of leptin and of its receptors has been described in other tissues such as heart, skeletal muscle, reproductive system, immune system, endothelium, and liver (39). Elevated levels of leptin, probably produced by hepatic cells in response to inflammation, have been described in experimental models of fibrosis and cirrhosis and in cirrhotic patients (5, 30, 31, 37).

Leptin has been shown to impair vascular tone and NO bioavailability. Leptin administration to obese rats impairs acetylcholine-mediated coronary vasodilatation, both in vivo and ex vivo (23). Recent studies demonstrate that leptin is able to increase O_2^- production in endothelial cells, leading to a marked decrease in NO levels together with an increase in peroxynitrite accumulation (24). On the other hand, leptin is actively involved in liver fibrogenesis. The absence of leptin or its receptor, as occurs in ob/ob mice or fa/fa rats, is associated with a significant reduction of fibrosis in experimental models of liver injury (2). Moreover, exogenous leptin administration potentiates progression of liver fibrosis (6, 19, 21, 28), a situation that is prevented administering leptin antagonists (7).

Given the above, the present study aimed at investigating whether 1-wk leptin blockade has a role modulating the increased hepatic vascular tone observed in cirrhosis. For this, we tested the effects of a nonselective blockade of leptin receptor using monoclonal antibodies (ObR-Ab) (32) on the hepatic and systemic hemodynamics and tested hepatic NO bioavailability in an experimental model of cirrhosis induced by CCl_4 in rats.

MATERIALS AND METHODS

Animals and induction of cirrhosis. Male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 50–75 g were induced to cirrhosis by inhalation of carbon tetrachloride (CCl_4) three times a week. Phenobarbital (0.3 g/l) was added to the drinking water as previously described (10). When cirrhotic rats developed ascites (after 14–16 wk), administration of CCl_4 and phenobarbital was stopped and treatment was started 1 wk later. Control animals only received phenobarbital. Animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

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Characterization of leptin and leptin receptor ObR in control and cirrhotic animals. Leptin levels were analyzed in serum from control and cirrhotic rats ($n = 12$ per group) by using a commercially available enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) following manufacturer's instructions. Leptin receptor protein expression was assessed by Western blot in hepatic homogenates from control and cirrhotic animals ($n = 6$ per group) as described below. ObR antibody specificity was validated by analyzing leptin receptor expression in liver tissue from db/db mice (Charles River Laboratories).

Leptin receptor antagonist ObR-Ab administration. SH-SY5Y human neuroblastoma cells (kindly provided by Dr. Sanfeliu, IIBB/CSIC/IDIBAPS) were used to test the specificity of the Ob receptor (all isoforms) blocker antibody (ObR-Ab, Alpha Diagnostic International, San Antonio, TX). These cells express active ObR that upon leptin administration signals through Signal Transducer Activator of Transcription 3 (STAT3). Cells cultured in DMEM supplemented with retinoic acid were starved for 16 h, incubated with ObR-Ab (200 nM) or its vehicle for 1 h, and afterward stimulated with leptin (100 ng/ml, R&D Systems, Minneapolis, MN), IL-6 (50 ng/ml, R&D Systems) or vehicle for 15 min (1). ObR blockade was defined as a reduction in the ratio of phosphorylated STAT3 (p-STAT3) to total STAT3 determined by Western blot (1, 32).

In addition, adequate hepatic leptin receptor blockade was analyzed by administrating ObR-Ab (4 or 8 μg/kg body wt) or its vehicle 2 h before administrating leptin (1 μg/g body wt) to control rats, and evaluating p-STAT3/STAT3 in liver samples.

Once ObR-Ab efficacy was confirmed, ObR-Ab (8 μg/kg, $n = 12$) or IgG (8 μg/kg, $n = 12$) was administered to cirrhotic rats via the tail vein every other day for 1 wk. Drug administration and subsequent experiments were performed blindly. In cirrhotic animals, hepatic ObR blockade was further validated by analyzing IL-6 mRNA expression (26).

Splanchnic and systemic in vivo hemodynamic studies. Two hours after the last dose of ObR-Ab or vehicle, rats were anesthetized with ketamine (100 mg/kg ip, Imalgene, Barcelona, Spain) and midazolam (5 mg/kg ip, Laboratorio Reig Jofre, Barcelona, Spain). A tracheotomy and cannulation with PE-240 catheter (Portex, Kent, UK) was performed to maintain adequate ventilation during the anesthesia. Then PE-50 catheters were introduced into femoral artery and ileocolic vein to continuously measure mean arterial pressure and portal pressure, respectively. A perivascular nonconstrictive ultrasonic flow probe (T206, Transonic Systems, Ithaca, NY) placed around the portal vein, as close as possible to the liver, measured the portal vein blood flow. Blood pressures and flows were registered on a multichannel computer-based recorder (Powerlab 4SP, ADInstruments; Mountain View, CA). Temperature was monitored and maintained at 37 ± 0.5°C during all procedure, and after 20 min of stabilization hemodynamic values were taken. At the end of the in vivo hemodynamic study, blood samples from cava vein were obtained and stored for further determinations. Intrahepatic vascular resistance was calculated as portal pressure/portal vein blood flow.

Nitric oxide bioavailability. NO bioavailability in livers from rats treated with ObR-Ab or vehicle was assessed by analyzing its surrogate markers cyclic guanosine monophosphate (cGMP) and phosphorylated vasodilator-stimulated phosphoprotein (p-VASP). cGMP was determined by enzyme immunoassay (Cayman Chemical) (14). p-VASP was determined by Western blot.

Nitric oxide signaling pathway characterization. Activity of hepatic NO synthase was evaluated through three different approaches. The phosphorylated form of eNOS at Ser1176 (active) was analyzed and referred to total eNOS protein expression by Western blot. NO synthase activity was also analyzed by determining the conversion of ^{14}C-labeled L-arginine to ^{14}C-labeled L-citrulline, according to a previously reported method (29), and expressed as picomoles per minute per gram protein. Additionally, eNOS dimer ("coupled" active form) and monomer ("uncoupled" inactive form) were determined using a nonreducing SDS-PAGE Western blot.

Evaluation of O_2^- and nitrotyrosine. Hepatic O_2^- levels were quantified using a commercially available assay (Sigma, Tres Cantos, Madrid, Spain) with minor modifications. Briefly, livers were homogenized in buffer containing 20 mM HEPES, 1 mM EDTA, 210 mM mannitol, and 70 mM sucrose. After centrifugation at 1,500 *g* for 5 min at 4°C, the supernatant was recuperated and incubated with WST-1 during 20 min at 37°C. O_2^--derived absorbance at 440 nm was proportional to the amount of O_2^- radical. Positive (exogenous O_2^- generating enzyme) and negative (samples with high antioxidant capacity) internal controls were included. Nitrotyrosine content, as secondary marker of the O_2^--mediated NO scavenging to form peroxynitrite, was analyzed in liver sections (10 μm) previously fixed in 10% formalin and embedded in paraffin, by use of a polyclonal rabbit anti-nitrotyrosine antibody (Millipore, Billerica, MA) as previously described (25).

Liver fibrosis evaluation. Paraffin-embedded liver slides were stained with Sirius red to detect fibrous tissue components. The degree of fibrosis was assessed by image analysis techniques with the freeware NIH Image J 1.38 (National Institutes of Health) as previously described (15). The results were expressed as a fibrosis ratio (%), calculated as the ratio of the Sirius red-positive area to the total area examined from 10 independent images for each animal. Additionally, α-smooth muscle actin (α-SMA) protein expression and procollagen I mRNA expression were analyzed by Western blot and quantitative real-time PCR, respectively.

Evaluation of hepatic function and injury. Albumin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured in serum by standard techniques.

Western blot analysis. Protein expression of ObR, STAT3, p-STAT3, eNOS, p-eNOS, p-VASP, and α-SMA was assessed by Western blot in livers from cirrhotic rats treated with ObR-Ab or IgG. Briefly, livers were collected, snap frozen in liquid N_2, and homogenized in Triton-lysis buffer as previously described (13). Aliquots from each sample containing equal amounts of protein (40–100 μg) were run on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. To evaluate the eNOS dimer-to-monomer ratio, low-voltage and low-temperature SDS-PAGE was run with non-heated tissue homogenates. After the transfer, the blots were subsequently blocked for 1 h with Tris-buffered saline containing 0.05% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk and were subsequently incubated with a primary antibody recognizing ObR (Alpha Diagnostic International), STAT3 (Cell Signaling Technology, Beverly, MA), p-STAT3 (Cell Signaling), eNOS (BD Transduction Laboratories, Lexington, KY), p-eNOS (Cell Signaling), p-VASP (Calbiochem, Darmstadt, Germany), or α-SMA (Sigma) overnight at 4°C. Then membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature.

Protein expression was determined by densitometric analysis using the Science Lab Image Gauge (Fuji Photo Film GMBH, Düsseldorf, Germany). After stripping, blots were assayed for GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) expression as standardization of sample loading. Quantitative densitometric values of all proteins were normalized to GAPDH.

Quantitative real-time TaqMan PCR. Hepatic RNA was isolated, and real-time TaqMan PCR was performed as previously described (15).

Statistical analysis. Statistical analysis was performed with the SPSS 18.0 for Windows statistical package (IBM, Armonk, NY). All results are expressed as means ± SE. Comparisons between groups were performed with the Mann-Whitney test for unpaired data. Differences were considered significant at a P value < 0.05.

RESULTS

Levels of leptin and its receptor in cirrhotic animals. Leptin circulating levels were significantly higher in serum from cirrhotic animals compared with controls (Fig. 1*A*). Similarly,

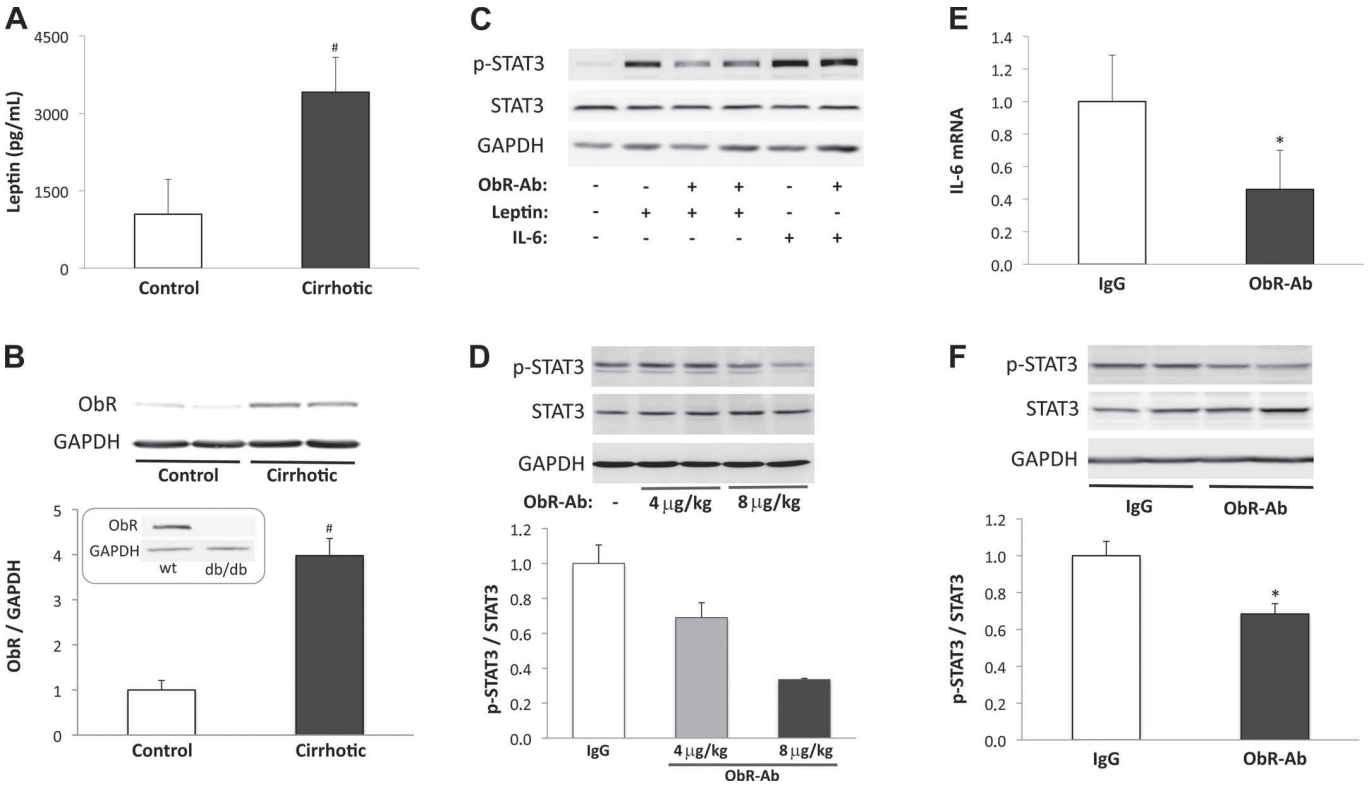

Fig. 1. Characterization of leptin, leptin receptor, and leptin receptor blocking antibody. *A*: levels of leptin in serum from control and cirrhotic animals ($n = 12$ per group). *B*: representative Western blots and densitometric quantification of leptin receptor (ObR), and housekeeping protein GAPDH in livers from control and cirrhotic rats ($n = 6$ per group) #$P < 0.05$ vs. control. *Inset*: representative ObR and GAPDH immunoblots in wild-type (wt) and db/db mice. *C*: Western blots of phosphorylated-STAT3 (active form; p-STAT3), total STAT3, and GAPDH in SH-SY5Y cells incubated with leptin, IL-6, or vehicle, in the presence of ObR-Ab or its vehicle (representative images from 3 independent experiments). *D*: blots of depicted proteins and densitometric quantification determined in livers from control rats pretreated with ObR-Ab and afterward treated with a single dose of leptin ($n = 3$ per group). *E*: hepatic IL-6 mRNA expression in cirrhotic rats receiving ObR-Ab or its vehicle IgG ($n = 8$ per group). *F*: representative Western blots and quantification of depicted proteins in livers from cirrhotic rats treated with ObR-Ab or its vehicle IgG ($n = 12$ per group). Values represent means ± SE. *$P < 0.05$ vs. IgG.

leptin receptor (ObR) expression in livers from cirrhotic animals was significantly higher than in control animals (Fig. 1*B*). As internal negative control, no ObR expression was detected in liver homogenates from db/db mice (Fig. 1*B*).

Effectiveness of ObR-Ab blocking leptin receptor. As previously described (1), leptin produced a significant upregulation in p-STAT3 in SH-SY5Y cells, which was blunted by pretreating cells with ObR-Ab (Fig. 1*C*). Contrarily, STAT3 activation induced by IL-6 was not affected by ObR-Ab (Fig. 1*C*), thus validating its specificity for ObR.

Similarly, after leptin administration to control animals, the hepatic expression of p-STAT3 was significantly lower in rats pretreated with ObR-Ab than in those treated with its vehicle, confirming an adequate leptin receptor blockade by ObR-Ab (Fig. 1*D*). Considering that the greater inhibition of p-STAT3 expression by ObR-Ab was achieved with the 8 μg/kg dose, this concentration was chosen for all subsequent experiments. In cirrhotic rats the ratio p-STAT3/STAT3, as well as the hepatic expression of IL-6, were significantly lower in the group treated with ObR-Ab compared with those treated with IgG, confirming an efficient blockade of the leptin receptor also in cirrhotic animals (Fig. 1, *E* and *F*).

Hemodynamic effects of ObR-Ab administration in cirrhotic rats. Cirrhotic rats receiving ObR-Ab exhibited significantly lower portal pressure than those receiving IgG-vehicle (mean decrease −19%; $P = 0.02$; Fig. 2) with no significant differ-

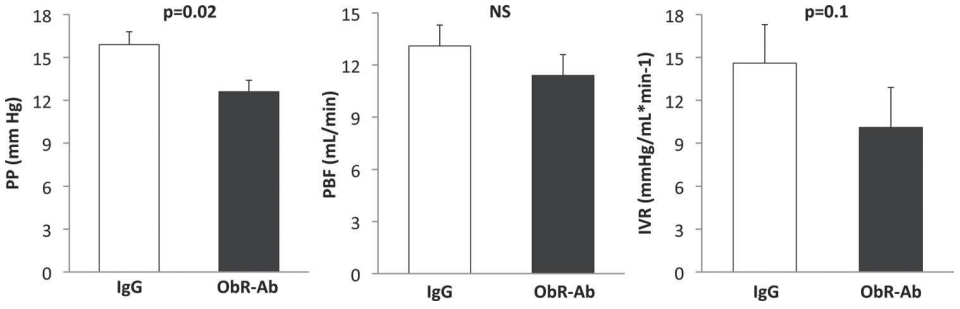

Fig. 2. Effects of leptin receptor blockade on hepatic and splanchnic hemodynamics. Portal pressure (PP), portal blood flow (PBF), and intrahepatic vascular resistance (IVR) measured in CCl_4-cirrhotic rats treated with ObR-Ab (8 μg/kg body wt) or its vehicle IgG every other day during 1 wk ($n = 12$ per group). Values represent means ± SE.

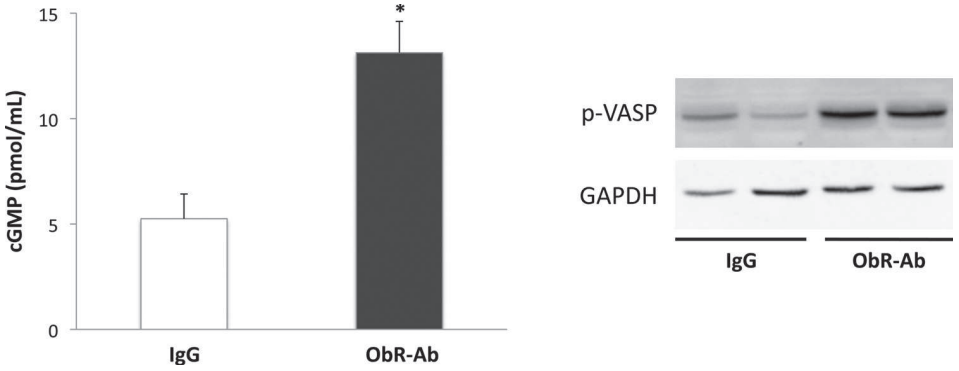

Fig. 3. Effects of leptin receptor blockade on hepatic nitric oxide bioavailability in cirrhotic rats. *Left*: hepatic cGMP levels from cirrhotic rats treated with the leptin receptor antagonist ObR-Ab or its vehicle IgG ($n = 8$ per group). Values represent means ± SE, $*P < 0.05$ vs. IgG. *Right*: representative Western blots of phosphorylated vasodilator-stimulated phosphoprotein (p-VASP) and GAPDH from livers described above.

ences in portal blood flow. As a consequence, portal vascular resistance was lower in cirrhotic animals receiving ObR-Ab than in those treated with IgG; however, this difference did not reach statistical significance ($P = 0.1$; Fig. 2). ObR-Ab administration did not significantly modify mean arterial pressure (102 ± 7 mmHg in IgG vs. 97 ± 9 mmHg in ObR-Ab group).

Effects of ObR-Ab administration on the nitric oxide pathway in the cirrhotic liver. To understand the molecular mechanisms partly responsible for the decrease in portal pressure, we characterized hepatic NO bioavailability, eNOS expression and activity, oxidative stress, and protein nitrotyrosination. Figure 3 shows that cirrhotic animals treated with ObR-Ab exhibited a significant increase in hepatic NO bioavailability, as indicated by the measurement of its secondary messenger cGMP, and by increased expression of p-VASP, a surrogate of NO activity, compared with cirrhotic animals receiving IgG. These observations were not associated with significant changes in eNOS or p-eNOS, in eNOS coupling status, or in eNOS activity (Fig. 4), altogether discarding a possible increase in NO biosynthesis by eNOS due to leptin receptor blockade. Contrarily, oxidative stress quantification evidenced that liver tissue from ObR-Ab-treated animals exhibited a significant reduction in O_2^- levels that was associated with a significant reduction in protein nitrotyrosination, as shown by immunohistochemistry (Fig. 5).

Leptin receptor blockade effects on liver fibrosis. Liver fibrosis levels were characterized by Sirius red staining followed by computational analysis, and by α-SMA and procollagen I quantification. No differences in percentage of fibrotic

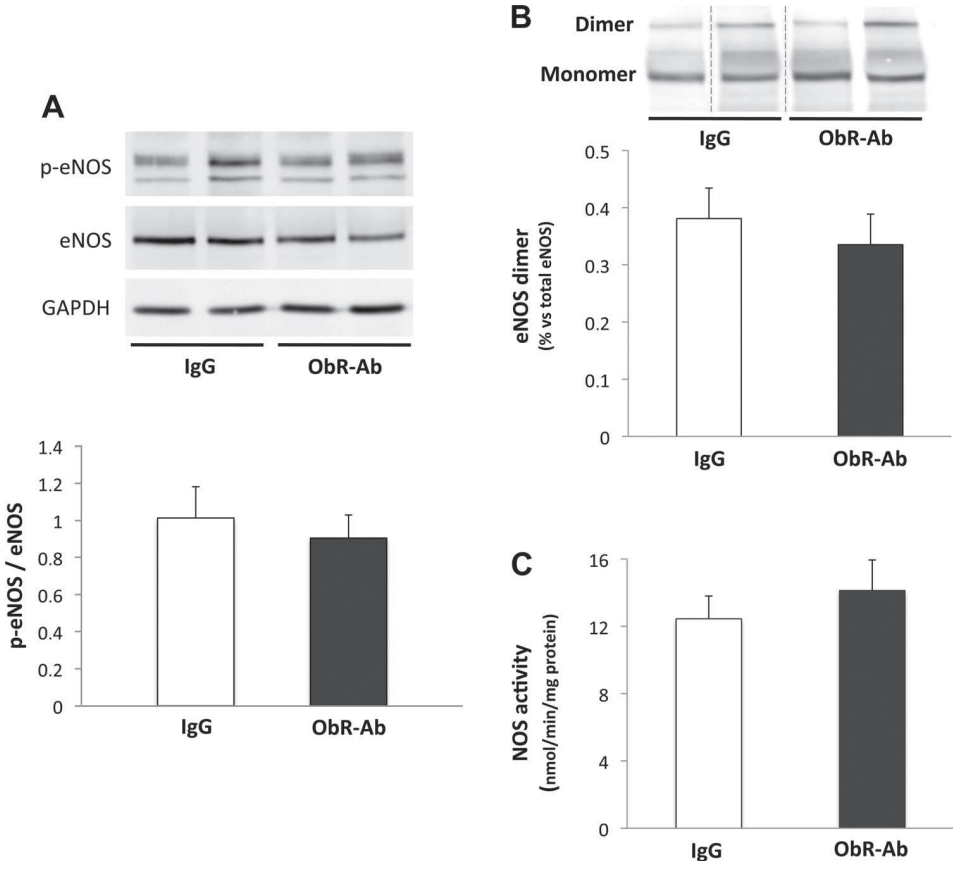

Fig. 4. Effects of leptin receptor blockade on hepatic nitric oxide synthase (NOS) expression and activity. *A*: representative Western blots and densitometric quantification of depicted proteins from cirrhotic rats treated with ObR-Ab or IgG-vehicle ($n = 8$ per group). *B*: representative Western blot and quantification of endothelial nitric oxide synthase (eNOS) dimer (active) and monomer (inactive) forms determined under the nonreducing SDS-PAGE preparation. Dotted lines separate independent samples from the same gel. *C*: nitric oxide synthase activity in livers described above. Values represent means ± SE.

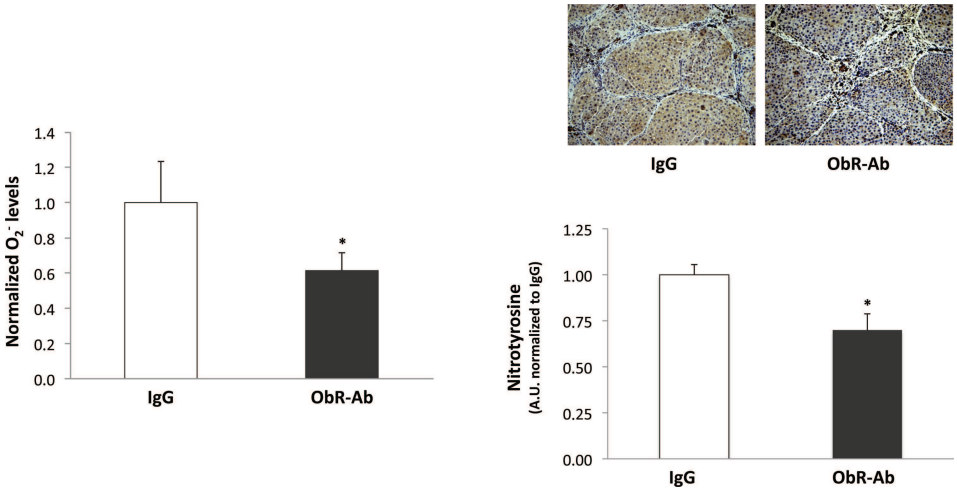

Fig. 5. Leptin receptor blockade effects on hepatic oxidative stress and protein nitrotyrosination. *Left*: superoxide (O_2^-) levels determined in livers from cirrhotic rats treated with ObR-Ab or its vehicle ($n = 8$ per group). *Right*: representative immunohistochemistry images, and semiquantitative analysis, of nitrotyrosinated proteins in livers described above. Values in arbitrary units (A.U.) represent means ± SE, $^*P < 0.05$ vs. IgG.

tissue areas or in α-SMA protein expression were observed when comparing cirrhotic animals that received ObR-Ab or IgG; however, livers from animals receiving ObR-Ab exhibited reduced levels of procollagen I mRNA expression (Fig. 6).

Hepatic function and liver injury in response to ObR-Ab administration. There were no significant differences in the values of AST (157 ± 69 in IgG vs. 180 ± 88 U/l in ObR-Ab group), ALT (73 ± 19 in IgG vs. 80 ± 18 U/l in ObR-Ab group), and albumin (25 ± 4 in IgG vs. 24 ± 3 g/l in ObR-Ab group) between cirrhotic rats receiving IgG and those who received ObR-Ab.

DISCUSSION

Leptin, a hormone encoded by the Ob gene, has six recognized receptor isoforms (Ob-Ra-Ob-Rf) (36), but only the long receptor (Ob-Rb) mediates the biological actions of leptin through the activation of the JAK/STAT pathway (9, 36). Specifically, in the liver, Ikejima et al. (20) showed the presence of Ob-Rb and Ob-Ra receptors. Subsequently, another study confirmed that sinusoidal endothelial cells also express the gene for leptin (33). In patients with cirrhosis, regardless of its etiology, high serum levels of leptin have been described (18, 30, 39); moreover, a role for leptin in hepatic fibrosis has been hypothesized (2, 6, 21).

We herein show that cirrhotic animals exhibit elevated levels of leptin and hepatic expression of its receptor compared with control rats, mirroring what has been described in human cirrhosis. In addition, the present study demonstrates for the first time that the blockade of the receptor of leptin in cirrhotic rats is associated with a significant decrease in portal pressure.

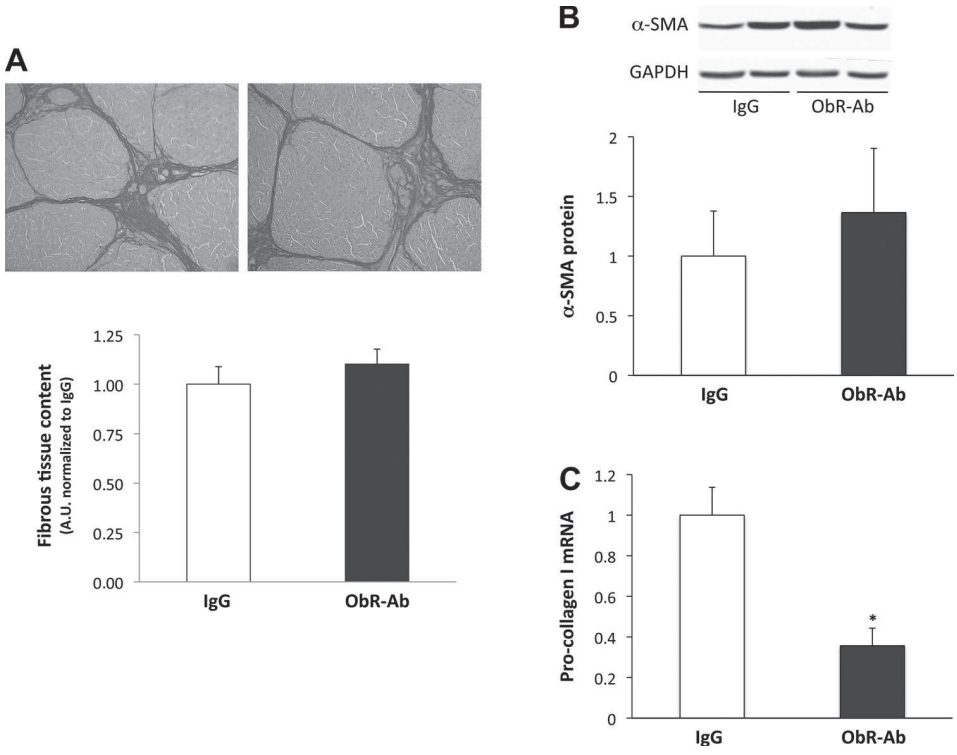

Fig. 6. Effects of ObR-Ab treatment on intrahepatic fibrosis in CCl_4-cirrhotic rats. *A*: representative histological images of livers stained with Sirius red from cirrhotic rats treated with vehicle IgG (left) or ObR-Ab (right, $n = 8$ per group; ×10), and quantification of liver fibrosis (Sirius red staining area per total area) from means of 8 pictures for each slide. *B*: representative α-smooth muscle actin (α-SMA) Western blot and densitometric quantification of livers described in *A*. *C*: procollagen I mRNA expression of livers described above. Values in arbitrary units (A.U.) represent means ± SE, $^*P < 0.05$ vs. IgG.

In fact, portal pressure in ObR-Ab-treated animals was 19% lower than in rats treated with IgG. This reduction in portal pressure was not related to significant changes in portal blood flow, suggesting that it was due to a reduction in intrahepatic vascular resistance.

As discussed below, the mechanism by which leptin-receptor blockade decreases portal pressure appears to be related to a reduced hepatic vascular tone: the so-called dynamic component of the increased resistance to portal flow through the cirrhotic liver. Low hepatic NO bioavailability, due to reduced eNOS activity and increased NO scavenging by an excess of radical oxygen species, has been described as an essential mechanism contributing to increase hepatic vascular tone in cirrhosis (3, 22). The results of our study suggest that increased levels of leptin may contribute to increased portal pressure precisely by influencing these pathophysiological alterations. Indeed, herein we demonstrate that ObR-Ab administration to cirrhotic rats produced a significant increase in hepatic NO bioavailability, as evidenced by the significantly increased levels of hepatic cGMP and VASP phosphorylation. Subsequently, we ascertained the underlying mechanisms of NO increment by analyzing eNOS expression and activity, and O_2^--mediated NO scavenging. Leptin receptor blockade was not associated with changes in eNOS protein expression or in eNOS activity, demonstrated by absence of changes in phosphorylated eNOS and in eNOS monomer/dimer expression. On the other hand, leptin receptor blockade resulted in a significant amelioration of oxidative stress and protein nitrotyrosination, which is a surrogate marker of scavenging of NO by O_2^-, producing peroxynitrite. Altogether these results suggest that the increment in hepatic NO bioavailability observed in rats treated with the leptin receptor-blocking antibody resulted from a reduction in O_2^- levels, and therefore in NO scavenging, with no increase in eNOS activity. Previous studies suggested that leptin-derived endothelial O_2^- mainly originates from NADPH oxidase and uncoupled eNOS (32). Our results showing no improvement in eNOS coupling status after leptin-receptor blockade, together with a previous report demonstrating a lack of role for NADPH oxidase in the pathogenesis of the increased hepatic vascular tone in cirrhosis (12), suggest that leptin may activate another prooxidant mechanism in cirrhotic hepatic sinusoidal cells, most likely by further decreasing hepatic superoxide dismutase activity. Although the present study mainly focused on characterizing the increment in hepatic NO bioavailability to explain the beneficial effects of ObR blockade, we cannot discard that the inhibition of other leptin-dependent pathways would also contribute to ameliorate portal hypertension in cirrhosis. As example, it is conceivable that leptin receptor blockade may downregulate VEGF-mediated angiogenesis (4), which we and others have demonstrated actively contributes to portal hypertension pathophysiology (8). Nevertheless future studies are required to validate this hypothesis. It is worthy to note that ObR-Ab administration had no deleterious effects on mean arterial pressure or in liver blood test.

As previously mentioned, elevated leptin levels in serum from cirrhotic patients have been considered as a mediator of enhanced liver fibrosis (21, 39). In the present study, no differences in hepatic fibrous tissue content or in hepatic stellate cells activation marker α-SMA were observed when comparing rats treated with ObR-Ab or its vehicle, indicating no net effects on liver fibrosis after 1-wk administration. Our results indicate that portal pressure reduction due to ObR-Ab administration is derived from an improvement in NO bioavailability rather than from a reduction in fibrosis. However, our data demonstrating reduced levels of procollagen I mRNA expression in livers from animals receiving ObR-Ab suggest that using higher doses or longer periods of treatment with ObR-Ab may have additional effects reducing liver fibrosis.

In conclusion, our study shows for the first time that leptin might be involved in the pathogenesis of portal hypertension in cirrhosis. Blockade of hepatic leptin receptors results in a decrease in portal pressure through a reduction in intrahepatic vascular resistance without modifying systemic hemodynamics. These data suggest that leptin can be a potential target in the treatment of portal hypertension.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.G.D., J.G.-S., and J.C.G.-P. conception and design of research; M.G.D. and G.M. performed experiments; M.G.D. and G.M. analyzed data; M.G.D., J.G.-S., and J.C.G.-P. interpreted results of experiments; M.G.D., J.G.-S., and J.C.G.-P. drafted manuscript; M.G.D., J.G.-S., G.M., A.R.-V., R.D., J.G.A., J.B., and J.C.G.-P. approved final version of manuscript; J.G.-S. prepared figures; J.G.-S., A.R.-V., R.D., J.G.A., J.B., and J.C.G.-P. edited and revised manuscript.

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Study 2:

THE TRANSCRIPTION FACTOR KLF2 MEDIATES HEPATIC ENDOTHELIAL PROTECTION AND PARACRINE ENDOTHELIAL–STELLATE CELL DE-ACTIVATION INDUCED BY STATINS.

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Research Article

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The transcription factor KLF2 mediates hepatic endothelial protection and paracrine endothelial–stellate cell deactivation induced by statins

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See Focus, pages 1–2

Background & Aims: Statins improve hepatic endothelial function and liver fibrosis in experimental models of cirrhosis, thus they have been proposed as therapeutic options to ameliorate portal hypertension syndrome. The transcription factor Kruppel-like factor 2 (KLF2) may be induced by statins in liver sinusoidal endothelial cells (SEC), orchestrating an efficient vasoprotective response. The present study aimed at characterizing whether KLF2 mediates statins-derived hepatic protection.

Methods: Expression of KLF2 and its vasoprotective target genes was determined in SEC freshly isolated from control or CCl_4-cirrhotic rats treated with four different statins (atorvastatin, mevastatin, simvastatin, and lovastatin), in the presence of mevalonate (or vehicle), under static or controlled shear stress conditions. KLF2-derived vasoprotective transcriptional programs were analyzed in SEC transfected with siRNA for *KLF2* or siRNA-control, and incubated with simvastatin. Paracrine effects of SEC highly-expressing KLF2 on the activation status of rat and human hepatic stellate cells (HSC) were evaluated.

Results: Statins administration to SEC induced significant upregulation of KLF2 expression. KLF2 upregulation was observed after 6 h of treatment and was accompanied by induction of its vasoprotective programs. Simvastatin vasoprotection was inhibited in the presence of mevalonate, and was magnified in cells cultured under physiological shear stress conditions. Statin-dependent induction of vasoprotective genes was not observed when KLF2 expression was muted with siRNA. SEC overexpressing KLF2 induced quiescence of HSC through a KLF2–nitric oxide–guanylate cyclase-mediated paracrine mechanism.

Conclusions: Upregulation of hepatic endothelial KLF2-derived transcriptional programs by statins confers vasoprotection and stellate cells deactivation, reinforcing the therapeutic potential of these drugs for liver diseases that course with endothelial dysfunction.

Keywords: Simvastatin; Liver; Cirrhosis; Portal hypertension; Endothelium; Kruppel-like factor 2.

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Abbreviations: KLF2, Kruppel-like factor 2; eNOS, endothelial nitric oxide synthase; SEC, sinusoidal endothelial cells; HSC, hepatic stellate cells; CCl_4, carbon tetrachloride; VEGF, vascular endothelial growth factor; α-SMA, alpha smooth muscle actin; IVR, intrahepatic vascular resistance; KLF4, Kruppel-like factor 4.

Introduction

Statins (3-hydroxy-3-methyl-glutaryl co-enzyme A reductase inhibitors) were originally designed to lower cholesterol levels, however, some beneficial effects of statin therapy are independent of lipid lowering and occur with no significant reduction in serum cholesterol [1]. Among the lipid-independent beneficial effects of statins, hepatic endothelial protection in cirrhosis represents a highly promising therapeutic approach to treat patients with portal hypertension. Indeed, studies from our group and others have demonstrated that statin administration to cirrhotic rats improves hepatic endothelial dysfunction and portal hypertension [2,3]. Moreover, a recent double-blind study has demonstrated that simvastatin administration decreases portal pressure and improves liver function in cirrhotic patients with portal hypertension [4]. Although these studies have reported positive results, the underlying mechanisms explaining such hepatic vascular protection are not completely understood.

Kruppel-like factor 2 (KLF2) is a transcriptional factor expressed in endothelium, lung, and lymphocytes [5]. KLF2 confers vasoprotection inducing the expression of vasodilator, antithrombotic, and anti-inflammatory genes, including endothelial nitric oxide synthase (eNOS) and thrombomodulin [6,7], and repressing the expression of adhesion molecules, such as vascular cell

adhesion molecule 1 and E-selectin [6,8]. Furthermore, KLF2 expression in endothelial cells inhibits oxidative stress-mediated cell injury and apoptosis [9,10]. Altogether, KLF2 is considered as a nuclear factor essential to maintain a functional endothelial phenotype. Experimental studies using macrovascular endothelial cells have demonstrated that KLF2 expression is induced by physiological blood flow-derived shear stress [11] and by exogenous administration of small molecules, including statins and resveratrol [12–14].

Considering that KLF2-derived vasoprotective pathways may contribute to the beneficial effects of statins in cirrhosis, we aimed at characterizing statin-derived vasoprotective pathways in the highly specialized sinusoidal endothelium from control and cirrhotic animals. Moreover, since recent data have demonstrated that sinusoidal endothelial cells (SEC) and hepatic stellate cells (HSC) regulate their phenotypes in a paracrine manner [15,16], and that simvastatin may reduce liver fibrosis [17], our second aim was to unravel the possible role of KLF2-derived endothelial protection in HSC deactivation due to statins treatment.

Materials and methods

Induction of cirrhosis by carbon tetrachloride (CCl_4)

Male Wistar rats weighing 50–75 g underwent inhalation exposure to CCl_4. Phenobarbital (0.3 g/L) was added to the drinking water as previously described [18]. When the cirrhotic rats developed ascites, administration of phenobarbital was stopped, and subsequent experiments were performed 1 week later. Control animals received phenobarbital only. Animals were kept in environmentally controlled animal facilities at the August Pi i Sunyer Institute for Biomedical Research. All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

Isolation and culture of sinusoidal endothelial cells

Rat liver sinusoidal endothelial cells were isolated as previously described [19]. Briefly, after perfusion of the livers with 0.015% collagenase A and isopycnic sedimentation of the resulting dispersed cells through a two-step density gradient of Percoll (25–50%), monolayer cultures of SEC were established by selective attachment on a collagen I substrate. Cells were cultured (37 °C, 5% CO_2) in Roswell Park Memorial Institute (RPMI) 1640 as previously described [19]. Experiments were performed after 12 h of cell isolation; highly pure (as determined by uptake of Ac-LDL) and viable (by trypan blue exclusion) cells were used [20]. In all experiments, except in those of co-culture, SEC capillarization was prevented by adding VEGF to the culture media [21,22].

Statin treatment

Cells were washed twice with phosphate buffered saline (PBS) and incubated for 24 h with atorvastatin, mevastatin, simvastatin or lovastatin (Calbiochem), at concentrations of 0.1 μM, 1 μM or 10 μM, or with vehicle (DMSO). In addition, the time-course response to simvastatin was assessed using SEC cultured in fresh media with 1 μM simvastatin or with vehicle for 6 h, 12 h or 24 h.

siRNA experiments

siRNA transfection was performed as previously described with minor modifications [23]. Briefly, SEC were transfected with a siRNA targeting rat KLF2 (5 nM, s157429, Life Technologies, Carlsbad, CA), or with a control siRNA (5 nM, 4390843, Life Technologies) using siPORT transfection agent (Life Technologies) according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were treated for an additional 24 h with either 1 μM simvastatin or its vehicle.

Isoprenoids experiments

SEC were treated with either vehicle (ethanol), 10 μM or 100 μM mevalonate (Sigma) for 1 h, afterwards 1 μM simvastatin or vehicle was added and cells were incubated for an additional 24 h. In additional experiments, SEC were incubated with either vehicle (methanol) or 10 μM geranylgeranyl pyrophosphate (GGPP; Sigma) together with 1 μM simvastatin or its vehicle for 8 h.

Shear stress experiments

SEC were plated on collagen-coated plastic flow chambers (IBIDI, Munich, Germany) and maintained at 37 °C, 5% CO_2 for 12 h of static culture. Afterwards, the SEC growth medium was exchanged for shear medium (RPMI medium, 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin plus 100 μg/ml streptomycin, and 2% dextran (Sigma)) supplemented with either 1 μM simvastatin or its vehicle. Cells were exposed for 24 h to static conditions or to previously characterized physiological laminar flow [24], using a computer-controlled cell culture flow system that maintains cells at 37 °C in a 5% CO_2 environment previously described and used in our laboratory [21].

Hepatic endothelial and stellate cells co-culture

SEC were plated in collagen-coated wells of 12-well plates and cultured for 24 h alone or in the presence of simvastatin (1 μM), simvastatin plus mevalonate (100 μM), simvastatin plus NG-nitro-L-arginine methyl ester (L-NAME; 3 mM; Sigma) or simvastatin plus ODQ (10 μM; Cayman Chemical Co., Ann Harbor, MI). Freshly isolated rat hepatic stellate cells (HSC) [25] and activated human HSC LX-2 (kindly provided by Dr. Bataller) were plated in transwell inserts with 0.4 μm pore size (Corning, Life Sciences) at a density of 130,000/cm^2. After 24 h of SEC culture, endothelial cells were washed and HSC-containing transwells were added to culture plates. Cells were cultured for additional 24 h in fresh media.

In an additional group of experiments, SEC were infected with the adenovirus codifying for mouse-KLF2–GFP or the adenovirus control-GFP for 2 h at 10 MOI, followed by 22-h incubation in media [8]. Afterwards, co-culture experiments were performed as described above.

RNA isolation and real-time TaqMan PCR

Cells were lysed, RNA isolated, and real-time TaqMan PCR performed as previously described [21].

Western blot and immunostaining

Cells were lysed, protein extracted and immunoblotting performed as previously described [26]. Primary antibodies for KLF2 (N-13, Santa Cruz Biotechnology), eNOS (BD Transduction Laboratories, Lexington, KY) and α-SMA (Sigma) were used.

α-SMA immunostaining was performed in human HSC as follows. Cells cultured on glass coverslips were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100. Cells were then washed with PBS, blocked with 1% BSA and incubated for 2 h with a mouse monoclonal anti-α-SMA (1:300; Sigma). After washing with PBS, cells were incubated with an Alexa 555-conjugated secondary antibody (1:300, Life Technologies). Negative controls stained with a primary antibody for GFP (1:300; Abcam, Cambridge, UK) were included. Slides were examined using a fluorescence microscope (Olympus BX51, Tokyo, Japan) equipped with a digital camera (Olympus, DP72).

Statistical analysis

Statistical analysis was performed with the SPSS 18.0 for Windows statistical package (IBM Corp., Armonk, NY). All results are expressed as mean ± standard error of the mean. Comparisons between groups were performed with the Student-*t* test or ANOVA followed by a *post hoc* test when adequate. Differences were considered significant at a p value <0.05.

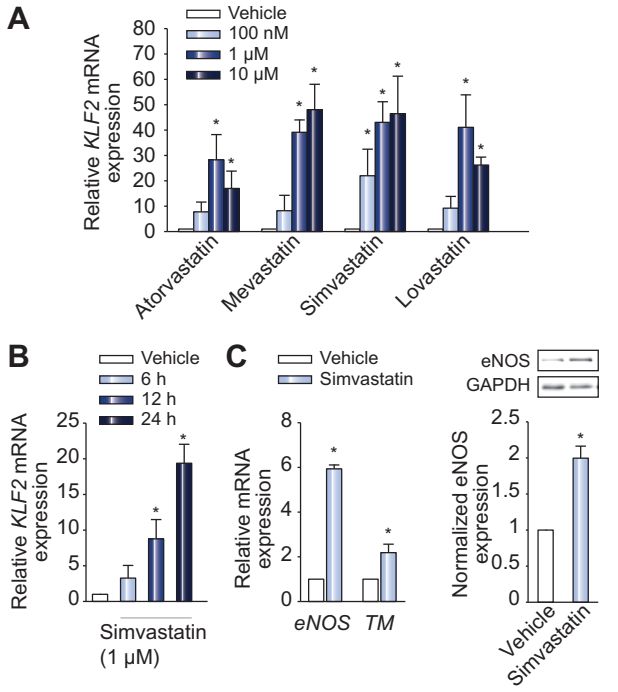

Fig. 1. Statins upregulate KLF2-derived vasoprotective programs in sinusoidal endothelial cells. (A) Concentration-dependent regulation of KLF2 expression in response to four different statins. (B) Time-dependent regulation of KLF2 expression in response to simvastatin (1 μM). (C) (Left panel) KLF2-transcriptional targets expression in response to 24 h of 1 μM simvastatin, eNOS (endothelial nitric oxide synthase), TM (thrombomodulin). (Right panel) Representative Western blot and densitometric quantification of eNOS and GAPDH protein expression determined in sinusoidal endothelial cells after 24 h-simvastatin or vehicle. n = 5 per condition, $^{*}p$ <0.05 *vs.* its corresponding vehicle.

Results

Statins induce hepatic endothelial KLF2 expression

Primary rat liver sinusoidal endothelial cells treated with increasing concentrations of four different statins exhibited an induction in the expression of KLF2 (Fig. 1A), being simvastatin the most effective one, followed by mevastatin. Since 1 μM simvastatin resulted in an upregulation of KLF2 similar to the higher dose, this concentration was used in all subsequent experiments. Time-course experiments demonstrated that simvastatin induction of KLF2 followed a time-dependent pattern (Fig. 1B). Indeed, KLF2 upregulation was detected after 6 h of simvastatin administration and reached a maximum increase after 24 h.

Hepatic endothelial expression of vasoprotective genes induced by simvastatin depends on KLF2 expression

Freshly isolated sinusoidal endothelial cells incubated for 24 h with 1 μM simvastatin exhibited significantly upregulated expression of its vasoprotective targets eNOS and thrombomodulin (Fig. 1C). These effects were abolished when KLF2 was muted using specific siRNA (Fig. 2A and B).

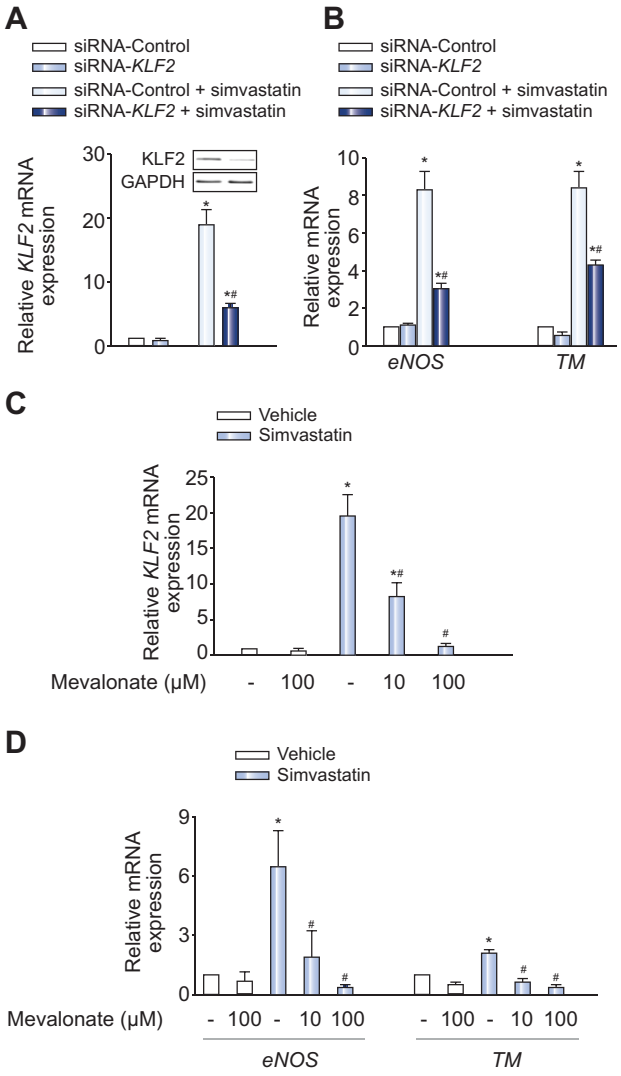

Fig. 2. KLF2 mediates statin-derived vasoprotection in sinusoidal endothelial cells. Effects of *KLF2* knockdown using siRNA on (A) *KLF2* and (B) its vasoprotective target genes in response to simvastatin. The insert shows a representative Western blot demonstrating *KLF2* silencing efficiency. (C) Endothelial expression of KLF2 and (D) its transcriptional targets in response to simvastatin (24 h, 1 μM) in the presence of the isoprenoid intermediate mevalonate or its vehicle. n = 5 per condition, $^{*}p$ <0.05 *vs.* its corresponding control, $^{\#}p$ <0.05 *vs.* control-simvastatin condition.

Simvastatin-derived hepatic vasoprotective phenotype is dependent on isoprenoids synthesis

Induction of KLF2 and its target genes expression was not observed when endothelial cells were treated with simvastatin and co-incubated with the immediate metabolite of the enzyme HMG-CoA reductase, mevalonate. As shown in Fig. 2C and D, 100 μM mevalonate completely inhibited the induction of KLF2, eNOS and thrombomodulin derived from simvastatin. Complementary experiments evidenced that simvastatin-derived KLF2 induction was totally prevented by GGPP (Supplementary Fig. 1).

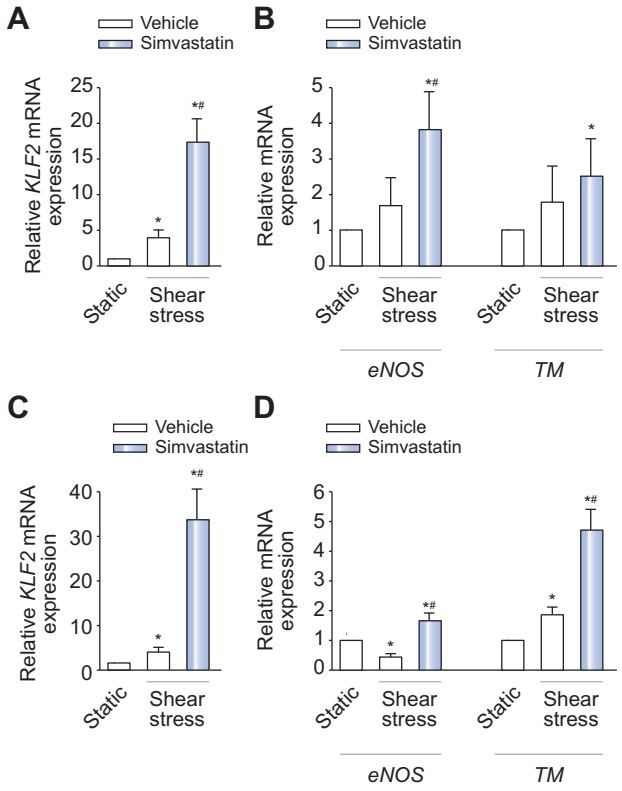

Fig. 3. Shear stress-derived vasoprotection on the hepatic endothelium is enhanced in combination with simvastatin. (A) *KLF2* and (B) its target genes expression in control sinusoidal endothelial cells exposed to shear stress stimuli (or static) with simvastatin or its vehicle. (C) and (D) Response of sinusoidal endothelial cells from cirrhotic rats to the experimental conditions described above. n = 5 per group, $^{*}p$ <0.05 *vs.* static-vehicle $^{\#}p$ <0.05 *vs.* shear stress-vehicle.

Simvastatin enhances the KLF2-derived vasoprotective phenotype induction by shear stress

Liver sinusoidal endothelial cells freshly isolated from control and cirrhotic rats exposed for 24 h to physiological shear stress stimuli exhibited a significant upregulation of KLF2 expression and that of its targets genes eNOS and thrombomodulin (Fig. 3). Addition of simvastatin to these cells further increased the vasoprotective effects induced by the shear stress. Interestingly, under shear stress conditions, cirrhotic SEC strongly responded to simvastatin and exhibited upregulated levels of KLF2 and its target genes expression in comparison to its shear stress-vehicle condition.

Simvastatin-treated endothelial cells paracrinally improve hepatic stellate cells phenotype through a KLF2-dependent mechanism

Activated human hepatic stellate cells co-cultured with SEC previously treated with simvastatin exhibited a significant improvement in their phenotype, expressed as reduction in the activation markers pro-collagen I (26 ± 6% of reduction; $p = 0.02$), and α-SMA in comparison to those cells co-cultured with vehicle-SEC (Fig. 4A and Supplementary Fig. 2). This beneficial effect was abolished when endothelial KLF2 expression was blunted using mevalonate, when nitric oxide production was inhibited by

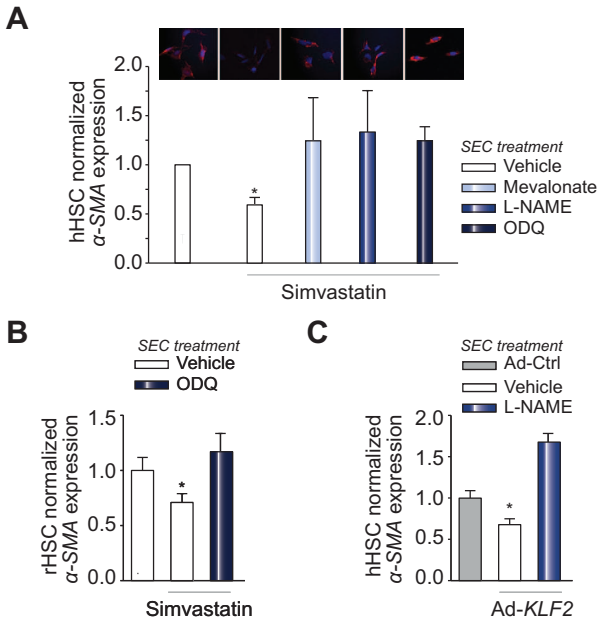

Fig. 4. Hepatic endothelial upregulation of KLF2-derived vasoprotective pathways deactivates hepatic stellate cells. (A) Top: Representative images of α-SMA (alpha smooth muscle actin; in red) protein staining in human LX-2 hepatic stellate cells (hHSC) co-cultured with primary sinusoidal endothelial cells (SEC) pre-treated with simvastatin or its vehicle, in the presence of mevalonate, L-NAME, ODQ or vehicle (DAPI nuclear counterstaining in blue; 40×). Bottom *α-SMA* mRNA expression in the cells described above. (B) α-SMA expression in freshly isolated rat HSC (rHSC) co-cultured with SEC pre-treated with simvastatin or its vehicle, in the presence of ODQ or vehicle. (C) α-SMA expression in hHSC co-cultured with SEC pre-infected with an adenovirus codifying for KLF2 (Ad-*KLF2*) or control adenovirus (Ad-Ctrl), in the presence of L-NAME or its vehicle. n = 5 per condition, $^{*}p$ <0.05 *vs.* other conditions.

L-NAME or when soluble guanylate cyclase was inhibited by ODQ (Fig. 4A). Similarly, freshly isolated rat hepatic stellate cells co-cultured with SEC pre-treated with simvastatin exhibited significantly lower expression of α-SMA in comparison to cells cultured with vehicle-SEC. ODQ co-incubation with simvastatin prevented the endothelial-mediated improvement in stellate cells phenotype (Fig. 4B).

SEC infected with Ad-KLF2 exhibited a markedly increased expression of KLF2 and its vasoprotective target genes in comparison to Ad-GFP cells (Supplementary Fig. 3). Hepatic stellate cells co-cultured with endothelial cells overexpressing KLF2 displayed a significant activation reversal, which was not observed in the presence of L-NAME (Fig. 4C).

Discussion

Portal hypertension and its derived complications represent the main cause of liver failure and transplantation in patients with cirrhosis [27]. Pathophysiologically, this syndrome may be improved by two strategies, amelioration of intrahepatic vascular resistance (IVR) or reduction of portal blood flow. Current treatments for portal hypertension focus mainly on decreasing portal blood flow using non-selective beta-blockers [28,29]. Nevertheless, great efforts to develop a safe pharmacological treatment that may improve intrahepatic microcirculation are being invested. Statins, primarily designed to reduce cholesterol levels,

confer hepatic endothelial protection and reduce IVR in experimental models of liver cirrhosis. Indeed, cirrhotic animals treated with statins exhibit improvements in endothelial dysfunction, portal pressure and liver fibrosis [2,3,17]. The present study aimed at further understanding of the underlying molecular mechanisms of the statins-derived hepatic circulation protection.

Previous studies from us and others demonstrated that the induction of the transcription factor KLF2 in endothelial cells conferred a highly vasoprotective phenotype [5,8,10,13,14]. Indeed, KLF2 expression can be stimulated by blood flow-derived shear stress and some chemical compounds [5]. Considering these data, we herein characterized the response of freshly isolated SEC to the first developed statin (mevastatin) and to three different FDA-approved statins (atorvastatin, simvastatin and lovastatin). These experiments showed that simvastatin was the most effective in inducing KLF2 expression in SEC, thus reinforcing the use of this particular formulation to specifically protect the hepatic endothelium. Subsequent experiments showed that the hepatic endothelium rapidly responded to simvastatin stimulation, upregulating KLF2 expression just after 6 h of simvastatin administration, and reaching a maximum peak at 24 h of treatment. It is worth noting that KLF2 induction after 24 h of simvastatin treatment was accompanied by a marked increase in its vasoprotective targets eNOS and thrombomodulin, thus confirming that statins confer vasoprotection to a highly specialized endothelium such as the liver sinusoidal endothelium. Our results are in agreement with previous reports demonstrating a significant upregulation of eNOS protein expression and NO levels in livers from statins-treated animals [2,3].

The exact role of KLF2 in statins-derived vasoprotection in SEC was defined using two experimental approaches. Firstly, we showed that reactivation of the isoprenoid pathway, administrating the metabolic intermediates mevalonate and GGPP, inhibited the simvastatin-derived induction of KLF2 and its target genes. These results are in agreement with previous data obtained in human umbilical vein endothelial cells [12], and suggest that simvastatin-derived KLF2 induction in SEC mainly depends on depleting cells of GGPP-mediated molecular pathways. In addition, we unequivocally confirmed KLF2 involvement in statins vasoprotection by the specific knockdown of KLF2 expression using siRNA. This second approach further demonstrated that simvastatin-derived hepatic endothelial vasoprotection depends on KLF2 expression. Although a major role for KLF2 is herein demonstrated, we cannot rule out that other mechanisms may also contribute to statin-derived hepatic endothelial protection, such as AKT-dependent activation of eNOS and/or KLF4-derived transcriptional protection [30,31].

We have recently demonstrated a differential expression of KLF2 in SEC from control and cirrhotic animals [21]. Indeed, SEC from cirrhotic rats exhibit higher levels of KLF2 expression than control SEC. Considering these data, we herein evaluated the effects of administrating simvastatin to control and cirrhotic SEC under shear stress stimuli, mimicking the real hemodynamic conditions by which statins may influence the liver endothelial phenotype *in vivo*. These experiments showed that simvastatin markedly magnified the upregulation of KLF2-derived vasoprotection in response to shear stress, especially in SEC from cirrhotic animals, reinforcing the concept that simvastatin administration to cirrhotic individuals exerts significant liver endothelial protection. Interestingly, in the absence of simvastatin, cirrhotic SEC did not respond to shear stress in terms of eNOS expression regulation. These findings are in line with previous data suggesting that hepatic eNOS transcriptional pathway is impaired in cirrhosis, and document that statins can restore some flow-dependent signaling affected in endothelial dysfunction [21].

Together with endothelial dysfunction, another important factor contributing to increase IVR in cirrhosis is hepatic stellate cell activation. Indeed, activated HSC exhibit a pro-fibrogenic and vasoconstrictor phenotype that further aggravates the increased intrahepatic vascular tone, contributing to worsen the portal hypertension [32]. Although a study from Moreno *et al.* failed to show an effect of atorvastatin on HSC activation [33], recent studies suggest that statin administration to cirrhotic animals reduces HSC activation, and liver fibrosis [17]. Here we demonstrate that SEC vasoprotective phenotype improvement due to simvastatin-dependent KLF2 upregulation is necessary to paracrinally ameliorate HSC phenotype. In fact, co-culture experiments have shown that induction of endothelial KLF2 programs by simvastatin results in HSC de-activation, which is mediated at least in part via a KLF2–NO–cGMP dependent mechanism. Indeed, the improvement in HSC phenotype was not observed when SEC were co-treated with simvastatin and mevalonate, the non-selective NO synthase inhibitor L-NAME, or the soluble guanylate cyclase inhibitor ODQ. These observations, further confirmed in SEC overexpressing KLF2 by adenoviral gene transfer, are in agreement with a recent report demonstrating that endothelial guanylate cyclase activation leads to HSC deactivation [16].

The mechanisms by which the downregulation of SEC KLF2-derived pathways abolishes the beneficial paracrine effect on HSC phenotype are unknown. Nevertheless, a recent study from Xie and colleagues has demonstrated that SEC treated with L-NAME or ODQ become capillarized, and lose their paracrine signaling to HSC [16]. These data suggest that capillarization of simvastatin-treated SEC due to mevalonate, L-NAME or ODQ co-treatment is responsible for losing their paracrine effect on HSC.

In conclusion, our study suggests that KLF2-dependent endothelial vasoprotection represents an underlying molecular mechanism for the beneficial effects of statins on liver microcirculation and liver fibrosis in cirrhosis, further supporting the use of this drug for the treatment of patients with chronic liver diseases, where it could promote a reduction in portal pressure and liver fibrosis.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.08.026.

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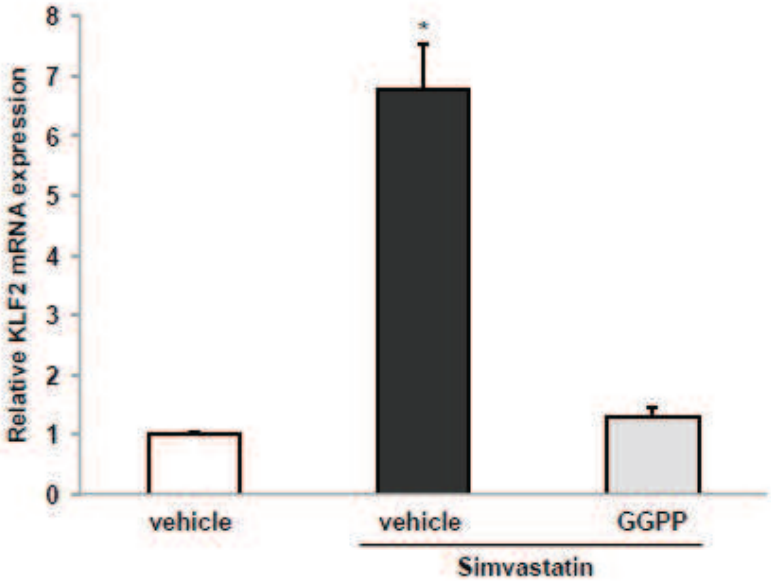
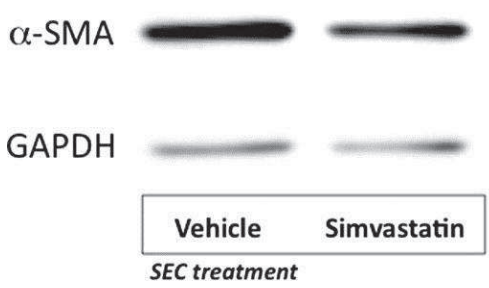
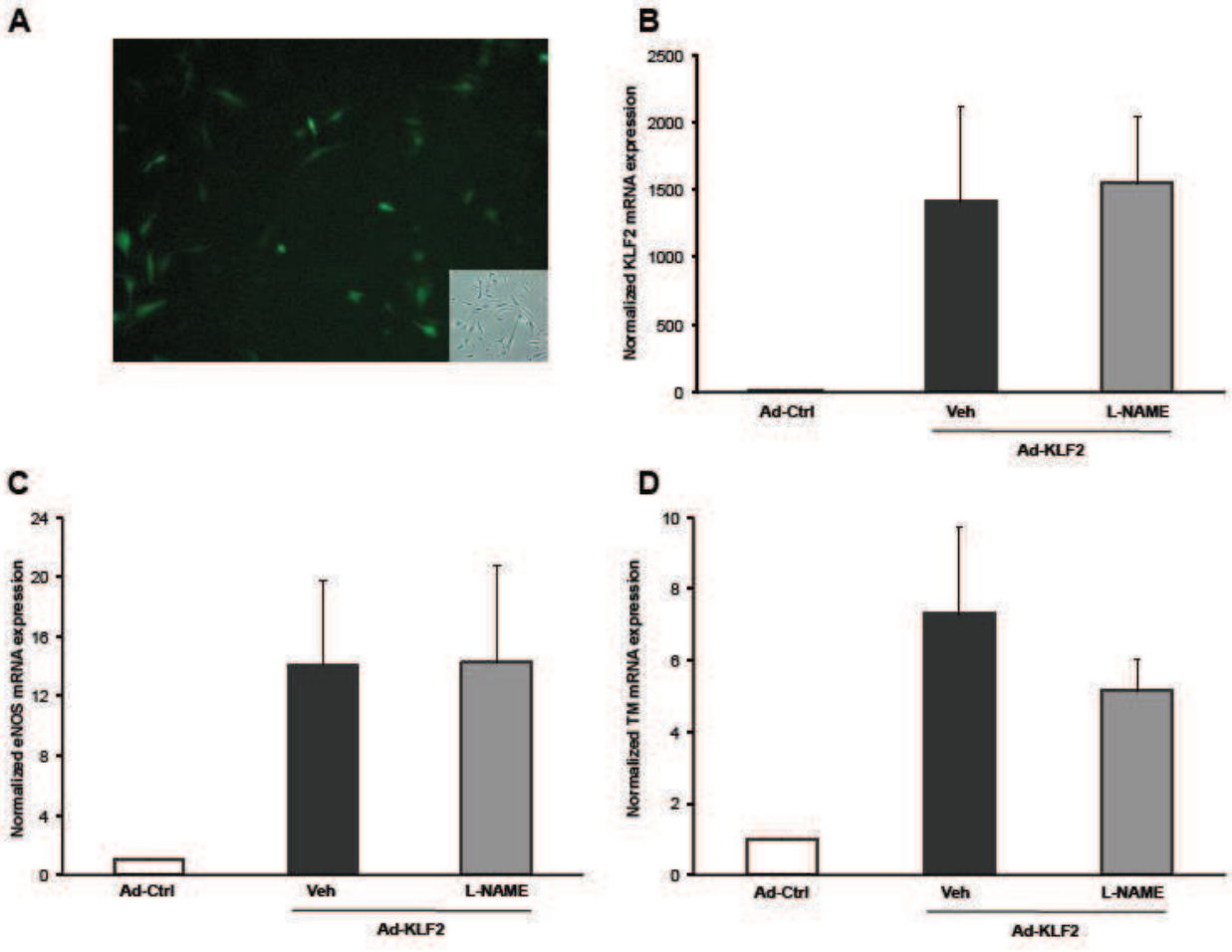
Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3



Study 3:

KLF2 EXERTS ANTI-FIBROTIC AND VASOPROTECTIVE EFFECTS IN CIRRHOTIC RAT LIVERS: BEHIND THE MOLECULAR MECHANISMS OF STATINS.

Giusi Marrone, Raquel Maeso-Díaz, Guillermo García-Cardena, Juan G Abraldes, Juan Carlos García-Pagán, Jaime Bosch* & Jordi Gracia-Sancho*

Submitted

KLF2 exerts anti-fibrotic and vasoprotective effects in cirrhotic rat livers: behind the molecular mechanisms of statins

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Keywords: cirrhosis; simvastatin; sinusoid; HSC.

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Abbreviations: α-SMA: alpha-smooth muscle actin; CBDL: common bile duct ligation; CCl_4: carbon tetrachloride; eNOS: endothelial nitric oxide synthase; GFP: green fluorescence protein; HO-1: heme oxygenase-1; HSC: hepatic stellate cells; IHVR: intrahepatic vascular resistance; KLF: kruppel-like factor; LSEC: liver sinusoidal endothelial cells; MAP: mean arterial pressure; NF-kB: nuclear factor kappa B; NQO1: NAD(P)H quinone oxidoreductase 1; Nrf2: NF-E2 related factor 2; O_2^-: Superoxide; PBF: portal blood flow; PP: portal pressure; SMABF: superior mesenteric artery blood flow; TGF-β: tissue growth factor-beta; VEGF: vascular endothelial growth factor.

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Authors Contributions: G.M. designed the research, conceived ideas, performed experiments, analyzed data and wrote the manuscript. R.M.-D. performed experiments and analyzed data. G.G.-C. & J.G.-A. provided essential materials. J.C.G.-P. critically revised the manuscript. J.B. conceived ideas, critically revised the manuscript, obtained funding and co-directed the study. J.G.-S. designed the research, conceived ideas, wrote the manuscript, obtained funding and co-directed the study. All authors edited and reviewed the final manuscript.

Abstract

In the liver, the transcription factor Kruppel-like factor 2 (KLF2) is early induced during the progression of cirrhosis and portal hypertension to lessen the development of vascular dysfunction. We recently demonstrated that KLF2 mediates statins beneficial effects on the hepatic endothelium, paracrinally improving hepatic stellate cells (HSC) phenotype. This led us to explore the effects and the underlying mechanisms of KLF2 up-regulation in *in vitro* and *in vivo* models of liver cirrhosis. Activation phenotype was evaluated in human and rat cirrhotic HSC treated with the pharmacological inductor of KLF2 simvastatin, with adenovirus codifying for this transcription factor (Ad-KLF2), or vehicle, in presence/absence of inhibitors of KLF2. Effects of hepatic KLF2 over-expression on liver fibrosis and systemic and hepatic hemodynamics were assessed in cirrhotic rats. KLF2 up-regulation profoundly ameliorated HSC phenotype (reduced α-SMA, pro-collagen I and oxidative stress) partly via the activation of the nuclear factor Nrf2. Co-culture experiments showed that improvement in HSC phenotype paracrinally ameliorated liver sinusoidal endothelial cells probably through a VEGF-mediated mechanism. Cirrhotic rats treated with simvastatin or Ad-KLF2 showed hepatic up-regulation in the KLF2-Nrf2 pathway, marked de-activation of HSC and prominent reduction in liver fibrosis. Hepatic KLF2 over-expression was associated with lower portal pressure (-15%) due to both attenuations in the increased portal blood flow and hepatic vascular resistance, together with a significant improvement in hepatic endothelial dysfunction. ***Conclusion:*** Hepatic KLF2 up-regulation improves liver fibrosis, endothelial dysfunction and portal hypertension in cirrhosis.

Maintaining a normal liver function requires a perfect sinusoidal environment in which hepatic cells, regularly communicating each other, control liver metabolism, homeostasis, and intrahepatic vascular tone. Modifications in liver circulation and de-regulations in the phenotype of hepatic cells lead to parenchymal and non-parenchymal dysfunction, characteristic of chronic liver diseases.

In cirrhosis, increased intrahepatic resistance to portal blood flow is in part due to structural abnormalities and in part to alterations in the liver sinusoidal milieu, primarily contributing to portal hypertension development(1). Among the non-parenchymal cells alterations, liver sinusoidal endothelial cells (LSEC) become dysfunctional and lose their unique characteristics to acquire a vasoconstrictor, pro-thrombotic and pro-inflammatory phenotype(2) while hepatic stellate cells (HSC), which modulate liver contractility and extracellular matrix deposition, trans-differentiate and proliferate, becoming activated and phenotypically different from the normal quiescent HSC(3, 4). Therefore, the efficient modulation of the injured hepatic microvascular phenotype, especially the activated HSC and the dysfunctional LSEC, may lead to a significant amelioration of liver cirrhosis. Along with this line, most of the reported anti-fibrotic treatments aimed at inhibiting HSC trans-differentiation and proliferation or at regulating HSC apoptosis(5-7). On the other hand, therapeutic approaches to improve LSEC phenotype and liver circulation in cirrhosis focused on the benefit of statins(8, 9) and anti-oxidant therapies(10).

Kruppel-like transcription factors (KLFs) are zinc finger proteins that act as activators or repressors of the expression of genes involved in cell proliferation and differentiation(11). We recently identified KLF2 as a key component of the hepatic endothelium, where it may act as a defense mechanism in response to damage that occurs during the progression of the disease(12). Simvastatin, through the activation of the endothelial KLF2-nitric oxide pathway, is the most effective statin protecting the hepatic endothelium in cirrhosis, which ultimately leads to HSC amelioration(13). Previous reports suggested that statins may improve HSC phenotype(6, 14), but the possible role of KLF2 mediating the beneficial effects of statins on HSC function and liver fibrosis has not been investigated.

Considering this background, we aimed at studying the effects, and the underlying molecular mechanisms, of hepatic KLF2 induction using pharmacological and adenoviral approaches in *in vitro* and *in vivo* models of liver cirrhosis.

Experimental procedures

Complete description of materials and methods can be found as supplementary material.

Animal models of liver cirrhosis – KLF2 over-expression

Induction of cirrhosis by carbon tetrachloride (CCl_4)

Male Wistar rats (50–75g) underwent inhalation exposure to CCl_4 three times a week and received phenobarbital (0.3g/L) in the drinking water as described(12). After 10 weeks of liver injury, toxicants administration was stopped and experiments were performed 1 week later. Hepatic KLF2 over-expression was achieved administrating a unique dose of 10^{11} adenoviral particles codifying murine KLF2 (Ad-KLF2)(15) or control (Ad-CTRL) through the penile vein. 3 days after intravenous injection of the adenoviral constructs, cells isolation, hemodynamic studies and molecular analysis were performed. Adenoviruses were prepared by a third person and kept under a code, therefore, the investigators performing the experiments were not aware of the treatment received by the rats. Animals were kept in environmentally controlled animal facilities at the University of Barcelona Animal Facilities. All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

Induction of cirrhosis by common bile duct ligation (CBDL)

Secondary biliary cirrhosis with intrahepatic portal hypertension was induced in male Sprague–Dawley rats (250–275g) by common bile duct ligation (CBDL). After four weeks of CBDL, animals received either simvastatin (25mg/kg/day p.o.) or its vehicle (n=6 per group), for 3 days. Liver histology and molecular analysis were performed as described below.

Hepatic stellate cells isolation, culture and treatments

Isolation and culture of hepatic stellate cells

Hepatic stellate cells (*rHSC*) were isolated from cirrhotic rats(13). Results using rHSC derived from at least 3 independent isolations and 3 replicates.

Immortalized human activated hepatic stellate cells LX-2, kindly provided by Dr Bataller, were cultured as described(13, 16). Results derived from at least 3 replicates per group.

Simvastatin treatment

LX-2 cells were treated with simvastatin (Calbiochem) or its vehicle, 0.05% DMSO, at different concentrations (0.1μM, 1μM and 10μM) for 24h and 72h. In addition, time-course experiments (8h, 24h, 72h) were performed in primary cultured rHSC incubated with 10μM simvastatin.

KLF2 inhibition

HSC-KLF2 knock-down was performed using siRNA technology and pharmacological approaches as described(13, 17). See supplementary material for more information.

KLF2 up-regulation using adenoviral vectors

In vitro: LX-2 were infected with Ad-KLF2 or Ad-CTRL at 10 MOI for 2h, rinsed with PBS and then incubated for additional 22h or 70h. Infection was estimated visually, evaluating adenoviral-encoded GFP expression by fluorescence microscopy, resulting in ≈90% positive cells.

In vivo: KLF2-over-expressing HSC were isolated from cirrhotic rats previously infected with Ad-KLF2 or Ad-CTRL.

Intracellular superoxide (O_2^-) content

O_2^- levels in HSC were assessed with the oxidative fluorescent dye dihydroethidium as described(18).

HSC apoptosis and necrosis

HSC were stained with acridine orange and propidium iodide to detect apoptosis and necrosis, respectively, as described in supplementary methods.

Liver Sinusoidal Endothelial Cells (LSEC) isolation and co-culture with HSC

LSEC were isolated and cultured as described(19). After 3 days, de-differentiated LSEC were co-cultured for 24h with LX-2 over-expressing KLF2 due to adenoviral or simvastatin pre-administration(13). VEGF was analyzed in culture media supernatants using a commercially available EIA kit (R&D systems) following manufacturer's instructions.

HSC and LSEC co-culture in a sinusoidal-like environment

Activated human HSC and de-differentiated LSEC were co-cultured using a cell culture chamber with microfluidics that mimics the liver sinusoidal architecture recently developed and validated by our group (Illa et al, currently under review). Briefly, the chamber contains up-to-three cell culture compartments, with porous membranes

separating each of them, thus allowing paracrine interactions. In the top compartment, LSEC are cultured under continuous and homogeneous laminar shear stress (5dyn/cm^2) generated by a microfluidic system. In a bottom compartment, HSC are cultured. Cells are maintained in 5% CO_2 and 37ºC during all the experiment.

Characterization of cirrhotic animals with hepatic KLF2 over-expression

Hepatic fibrosis and HSC status

Liver fibrosis was assessed in cirrhotic rats infected with Ad-KLF2 or Ad-CTRL (n=10 per group), and in cirrhotic rats receiving simvastatin or vehicle (n=6 per group) using Sirius Red staining and computerized analysis(20).

a-SMA and desmin expression was determined in paraffin-embedded liver sections as previously described(21).

In vivo hemodynamic studies

Mean arterial pressure (MAP), portal pressure (PP), portal blood flow (PBF) and superior mesenteric artery blood flow (SMABF) were measured in Ad-KLF2 and Ad-CTRL treated cirrhotic rats using microcatheters and flow probes as previously described(22) (n=10 per group).

Evaluation of hepatic endothelial function

After *in vivo* hemodynamic measurements, livers were quickly isolated and perfused using a flow-controlled perfusion system as previously described(22). Liver endothelial function was determined as response to incremental doses of the endothelium-dependent vasodilator acetylcholine(23).

Statistical analysis

Statistical analysis was performed with the SPSS 19.0 for Windows statistical package (IBM Corp., Armonk, NY). All results are expressed as mean ± standard error of the mean. Comparisons between groups were performed with the Student-t test or ANOVA followed by a post hoc test when adequate. Differences were considered significant at a p value <0.05

Results

Simvastatin induces KLF2 expression in activated HSC and concomitantly improves their phenotype

Human activated HSC treated for 24h with increasing concentrations of simvastatin exhibited an induction in the expression of the transcription factor KLF2 at a concentration of 10μM (Fig. 1A), which was associated with a marked down-regulation in the activation marker α-SMA both at mRNA and protein levels (Fig. 1A). Simvastatin effects on KLF2 and α-SMA was maintained, or even increased, after 3 days of treatment (Supplementary fig. 1A). Primary cirrhotic rHSC incubated with simvastatin during 24h also showed a significant up-regulation of KLF2 mRNA expression (Fig. 1B) and reduction in α-SMA and pro-collagen I levels (Fig. 1B). Time-course experiments showed a marked and time-dependent α-SMA and pro-collagen I down-regulation up to 3 days of treatment (Supplementary fig. 1B).

Amelioration of HSC activation phenotype due to simvastatin is mediated by KLF2

To investigate whether the α-SMA and pro-collagen I reduction observed in simvastatin treated HSC was dependent on KLF2 expression, we performed KLF2 knock-down experiments through two approaches. First, siRNA against KLF2 was used. Surprisingly, these experiments showed that abrogation of simvastatin-derived KLF2 up-regulation due to siRNA treatment was not associated with an inhibition in simvastatin-derived improvement of HSC phenotype (Fig. 2A and 2B). However, a concomitant induction in KLF4 and KLF6 expression was observed in simvastatin treated cirrhotic siKLF2 rHSC, in comparison to simvastatin-treated siCTRL rHSC (25% and 68%, respectively).

As a second approach, LX-2 incubated with simvastatin or vehicle were co-treated with two KLF2 inhibitors, namely mevalonate and GGPP. As demonstrated in Figure 2C and 2D, isoprenoids attenuated or even inhibited simvastatin-mediated KLF2 induction and reduced α-SMA diminution. Similar results were obtained in primarily cultured cirrhotic rHSC (Supplementary fig. 2).

To further understand the role of KLF2 on HSC improvement, we decided to analyze HSC phenotype in cells selectively over-expressing KLF2 due to adenoviral administration. LX-2 infected with Ad-KLF2 exhibited markedly increased expression of KLF2 (data not shown) that came along with a time-dependent decrease in the expression of α-SMA, in comparison to Ad-CTRL cells (Fig. 2E). In addition, primary

HSC isolated from cirrhotic rats previously infected with Ad-KLF2 displayed a significant HSC activation reversal, compared to cells from Ad-CTRL cirrhotic animals (Fig. 2F).

KLF2 up-regulation decreases intrahepatic fibrosis in cirrhotic rats both by HSC de-activation and apoptosis

The potential effects of KLF2 up-regulation as a new anti-fibrotic strategy were analyzed in cirrhotic animals. Rats infected with Ad-KLF2 showed increased expression of hepatic KLF2, compared to Ad-CTRL rats (Supplementary fig. 3A-B). This increment in KLF2 levels was associated with a 41% reduction in intrahepatic fibrosis, as proved by Sirius Red staining (Fig. 3A), as well as a decrease in pro-collagen I and α-SMA expression (Fig. 3A-C) in comparison to livers from cirrhotic animals receiving Ad-CTRL. Moreover, a profound decline in desmin protein expression (Fig. 3A) was observed in Ad-KLF2 cirrhotic animals, suggesting that decrease in HSC activation markers may be mostly due to both apoptosis of activated HSC and to their de-activation. Indeed, we observed augmentation of the apoptosis marker cleaved caspase 3 and a reduction of phosphorylated-Bad, and a significant decrease in HSC activation measured as Rho Kinase activity (Fig. 3D). *In vitro* studies confirmed that simvastatin diminishes HSC proliferation (43.7% reduction after 24h of treatment vs. vehicle) and promotes their death (28% increase in non-viable cells) through a KLF2-dependent apoptotic mechanism (Supplementary fig. 4).

In addition, the anti-fibrotic effects of KLF2 were evaluated in a group of cirrhotic animals where hepatic KLF2 expression was induced using a pharmacological approach. These experiments showed that cirrhotic rats treated during 3 days with simvastatin exhibited up-regulation in KLF2 expression (4 fold higher vs. vehicle-cirrhotic rats), diminution in fibrosis, and HSC de-activation (Fig. 3E).

KLF2 up-regulation decreases HSC oxidative stress, probably via NF-E2 related factor 2 (Nrf2) mediated pathway

In vitro studies showed that the improvement in HSC activation phenotype due to KLF2 up-regulation was accompanied by a significant increment in the expression of the anti-oxidant and detoxifying genes heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase quinone 1 (NQO1) (Fig. 4A and supplementary fig 5A), overall reducing the intracellular levels of O_2^-, through a KLF2-dependent mechanism (Fig. 4B). Similarly, induction of hepatic KLF2 in cirrhotic animals due to simvastatin or adenoviral

administration was associated with increased hepatic expression of HO-1 and NQO1, which may be derived from increased nuclear levels of the KLF2-derived antioxidant transcription factor Nrf2 (Fig. 4C and supplementary fig. 5B).

KLF2 over-expressing HSC paracrinally improve LSEC phenotype

Primary cultured de-differentiated LSEC exhibited a dysfunctional phenotype defined as diminished expression of eNOS and up-regulated endothelin-1, in comparison to healthy LSEC (Supplementary fig. 6)(24). De-differentiated LSEC co-cultured with LX-2 over-expressing KLF2 (due to previous treatment with simvastatin or Ad-KLF2) significantly improved their phenotype, as demonstrated by an increment in eNOS levels and reduction in endothelin-1 expression, in comparison to LSEC co-cultured with vehicle-LX-2 (Fig. 5A-B). This amelioration in LSEC phenotype was probably due, at least in part, to an increment in VEGF release from KLF2-over-expressing LX-2 (3.2 fold induction in simvastatin pre-treated cells and 2 fold induction in Ad-KLF2 cells; $p<0.05$).

Simvastatin enhances the KLF2-derived protective phenotype in HSC and LSEC cultured in an in vitro sinusoidal-like environment

Primary cultured de-differentiated LSEC were co-cultured with activated HSC in a 3D cell culture chamber with microfluidics that mimics the sinusoidal architecture. Cells were simultaneously treated with simvastatin and LSEC exposed to physiological shear stress stimuli. After 24h of co-culture and biomechanical stimulation, LSEC exhibited a significant improvement in their phenotype defined by up-regulation in KLF2 (Fig. 5C) and HSC showed a further down-regulation of α-SMA (Fig. 5D), in comparison to simvastatin treated sinusoidal cells cultured under static conditions or cells cultured within the sinusoidal microchamber but treated with vehicle.

Hepatic KLF2 over-expression improves portal hypertension

To ultimate validate the potential of KLF2 up-regulation as new therapeutic strategy for portal hypertension, the hepatic and systemic hemodynamics were evaluated in cirrhotic animals infected with adenoviral constructs. When compared to control rats, cirrhotic rats transfected with Ad-CTRL exhibited arterial hypotension and portal hypertension derived from both increased portal blood flow (PBF) and intrahepatic vascular resistance (IHVR) (data not shown). Cirrhotic rats over-expressing hepatic KLF2 due to adenoviral administration showed a significant improvement in portal pressure (-15%), due to both an improvement in PBF and IHVR, in comparison to cirrhotic Ad-CTRL

rats (Fig. 6). No differences in SMABF (2.1±0.3 vs. 2.2±0.2 mL/min*100g bw; p=0.7) or MAP were observed comparing groups.

Hepatic KLF2 over-expression improves liver endothelial dysfunction of cirrhotic rats

To further characterize the effects of KLF2 up-regulation on liver vasculature, livers from cirrhotic rats treated with Ad-KLF2 or its control, were isolated and perfused. Basal ex vivo IHVR was significantly lower in cirrhotic rats receiving Ad-KLF2 than Ad-CTRL animals (1.05±0.09 vs. 1.37±0.08 mmHg*min/mL*g; p=0.02). Endothelial function evaluation revealed that livers for cirrhotic animals treated with Ad-CTRL showed endothelial dysfunction, defined as a deficient response to the vasodilator acetylcholine in comparison to control rats, which was not observed in cirrhotic animals treated with Ad-KLF2 (Fig. 7A). Endothelial function amelioration was accompanied by an increase in the expression of eNOS and its phosphorylated form (Fig. 7B).

Discussion

Cirrhosis is the principal cause of intrahepatic portal hypertension, a deleterious syndrome derived from increments in intrahepatic vascular resistance (IHVR) and portal blood flow (PBF)(1). Pharmacological treatments currently available for portal hypertension, non-selective β blockers, lower cardiac output and cause splanchnic vasoconstriction reducing PBF, but they are not liver specific and less than half of patients under β blockade are protected from the portal hypertension-derived complications, mainly variceal bleeding and splanchnic vasodilation(25). Therefore, new strategies that may improve portal hypertension through a reduction in intrahepatic vascular resistance are highly necessary.

In cirrhosis, LSEC lose their highly specialized phenotype and produce diminished vasodilators and large levels of vasoconstrictors, contributing to increase IHVR and consequently to aggravate the portal hypertension syndrome(1,26). On the other hand, perisinusoidal HSC activation, proliferation and contraction increase dynamically the hepatic vascular resistance(27). Moreover, activated HSC produce large amounts of extracellular matrix components that result in increased structural resistance to liver perfusion(27).

We recently demonstrated that the transcription factor KLF2 is highly expressed in cirrhotic rats' liver, particularly in the hepatic endothelium(12). However, this endogenous protective mechanism is insufficient to prevent sinusoidal endothelial dysfunction and HSC activation in cirrhotic animals. Thus, considering that KLF2 confers endothelial protection against inflammation, thrombosis and vasoconstriction, and it is a regulator of hemodynamics, we hypothesized that increasing the expression of this transcription factor could be beneficial in cirrhosis.

In the present study we demonstrate for the first time that hepatic KLF2 over-expression in cirrhotic animals de-activates HSC and ameliorates the dysfunctional hepatic endothelium, which leads to a significant improvement in liver cirrhosis and portal hypertension. KLF2 expression was exogenously induced through two approaches: using simvastatin, the generic statin with major vasoprotective effects in the liver(13), and specifically by the administration of an adenoviral construct codifying for KLF2.

We herein demonstrate that simvastatin induces KLF2 expression both in human and rat activated HSC, which is accompanied by a time-dependent decrease in α-SMA and pro-collagen I expression. The role of KLF2 in HSC de-activation was firstly studied using siRNA-KLF2, however this methodology failed in demonstrating whether simvastatin

effects on HSC were KLF2-dependent most likely because of a compensatory increment in the expression of other members of the KLF family with anti-fibrogenic properties, specifically KLF4 and KLF6 (28,29), thus suggesting a possible KLFs on-off transcriptional switch(30). Nevertheless, pharmacological blockade of KLF2 using mevalonate and GGPP resulted in inhibition of the simvastatin-derived HSC amelioration. The experiments using GGPP further demonstrated that the KLF2-mediated effects of simvastatin on sinusoidal cells are independent on cholesterol since GGPP is away from being an intermediate for cholesterol synthesis.

To confirm the importance of KLF2 on HSC phenotype improvement, we decided to study the effects of over-expressing KLF2 in activated HSC through adenoviral technology. Specific KLF2 up-regulation significantly reversed HSC activation, both *in vitro* and *in vivo*. This amelioration is probably due to the fact that KLF2 suppresses TGF-beta signaling preserving a quiescent and atheroprotective status of the vascular endothelium(31); this could also be herein true because KLF2 over-expressing HSC showed reduced TGF-beta mRNA (data not shown).

To validate the KLF2-mediated anti-fibrotic effects in the cirrhotic liver, hepatic KLF2 expression was augmented in cirrhotic animals via adenoviral administration. KLF2 up-regulation led to a significant regression in fibrosis, defined by a marked decrease in collagen and in α-SMA and desmin expression, suggesting that fibrosis resolution was therefore associated to both HSC phenotype improvement and apoptosis. These results were corroborated finding a reduction in phosphorylated-Bad and increased cleaved caspase 3, markers of apoptosis, together with a significant decrease in the HSC activation marker Rho Kinase. The definition of KLF2 as a pro-apoptotic factor in certain situations is of interest. Indeed, our findings reveal that KLF2 *per se* induces HSC apoptosis (*in vivo* and *in vitro*) and that simvastatin-derived HSC apoptosis would be due to KLF2 up-regulation (*in vitro*). Considering our results, and previously published data(17,32), it can be proposed that KLF2 may have a dual role in terms of apoptosis, depending on the cell type and the basal phenotype of such cell. In the particular case of activated HSC, apoptosis might be mediated by a KLF2-derived down-regulation in NF-kB (data not shown), which tightly regulates the expression of different anti-apoptotic genes(33). In addition, our findings suggest that the pro-apoptosis and anti-fibrotic properties of other KLF2 inducers, such as resveratrol(21) and curcumin(34), may also be mediated by KLF2. In parallel experiments, KLF2 hepatic up-regulation observed in simvastatin treated cirrhotic rats was associated with a

decline in liver fibrosis (reduced α-SMA and collagen amount) but no differences in desmin were observed, suggesting that a 3-day pharmacological treatment would mainly modulate HSC phenotype. Taken together, our data define KLF2 up-regulation as an efficient mechanism to reduce hepatic fibrosis by improvement in HSC phenotype and promoting their apoptosis.

One of the mechanisms by which simvastatin may improve HSC phenotype might be related to an amelioration in intracellular oxidative stress. Indeed, simvastatin triggers nuclear translocation of the antioxidant transcription factor Nrf2(35), which has been shown to play a critical role attenuating liver fibrosis(36). Under basal conditions, Nrf2 is retained in the cytoplasm bound to Keap1 that promotes its proteasomal degradation. However, upon stimulation Nrf2 is released and translocates to the nucleus where it binds to the antioxidant responsive elements of cytoprotective genes such as NQO1 and HO-1, promoting their transcription. We herein demonstrate that HSC activation reversal due to KLF2 up-regulation was accompanied by increments in the expression of the Nrf2-targets HO-1 and NQO1, altogether promoting a marked decline in intracellular oxidative stress. It has been reported that KLF2 enhances the antioxidant activity of Nrf2 by increasing its nuclear localization and activity(37,38) but also that a specific Nrf2-activating stimulus, apart from KLF2, is required for full transcriptome effects(38). This could explain the increased hepatic nuclear accumulation of Nrf2, with a concomitant activation of its pathway, observed in Ad-KLF2 and simvastatin-treated cirrhotic animals in which the activating stimulus may probably be the elevated oxidative stress characteristic of cirrhosis(39). Taken together, these results suggest that KLF2 improves HSC phenotype by reduction in intracellular oxidative stress through Nrf2 activation.

We have recently demonstrated that the preservation of a normal LSEC phenotype, via KLF2 up-regulation, maintains HSC in a quiescence status or even promote their de-activation(13). However, it is unknown whether therapeutic strategies that de-activate HSC may impact on the phenotype of de-differentiated LSEC. We herein observe that HSC de-activation due to KLF2 over-expression paracrinally improves LSEC. Indeed, de-differentiated LSEC recover eNOS and lose endothelin-1 mRNA expression (genes profoundly de-regulated during *in vitro* capillarization(24)) when co-cultured with HSC pre-treated with simvastatin or Ad-KLF2. We suggest that this improvement in LSEC phenotype would partly be due to HSC-derived VEGF that may act via a microvascular internal loop mechanism. VEGF might bind to its receptor in LSEC determining

amelioration in their phenotype, but it also could induce an attenuation of the contractile properties of HSC via up-regulation of VEGFR-1(40). Although these data seem to be of great value, more investigations are required.

To better understand the paracrine interactions between LSEC and HSC in response to simvastatin, we *in vitro* reproduced the liver sinusoid and analyzed the effects of simvastatin on both cell types. These experiments showed a global improvement in the sinusoidal microenvironment as consequence of cells paracrine communications. Indeed, both LSEC and HSC exhibited a more profound amelioration in their phenotypes that those cells co-cultured without physiological shear stress stimulation or without simvastatin, thus validating the hypothesis that simvastatin administration to individuals with sinusoidal microvascular injuries (i.e. cirrhosis or ischemia/reperfusion) exerts strong liver protection(41,42).

Finally, the effects of KLF2 over-expression on hepatic and systemic hemodynamics were determined. Improvement in liver sinusoidal cells due to KLF2 induction promoted a significant reduction in portal pressure, consequence of both reductions in PBF and IHVR, without changes in systemic hemodynamic parameters. In addition, KLF2-over-expressing cirrhotic rats exhibited restored liver endothelial function, associated with an increase in the KLF2 transcriptional target eNOS. Thus, the mechanisms by which KLF2 up-regulation improves portal hypertension go back to the recovery of sinusoidal function and the restoration of paracrine signaling. Although we did not appreciate the reestablishment of fenestra in these improved LSEC or differences in analytical biochemistry data (data not shown), we do not discard that a more prolonged treatment could lead to even better results.

In conclusion, this study provides evidence that increasing KLF2 in cirrhotic animals leads to an improvement in liver sinusoidal cells phenotype, de-activating HSC, ameliorating the dysfunctional endothelium, and reducing oxidative stress, that turns into an amelioration of cirrhosis and portal hypertension. The use of simvastatin or other drugs capable of augmenting KLF2 expression might be an appealing proposition to treat portal hypertension in cirrhosis.

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Figure Legends

Figure 1. Simvastatin ameliorates HSC phenotype. Expression of KLF2 and HSC activation markers (α-SMA and pro-collagen I) in response to 24h of simvastatin in LX-2 (**A**) and primary cirrhotic rat HSC (**B**). Left y-axis indicates KLF2 fold induction. Right y-axis indicates mRNA changes of HSC activation markers. Inserts show representative western blots of the corresponding protein. Percentage under the inserts refers to protein increase (↑) or decrease (↓) due to simvastatin treatment, normalized to GAPDH and compared to vehicle-cells. n=3 per condition. $^{*}p<0.01$ vs. vehicle-cells.

Figure 2. Effects of KLF2 modulation on HSC phenotype. Effects of KLF2 silencing using siKLF2 (LX-2, **A**; rHSC, **B**), mevalonate (LX-2, **C**) or GGPP (LX-2, **D**) on HSC phenotype in response to simvastatin. Left y-axis indicates KLF2 fold induction. Right y-axis indicates mRNA changes of the activation markers. n=3 per condition, $^{*}p<0.01$ vs. vehicle-cells, $^{\#}p<0.01$ vs. simvastatin. (**E**) α-SMA expression in LX-2 after 24h or 72h of incubation with adenovirus codifying for KLF2 (*k*) or control adenovirus (*c*). Inserts show a representative western blot with its quantification normalized to GAPDH. n=3 per condition. $^{*}p<0.01$ vs. Ad-CTRL. (**F**) α-SMA and pro-collagen I expression in HSC isolated from cirrhotic rats previously infected with Ad-KLF2 or Ad-CTRL. n=3 per condition. $^{*}p<0.01$ vs. Ad-CTRL.

Figure 3. KLF2 up-regulation decreases intrahepatic fibrosis in cirrhotic rats. (**A**) *Top*, representative images of liver fibrosis assessment using Sirius Red staining (10X), and α-SMA and desmin immunohistochemistry (20X) in Ad-KLF2 (*k*) and Ad-CTRL (*c*) cirrhotic animals (10X). *Bottom*, corresponding quantifications (n=10 per group). (**B**) Representative western blot and densitometric quantification of α-SMA determined in livers from rats described in A. (**C**) Hepatic pro-collagen I mRNA expression in rats described in A. (**D**) Representative western blots and densitometric quantifications of depicted proteins determined in livers from rats described in A. $^{*}p<0.05$ vs. Ad-CTRL. (**E**) Representative images of Sirius Red staining and α-SMA and desmin immunohistochemistry in livers from cirrhotic rats treated with simvastatin or its vehicle for 3 days, and corresponding quantifications. n=6 per group. $^{*}p<0.05$ vs. vehicle.

Figure 4. KLF2 reduces HSC oxidative stress through Nrf2. (**A**) Relative expression of Nrf2 and its target genes, HO-1 and NQO1, in simvastatin treated LX-2 cells in the presence of the KLF2 inhibitor, GGPP. (**B**) *Top*, representative fluorescence images of superoxide (O_2^-) in LX-2 and rHSC incubated as depicted. *Bottom*, quantitative analysis of the fluorescent intensity normalized to the number of cells. Data obtained in 3 independent experiments. $^*p<0.05$ vs. vehicle. $^\#p<0.05$ vs. simvastatin. (**C**) Expression of Nrf2 (left) and its target genes HO-1 and NQO1 (right) in livers from cirrhotic rats receiving Ad-KLF2 (*k*) or Ad-CTRL (*c*) $^*p<0.05$ vs. Ad-CTRL.

Figure 5. HSC and LSEC paracrinally improve their phenotype. Relative expression of eNOS (**A**) and endothelin-1 (**B**) in de-differentiated LSEC co-cultured with LX-2 cells previously treated with simvastatin, Ad-KLF2, or vehicle. n=3 per condition. $^*p<0.05$ vs. vehicle. (**C**) Expression of KLF2 in de-differentiated LSEC co-cultured with LX-2 under physiological conditions (shear stress), or static, and treated with simvastatin, or vehicle. (**D**) Relative α-SMA expression in LX-2 described in C. n=3 per condition. $^*p<0.05$ versus vehicle-static. $^\#p<0.05$ vs. simvastatin-static. $^\dagger p<0.05$ vs. vehicle-shear stress.

Figure 6. Hepatic KLF2 up-regulation improves portal hypertension. (**A**) Mean arterial pressure (*MAP*), (**B**) portal pressure (*PP*), (**C**) portal blood flow (*PBF*) and (**D**) intrahepatic vascular resistance (*IHVR*) determined in cirrhotic rats over-expressing hepatic KLF2 due to adenovirus administration (Ad-KLF2) compared to cirrhotic rats receiving control adenovirus (Ad-CTRL). n=10 per group. $^*p<0.05$ vs Ad-CTRL.

Figure 7. KLF2 up-regulation restores hepatic endothelial function in cirrhotic rats. (**A**) Endothelial function evaluation in livers from cirrhotic rats infected with Ad-KLF2 (*k*) or Ad-CTRL (*c*). (**B**) Representative western blots and densitometric quantifications of hepatic eNOS (top) and p-eNOS (bottom) in cirrhotic rats described in A. n=10 per group. $^*p<0.05$ vs. Ad-CTRL.

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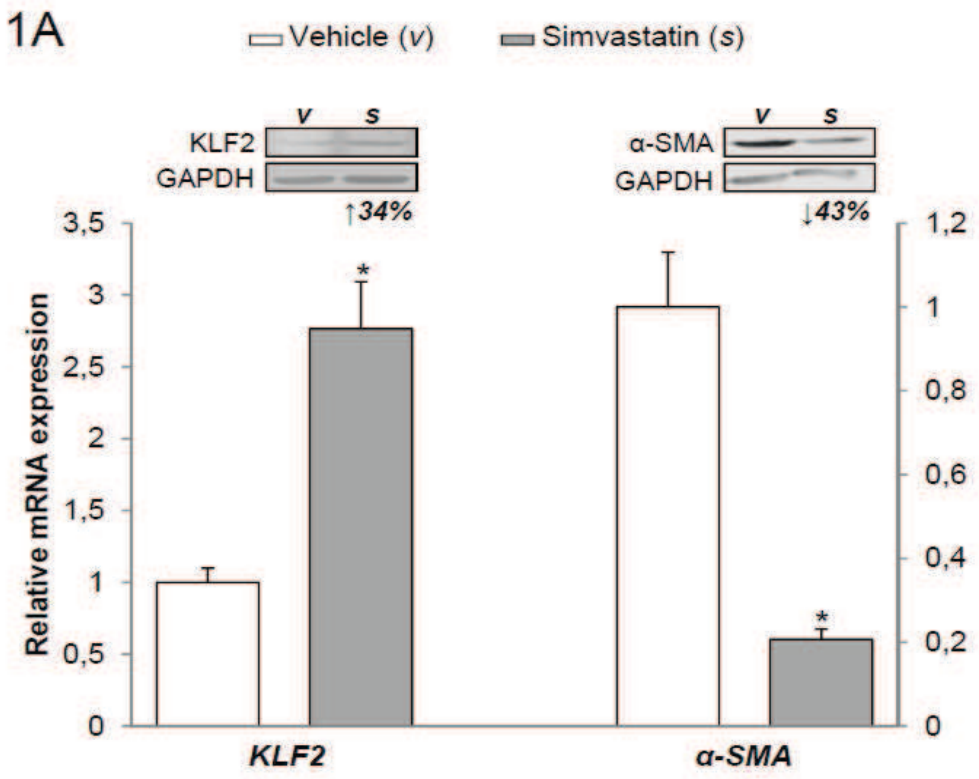
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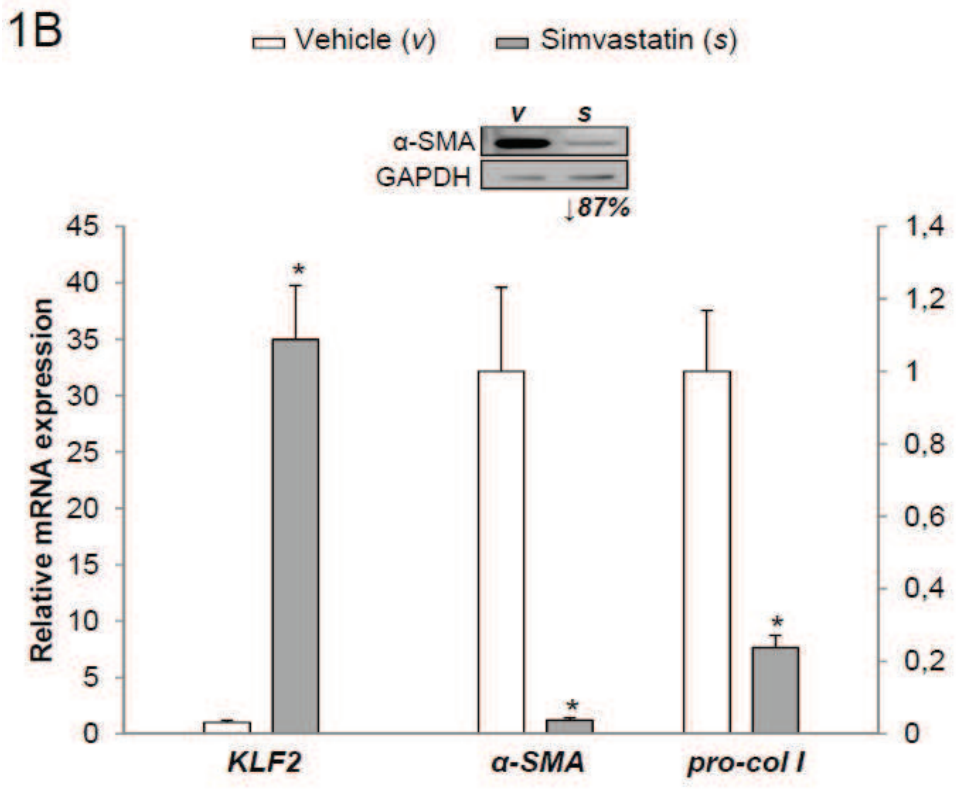
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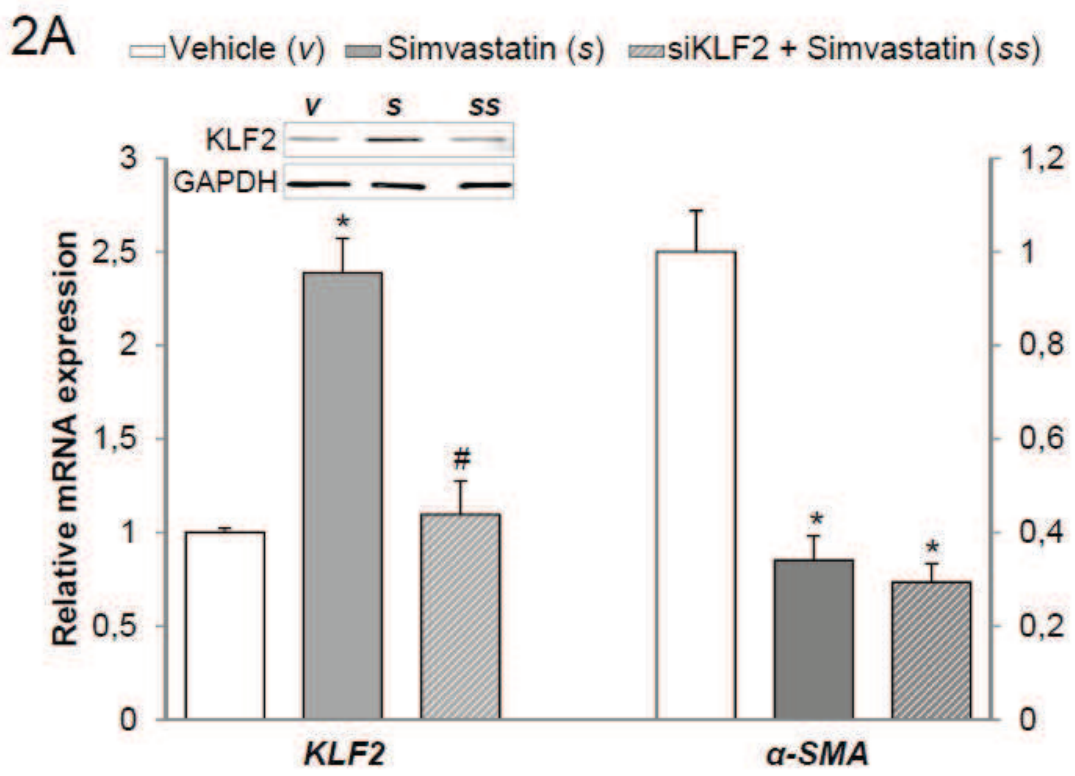
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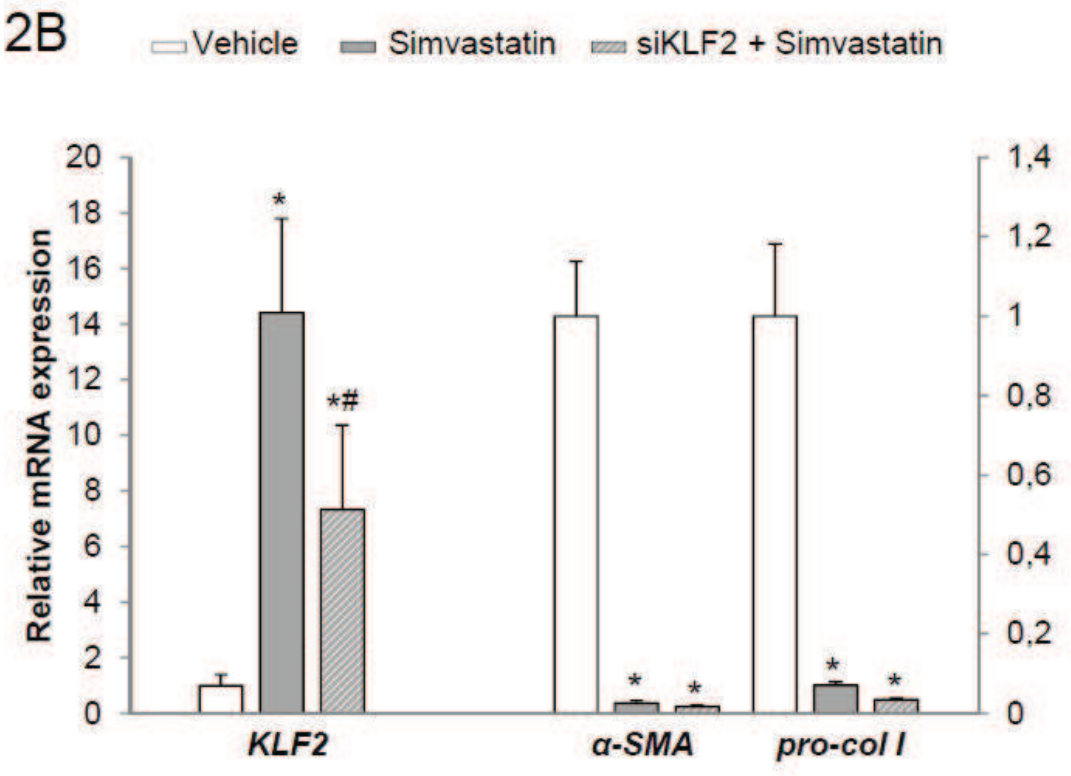
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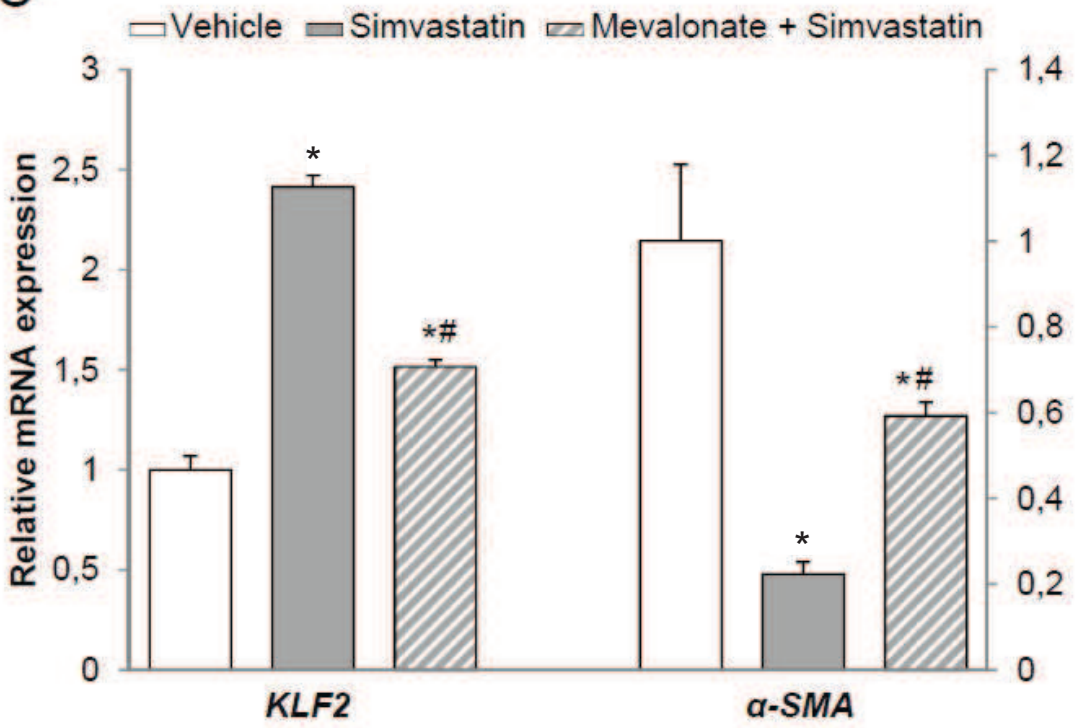
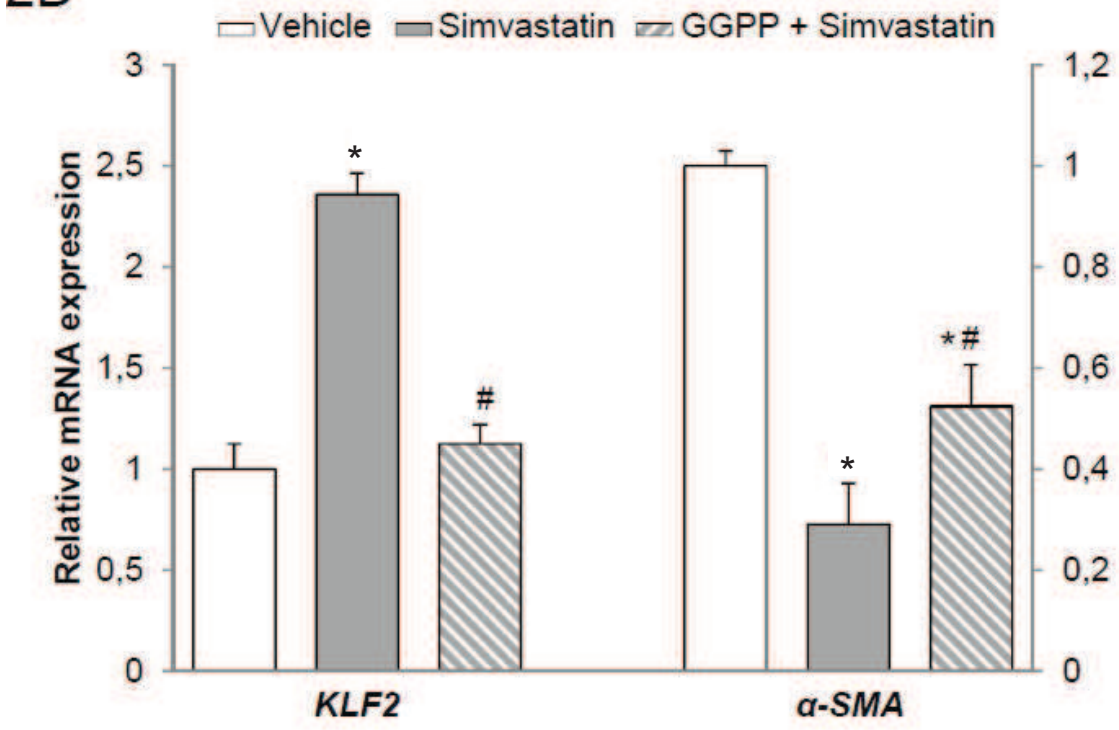
1,2
1
0,8
0,6
0,4
0,2
0
*
*#
*
*
*
*
KLF2
α-SMA
pro-col I

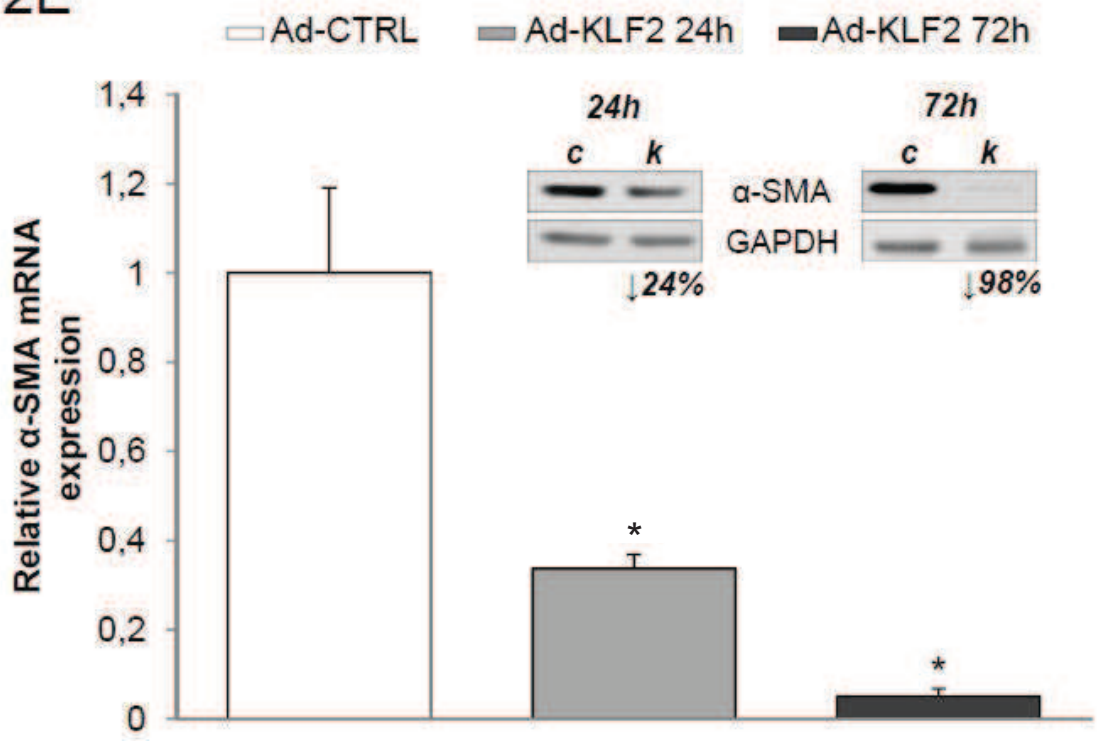
2C


Vehicle
Simvastatin
Mevalonate + Simvastatin
Relative mRNA expression
3
2,5
2
1,5
1
0,5
0
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*#
*
*#
KLF2
α-SMA

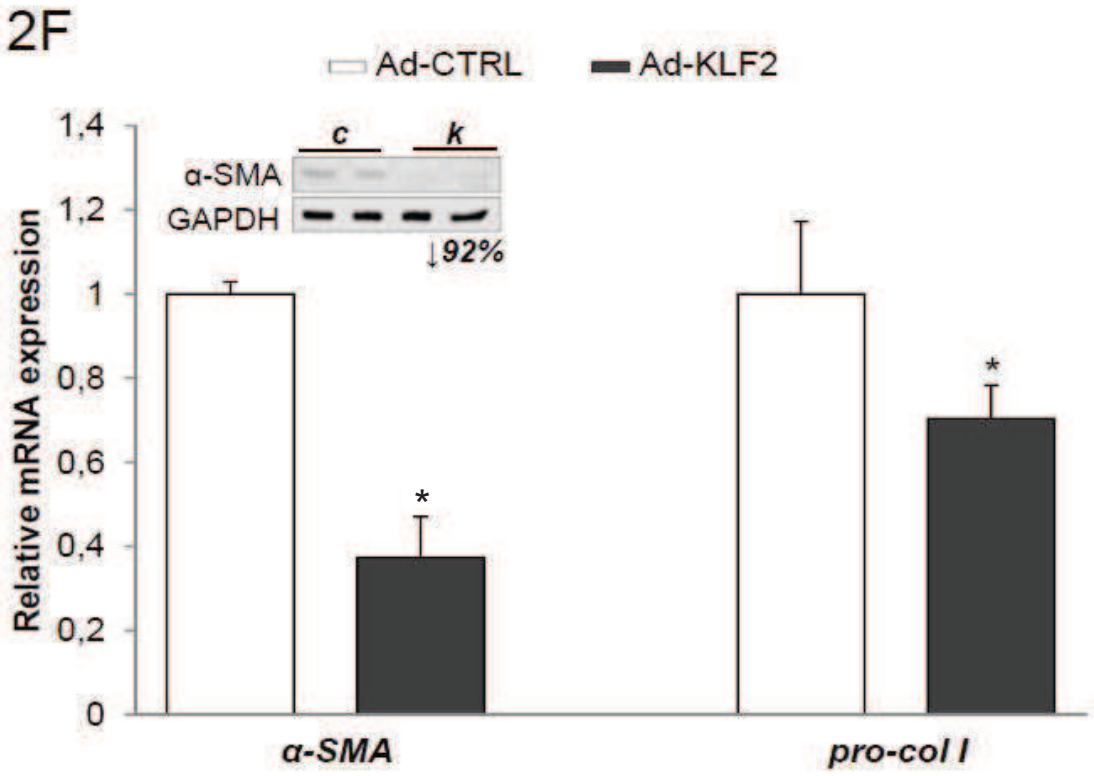
2D


Vehicle
Simvastatin
GGPP + Simvastatin
Relative mRNA expression
3
2,5
2
1,5
1
0,5
0
1,2
1
0,8
0,6
0,4
0,2
0
*
#
*
*#
KLF2
α-SMA

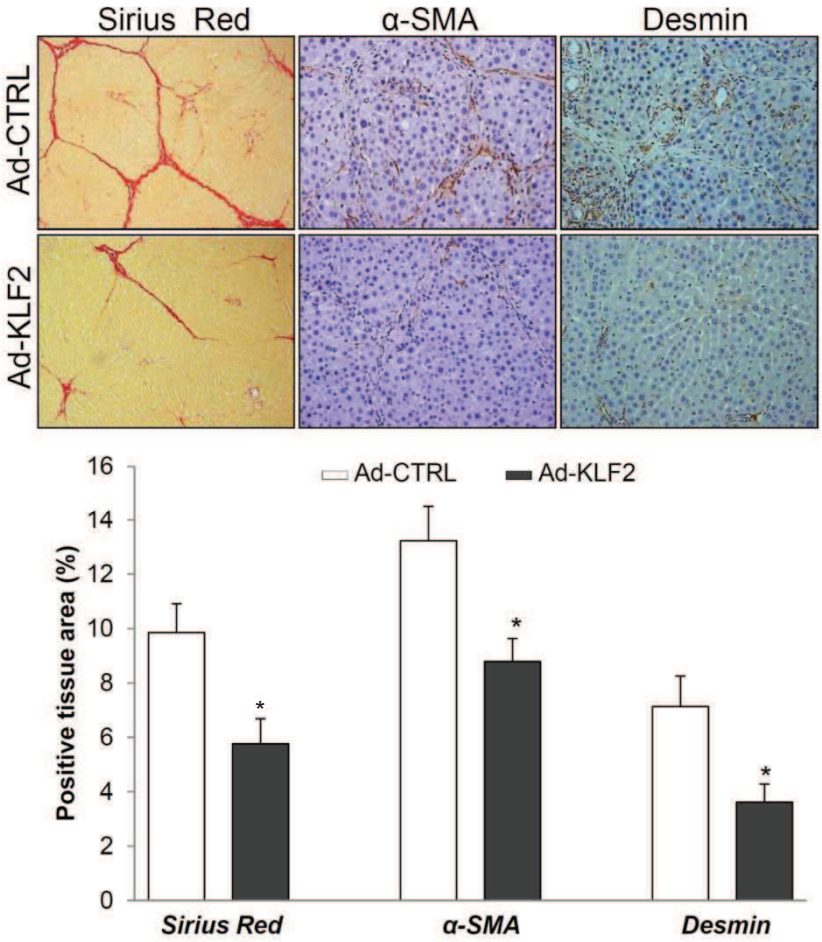
2E


Ad-CTRL
Ad-KLF2 24h
Ad-KLF2 72h
Relative α-SMA mRNA expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
24h
72h
c
k
c
k
α-SMA
GAPDH
↓24%
↓98%
*
*

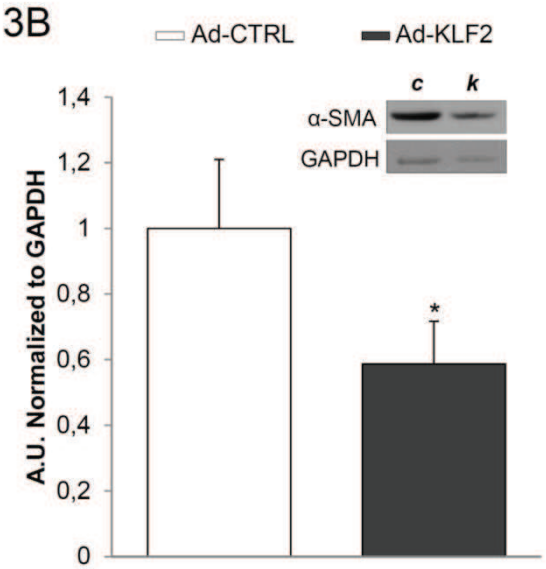
2F


Ad-CTRL
Ad-KLF2
Relative mRNA expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
c
k
α-SMA
GAPDH
↓92%
*
*
α-SMA
pro-col I

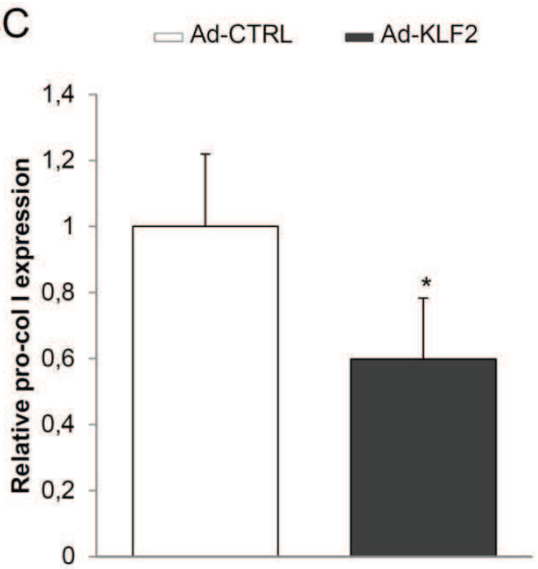
3A


Sirius Red
α-SMA
Desmin
Ad-CTRL
Ad-KLF2
Ad-CTRL
Ad-KLF2
Positive tissue area (%)
16
14
12
10
8
6
4
2
0
*
*
*
Sirius Red
α-SMA
Desmin

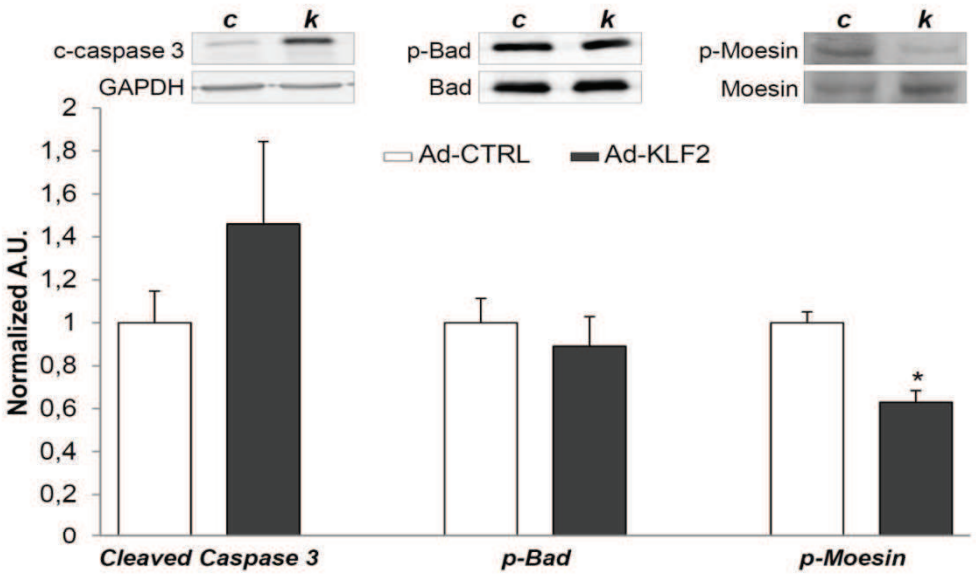
3B


Ad-CTRL
Ad-KLF2
c
k
α-SMA
GAPDH
A.U. Normalized to GAPDH
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*

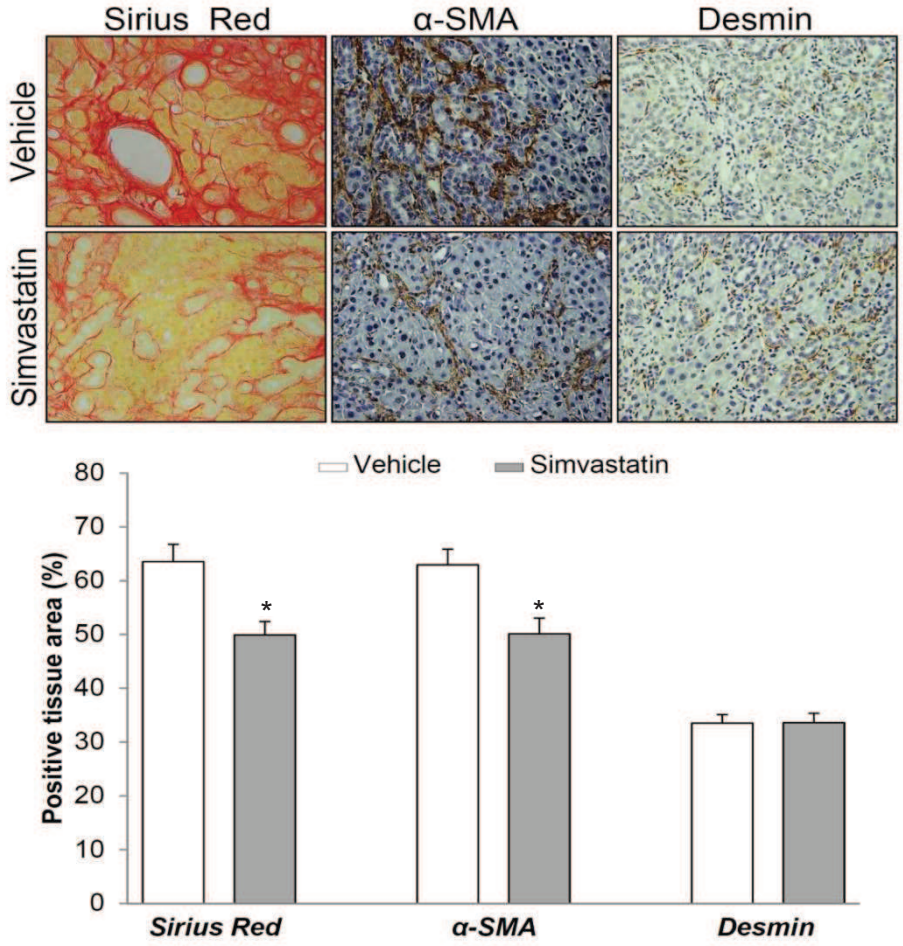
3C


Ad-CTRL
Ad-KLF2
Relative pro-col I expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*

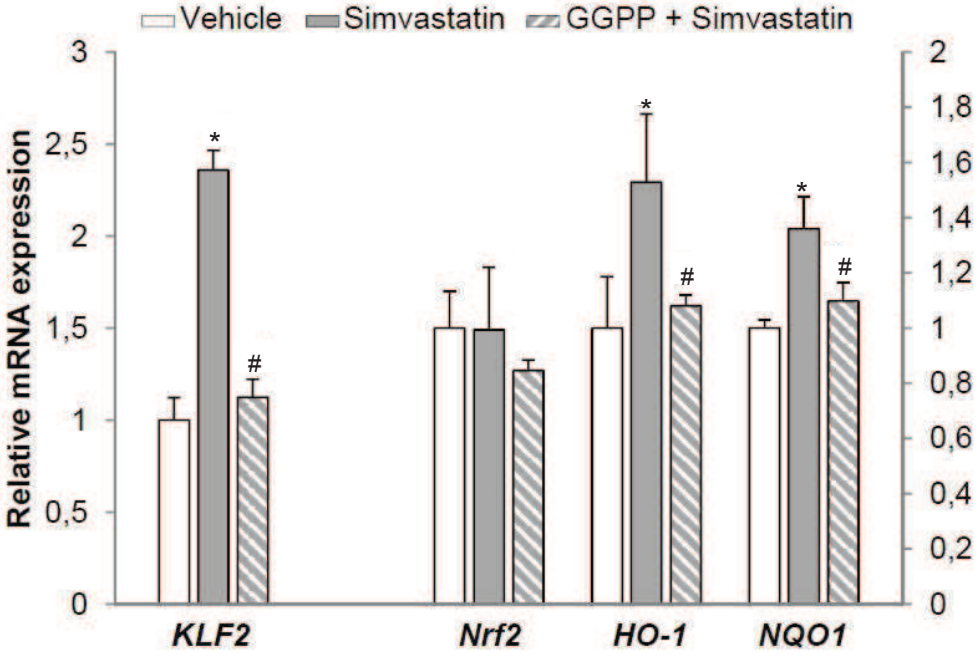
3D


c
k
c-caspase 3
GAPDH
p-Bad
Bad
p-Moesin
Moesin
Ad-CTRL
Ad-KLF2
Normalized A.U.
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
Cleaved Caspase 3
p-Bad
p-Moesin

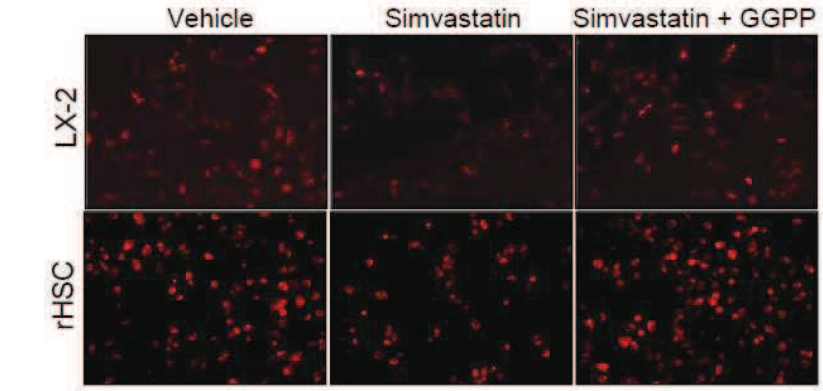
3E

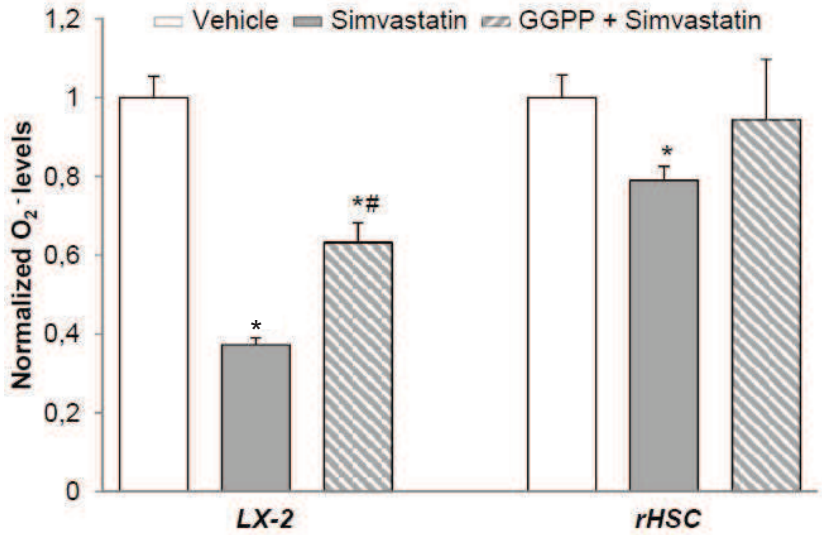

Sirius Red
α-SMA
Desmin
Vehicle
Simvastatin
Vehicle
Simvastatin
Positive tissue area (%)
80
70
60
50
40
30
20
10
0
*
*
Sirius Red
α-SMA
Desmin

4A

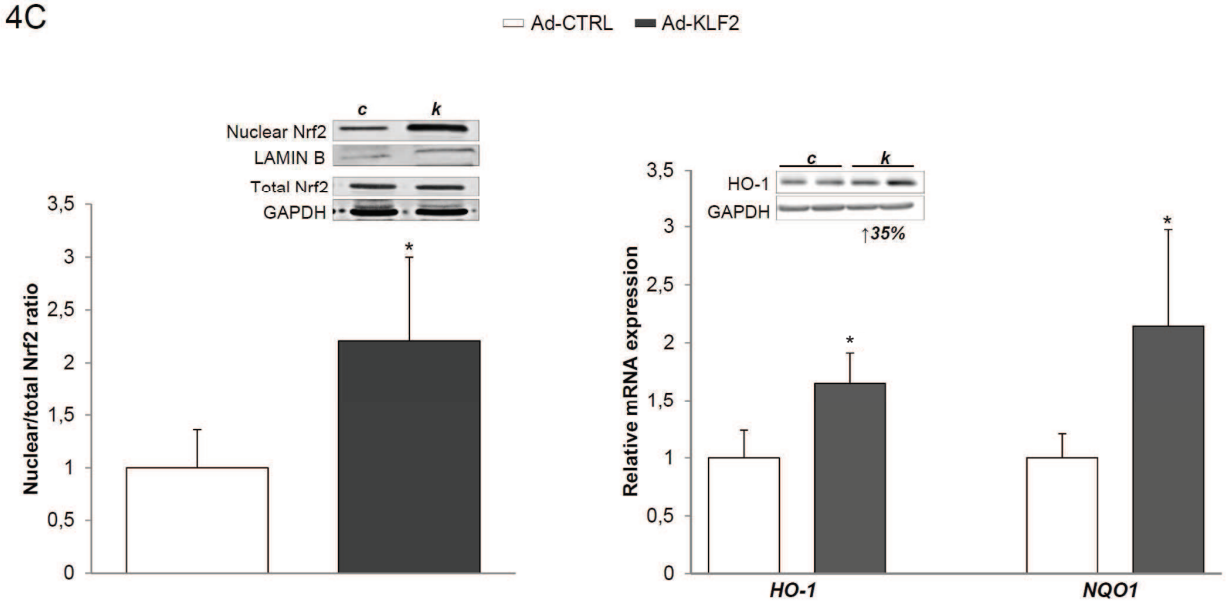

Vehicle
Simvastatin
GGPP + Simvastatin
Relative mRNA expression
3
2,5
2
1,5
1
0,5
0
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
#
KLF2
Nrf2
HO-1
NQO1

4B

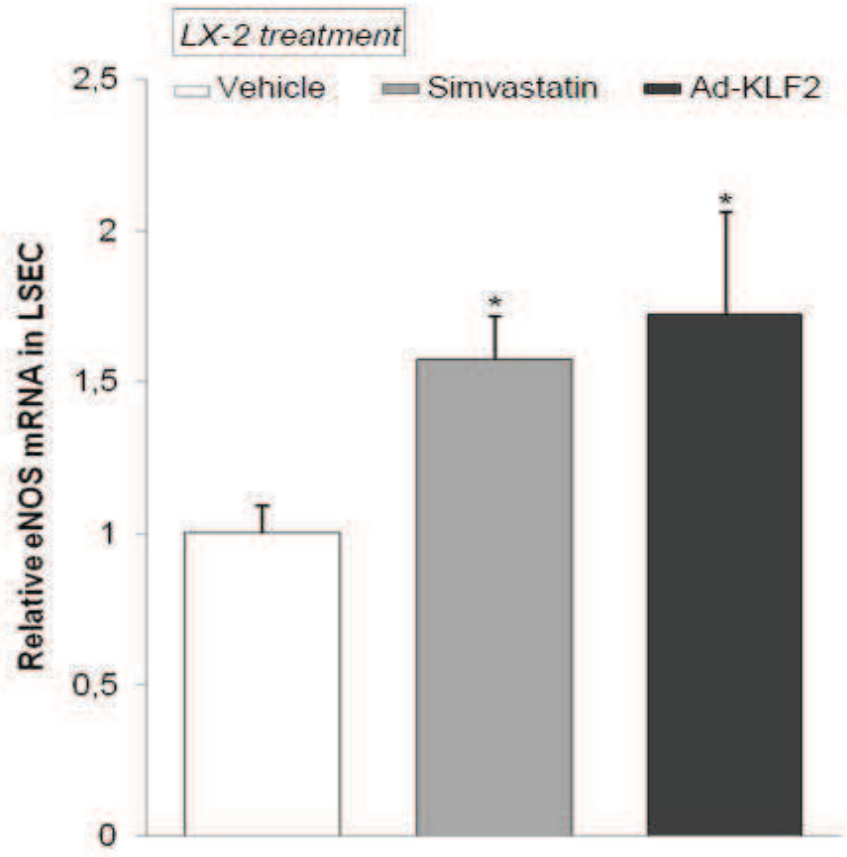

Vehicle
Simvastatin
Simvastatin + GGPP
LX-2
rHSC


Vehicle
Simvastatin
GGPP + Simvastatin
Normalized O2- levels
1,2
1
0,8
0,6
0,4
0,2
0
*
*#
LX-2
rHSC

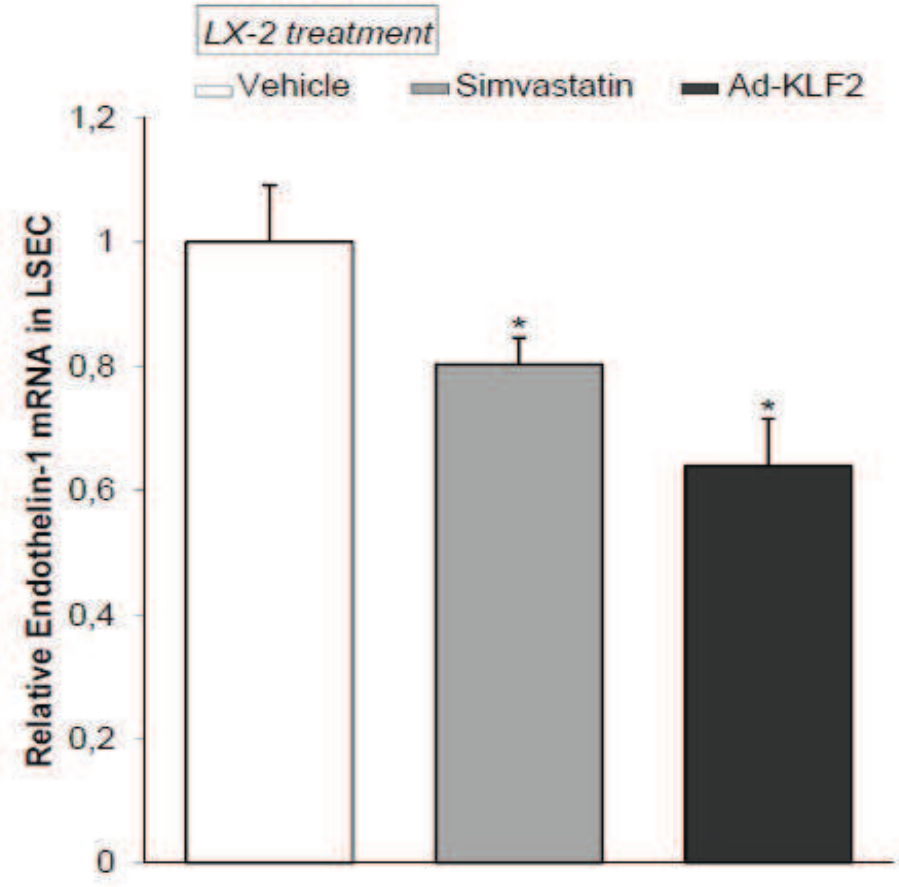
4C


Ad-CTRL
Ad-KLF2
c
k
Nuclear Nrf2
LAMIN B
Total Nrf2
GAPDH
Nuclear/total Nrf2 ratio
3,5
3
2,5
2
1,5
1
0,5
0
*
HO-1
GAPDH
↑35%
Relative mRNA expression
HO-1
NQO1

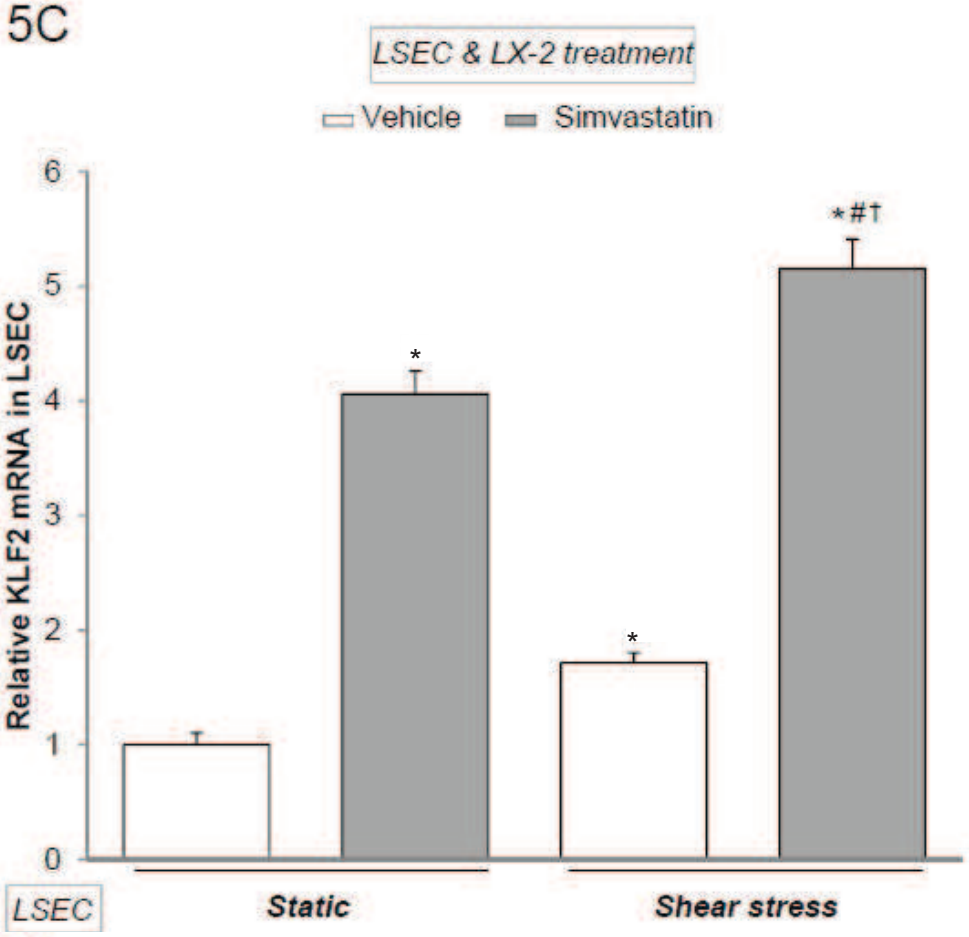
5A


LX-2 treatment
Vehicle
Simvastatin
Ad-KLF2
Relative eNOS mRNA in LSEC
2,5
2
1,5
1
0,5
0
*
*

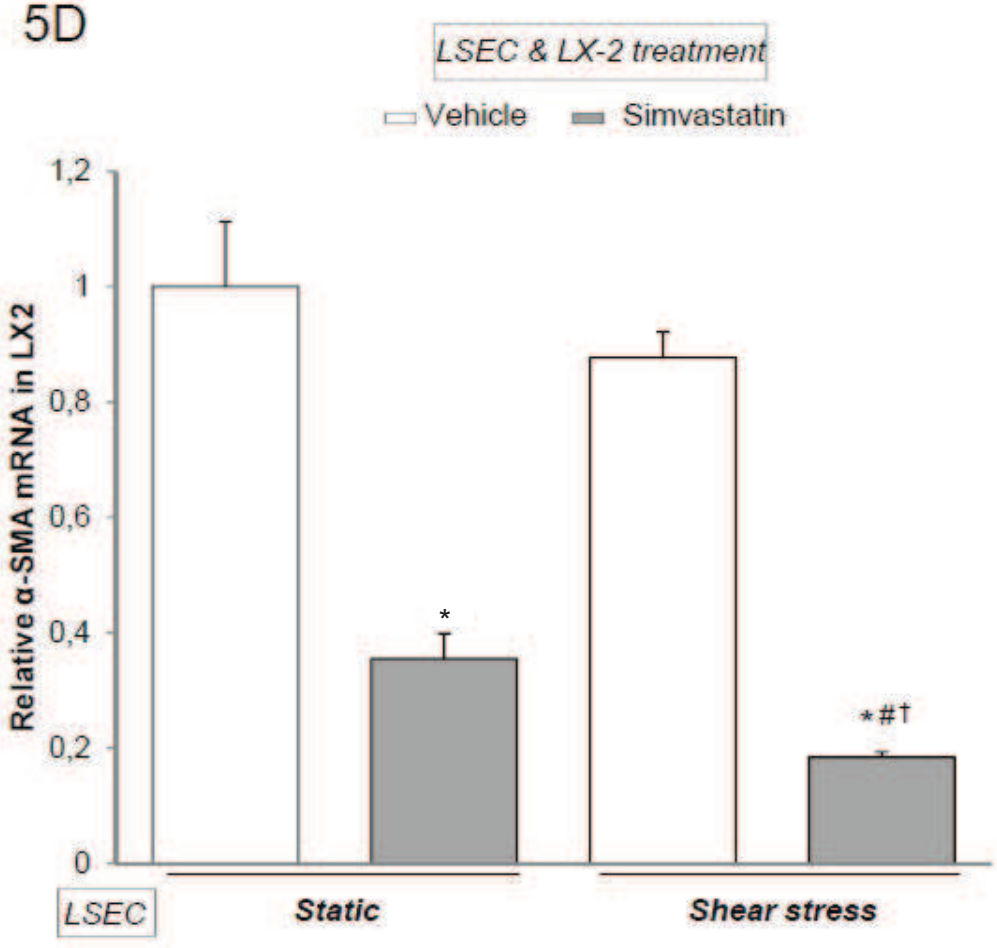
5B

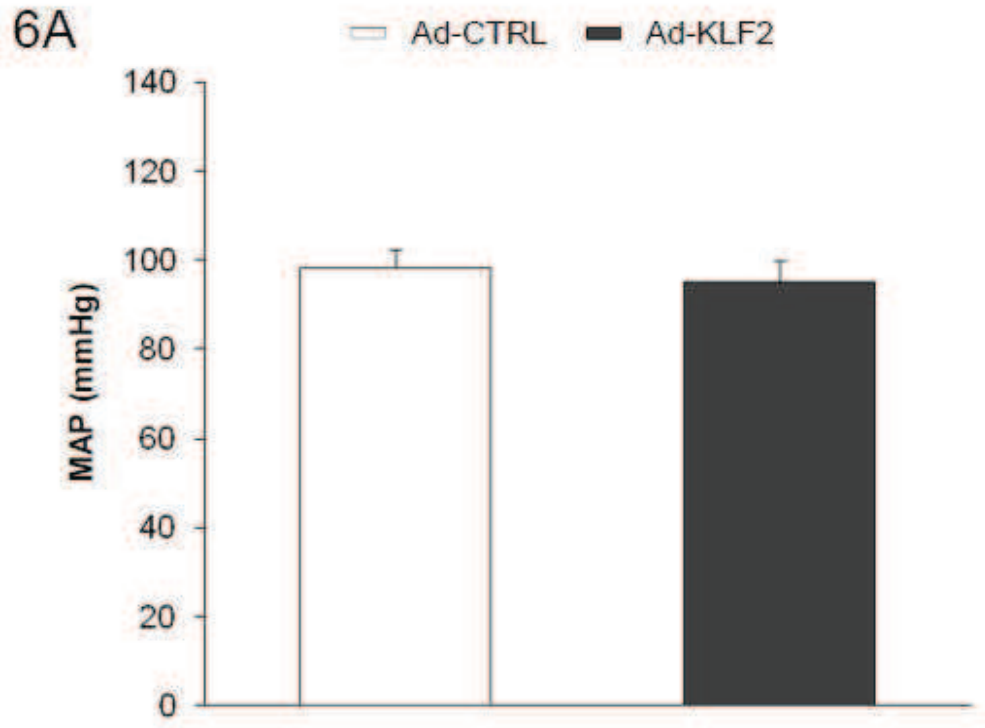

LX-2 treatment
Vehicle
Simvastatin
Ad-KLF2
Relative Endothelin-1 mRNA in LSEC
1,2
1
0,8
0,6
0,4
0,2
0
*
*

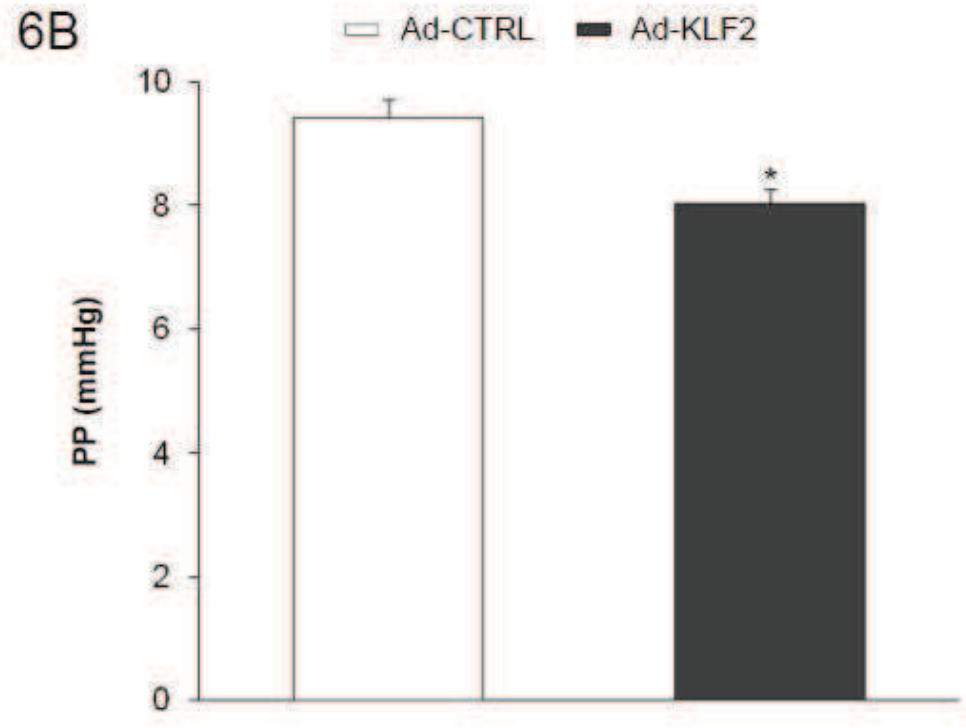
5C


LSEC & LX-2 treatment
Vehicle
Simvastatin
Relative KLF2 mRNA in LSEC
0
1
2
3
4
5
6
*
*
*#†
LSEC
Static
Shear stress

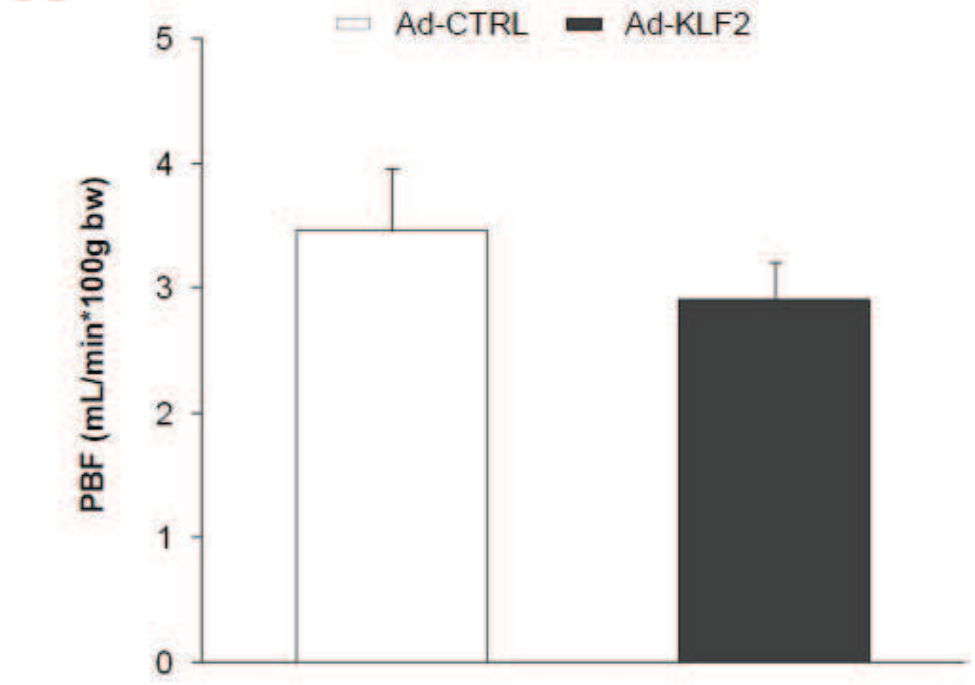
5D


LSEC & LX-2 treatment
Vehicle
Simvastatin
Relative α-SMA mRNA in LX2
0
0,2
0,4
0,6
0,8
1
1,2
*
*#†
LSEC
Static
Shear stress

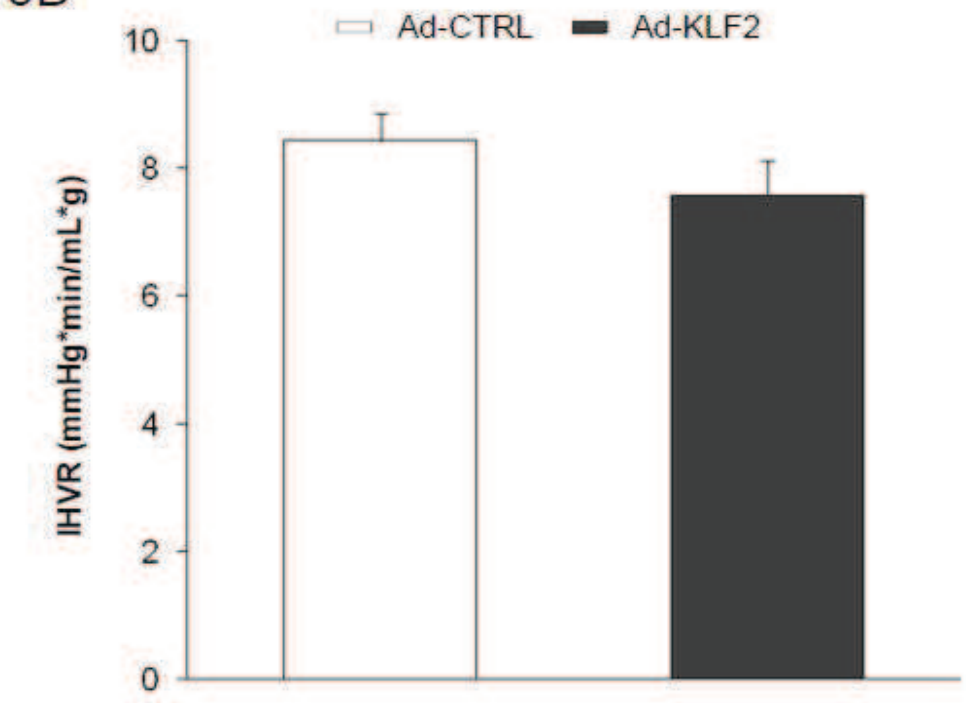

6A
Ad-CTRL
Ad-KLF2
MAP (mmHg)
140
120
100
80
60
40
20
0


6B
Ad-CTRL
Ad-KLF2
PP (mmHg)
10
8
6
4
2
0
*

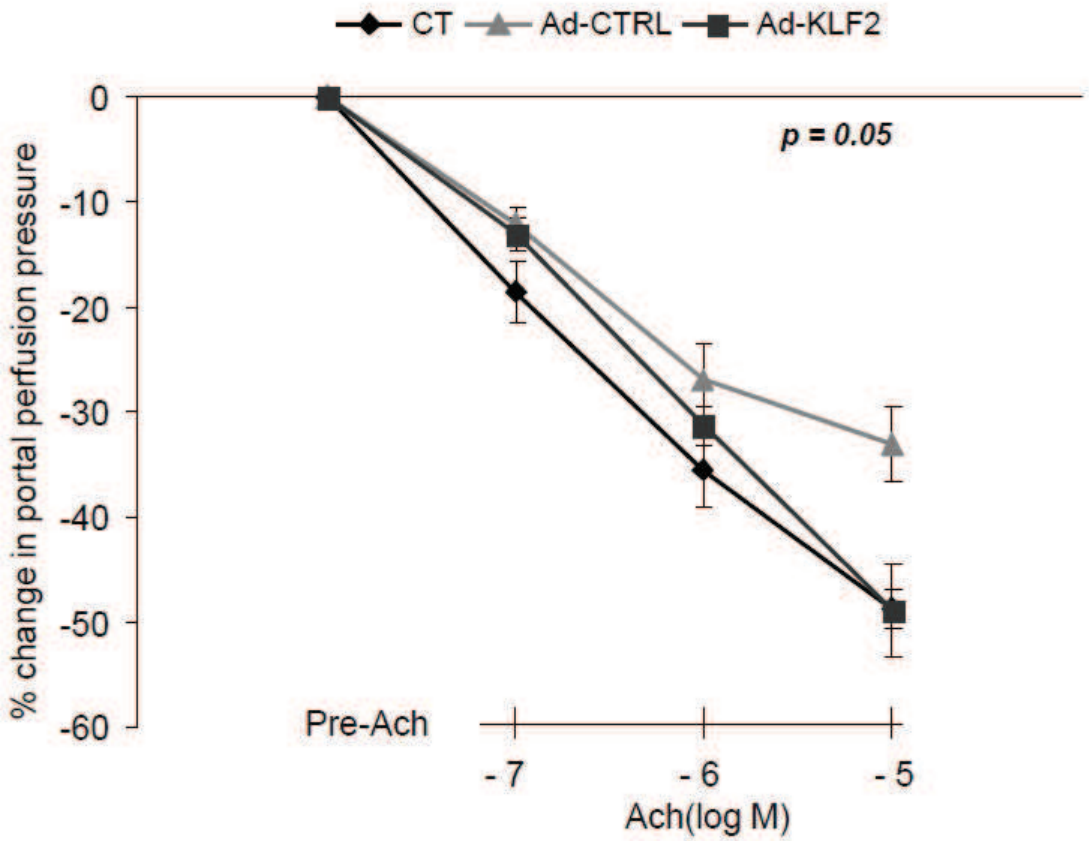
6C


Ad-CTRL
Ad-KLF2
PBF (mL/min*100g bw)
0
1
2
3
4
5

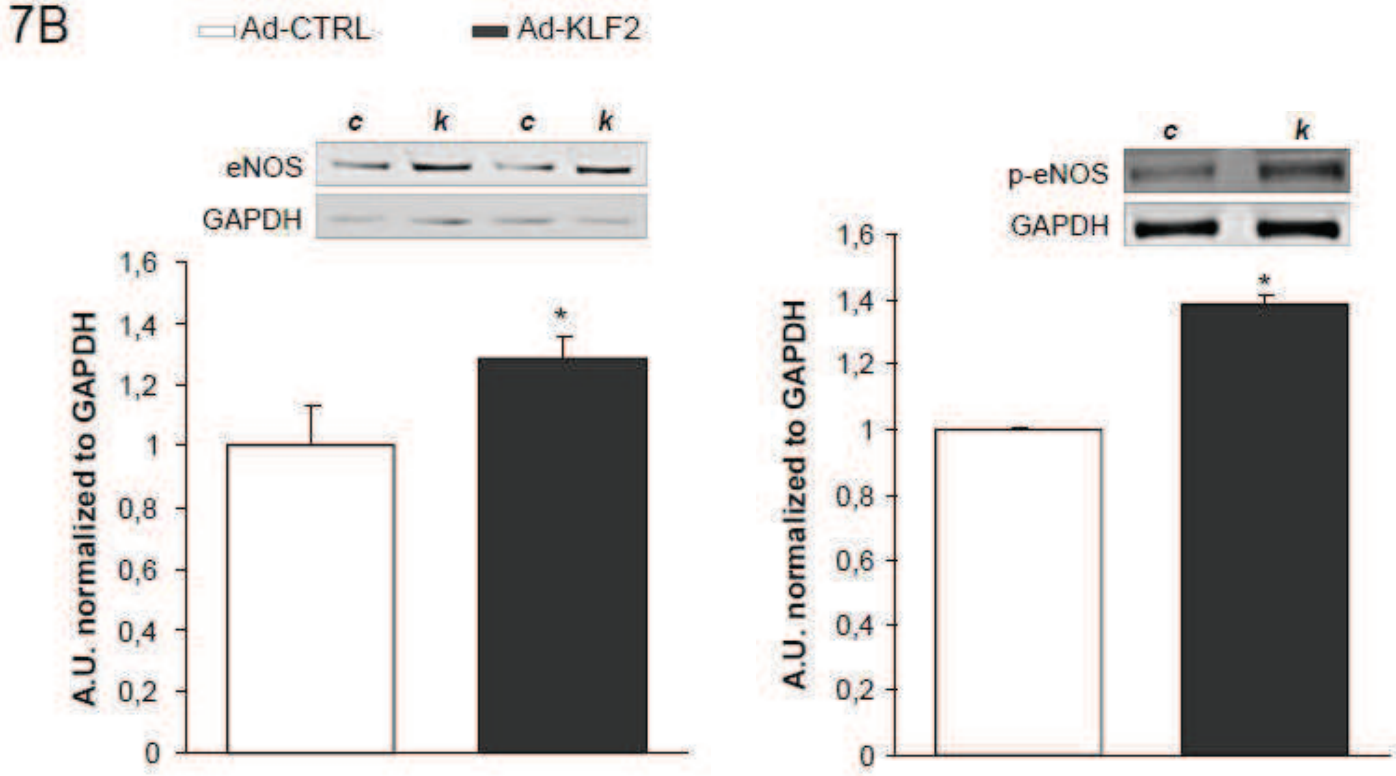
6D


Ad-CTRL
Ad-KLF2
IHVR (mmHg*min/mL*g)
0
2
4
6
8
10

7A


CT
Ad-CTRL
Ad-KLF2
p = 0.05
% change in portal perfusion pressure
0
-10
-20
-30
-40
-50
-60
Pre-Ach
- 7
- 6
- 5
Ach(log M)

7B


Ad-CTRL
Ad-KLF2
c
k
c
k
eNOS
GAPDH
A.U. normalized to GAPDH
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
c
k
p-eNOS
GAPDH
A.U. normalized to GAPDH
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*

Supplementary Fig. 1. Simvastatin maintains HSC phenotype amelioration after 72h of treatment. (**A**) KLF2 and α-SMA expression in response to 72h of simvastatin in LX-2. Inserts show a representative western blot of α-SMA with its percentage of decrease (↓), normalized to GAPDH, compared to vehicle-cells. (**B**) Time dependent regulation of KLF2, α-SMA and pro-collagen I expression in response to simvastatin in cirrhotic rat HSC. Left y-axis indicates KLF2 fold induction. Right y-axis indicates mRNA changes of the HSC activation markers. n=2 per condition. $^{*}p < 0.001$ vs. all.

Supplementary Fig. 2. KLF2 inhibition in rHSC. Effects of KLF2 knock-down using GGPP on HSC phenotype in response to simvastatin. Left y-axis indicates KLF2 fold induction. Right y-axis indicates mRNA changes of the activation markers. n=3 per condition, $^{*}p< 0.01$ vs. vehicle-cells, $^{\#}p<0.01$ vs. simvastatin

Supplementary Fig. 3. Efficacy of hepatic KLF2 up-regulation after systemic administration of adenovirus codifying for KLF2 (Ad-KLF2) to cirrhotic animals. (**A**) Relative KLF2 mRNA expression in livers from cirrhotic animals previously treated with a unique dose of Ad-KLF2, and compared to rats receiving control adenovirus (Ad-CTRL). (**B**) *Left*, representative western blot of KLF2 with its densitometric analysis normalized to GAPDH. *Right*, representative histological images of liver tissues immunostained for the adenovirus marker green fluorescence protein in rats described in A. (10X). n=10 animals per group. $^{*}p < 0.05$ vs. Ad-CTRL.

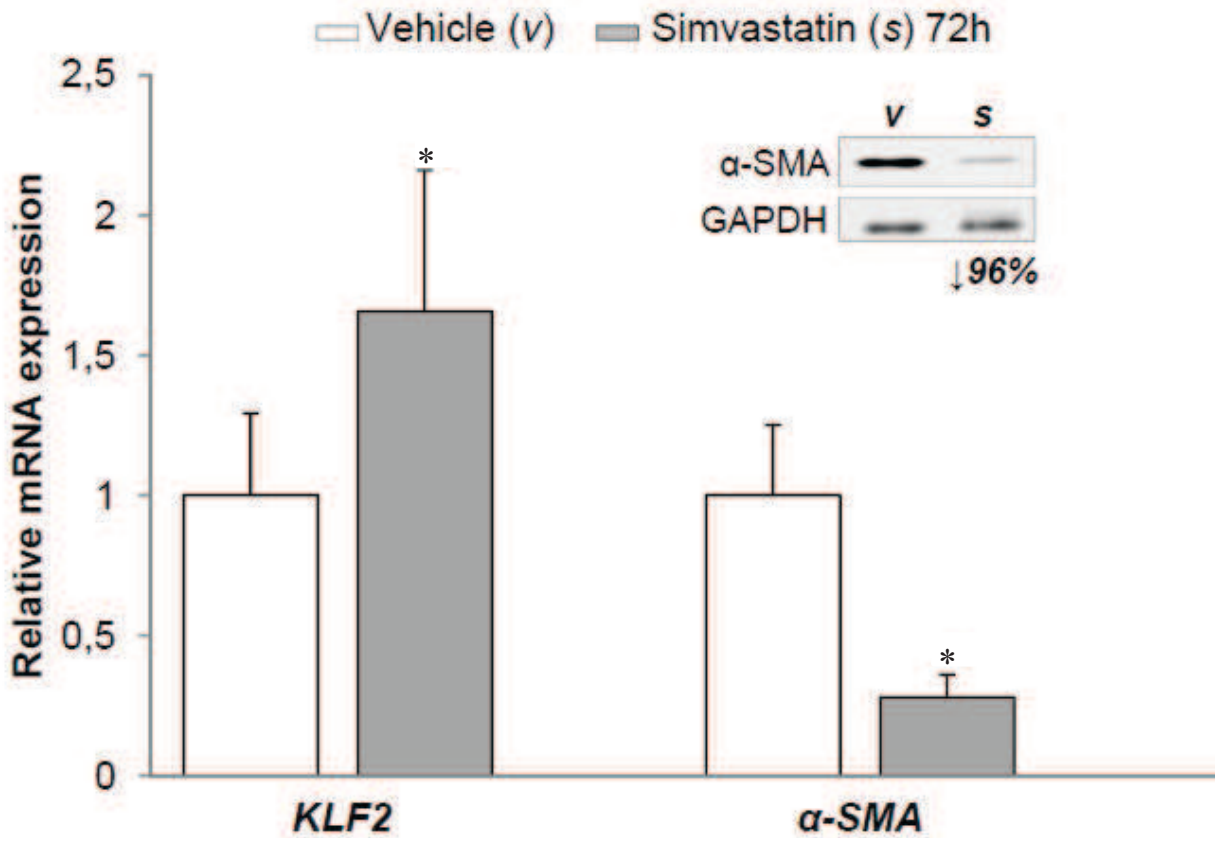
Supplementary Fig. 4. KLF2 mediates HSC apoptosis due to simvastatin treatment. *Top*, representative fluorescence images of acridine orange, marker of viable cells (diffuse staining) and apoptotic cells (nuclear condensate staining), in LX-2 treated for 24h with simvastatin, or vehicle, in presence/absence of GGPP. *Bottom*, % of apoptotic cells normalized to total number of cells from 3 independent experiments. HSC death due to necrosis was not detected. $^{*}p< 0.05$ vs. all.

Supplementary Fig. 5. KLF2 activates the Nrf2 pathway. (**A**) Relative mRNA expression of HO-1, one target of the Nrf2 mediated pathway, in LX-2 infected with adenovirus codifying for KLF2 (Ad-KLF2) or control adenovirus (Ad-CTRL), and in HSC isolated from cirrhotic rats (rHSC) previously infected with Ad-KLF2 or Ad-CTRL. n=3 per condition. $^{*}p< 0.05$ vs. Ad-CTRL. (**B**) Representative western blots and

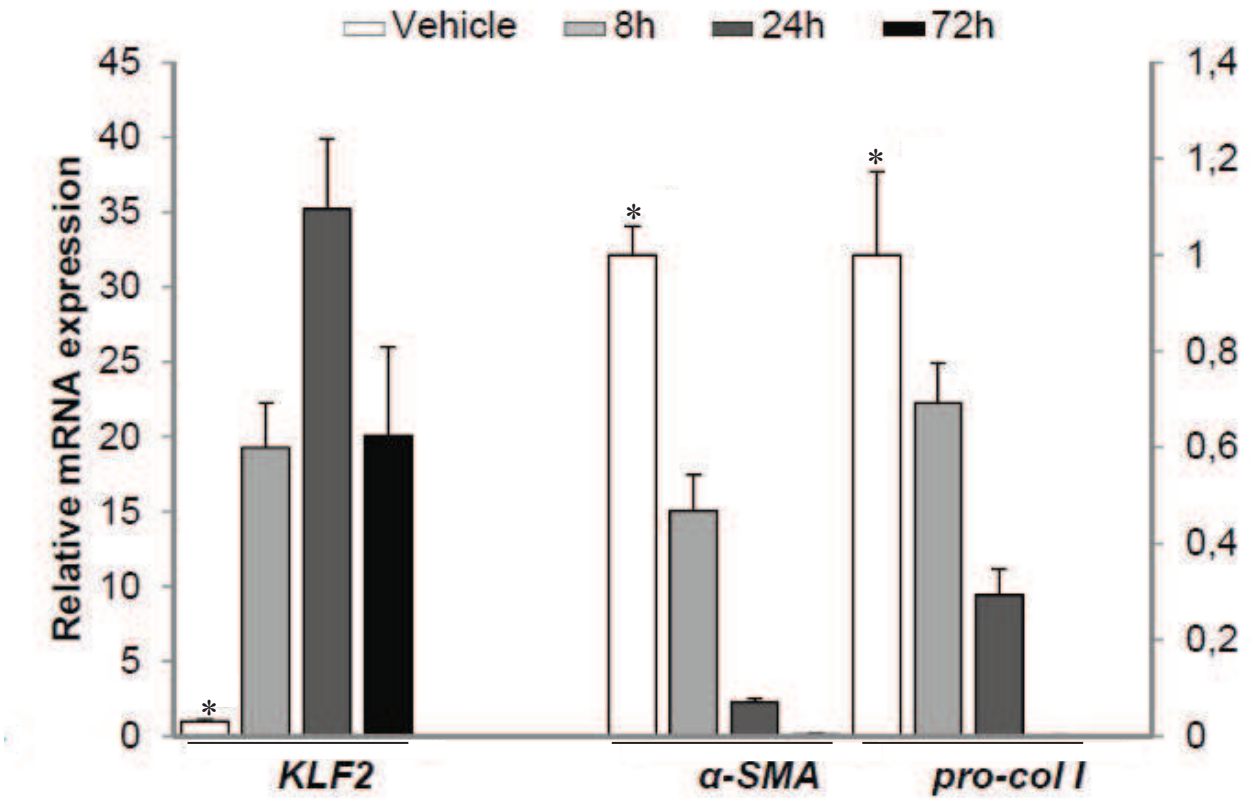
densitometric quantifications of depicted proteins, with their corresponding housekeeping controls, determined in livers from cirrhotic rats treated with simvastatin (*s*) or vehicle (*v*) for 3 days. n=6 per group. $^{*}p< 0.05$ vs. vehicle.

Supplementary Fig. 6. Evidence of liver sinusoidal endothelial cells (LSEC) capillarization *in vitro*. Relative eNOS and endothelin-1 mRNA expression in primary LSEC after 2h of culture (differentiated) or 72h (de-differentiated). Left y-axis indicates eNOS while right y-axis indicates endothelin-1. Data derive from 3 independent experiments. $^{*}p< 0.05$ vs. differentiated LSEC.

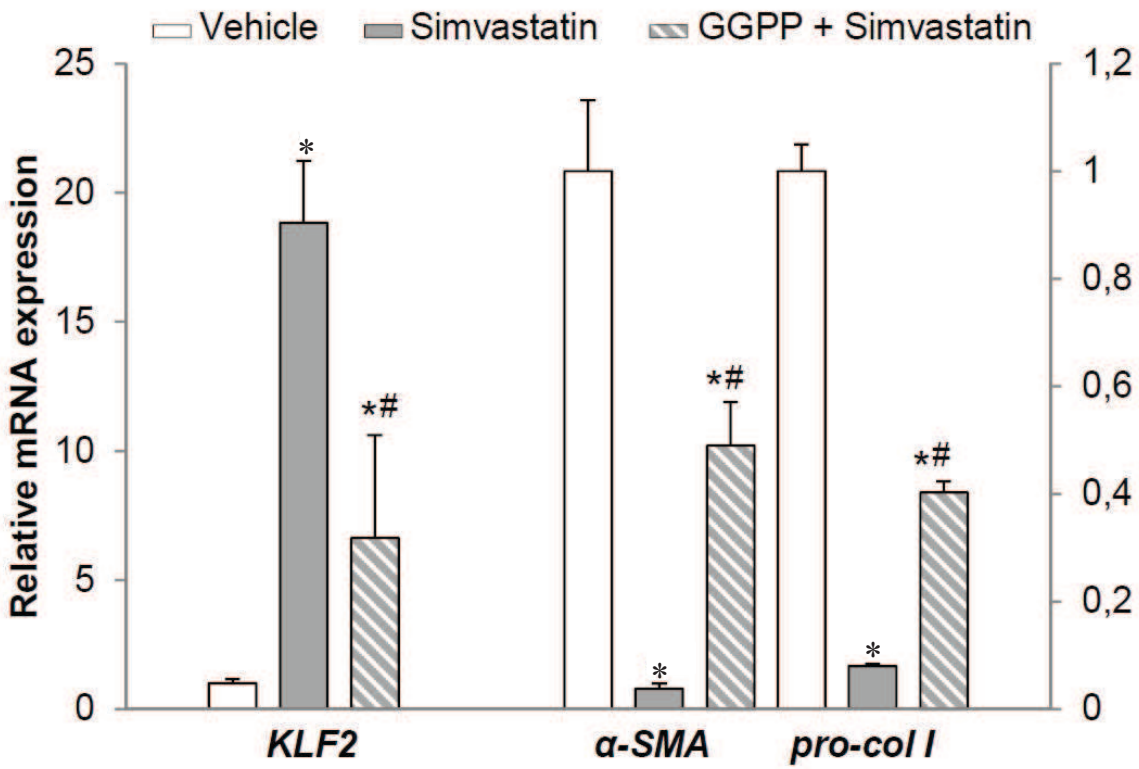
Suppl. 1A



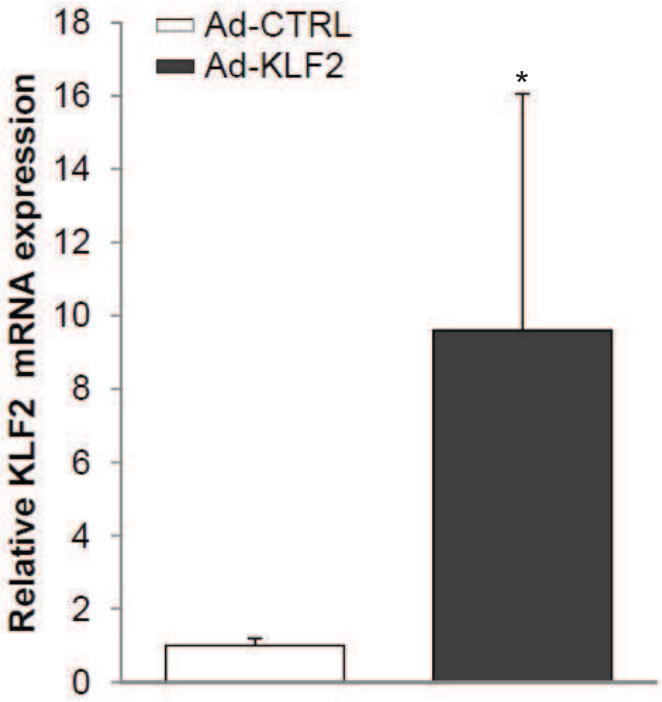
Suppl. 1B



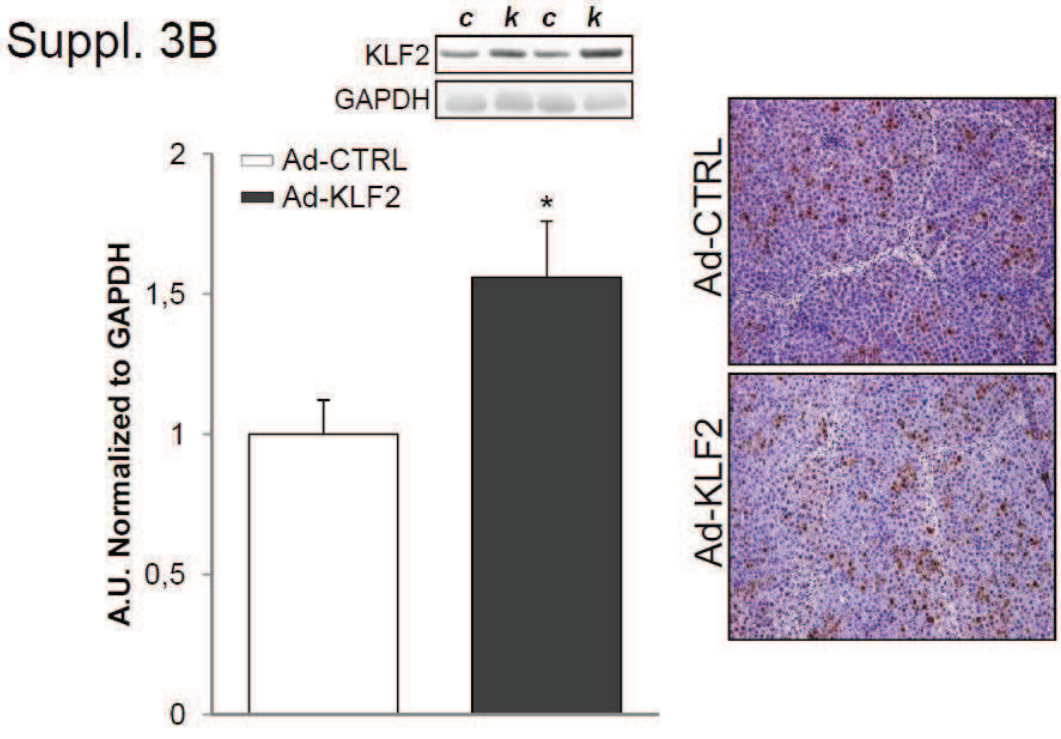
Suppl. 2


Vehicle
Simvastatin
GGPP + Simvastatin
Relative mRNA expression
25
20
15
10
5
0
1,2
1
0,8
0,6
0,4
0,2
0
*
*#
*
*#
*
*#
KLF2
α-SMA
pro-col I

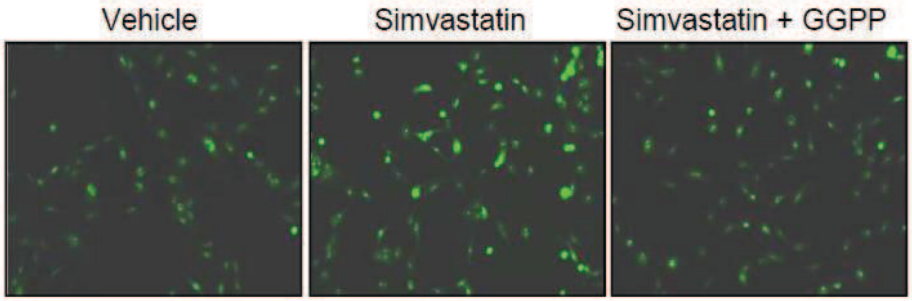
Suppl. 3A

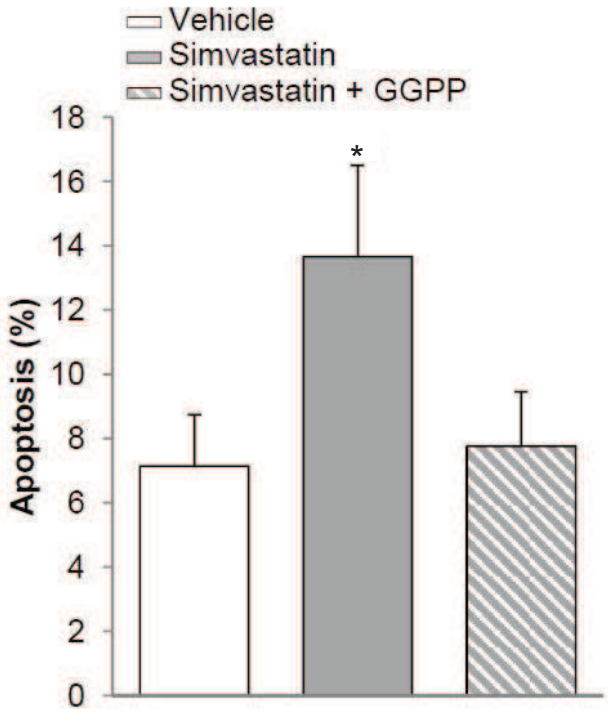


Suppl. 3B

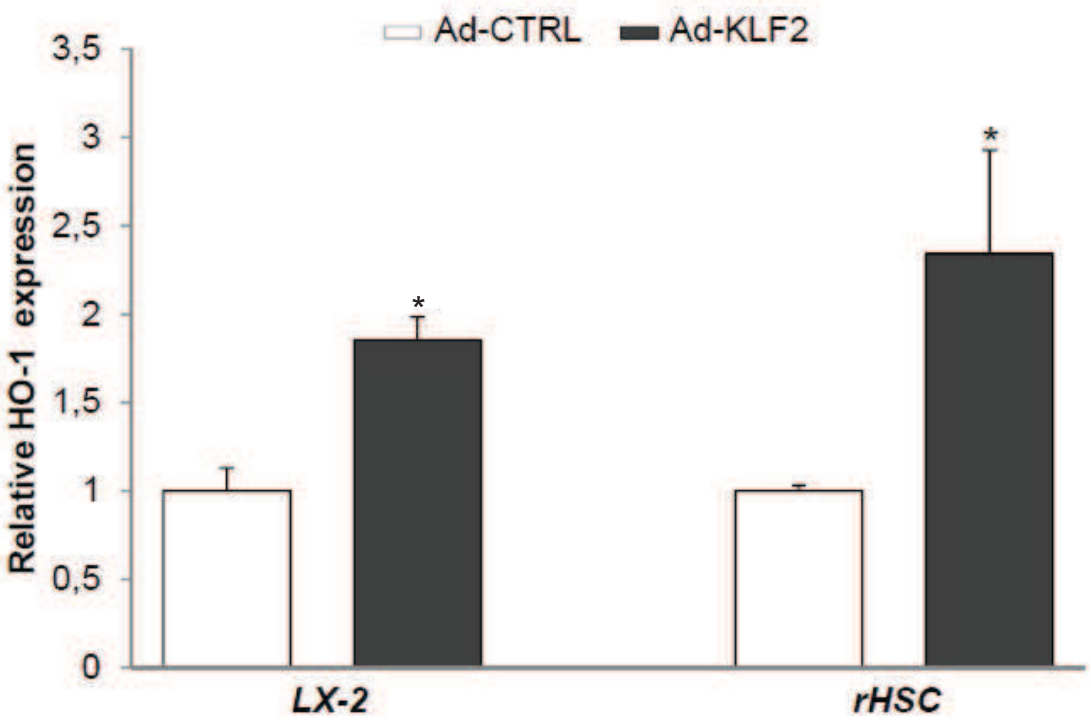


Suppl. 4

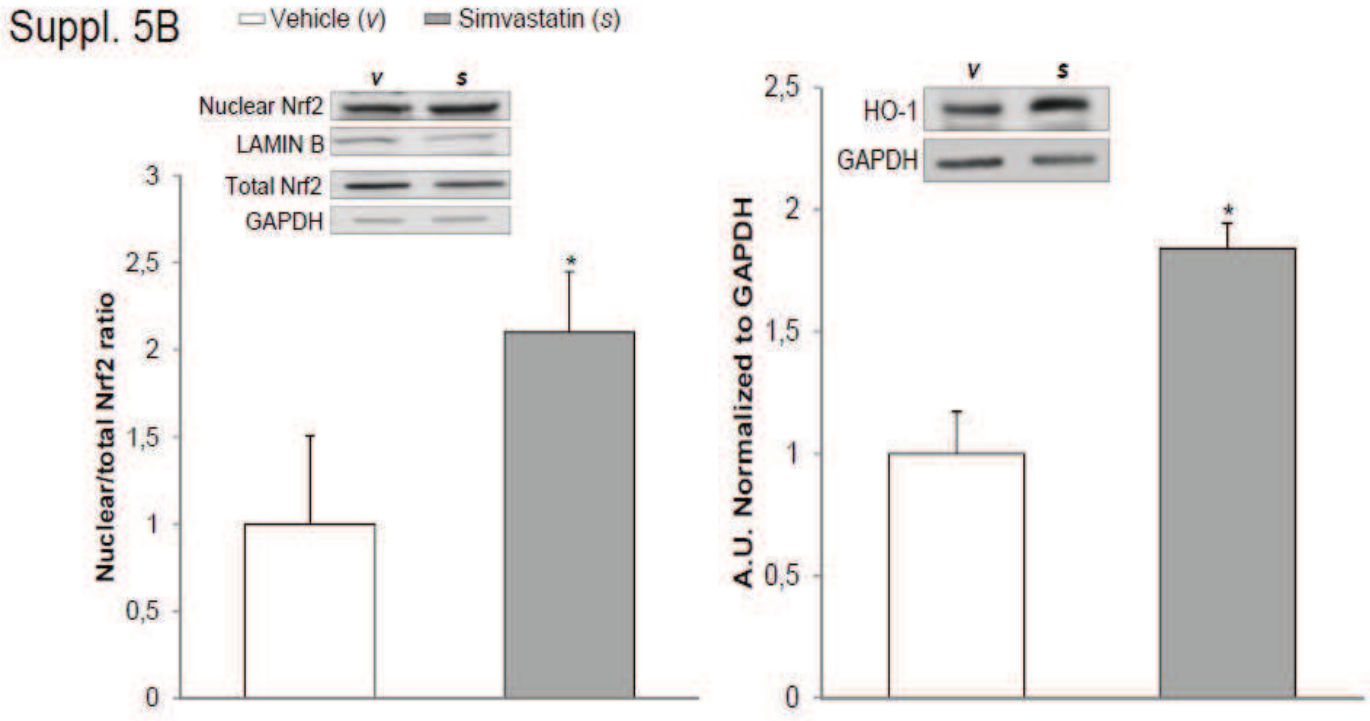

Vehicle
Simvastatin
Simvastatin + GGPP


Vehicle
Simvastatin
Simvastatin + GGPP
Apoptosis (%)
0
2
4
6
8
10
12
14
16
18
*

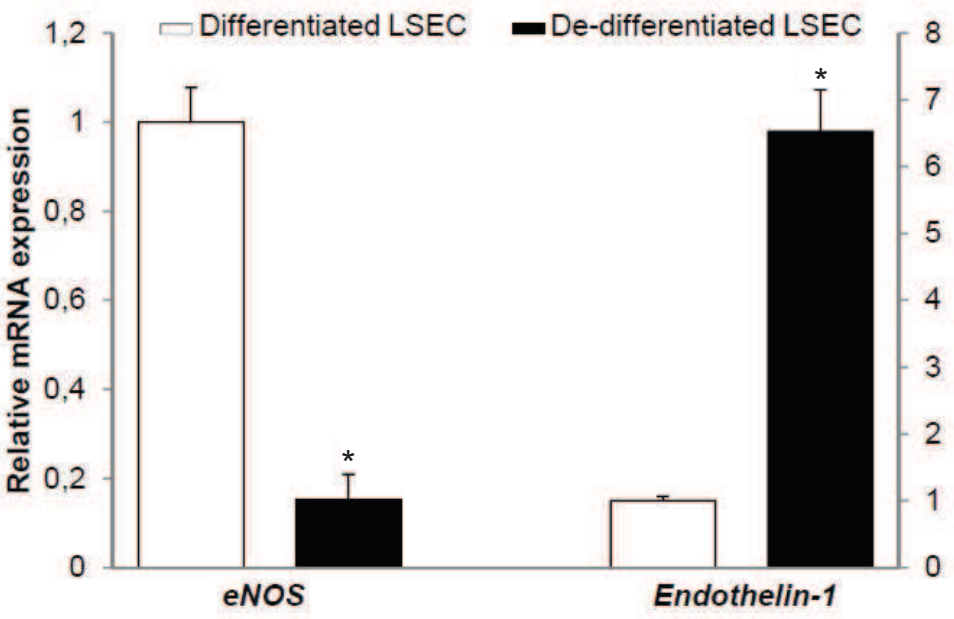
Suppl. 5A



Suppl. 5B



Suppl. 6


Differentiated LSEC
De-differentiated LSEC
Relative mRNA expression
1,2
1
0,8
0,6
0,4
0,2
0
8
7
6
5
4
3
2
1
0
*
*
eNOS
Endothelin-1

Supplementary methods

Experimental models of liver cirrhosis

CCl_4-derived and CDBL cirrhosis were performed as previously described (1,2).

HSC isolation

HSC from cirrhotic rats were isolated through a sequential *in situ* perfusion of the liver with 0.195 mg/mL collagenase A (Roche), 1.5 mg/mL pronase (Roche) and 0.05 mg/mL Dnase (Roche) in Gey's Balanced Salt Solution (GBSS; Sigma), and a digestion *ex vivo* with GBSS containing 0.13 mg/mL collagenase A, 0.4 mg/mL pronase and 0.1 mg/mL Dnase. Dispersed cells were fractionated by density gradient centrifugation using 22% Histodenz™ (Sigma) and cultured in Iscove's Modified Dulbecco's Media (IMDM, Invitrogen, Gibco) supplemented with 10% fetal bovine serum, 1% glutamine, 1% antibiotic solution and with 1% amphotericin B (Reactiva). Viability and purity were systematically over 95%.

KLF2 inhibition

HSC were transduced at a 40%-70% confluency with siRNA targeting KLF2 (50nM, s20270 for LX-2; s157429 for rHSC, Life Technologies), or with a control siRNA (50nM, 4390843, Life Technologies) using siPORT™ NeoFX™ transfection agent (Life Technologies) according to the manufacturer's instructions. At 42 hours post-transfection, cells were treated for an additional 24 hours with either 10μM simvastatin, or vehicle, and then harvested for RNA/protein extraction. For pharmacological inhibition, HSC were treated with either vehicle (0.05% ethanol) or 100μM mevalonate for 1 h, afterwards 10μM simvastatin, or vehicle, was added and cells were incubated for additional 24h. 10μM geranylgeranyl pyrophosphate (GGPP), or its vehicle (methanol), was added simultaneously with simvastatin for 24h.

Acridine Orange and Propidium Iodide staining

Cells were incubated with fresh medium containing 800ng/mL Acridine Orange (AO) and 5μg/mL of Propidium Iodide (PI) for 10min at 37ºC and then washed with PBS to eliminate the unincorporated dye. Fresh medium without phenol red was added and cell death was examined using a fluorescence microscope (Olympus BX51, Tokyo, Japan) equipped with a digital camera (Olympus, DP72). AO is a metachromatic dye that stains both viable and apoptotic cells by intercalate into DNA and emits green fluorescence

upon excitation at 480-490 nm. Nevertheless, nuclear condensation that occurs during apoptosis glares a more intense fluorescence. PI is excluded by viable cells but can penetrate cell membranes of dying or dead cells due to necrosis, emitting red fluorescence (3,4). Positive controls (starvation for apoptosis, medium dryness for necrosis), and negative controls (without dye) were included.

Cell Viability and Proliferation

Equal number of LX-2 were seeded and after 24h of simvastatin treatment, or vehicle, floating and adhered cells were collected and counted using trypan blue staining (FLUKA). Cell viability was expressed as % of viable cells vs. total number of cells after treatment. Cell proliferation was calculated as % of total number of cells after treatment vs. initial number of seeded cells.

Immunohistochemistry for GFP

Hepatic adenovirus infection was confirmed in all rats included in the study by immunostaining of paraffin-embedded liver sections with a rabbit polyclonal antibody for GFP. Briefly, sections were treated twice with PBS containing 0.3% hydrogen peroxide, blocked with 5% horse serum for 1h at RT and incubated with the primary antibody (Abcam). Bound antibodies were visualized using diaminobenzidine as the chromogen (Dako), and slides were then counterstained with hematoxylin solution for 1min before being mounted and examined using light microscopy (Zeiss Axiovert) for a qualitative analysis. For the negative control, PBS was used instead of the primary antibody.

Superoxide (O_2^-) detection

HSC were washed in medium without phenol red and loaded with dihydroethidium (DHE; Molecular Probes Inc.; 10 μM for 20 min 37°C). After that, cells were rinsed with PBS and kept in the dark before obtaining fluorescence images (Olympus BX51 fluorescence microscope, Tokyo, Japan; Olympus digital camera, DP72). Quantitative analysis was obtained by averaging of the peak relative fluorescent intensity (optical density arbitrary units) of each image (Image J 1.43m software, National Institutes of Health) and normalization of the fluorescent result by the total number of cultured cells. Specificity controls (exogenous SOD co-incubation) were included.

RNA isolation and Quantitative Real-Time PCR

RNA isolation, reverse transcription and quantification by RT-PCR (ABI PRISM® 7900HT Fast Real-Time PCR System) were performed as described previously (5). Primers for RT-PCR were obtained from Applied Biosystems. 18S or GAPDH served as endogenous controls. Results, expressed as $2^{-\Delta\Delta Ct}$, represent the x-fold increase of gene expression compared with the corresponding control group.

Western blot analysis

Cells were lysed using triton-lysis buffer for whole protein extraction. Livers were collected, snap frozen in liquid nitrogen and homogenized in triton-lysis buffer for whole protein extraction. For nuclear protein extraction, 8 volumes of a cytoplasmic protein extraction buffer (CPEB: 10mM Tris-HCl pH 7.0, 10mM Tris-HCl pH 8.0, 150mM NaCl, 5mM $MgCl_2$, 1mM DTT, 0.5% Triton X-100 and protease inhibitors) were added to ≈30 mg of liver samples and kept 10 minutes on ice. After that, the samples were centrifuged at 2500rpm for 3 minutes at 4ºC. The supernatant represented the cytoplasmatic proteins and was washed, centrifuged again at 13000rpm for 15 minutes at 4ºC and recovered for the quantifications. Four volumes of a nuclear proteins extraction buffer (NPEB: 10mM Tris-HCl pH 7.0, 10mM Tris-HCl pH 8.0, 50mM KCl, 400mM NaCl, 1 mMEDTA, 1mM DTT, 0.5% Triton X-100, 20% Glycerol and protease inhibitors) were added to the nuclear pellet and kept on ice for 40 minutes before centrifuged at 12000rpm for 10 minutes at 4ºC. The supernatant containing the nuclear proteins was subsequently quantified. Aliquots from each sample containing equal amounts of protein (50μg) were run on a sodium dodecylsulphate polyacrylamide gel, and transferred to a nitrocellulose membrane. After the transfer, the blots were subsequently blocked for 2h with Tris buffered saline containing 0.05% (vol/vol) Tween 20 and 5% (wt/vol) non-fat dry milk or 5% albumin fraction V (for nuclear extracts) and subsequently incubated with primary antibodies overnight at 4ºC. Then membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. Primary antibodies for KLF2 (N-13, Santa Cruz Biotechnology), eNOS (BD Transduction Laboratories, Lexington, KY), phosphorylated eNOS (Ser1176, P-eNOS, Cell Signaling), α-SMA (Sigma), Nrf2 (C-20, Santa Cruz Biotechnology), Hmox-1 (Hsp31, Enzo Life Sciences), moesin (E-10, Santa Cruz Biotechnology), phosphorylated moesin (Thr588, P-moesin, Santa Cruz Biotechnology), Bad (Cell Signaling), phosphorylated Bad (Ser112, P-Bad, Cell Signaling), cleaved caspase-3 (Asp175, Cell Signaling) and GAPDH (Santa Cruz

Biotechnology) or Lamin B (Santa Cruz Biotechnology), as standardization of sample loading, were used. Protein expression was determined by densitometric analysis using the ImageQuant™ LAS4000 (GE Healthcare). Quantitative densitometric values of all proteins were normalized to GAPDH or Lamin B.

Sirius Red staining and Immunohistochemistry

Livers were fixed in 10% formaldehyde, embedded in paraffin, sectioned and stained with 0.1% Sirius Red, photographed, and analyzed using a microscope equipped with a digital camera. Ten fields from each slide were randomly selected, and the red-stained area per total area was measured using AxioVision software. For immunostaining, liver sections were processed as described above and stained using a α-SMA (Dako) and desim (Dako) antibodies. Fifteen fields were counted in each liver by two blinded observers. The relative volume was calculated by dividing the number of points over that particular cell type by the total number of points over liver tissue. Results are normalized to vehicle/Ad-CTRL.

In vivo hemodynamics

Rats were anesthetized with ketamine hydrochloride (100 mg/kg; Merial Laboratories, Barcelona, Spain) plus midazolam (5 mg/kg; Laboratorios Reig Jofré, Barcelona, Spain) intraperitoneally. A tracheotomy was performed and a polyethylene tube PE-240 was inserted into the trachea to ensure a patent airway. PE-50 catheters were introduced into the femoral artery to measure mean arterial pressure (MAP; mmHg) and into the ileocolic vein to measure portal pressure (PP, mmHg). Perivascular ultrasonic flow probes connected to a flow meter (Transonic Systems Inc., Ithaca, NY, USA) were placed around the portal vein, as close as possible to the liver to avoid portal-collateral blood flow, in order to measure portal blood flow (PBF; ml min^{-1}) going through the liver, and at the superior mesenteric artery to measure superior mesenteric artery blood flow (SMABF; ml min^{-1}). Blood pressures and flows were registered on a multichannel computer-based recorder (PowerLab; AD Instruments, Colorado Springs, CO). The temperature of the animals was maintained at 37 ± 0.5 ºC and hemodynamic data were collected after a 20 min stabilization period.

Liver endothelial function

Rat livers were isolated and perfused at 35mL/min with Krebs buffer. The perfused rat liver preparation was allowed to stabilize for 20 min before vasoactive substances were added. The intrahepatic microcirculation was pre-constricted by adding the α1-

adrenergic agonist methoxamine (Mtx; 10^{-4}M; Sigma) to the reservoir. After 5 min, concentration–response curves to cumulative doses of acetylcholine (Ach; 10^{-7} - 10^{-5}M; Sigma) were evaluated. The concentration of Ach was increased by 1log unit every 1.5 min interval. Responses to Ach were calculated as the percentage change in portal perfusion pressure. The gross appearance of the liver, stable perfusion pressure, bile production over 0.4µl/min/g of liver and a stable buffer pH (7.4 ± 0.5) were monitored during this period. If any viability or stability criteria were not satisfied, the experiment was discarded.

Supplementary references

1. Gracia-Sancho J. et al. Endothelial expression of transcription factor Kruppel-like factor 2 and its vasoprotective target genes in the normal and cirrhotic rat liver. Gut 2011;60:517-524.

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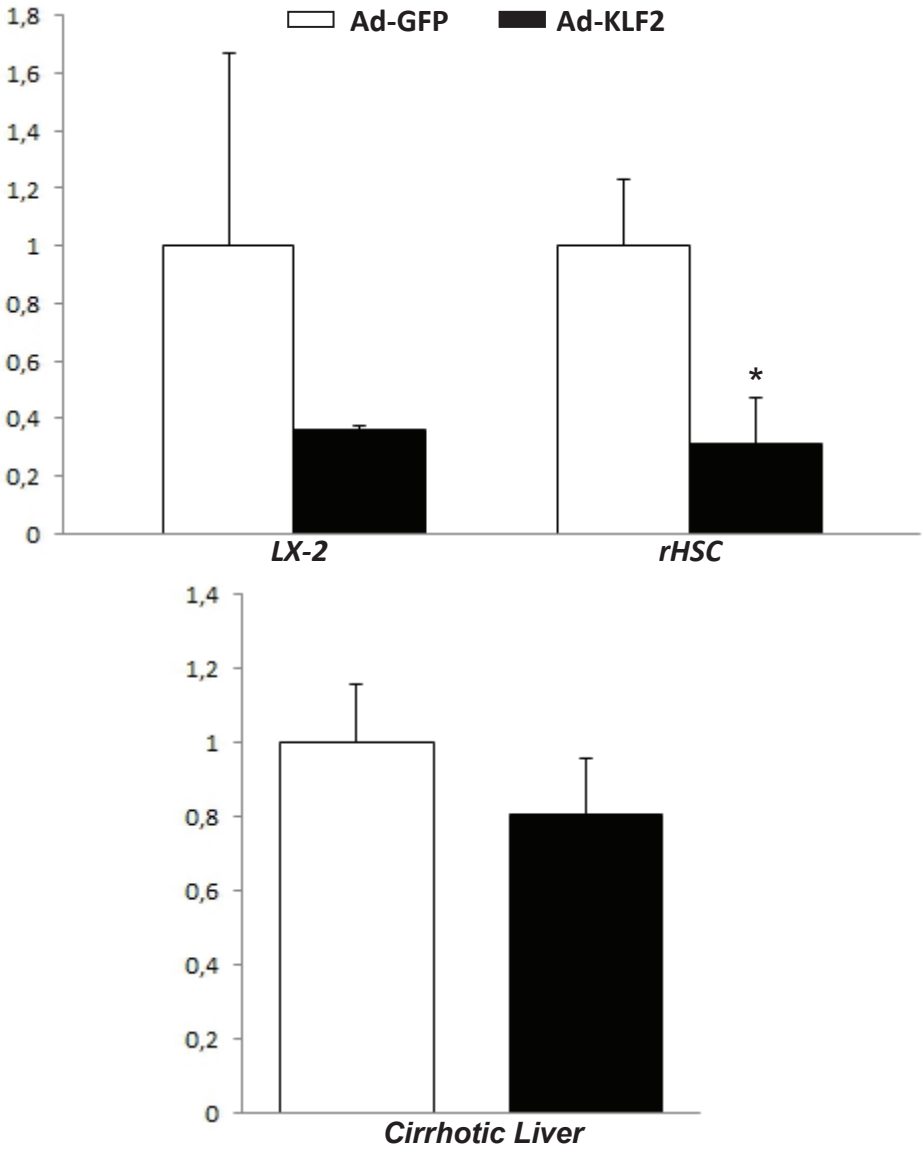
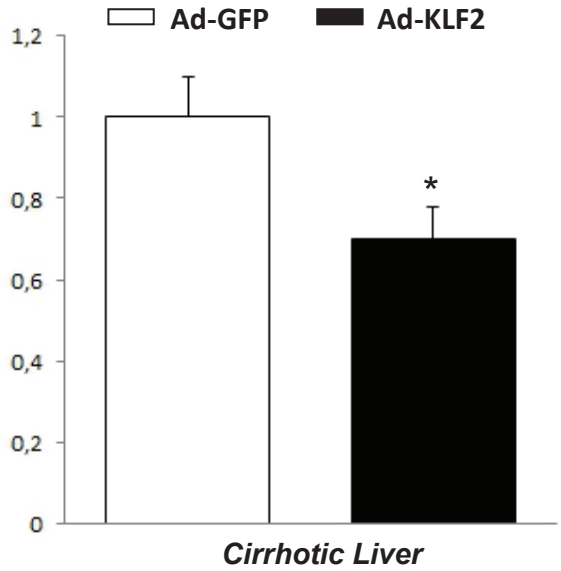
Supplementary Ph.D. thesis Figure 1: TGFβ mRNA relative expression



Supplementary Ph.D. thesis Figure 2: NFκB mRNA relative expression



Summary of results

Summary of results

Study 1. Leptin receptor blockade reduces intrahepatic vascular resistance and portal pressure in an experimental model of rat liver cirrhosis.

- Serum leptin levels and hepatic expression of the leptin receptor (ObR) are increased in cirrhotic animals, in comparison to control rats.
- The hepatic blockade of the ObR in cirrhotic rats translates into a 19% decrease in PP, without modification in systemic hemodynamics.
- Cirrhotic animals in which the ObR is blockade exhibit an increment in NO bioavailability, expressed as an increment in the levels of its markers cGMP and p-VASP, derived from a reduction in the levels of the NO scavenger O_2^-.
- Cirrhotic animals receiving the ObR-Ab show a decrease in pro-collage I mRNA levels, but no changes in fibrotic tissue area percentage or α-SMA protein expression is reported, in comparison to vehicle-cirrhotic animals.

Study 2. The transcription factor KLF2 mediates hepatic endothelial protection and paracrine endothelial–stellate cell deactivation induced by statins.

- Four different statins induce KLF2 expression in LSEC. Simvastatin is the most effective one.
- Simvastatin activates the KLF2-mediated vasoprotective pathway in LSEC, expressed as an induction in eNOS and TM expression.
- The knockdown of KLF2 in LSEC, obtained using specific siRNA or isoprenoids, inhibits eNOS and TM induction due to simvastatin treatment.
- Simvastatin enhances shear stress up-regulation of KLF2 and its target genes, eNOS and TM, in LSEC isolated from normal or cirrhotic animals, compared to LSEC cultured under static conditions.
- KLF2 up-regulation in LSEC, due to simvastatin treatment or KLF2-adenoviruses administration, paracrinally improves the HSC phenotype, as shown by a reduction in the expression of the HSC activation marker α-SMA. This improvement partly depends on a KLF2-NO-cGMP mediated pathway.

Study 3. KLF2 exerts anti-fibrotic and vasoprotective effects in cirrhotic rat livers: behind the molecular mechanisms of statins.

- Simvastatin induces KLF2 and reduces α-SMA and pro-collagen I expression in HSC. The same effects are obtained with the administration of an adenovirus-codifying for this transcription factor (Ad-KLF2).

- HSC-KLF2-knockdown, obtained using specific siRNA against KLF2, does not abolish the beneficial effect of simvastatin on HSC phenotype. In these KLF2-knocked-down-HSC, simvastatin concomitantly induces KLF4 and KLF6 expression.

- HSC-KLF2-knockdown, obtained using isoprenoids, does attenuate the amelioration in HSC phenotype due to simvastatin treatment.

- Cirrhotic rats infected with Ad-KLF2 or treated with simvastatin exhibit a marked induction in KLF2 expression and a reduction in liver fibrosis (sirius red and α-SMA), compared to cirrhotic animals receiving Ad-CTRL. In addition, Ad-KLF2 administration leads to an induction in cleaved caspase-3 protein expression and a reduction in p-moesin/moesin and p-bad/bad ratio.

- KLF2 up-regulation is also associated with a marked increase in HSC apoptosis, as demonstrated in Ad-KLF2 treated cirrhotic animals (desmin) and in simvastatin treated HSC (acridine orange). KLF2 knockdown in HSC prevents the apoptotic effects of simvastatin.

- KLF2 over-expression (due to simvastatin or Ad-KLF2) activates the antioxidant Nrf2-mediated pathway, expressed as an induction in Nrf2 targets genes (HO-1 and NQO1), both in HSC and in cirrhotic animals. HSC-KLF2-knockdown, obtained using isoprenoids, attenuates or even inhibits the reduction in O_2^- levels and the activation of the Nrf2-mediated pathway due to simvastatin treatment.

- *In vitro* de-differentiated (dysfunctional) rat LSEC exhibit an increment in endothelin-1 and a reduction in eNOS mRNA levels, compared to differentiated (functional) rat LSEC. These de-differentiated LSEC produce more VEGF and show an improvement in their phenotype, expressed as a reduction in endothelin-1 and an induction in eNOS, when co-cultured with KLF2-over-expressing HSC.

- Simvastatin enhances shear stress up-regulation of KLF2 in de-differentiated LSEC. This amelioration of LSEC phenotype leads to an improvement in HSC
- KLF2 over-expression in cirrhotic animals through Ad-KLF2 administration translates into a 15% decrease in PP, without changes in systemic haemodynamics. Moreover, basal *ex vivo* intrahepatic vascular resistance is significantly lower in cirrhotic rats receiving Ad-KLF2, in which there is also an improvement in endothelial dysfunction, in comparison to Ad-CTRL cirrhotic animals.

- KLF2 over-expressing cirrhotic animals exhibit an induction in eNOS and p-eNOS protein expression, compared to cirrhotic animals receiving Ad-CTRL.

Discussion

Discussion

Portal hypertension complications (mainly bleeding from ruptured esophagogastric varices) are the first cause of death in about one third of patients with decompensated cirrhosis (237). Pathophysiologically, this syndrome may be improved by two strategies, amelioration of intrahepatic vascular resistance (IHVR) or reduction of portal blood flow (PBF). An early intervention to stabilize disease progression, and to avoid or delay clinical decompensation could be of great advantage in the management of cirrhosis.

Two different components contribute to increase the IHVR that leads to the development of cirrhotic portal hypertension: a mechanical component (fibrogenesis, angiogenesis, vascular occlusion and sinusoidal capillarisation) and a dynamic component (functional abnormalities mainly derived by increased production and response to vasoconstrictors such as TXA_2 and ET-1, and low NO bioavailability). As discussed in previous sections of this Ph.D. thesis, the functions of the liver sinusoid are impaired in cirrhosis, in particular the regulation of the hepatic vascular tone and of the hepatic oxidative stress. This explains part of the pathophysiological mechanisms that are the basis of the dynamic component of the increased hepatic vascular resistance.

Substantial progress has been made over the last 40 years in understanding the pathophysiology of portal hypertension and in the attempt of develop new possible pharmacological therapies. Although many advances have been made in this field, non-selective β-blockers remain the cornerstone of therapy in cirrhotic patients with portal hypertension. For this reason, identification of key molecules that have a role in impairing hepatic sinusoidal functions or that are involved in the modulation of the microvascular environment are highly necessary to find new molecular targets for therapy, and are the main topic of this Ph.D. thesis.

The results of the **study 1** provide the first evidence regarding the involvement of leptin in the pathogenesis of portal hypertension in the CCl_4-model of cirrhosis, demonstrating for the first time that the blockade of the receptor of leptin leads to a reduction in portal pressure due to an amelioration in the dynamic component of the IHVR, with no deleterious effects on mean arterial pressure or in liver blood test.

Hyperleptinemia is characteristic of non-alcoholic fatty liver disease (NAFLD) (238) and of non-alcoholic steatohepatisis (NASH) (239), conditions indeed related to obesity. However, since serum leptin levels have been found increased in patients with different causes of liver cirrhosis (185, 186, 240, 241), it has been demonstrated that this hormone is essential to the aggravation

of hepatic fibrosis and development of cirrhosis (188, 195), and regulates the hepatic vascular tone in a model of cirrhotic NASH (242), we decided to evaluate if the blockade of leptin signaling could affect the hemodynamic parameters in cirrhotic portal hypertension.

In this study, we first observed that cirrhotic animals expressed elevated serum levels of leptin, mirroring what has been described in human cirrhosis, and exhibited increased hepatic expression of the leptin receptor ObR, compared to control animals. We then showed that the blockade of the ObR in cirrhotic rats, using an ObR-Ab, determined a 19% decrease in the portal pressure, compared to cirrhotic animals receiving vehicle, that derived from a reduction in the IHVR. This marked improvement in portal hypertension seemed to be related to the dynamic component of IHVR, in particular to reduced hepatic vascular tone. Indeed, the ObR-Ab administration produced a significant increase in hepatic NO bioavailability (increased cGMP levels and p-VASP protein), mostly due to blunted scavenge by O_2^- (that resulted indeed decreased in ObR-Ab-treated animals, compared to vehicle-IgG cirrhotic animals) than by an increased eNOS expression or activity (no changes in eNOS, p-eNOS or eNOS monomer/dimer expression between cirrhotic animals receiving the ObR-Ab or IgG). The decreased production of O_2^-, and the consequently reduced NO scavenging in ObR-Ab cirrhotic rats, translated into a decreased production of peroxynitrites and an amelioration of oxidative stress. How leptin produce O_2^- in cirrhotic livers is unknown. It is possible that it may activate a pro-oxidant mechanism, most likely by further decreasing hepatic superoxide dismutase activity in sinusoidal endothelial cells, since eNOS uncoupling seems to have no role and NADPH oxidase is not the source of O_2^- in cirrhosis (174).

Although this study focus on the dynamic component of the increased IHVR, the amelioration of portal hypertension in cirrhotic rats treated with the ObR-Ab could be also due to a possible decreased angiogenesis, which we and others have demonstrated that actively contributes to portal hypertension pathophysiology (243). Indeed it has been reported that leptin receptor blockade may down-regulate VEGF-mediated angiogenesis (244). Nevertheless future studies are required to validate this hypothesis.

Despite the fact that leptin has also been associated with enhanced hepatic fibrosis (195), we observed that 1-week administration of ObR-Ab had no effects on liver fibrosis (same liver collagen deposition and α-SMA expression between cirrhotic animals receiving the ObR-Ab or IgG), further pointing out that the amelioration of cirrhotic portal hypertension, obtained blocking the ObR, derived from the modulation of the dynamic component of IHVR rather than the modulation of architectural distortion due to the fibrotic process. However, our data

demonstrating reduced levels of pro-collagen I mRNA expression in livers from animals receiving ObR-Ab suggest that using higher doses or longer periods of treatment with ObR-Ab may have additional effects reducing also liver fibrosis.

Taken together, our data demonstrate that the blockade of hepatic leptin receptors results in decreased portal pressure that derives from a reduction in IHVR, without modifying systemic hemodynamics. These data suggest that leptin can be a potential target in the treatment of portal hypertension.

The **studies 2 and 3** focus on the underlying molecular mechanisms of statins in healthy and cirrhotic livers, highlighting the role of the transcription factor KLF2 in the liver sinusoid. Up-regulation of KLF2 improves LSEC and HSC phenotypes, and restores the cross-talk between these cells. It ensues an overall amelioration of the liver sinusoidal microenvironment, with less fibrosis and better endothelial function, that leads to amelioration of cirrhotic portal hypertension.

Statins, primarily designed to reduce cholesterol levels, improve endothelial dysfunction, portal pressure, and liver fibrosis in experimental models of hepatic cirrhosis (136, 137, 237). In addition, a decade ago, our group was the first approaching statins pharmacological therapy to cirrhotic patients with portal hypertension, demonstrating their beneficial effects (224). Later on, controlled clinical trials led by our team further supported the use of simvastatin to treat portal hypertension (224, 225).

As explained in the introduction of the present Ph.D. thesis, KLF2 expression can be stimulated by blood flow-derived shear stress and by some chemical compounds, like statins, conferring to endothelial cells a highly vasoprotective phenotype (41, 51, 56, 235, 245). Moreover, although we recently demonstrated that this transcription factor is highly expressed in cirrhotic rats' liver, particularly in the hepatic endothelium (42), and that simvastatin induces the activation of the hepatic KLF2-vasoprotective pathway (42), we hypothesized that this endogenous protective mechanism might be insufficient to prevent liver sinusoidal endothelial dysfunction and microcirculatory impairment in cirrhotic animals. Therefore, that a sustained external up-regulation of KLF2 may provide stronger protection to the liver sinusoid in cirrhosis.

Considering these data, our first objective was to characterize the response of freshly isolated LSEC to four different FDA-approved statins (mevastatin, atorvastatin, simvastatin and

lovastatin). These experiments showed that simvastatin was the most effective one inducing KLF2 expression in LSEC, thus reinforcing the use of this particular formulation to specifically protect the hepatic endothelium. As expected, KLF2 induction after 24h of simvastatin was associated to a marked increase in its vasoprotective target genes eNOS and thrombomodulin, an increment that depended on KLF2 function, since KLF2-knockdown experiments attenuated or even inhibited simvastatin vasoprotective beneficial effects. Our results were in agreement with previous reports demonstrating a significant up-regulation of eNOS protein expression and NO levels in livers from statins-treated animals (136, 137), and allowed us to frame out KLF2 in LSEC for the first time. Indeed, these results confirmed that statins also confer vasoprotection to a highly specialized endothelium such as the liver sinusoidal endothelium.

As previously explained in the introduction of this thesis, together with endothelial dysfunction, another important factor contributing to increase IHVR in cirrhosis is hepatic stellate cell activation. Indeed, activated HSC exhibit a pro-fibrogenic and vasoconstrictor phenotype that further aggravates the increased intrahepatic vascular tone, contributing to aggravate portal hypertension (246).

We herein demonstrated that simvastatin directly improved HSC phenotype, as shown by reduced expression of the HSC activation markers α-SMA and pro-collagen I, in part through the induction of KLF2. Indeed KLF2-knocked-down HSC exhibited an attenuation or even inhibition in the simvastatin beneficial effects on HSC phenotype. The importance of KLF2 in HSC de-activation was also corroborated infecting HSC with Ad-KLF2. We propose that this amelioration could derive from the ability of KLF2 to suppress TGF-β signaling (247). Indeed, KLF2 over-expressing HSC showed reduced TGF-β mRNA levels (supplementary Ph.D. thesis figure 1).

Beside the direct effects of statins on sinusoidal cells phenotype, we aimed to understand the possible paracrine interactions between LSEC and HSC in a KLF2-up-regulated situation. Indeed, performing co-cultured experiments we demonstrated that KLF2-overexpressing LSEC (due to simvastatin or Ad-KLF2) paracrinally ameliorated HSC phenotype through a KLF2-NO-cGMP mechanism. Indeed, inhibitions at any point of this pathway blunted simvastatin KLF2-mediated effects on HSC. These observations were in agreement with a recent report demonstrating that endothelial guanylate cyclase activation leads to HSC deactivation (11).

The LSEC-HSC cross-talk is not unidirectional. We were the first demonstrating that HSC de-activation due to KLF2 up-regulation (using simvastatin or Ad-KLF2) paracrinally improved

LSEC phenotype, in part through an increased HSC-derived VEGF production. VEGF might bind to its receptor in LSEC determining amelioration in their phenotype, but it also could induce an attenuation of the contractile properties of HSC via up-regulation of VEGFR-1 (248). Although these data seem to be of great value, more investigations are required.

To mimic *in vitro* the real hemodynamic conditions by which statins may influence the liver endothelial phenotype, we cultured LSEC from control and cirrhotic animals under shear stress stimuli. We observed that simvastatin markedly magnified the up-regulation of KLF2-derived vasoprotection in response to shear stress, especially in LSEC from cirrhotic animals. Moreover, using an *in vitro* reproduction of the liver sinusoid, consisting in a 3D cell culture chamber with microfluidics that mimics the sinusoidal environment, we observed a global improvement in the impaired sinusoidal phenotype in response to simvastatin treatment (amelioration of both dysfunctional LSEC and activated HSC). These data reinforce the concept that simvastatin administration to cirrhotic individuals may exert significant liver protection.

In fact, evaluation of hepatic KLF2-up-regulation *in vivo* revealed that the amelioration of the hepatic sinusoidal microenvironment observed *in vitro* translated into a decreased portal pressure (↓15%) in cirrhotic animals receiving Ad-KLF2, compared to Ad-CTRL cirrhotic animals. This improvement in portal hypertension derived from both an amelioration of the IHVR and PBF, without changes in systemic hemodynamics. In addition, Ad-KLF2 treated cirrhotic rats exhibited restored liver endothelial function, associated with an increase in the KLF2 transcriptional target eNOS, similarly to previously demonstrated in simvastatin treated cirrhotic animals (136).

Nevertheless, to deeper characterize the effects of KLF2 up-regulation on hepatic hemodynamics, we aimed to study two additional major players in the pathophysiology of portal hypertension: hepatic oxidative stress and liver fibrosis. We observed a marked activation of the anti-oxidant Nrf2 mediated pathway, which turned into increased expression of its targets genes HO-1 and NQO1, and an improvement in the hepatic fibrosis, in KLF2 over-expressing cirrhotic animals.

The involvement of KLF2 in the activation of Nrf2 has been previously described (208), however we corroborated it revealing that the marked decrease in O_2^- levels observed in simvastatin treated HSC, compared to vehicle cells, was not observed in KLF2-knocked-down simvastatin treated HSC, underlying the role of KLF2 in the mediation of the Nrf2-derived anti-oxidant effect.

On the other hand, we observed that Ad-KLF2 cirrhotic animals showed a reduction in liver fibrosis, that was both due to HSC apoptosis, probably mediated by a KLF2-derived down-regulation in NF-kB (supplementary Ph.D. thesis figure 2) (249), and to HSC de-activation (reduced α-SMA, collagen levels and Rho kinase activity). The activation of the Nrf2 pathway could partly explain the anti-fibrotic properties of KLF2, therefore linking the decreased oxidative stress with the decreased fibrosis observed in KLF2 over-expressing cirrhotic rats. Although these results seem to be promising, further studies designed to inhibit the Nrf2 pathway in cirrhosis would be desirable.

The anti-fibrotic effects of KLF2 were also evaluated using a pharmacological approach and in a different experimental model of cirrhosis. These experiments evidenced that hepatic KLF2 up-regulation in simvastatin-treated CBDL-cirrhotic rats was also associated with a decline in liver fibrosis (reduced α-SMA and collagen amount) but no differences in desmin were observed, suggesting that a 3-day pharmacological treatment would mainly modulate HSC phenotype *in vivo*. Indeed, *in vitro* experiments clearly demonstrated that 24h of simvastatin treatment were sufficient to promote activated HSC death, and that these apoptotic properties of simvastatin were partly mediated by KLF2. Considering our results, and previously published data (245, 250), it can be proposed that KLF2 may have a dual role in terms of apoptosis, depending on the cell type and the basal phenotype of each cell. In addition, our findings suggested that the pro-apoptosis and anti-fibrotic properties of other KLF2 inducers, such as resveratrol (229) and curcumin (251), may also be mediated by KLF2.

Taken together, the studies 2 and 3 of this Ph.D. thesis suggest that KLF2-mediated sinusoidal protection represents the underlying molecular mechanism for the beneficial effects of statins on liver microcirculation and liver fibrosis in cirrhosis. We provide evidences that increasing KLF2 in cirrhotic animals leads to an improvement in liver sinusoidal cells phenotype, de-activating HSC, ameliorating the dysfunctional endothelium, and reducing oxidative stress. This turns into an amelioration of cirrhosis and portal hypertension. The use of simvastatin or other drugs capable of augmenting KLF2 expression might be an appealing proposition to treat portal hypertension in cirrhosis.

Conclusions

Conclusions

Study 1: Leptin receptor blockade reduces intrahepatic vascular resistance and portal pressure in an experimental model of rat liver cirrhosis.

- Blockade of hepatic leptin receptors leads to a reduction in portal pressure.
- The amelioration of portal hypertension derives from a reduction in intrahepatic vascular resistance, without modifying systemic hemodynamics.
- The decreased intrahepatic vascular resistance is related to the anti-oxidant effects derived from the blockade of the ObR, and to the consequent increase in NO bioavailability and decreased vascular tone.

These data suggest that leptin can be a potential target in the treatment of portal hypertension.

Study 2: The transcription factor KLF2 mediates hepatic endothelial protection and paracrine endothelial–stellate cell deactivation induced by statins.

- Statins confer vasoprotection to the highly specialized liver sinusoidal endothelium, inducing KLF2-mediated vasoprotective programs.
- Simvastatin markedly magnifies the up-regulation of KLF2-derived vasoprotection in response to shear stress, especially in LSEC from cirrhotic animals.
- LSEC vasoprotective phenotype improvement due to simvastatin-dependent KLF2 up-regulation paracrinally ameliorates HSC phenotype, partly via a KLF2–NO–cGMP dependent mechanism.

Our study suggests that KLF2-dependent endothelial vasoprotection represents an underlying molecular mechanism for the beneficial effects of statins on liver microcirculation and liver fibrosis in cirrhosis

Study 3: KLF2 exerts anti-fibrotic and vasoprotective effects in cirrhotic rat livers: behind the molecular mechanisms of statins.

- The induction of KLF2 in HSC leads to a reversion in their activated phenotype, partly due to a KLF2-Nrf2-mediated anti-oxidant mechanism. This HSC de-activation determines a paracrine amelioration of dysfunctional LSEC, in part through a VEGF mediated signaling.

- In an *in vitro* sinusoidal-like environment, simvastatin markedly magnifies the up-regulation of KLF2 in response to shear stress in dysfunctional LSEC, leading to an even more pronounced HSC de-activation. Thus, KLF2 over-expression overall improves the impaired hepatic sinusoid, restoring the paracrine signaling between LSEC and HSC.

- Pharmacological or adenoviral-mediated hepatic KLF2 over-expression in cirrhotic animals leads to a significant improvement in liver cirrhosis and portal hypertension, as a result of KLF2-mediated HSC de-activation and apoptosis, amelioration of the dysfunctional hepatic endothelium, and reduced oxidative stress.

The use of simvastatin or other drugs capable of augmenting KLF2 expression might be an appealing proposition to treat portal hypertension in cirrhosis.

Other publications

- ADDITION OF SIMVASTATIN TO COLD STORAGE SOLUTION PREVENTS ENDOTHELIAL DYSFUNCTION IN EXPLANTED RAT LIVERS.

  Russo L*, Gracia-Sancho J*, García-Calderó H, **Marrone G**, García-Pagán JC, García-Cardeña G, Bosch J.

  Hepatology. 2012 Mar; 55(3):921-30.
  IF: 12.003

- SIMVASTATIN MAINTAINS FUNCTION AND VIABILITY OF STEATOTIC RAT LIVERS PROCURED FOR TRANSPLANTATION.

  Gracia-Sancho J, García-Calderó H, Hide D, **Marrone G**, Guixé-Muntet S, Peralta C, García-Pagán JC, Abraldes JG, Bosch J.

  J Hepatol. 2013 Jun; 58(6):1140-6.
  IF: 9.858

- DNA DAMAGE LINKS WNT SIGNALING TO FIBROSIS

  Silvia Svegliati*, **Giusi Marrone***, Antonio Pezone*, Tatiana Spadoni, Antonella Grieco, Gianluca Moroncini, Domenico Grieco, Maria Vinciguerra, Savina Agnese, Astrid Jüngel, Oliver Distler, Anna Maria Musti, Armando Gabrielli and Enrico V. Avvedimento.

  Currently, under review in Science Signaling

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