UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Bioquímica y Biología Molecular II

TESIS DOCTORAL

Papel de BMP9 en la patología hepática crónica: estudios *in vivo* e *in vitro*

Role of BMP9 in chronic liver disease: *in vivo* and *in vitro* studies

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Analisa Addante

Directores

Aránzazu Sánchez Muñoz
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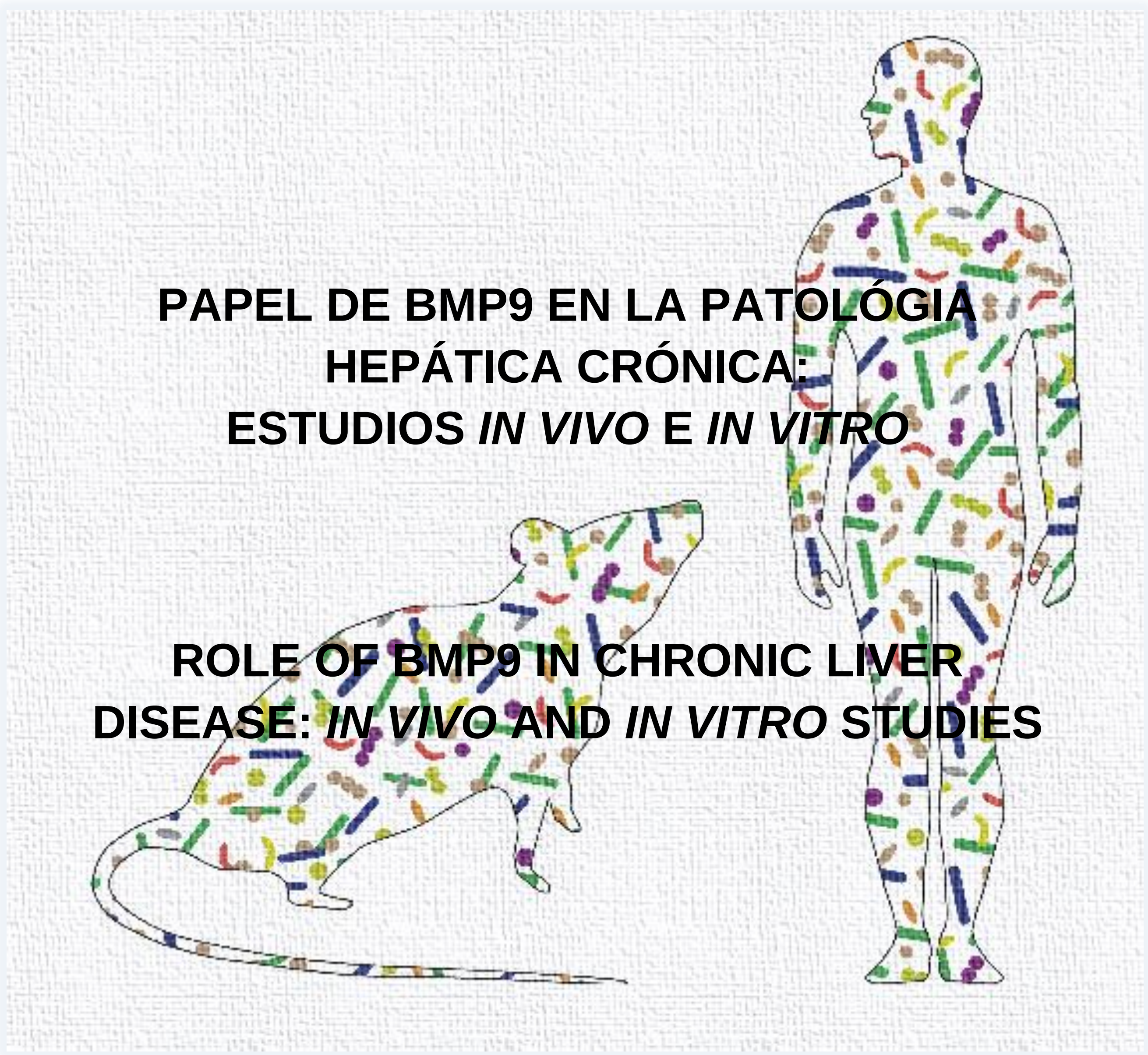
Madrid, 2018

Annalisa Addante

Tesis Doctoral



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El presente trabajo ha sido realizado en el Departamento de Bioquímica y Biología Molecular II de la Facultad de Farmacia de la Universidad Complutense de Madrid, bajo la dirección de las **Dra. Aránzazu Sánchez Muñoz** y **Dra. Blanca Herrera González.**

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OPTA AL GRADO DE DOCTOR CON MENCIÓN EUROPEA:

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Ai miei genitori,

ad Angela e Daniele,

a Dino.

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ABREVIATIONS

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AAF/PH: 2-acetylaminofluorene/Partial Hepatectomy
AFP: α-fetoprotein
AKT: AK strain Transforming
ALB: Albumin
ALD: Alcoholic Liver Disease
ALK1: Activin A receptor, type II-like 1
ALK2: Activin A receptor, type 1
ALT: Alanine aminotransferase
α-SMA: α-smooth muscle actin
AST: Aspartate aminotransferase
BDL: Bile Duct Ligation
BMP: Bone Morphogenetic Protein
BMP9: Bone Morphogenetic Protein 9
BMP9-KO: BMP9 knockout mouse
BMPR2: Bone Morphogenetic Protein Receptor, type II
BSA: Bovine Serum Albumin
CAM: Chorioallantoic Membrane
CBL: Bile Duct Ligation
CCl_4: Carbon Tetrachloride
CD34: Cluster of Differentiation 34
CK8: Cytokeratin 8
CK18: Cytokeratin 18
CK19: Cytokeratin 19
Col4: Collagen type IV
CRKL: CRK-like
CDE: Choline-Deficient, Ethionine-supplemented diet
CLD: Chronic Liver Disease
CXC: Chemokine
CV2: Crossveinless2
DDC: 3,5-diethoxycarbonyl-1, 4-dihydrocollidine
DEN: Diethyl Nitrosamine
DEP1: Density-Enhanced Phosphatase-1
Dlk1: Delta-like protein 1
DMEM: Dulbecco’s Modified Eagle Medium
DMN: Dimethyl Nitrosamine
DMSO: Dimethyl sulfoxide
DSS: Disuccinimidyl Suberate
DTT: Dithiothreitol
EC: Endothelial Cell
ECACC: European Collection of Cell Cultures
EC50: Half maximal Effective Concentration
ECM: Extracellular Matrix
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor receptor
EMT: Epithelial to Mesenchymal Transition
EndMT: Endothelial-to-Mesenchymal Transition
EOC: Epithelial Ovarian Cancer cell
EPCAM: Epithelial Cell Adhesion Molecule

FAK: Focal Adhesion Kinase
FGF: Fibroblast Growth Factor
FSB: Fetal Bovine Serum
FSP-1: Fibroblast-specific protein 1
GAB1: GRB2 Associated Binding Protein 1
GDF: Growth and Differentiation Factor
GRB2: Growth factor receptor-bound protein 2
HAI: Histology Activity Index
HBV: Hepatitis B virus
HCC: Hepatocellular Carcinoma
HCSC: Hospital Clinico San Carlos
HCV: Hepatitis C virus
HE: Hematoxylin and Eosin
HGF: Hepatocyte Growth Factor
HGFA: Hepatocyte Growth Factor Activator
HPC: Hepatic Progenitor Cell
HSC: Hepatic Stellate Cell
ID1: Inhibitor of DNA binding 1
IFN-γ: Interferon-gamma
IGF-1: Insulin-like Growth Factor I
IL: Interleukin
IOSE: Immortalised Ovarian Surface Epithelial cell
IPT: Immunoglobulin–Plexin–Transcription
JNK: c-Jun N-terminal kinase
KC: Kupffer Cell
LIF: Leukemia Inhibitory Factor
LSEC: Liver Sinusoidal Endothelial Cell
MAPK: Mitogen-Activated Protein Kinase
MCDA: Methionine Choline-Deficient diet supplemented with ethionine
MESEC: Mouse Embryonic-Stem-cell-derived Endothelial Cell
MMP: Matrix Metalloproteinase
mTOR: Mammalian Target of Rapamycin
NAFLD: Nonalcoholic Fatty Liver Disease
NASH: Nonalcoholic Steatohepatitis
N-CAM: Neural Cell Adhesion Molecule
NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells
NPC: Non-Parenchymal Cell
OC: Oval Cell
OD: Optical Density
OSM: Oncostatin M
OSMR: OSM Receptor
PAI-1: Plasminogen Activator Inhibitor 1
PCR: Polymerase Chain Reaction
PDGF: Platelet-Derived Growth Factor
PFA: Paraformaldehyde
PH: Partial Hepatectomy
PI3K: Phosphatidylinositol 3-kinase
PIP: Phosphatidylinositol-phosphate
PTP1B: Protein-Tyrosine Phosphatase 1B

Pref-1: Preadipocyte Factor 1
PSI: Plexin–Semaphorin–Integrin
ROS: Reactive Oxygen Species
RTK: Receptor Tyrosine Kinase
Sca-1: Stem cell antigen 1
SCF: Stem Cell Factor
SEC: Sinusoidal Endothelial Cell
SF: Scatter Factor
SHC: Src homology-2-containing
SHIP-2: Src homology domain-containing 5' inositol phosphatase
SNA1: Snail family zinc finger 1
SOS: Son of Sevenless
STAT3: Signal Transducer and Activator of Transcription 3
TAA: Thioacetamide
TIMP: Tissue Inhibitor of metalloproteinase
TDO: Tryptophan 2,3-dioxygenase
TGF-α: Transforming Growth Factor alpha
TGF-β1: Transforming Growth Factor beta 1
TGF-β2: Transforming Growth Factor, beta 2
TEC: Tumor associated Endothelial Cell
Thy1: Thy-1 Cell Surface Antigen
TIC: Tumor-initiating cells
TK: Tyrosine Kinase
TNF-α: Tumor Necrosis Factor alpha
TNFR1: Tumor Necrosis Factor Receptor 1
tPA: Tissue-type Plasminogen Activator
uPA: Urokinase-type Plasminogen Activator
uPAR: Urokinase-type plasminogen activator receptor
TWEAK: Tumor Necrosis factor-like weak inducer of apoptosis
VEGF: Vascular Endothelial Growth Factor
VEGFR: Vascular Endothelial Growth Factor Receptor
WHO: World Health Organization
WR: Working Reagent

INDEX

INTRODUCTION

1. THE LIVER

1.1 Function and structure: generalities

The liver, the largest internal organ in the body, is situated under the diaphragm. It is divided into two portions: a right lobe and a left lobe. The right lobe is much larger and it is further separated in two smaller lobes: the caudate and quadrate lobes. Liver has a central role in metabolic homeostasis, being responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. Its “strategic” location in relation to the food supply via the portal vein, and the unique gene-and protein-expression patterns of hepatocytes (the main functional cells of the liver) allow it to function as a biochemical defense against toxic chemicals entering through the food and as a processor of absorbed food ingredients (Michalopoulos 2007). Importantly, it is the main detoxifying organ of the body, which removes wastes and xenobiotics by metabolic conversion and biliary excretion (Hata *et al.* 2007).

In its simplest formulation, the histological units of the liver are the lobules (**FIGURE 1A and B**), randomly oriented structures that contain rows of hepatocytes (hepatocyte plates or cords). The lobules are demarcated by peripheral spaces (portal spaces or portal triads) that contain small branches of the portal vein, hepatic artery and bile ducts. In the portal spaces, the terminal segments of the biliary system connect with hepatocytes in the liver parenchyma through a terminal bile ductule known as the canal of Hering (Fausto and Campbell 2003). The hepatocytes plates extend from the periphery of the portal tracts to the central vein. Blood flows in sinusoids between one-cell thick hepatocyte plates from portal spaces to central vein; bile flows in the opposite direction.

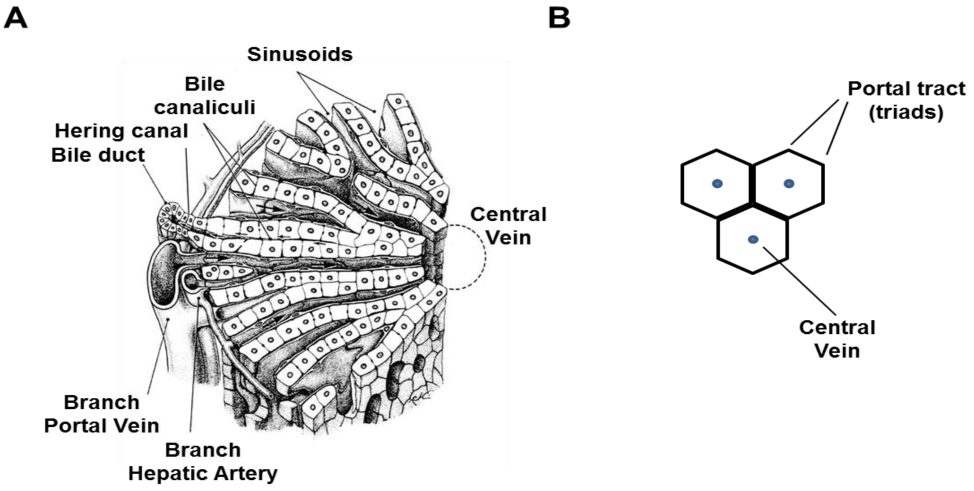


FIGURE 1. Lobular structure of the liver

A. Portal tracts contain branches of the portal vein and hepatic artery, as well as bile ducts. Hering canals and bile canaliculi are also indicated. **B.** Three lobules are shown as hexagonal structures demarcated by portal tracts or triads in the periphery and containing a central vein at the center.

(Modified from Fausto and Campbell, 2003)

1.2 Hepatic cell types

At least 15 different cell types can be found in normal liver (Kmiec 2001; Malik *et al.* 2002).

TABLE 1. Cells types found in the liver
Hepatocytes
Biliary epithelial cells (also known as cholangiocytes)
Endothelial cells
Sinusoidal endothelial cells
Arterial and venous endothelial cells
Lymphatic endothelial cells
Kupffer cells
Hepatic stellate cells (also known as Ito or fat-storing cells)
Lymphocytes (Pit cells)
Progenitor cells (fetal, also known as hepatoblasts, and adult, known as oval cells in rodent models)
Fibroblasts
Smooth muscle cells (blood vessels)
Mesothelial cells
Nerve cells (unmyelinated)
Neuroendocrine cells
Hematopoietic cells
Blood (erythrocytes, leukocytes, etc.)

(Modified from Malarkey et al., 2005)

Hepatocytes are the most abundant liver cell type and comprise 60% of the total cells and 80% of the volume of liver. In the liver plate, hepatocytes are referred to as periportal or centrolobular depending on their distance from the portal spaces (Malarkey *et al.* 2005). Hepatocytes carry out all the essential functions of the organ and are the cells responsible for bile production that is secreted from the apical membrane into the bile canaliculi that run between hepatocytes and merge to form bile ducts, which are lined by cholangiocytes. Cholangiocytes and hepatocytes, the epithelial cells of the liver, share a common precursor cell, the hepatoblast (Kang *et al.* 2012).

The remaining 40% of cells (the rest 20% of the total liver cell mass) comprises other cell types, collectively termed as *non*-parenchymal cells (NPCs), which include sinusoidal endothelial cells (SECs), Kupffer cells (KCs) (functioning as *in situ* macrophages), hepatic stellate cells (HSCs) and cholangiocytes as the prevalent populations of NPCs (Racanelli and Rehermann 2006; Godoy *et al.* 2013). Lymphocytes are also part of the innate immune system that resides within the liver to help resist infection and are included into the NPCs. NPCs play important roles in nutrients transport, metabolism, inflammatory response, growth functions and therefore they are involved in several pathophysiological processes in the liver, which makes them interesting for pharmaceutical research.

Different evidences based primarily on experimental models of liver injury and carcinogenesis have led to the concept that in the adult liver there are cells that function as stem cells or stem-like cells. Upon severe liver injury these cells, which are believed to be located in the canals of Hering, expand and give rise to a heterogeneous multipotent transient amplifying cell population known as hepatic progenitor cells (HPCs), known as “oval cells” in mouse and “intermediate hepatobiliary cells” in humans (Fausto and Campbell 2003; Esrefoglu 2013). We will study thoroughly this population in next chapters of this thesis.

1.3 Hepatic Extracellular Matrix (ECM)

The hepatic ECM is a complex macromolecular structural network surrounding stromal cells and underlying the majority of endothelial and epithelial cells. It is not only a physical scaffold that supports the parenchyma and maintains hepatic integrity but also a crucial modulator of biological processes including cell attachment, migration, differentiation and survival (Schuppan 1990; Martinez-Hernandez and Amenta 1995; Frantz *et al.* 2010). Recent studies indicate that the ECM modulates hepatic development, regeneration, and even the maintenance of the normal architecture and differentiated state in a variety of ways: by acting as a solid phase agonist, as polypeptides interacting with cell surface receptors, or by facilitating the maximal exchange of nutrients or cytokines between circulating blood and hepatocytes during liver regeneration (Kim *et al.* 1997; Michalopoulos 2010). Thus, the ECM creates a complex and naturally dynamic microenvironment that undergoes continuous remodeling.

In normal liver, ECM proteins are distributed mainly in the liver capsule, in the portal tracts and in the ducts and ductules of the biliary tree, whereas a basement-like matrix is located in the sinusoidal space or space of Disse (the small space between hepatocytes and sinusoidal endothelial cells), where the most prevalent ECM components are the collagens, elastin, structural glycoproteins and proteoglycans (**FIGURE 2)**.

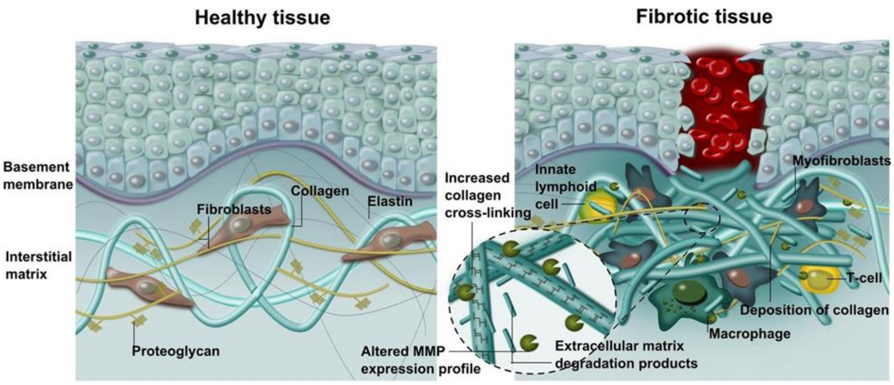


FIGURE 2. The hepatic ECM remodeling during the development of fibrosis

The main ECM structural components are shown in healthy and fibrotic tissue. ECM remodeling is tightly controlled to ensure homeostasis; during fibrogenesis, however, this process is disturbed.

(Modified from Morten A. Karsdal, 2014)

Interestingly, the structure of the sinusoidal space of Disse is different from other organs because it lacks a true basement membrane (Friedman 2003).

The ECM remodeling is a complex and highly coordinated process resulting from the balance between synthesis, degradation and reorganization of its components, in response to signals conveyed by cell surface receptors, among which integrins seem to play a major role (Arriazu *et al.* 2014). Matrix endopeptidases play a crucial role in this process and, not surprisingly, also in the liver regeneration process. Matrix metalloproteinases (MMPs) are the most relevant ECM-modifying enzymes. MMPs are a family of over 24 zinc-dependent endopeptidases capable of degrading virtually any component of the ECM (Kessenbrock *et al.* 2010). MMP-1 (collagenase-1), MMP-2 (gelatinase-A), MMP-3 (stromelysin-1), MMP-11 (stromelysin-3) and MMP-13 (collagenase-3) are among the MMPs constitutively expressed in normal liver, although other different MMPs can be secreted in several acute or chronic liver injury states contributing to the changes in the ECM (Duarte *et al.* 2015).

Significant ECM alterations are observed in liver cirrhosis and fibrosis (Schuppan *et al.* 2001; Wells 2008; Morten A. Karsdal 2014), in particular the balance between matrix formation and degradation in these pathological disorders is shifted leading to an abnormal and progressive deposition of ECM in the liver.

In spite of the fact that the mechanisms of ECM remodelling in development and disease have been thoroughly studied, the role of ECM in liver regeneration is still poorly understood; in particular the study of the potential mechanisms by which these changes regulate the proliferative properties of liver cells during the regenerative process would be very relevant. Recently, a very interesting work has demonstrated selective growth stimulation properties for ECM components; thus, collagen type IV (Col4) was shown to efficiently support hepatocyte proliferation *in vitro*, and high Col4 expression coincided with proliferating hepatocytes also in regenerating liver (Klaas *et al.* 2016). Moreover, it has been demonstrated that matrix components have also important effects in hepatic progenitor cells. For example, collagen I favours the differentiation of hepatic progenitor cells, while laminin maintains their stemness properties (McClelland *et al.* 2008); in addition, it has been shown that fibronectin promotes the differentiation of liver progenitor cells towards the hepatocyte phenotype (Lorenzini *et al.* 2010).

1.4 Liver regeneration

The liver has a remarkable regenerative capacity. In adult human, the liver is a mitotically quiescent organ with most hepatocytes in the G0 phase of the cell cycle and mitotically inactive (Thorgeirsson 1996). Under physiological conditions, mature hepatocytes and biliary epithelial cells maintain the balance between cell loss and cell gain by dividing themselves (Knight *et al.* 2005). Additionally, following partial removal of tissue, the remaining population of hepatocytes and cholangiocytes have a remarkable capacity to meet replacement demands of cellular loss accounting for the extraordinary regenerative capacity of the liver. Specifically, the regeneration of the liver can be more correctly defined as compensatory hyperplasia as the remaining liver tissue expands to meet the metabolic needs of the organism although the expanding liver does not regain its original gross anatomical structure (Mao *et al.* 2014).

A number of models have been used for the study of liver regeneration, but the best studied model is the partial hepatectomy (PH) in which two-thirds of the rodent liver are removed (Michalopoulos 2010; Gilgenkrantz and Collin de l'Hortet 2011; Michalopoulos 2014). This loss of liver mass leads to a rapid cellular proliferation and restoration of functional liver tissue. 24 hours after PH in the rat, more than 90% of the hepatocytes in the remnant 30% liver tissue are in the process of dividing and the liver mass is fully restored 7 to 10 days later (Higgins GM 1931). This process also occurs in human, although the precise timing of events is currently less clear: the available evidence suggests there is rapid regeneration during the first two weeks after major hepatectomy or transplantation in both donors and recipients, followed by a more gradual increase in size which ceases about 6 to 12 months later (Marcos *et al.* 2000).

Despite the high regenerative capacity of the liver, there are several situations where the hepatocyte proliferation is blocked or cannot be sufficient to prevent the damage, as it is the case in chronic liver disease or in conditions of replicative senescence of the majority of hepatocytes. In these situations an alternative regenerative pathway is activated in which the main mediators are the HPCs (Tanaka *et al.* 2011), process that will be discussed in detail in next sections.

2. CHRONIC LIVER DISEASES (CLDs)

Chronic liver diseases (CLDs) constitute a major medical and public health problem worldwide. Currently, liver diseases are the tenth most common cause of death in the USA and, in Europe, are at least comparable with other diseases that are considered to be of major public health concern (Blachier *et al.* 2013). This situation is unlikely to change in the next decade since recent World Health Organization (WHO) projections have demonstrated that cirrhosis is one of the top 10 causes of death in the western world. Moreover, if we take a look at data from Asian and African countries where liver diseases are more prevalent, there is no doubt that liver disease is becoming a global problem. While the mortality rates for most other leading causes of death declined significantly over the last 40 years, the age-adjusted mortality rate for CLD has changed little. The large number of people affected worldwide and the lack of effective anti-fibrotic treatment make a deeper understanding of mechanism driving CLD an absolute requirement in order to deliver novel and more effective therapies.

2.1 The natural history of CLDs

CLD refers to a disease process that involves progressive destruction and regeneration of liver parenchyma leading to fibrosis and cirrhosis. Liver fibrosis is the first stage of CLDs; if the cause of fibrosis is eliminated, resolution (that is, complete reversal to near-normal liver architecture) of early hepatic fibrosis can occur. In general, in the early stages of fibrosis, the liver functions relatively well and few people experience symptoms. But as the inflammation and liver injury continue, scar tissue builds up and connects with existing scar tissue, which can eventually disrupt the metabolic functions of the liver. If the disease progresses, it can lead to cirrhosis, a condition in which tissue architecture is disrupted, and the liver is severely scarred with fibrotic bands, parenchymal nodules, and vascular distortion. Pathophysiologically, this often leads to portal hypertension and hepatic failure. Therefore, cirrhosis represents the final stage of liver fibrosis and the final histological pathway for a wide variety of liver diseases (**FIGURE 3**).

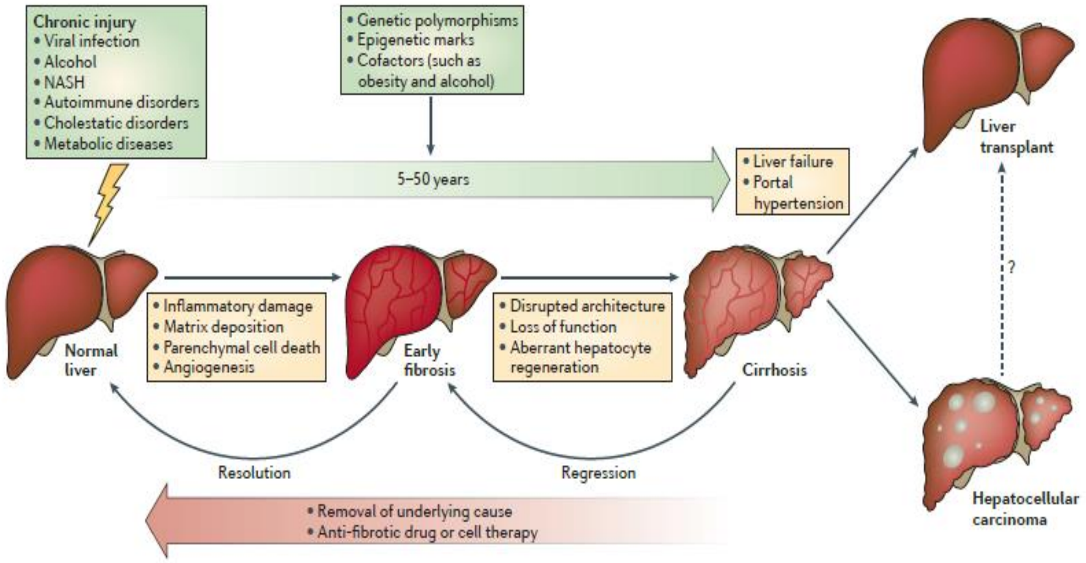


FIGURE 3. Natural history of chronic liver disease

Hepatic fibrosis is the wound-healing response of the liver to many causes of chronic injury. Regardless of the underlying cause, iterative injury causes inflammatory damage, matrix deposition, parenchymal cell death and angiogenesis leading to progressive fibrosis. If the cause of fibrosis is eliminated, resolution of early hepatic fibrosis can occur. In cirrhosis, although resolution is not possible, regression of fibrosis improves clinical outcomes. Currently, liver transplantation is the only available treatment for liver failure or for some cases of primary liver cancer. Hepatocellular carcinoma (HCC) is rising in incidence worldwide and is a major cause of liver-related death in patients with cirrhosis.

(Pellicoro et al., 2014)

The progression to cirrhosis is very variable and may occur over weeks or many years. Furthermore, in this stage, although resolution is not possible, regression (that is improvement but not reversal) of fibrosis improves clinical outcomes.

Importantly, cirrhosis can be considered a premalignant condition and patients with liver cirrhosis are usually at high risk of developing hepatocellular carcinoma (HCC), which ranks as the fifth most common cancer, causing over 600 thousand deaths *per annum* worldwide.

2.2 Causes of CLDs

The major causes of CLDs include chronic hepatitis virus infections, alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD). Other less common causes of cirrhosis may include: autoimmune disorders, where the immune system attacks healthy tissue; blocked or damaged bile ducts; use of certain medicines; exposure to certain toxic chemicals; repeated episodes of heart failure with blood buildup in the liver and parasitic infection such as schistosomiasis in developing countries (Sun and Tang 2004; Liu *et al.* 2013; Pellicoro *et al.* 2014). In many patients with chronic hepatitis, no obvious cause can be identified. In some of these patients, the sustained hepatic inflammation is the key element (Marra and Tacke 2014).

Hepatitis viruses have a special tropism toward hepatocytes and an ability to cause both acute and chronic liver diseases. There are 5 different hepatitis viruses from A to E of which predominantly B and C are the one causing CLDs and HCC. Specifically, chronic hepatitis B virus infection (HBV), in spite of the availability of an efficient vaccine, is considered a global public issue with more than 78.000 deaths per year.

Alcohol consumption and abuse are also major causes of CLD, which is a significant health problem in the United States and around the world. The pathogenesis of ALD in humans is characterized by steatosis (accumulation of fat in hepatocytes) and a concomitant inflammatory process (Park *et al.* 2014; Williams *et al.* 2014; Chatterjee and Mitra 2015).

NAFLD/nonalcoholic steatohepatitis (NASH) is considered to be a hepatic manifestation of metabolic syndrome, and its incidence is rapidly increasing worldwide. Sedentary lifestyles, poor food habits and a silent progression have contributed to the fatty liver disease epidemic in the Western world. NAFLD is a broad spectrum of diseases characterized by accumulation of triglycerides in the liver of patients without a history of excessive alcohol consumption. It is classified into simple steatosis and NASH, in which in addition to hepatic steatosis, intralobular inflammation and ballooning degeneration of hepatocytes are observed. NASH is a progressive disease and may lead to liver cirrhosis and HCC (Takahashi *et al.* 2015).

2.3 Liver fibrogenesis

Liver fibrogenesis can be considered the major pathophysiological event driving the progression of CLDs, irrespective of etiology (Friedman 2008).

Liver fibrogenesis is a dynamic tissue, cellular and molecular process that leads progressively to an excess deposition of ECM components in an attempt to limit the consequences of chronic parenchymal injury (Novo *et al.* 2015). This process is sustained and modulated by a crosstalk occurring between different hepatic cell populations, resident or recruited into chronically injured liver, that are involved in the synthesis and release of several mediators, including growth factors, cytokines, chemokines, adipokines, reactive oxygen species (ROS), vasoactive agents and plasma proteins. The cellular and molecular mechanisms of hepatic fibrogenesis have been extensively investigated using multiple complementary experimental animal model systems (Liu *et al.* 2013) that have led to the identification of different pro-fibrogenic mechanisms including chronic activation of the wound healing response; oxidative stress; a derangement of epithelial-mesenchymal interactions; epithelial to mesenchymal transition (EMT) of parenchymal cells; hepatocytes loss and chronic inflammation (Novo *et al.* 2015).

The major cell type responsible for cell repair appears to be the activated myofibroblast. There are several potential sources of myofibroblasts, including resident cells, periportal and pericentral fibroblasts, or quiescent HSC and bone marrow-derived cells or circulating mesenchymal cells, which can migrate through the injured liver and become myofibroblasts. It is still controversial whether hepatocytes, cholangiocytes or

SECs may undergo phenotypic transformation into activated myofibroblats. Evidence support a central role for quiescent HSCs that upon activation by cytokine signalling, turn into myofibroblast, which then suffer profound changes in behaviour, including proliferation, chemotaxis, contractility, altered matrix degradation and production of fibrous scar, retinoid loss, and inflammatory signaling (Friedman 2008; Rockey 2008; Zhou *et al.* 2014; Novo *et al.* 2015).

Emerging evidence from studies of renal fibrosis suggests that more than a third of all disease-related fibroblasts originate from EMT and EndMT (endothelial-to-mesenchymal transition) (Kalluri and Neilson 2003; Usunier *et al.* 2014). EMT and EndMT are biological processes by which an epithelial or endothelial cells undergo multiple biochemical changes that enable them to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, expression of typical fibroblast markers such as FSP-1 (fibroblast specific protein-1), *α*-SMA (α-smooth muscle actin), and vimentin and greatly increased production of ECM components (Kalluri and Weinberg 2009). Interestingly, in the context of liver fibrosis, Zeisberg et al. demonstrated that up to 45% of fibroblasts in the fibrotic liver derive from hepatocytes via TGF-β (Transforming growth factor beta)-induced EMT (Zeisberg *et al.* 2007).

TGF-β is a key player during liver fibrosis. It is known that TGF-β triggers the activation of HSCs, mediates ECM synthesis, provides cell contraction and migration, induces apoptosis in hepatocytes, and its signaling induces oxidative stress and consequently inflammation (Fabregat *et al.* 2016). Importantly, recent evidences show that TGF-β also participates in the development of fibrosis through signaling in other liver cell types, such as HPCs (Yang *et al.* 2016).

2.4 Hepatic inflammation

Acute inflammation as a defense against noxious stimuli is very important for homeostasis in the body, whereas chronic exposure to an inflammatory agent may cause a dysregulated or unresolved inflammatory response, which causes chronic inflammation. In sustained liver injury, persistent inflammation response is considered as one of the major ‘driving forces’ sustaining fibrogenesis, as it precedes and accompanies liver fibrosis (Forbes and Parola 2011). Both the innate and the adaptive immune systems contribute to the process. Damaged hepatocytes release apoptotic bodies, ROS, cytokines such as tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-1); and chemokines (CXC) that activate endothelial cells, HSCs, and KCs by paracrine stimulation, which can in turn maintain/promote inflammatory and fibrogenic responses. Additionally, these signals may lead to recruitment and activation of the inflammatory cells.

It seems clear that liver inflammation during CLD is orchestrated by a complex network of cytokine-mediated signaling pathways that contribute to fibrogenesis.

TNF-α is mainly produced by monocytes, macrophages, HSCs and KCs. It has pro-inflammatory activities and cytotoxic effects in the cells that produce it; furthermore, TNF-α activates HSCs that increases ECM synthesis thus contributing to liver fibrosis (Connolly *et al.* 2009).

Interleukins (ILs) are a group of cytokines expressed by CD4 T lymphocytes, monocytes, macrophages and endothelial cells. Both KCs and SECs produce ILs in response to liver tissue damage (Zhou *et al.* 2014). ILs can exert both pro-fibrogenic and anti-fibrogenic activities. Among the pro-fibrogenic interleukins are IL-1, IL-17 and IL-6, although specific roles of IL-6 are controversial (Gieling *et al.* 2009; Du *et al.* 2013). IL-6 has been shown to be produced by hepatoma cells, suggesting that it may also be produced by hepatocytes (Michalopoulos 2007). The underlying molecular mechanism of the pro-fibrogenic role of IL-6 is not clear, but *in vitro* studies in dermal fibroblasts have associated IL-6 with a hyperactivation of the TGF-β/Smad3 signaling (O'Reilly *et al.* 2014). IL-6 anti-fibrotic actions have been reported in liver; thus IL-6 reduces CCl_4-induced acute and chronic liver injury and fibrosis (Kovalovich *et al.* 2000). In addition, pretreatment of fibrotic liver with IL-6 improves hepatic microenvironment and primes it for mesenchymal stem cell transplantation, leading to improvement in liver injury after fibrosis (Nasir *et al.* 2013).

On the other side, IL-10 negatively regulates the inflammatory response and present anti-fibrotic effects being its collagenolytic effect attributed to MMP and tissue inhibitor of metalloproteinase (TIMP) modulation (Chou *et al.* 2006). IL-22 has also been shown to restrict liver fibrosis and accelerate the resolution of liver fibrosis by inducing HSC senescence (Kong *et al.* 2012).

2.5 Cirrhosis

Liver cirrhosis is the final pathological result of CLD. It is characterized by degeneration and necrosis of hepatocytes, replacement of liver parenchyma by fibrotic tissue and regenerative nodules, and loss of liver function (Zhou *et al.* 2014). Together with mechanisms operating during the progression of liver fibrosis, such as activation of myofibroblasts, the major contributor to liver cirrhosis are repeated cycles of apoptosis and regeneration of hepatocytes and defenestration of liver sinusoidal endothelial cells, indicating an exhaustion of the regenerative capacity of the liver.

The progression from fibrosis to cirrhosis occurs over decades, with an average of 20–30 years from the time of initiation of injury (Cohen-Naftaly and Friedman 2011).

Liver cirrhosis is the main risk factor for the development of HCC, however, the mechanism by which liver cirrhosis leads to HCC has not been clarified completely (Fattovich *et al.* 2004).

2.6 Hepatocellular carcinoma (HCC)

HCC is the most common primary malignancy of the liver. It is the fifth most common cancer in men, worldwide, and seventh among women, with over half a million new cases diagnosed annually worldwide. It is the second leading cause of cancer related mortality in the world. Approximately 75–80% of HCC frequently occurs in HBV- or Hepatitis C virus (HCV)-infected individuals whose disease has progressed to chronic stage such as cirrhosis, so that it can be considered the end-stage of CLDs. Any condition related to chronic liver inflammation is considered a risk factor because it is capable of causing genomic and mitochondrial damage facilitating the development of cancer (Berasain *et al.* 2009; Lachenmayer *et al.* 2010).

Interestingly, Berasain et al. have demonstrated that TNF-α constitutes a novel link between inflammatory signals and pro-tumorigenic mechanisms in liver cells; in particular, using human HCC and mouse hepatocytes they have shown the ability of this inflammatory cytokine to activate the epidermal growth factor receptor (EGFR), a key factor in both liver regeneration and hepatocarcinogenesis (Berasain *et al.* 2012; Lopez-Luque *et al.* 2016).

Multiple signaling pathways involved in several cellular functions have been described to be deregulated a in HCC including Wnt/β-catenin, p53, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), hepatocyte growth factor (HGF)/c-Met signaling pathway and TGFβ (Calvisi *et al.* 2007; Torrecilla and Llovet 2015). Moreover, although the relevance of the EMT in liver fibrogenesis remains controversial, there are no doubts that this process is involved in cancer progression (Giannelli *et al.* 2016).

2.7 How to study CLDs

Considering all the above, it seems clear that additional studies are required for a better understanding of the molecular process contributing to CLD development and progression to develop new therapeutic strategies. Experimental animal models offer researchers irreplaceable opportunities to study biological, pathological, and histological characteristics of human CLDs. However, although no single animal model exhibits all attributes of human liver diseases, it should be recognized that mouse models of liver fibrosis that mimic human liver fibrosis have aided study liver fibrogenesis (Urtasun *et al.* 2011; Liedtke *et al.* 2013).

According to Liu et al. (Liu *et al.* 2013), the most commonly used rodent models for studying CLDs can be divided into two groups:

1. Those generally used for studying mechanisms of liver fibrosis irrespective of disease etiologies that include: chemically-induced liver fibrosis models using hepatotoxins, such as carbon tetrachloride (CCl4), thioacetamide (TAA) and dimethyl or diethyl nitrosamine (DMN and DEN); and genetically engineered (transgenic/knockout) mouse models.

2. Those used to mimic specific CLDs including autoimmune and cholestatic liver diseases, chronic viral infection, NAFLD, and ALD that include: animal models of schistosomiasisl; autoimmune hepatitis; primary biliary cirrhosis; and progressive cholestatic liver disease (such as Mdr2 $^{-/-}$ mice and 3,5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) diet).

Animal models allow to (i) comprehensive study of questions that cannot be addressed in human studies, (ii) multiple sampling at strategic times during the evolution/resolution, (iii) experimental testing with restriction of the number of variables to a minimum. In the *in vivo* model, the whole organ is intact (interactions between various cell types and matrix) and the possible influences derived from other parts of the body, such as the nervous system, the endocrine system, the neuroendocrine system, the circulatory system, the diet, or the influences gut-derived are taken into consideration (Starkel and Leclercq 2011).

2.7.1 Carbon tetrachloride (CCl_4)

CCl_4 is one of the most widely used hepatic toxins for experimental induction of liver injury in laboratory animals. CCl_4 directly impairs the functionality of the hepatocytes by altering the permeability of different cellular membranes, the plasma, lysosomal and mitochondrial membranes.

CCl_4 is transformed into a toxic CCl3- radical by CYP2E1, an enzyme expressed in perivenular hepatocytes (Boll *et al.* 2001). In the presence of oxygen, the CCl3− radical is converted to the trichloromethyl peroxy radical, CCl3OO*. This radical, more reactive and thus with a shorter half-life than the CCl3− radical, is responsible for initiating the process of lipid peroxidation. It is of interest to note that lipid peroxidation may damage cellular functions in two ways: by compromising membrane function and by covalent binding of reactive intermediates formed during the toxicity reaction (Weber *et al.* 2003). Thus, it induces an acute centro-lobular necrosis that triggers a wound healing response with recruitment of phagocytic and inflammatory cells to clear necrotic zones, activation of fibrogenesis and increased ECM deposition and proliferation of parenchymal and non-parenchymal cells to replace dead cells. When the insult is repeated, successive rounds of wound healing occur prior to resolution of the previous one, resulting in fibrosis accumulation (Weber *et al.* 2003; Starkel and Leclercq 2011).

2.7.2 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet

DDC feeding in mice is a well-established animal model generally included in the second group of rodent models for studying CLDs, particularly for its use in the study of Mallory-Denk body formation which are inclusion bodies found in liver cells associated with alcoholic and nonalcoholic steatohepatitis, metabolic liver diseases, and chronic cholestatic liver diseases (Stumptner *et al.* 2001; Fickert *et al.* 2002; Zatloukal *et al.* 2004).

It is known the ability of this compound to induce porphyria in primary cultures of mouse hepatocytes, in which DDC treatment results in a marked inhibition of ferrochelatase and accumulation of protoporphyrin (Brady and Lock 1992). *In vivo*, DDC feeding leads to increased biliary porphyrin secretion and induction of vascular cell adhesion molecule, osteopontin, and TNF-α expression in cholangiocytes (Fickert *et al.* 2007). This xenobiotic-induced liver injury model is useful to investigate the mechanisms of chronic cholangiopathies and their consequences, including biliary fibrosis (Fickert *et al.* 2002; Fickert *et al.* 2007). Moreover, feeding a DDC-containing diet is one of the most efficient mouse models for chemical-induced oval cell proliferation, although the molecular mechanism remains unknown (Yokoo *et al.* 1982; Wang *et al.* 2003; Jakubowski *et al.* 2005; Malato *et al.* 2011; Yamazaki *et al.* 2011; Weng *et al.* 2013).

2.8 The treatment of CLDs

Treatment of patients with CLDs has been a big challenge for physicians because CLD is usually diagnosed at the irreversible stages such as cirrhosis, cancer or liver failure. Since the progress of fibrosis is basically similar in all liver diseases regardless of the etiology, the development of anti-fibrotic therapies should benefit all patients with fibrotic liver injury. The liver's ability to regenerate and its first-pass metabolism allow lower doses of orally administered compounds to achieve a therapeutic response, minimizing systemic distribution and non-hepatic side effects, which makes liver fibrosis an attractive target for therapy. Potential therapeutic approaches for liver fibrosis include: treatment of the primary disease, suppression of hepatic inflammation, inhibition of HSCs activation, promotion of matrix degradation, stimulation of HSCs apoptosis, and targeted anti-fibrotic therapies. The emerging anti-fibrotic therapies can slow, halt or reverse fibrosis progression. Inhibition of TGF-β1 signaling cascade is one of the most appealing therapeutic strategies of anti-fibrotic efforts in liver to combat CLDs progression, since TGF-beta is a master regulator of the fibrogenic process (Fabregat *et al.* 2016; Giannelli *et al.* 2016).

Elimination of the trigger(s) that lead to cirrhosis is likely to retard progression and to reduce the incidence of HCC. There is evidence that causal treatment may even reverse cirrhosis, although in some of the reports sampling variability cannot be excluded. Specifically, it has been demonstrated that alcohol withdrawal, interferon-based antiviral treatment and long-term treatment with oral inhibitors of HBV polymerase may not only retard or reverse cirrhosis but prevent complications of end-stage liver disease (Schuppan and Afdhal 2008).

Regarding HCC, a wide variety of non-surgical therapies can result in clinical responses in patients with this type of cancer, including ablative techniques, intra-arterial therapies or chemotherapy (Johnson 2005). No other approaches have demonstrated convincing evidence of survival advantage. To date, surgical resection is currently considered to be the definitive treatment for HCC and the only one that offers the prospect of cure or at least long-term survival (Liu *et al.* 2013).

3. HEPATIC PROGENITOR CELLS (HPCs)

3.1 Generalities

We previously mentioned that under situations where hepatocytes or biliary epithelial cells regenerative capacity is compromised, such as chronic injury states or in the presence of substances inhibiting adult hepatocyte proliferation, HPCs are activated to support or take over the regenerative process (Falkowski *et al.* 2003; Marshall *et al.* 2005).

These cells were first described by E. Faber in 1956 as “small oval cells with scant lightly basophilic cytoplasm and pale blue-staining nuclei” with a high nuclear/cytoplasmic ratio and an ovoid nucleus, which gave them the name “oval cells”. The oval cell-mediated regenerative process is often referred to as “oval cell response” or “ductular reaction” due to the fact that proliferating oval cells organize into ductular structures or ductules.

During the oval cell-mediated regeneration, oval cells arise in the periportal region of the liver. Although their origin is still under debate, the most accepted theory is that they derive from adult liver stem cells located at the canal of Hering, in the smallest branches of the biliary tree, therefore considered the niche for the oval cell precursors. As other stem cell niches, this is a restricted compartment in a tissue that maintains and regulates stem cell behaviour, supporting self-renewal and maintaining the balance between quiescence, proliferation and differentiation required in response to injury (Lorenzini *et al.* 2010; Hsieh *et al.* 2015).

Oval cells are bipotential progenitors able to differentiate into both hepatocytes and cholangiocytes and therefore replenish cellular loss both in rodents and humans (Okabe *et al.* 2009; Shin *et al.* 2011). These properties support their role as HPCs (Alison *et al.* 1996; Yang *et al.* 2002; Roskams *et al.* 2004; Duncan *et al.* 2009; Tanaka *et al.* 2011). It is interesting to mention that, apart from their bipotential nature, these cells can differentiate in a non-lineage-restricted manner, generating cells of other organs when placed in the environment of a different organ, which would evidence their multipotency (Cardinale *et al.* 2011).

Because of their bipotential nature, oval cells have a unique phenotype and coexpress bile duct epithelium markers, as CK19 (cytokeratin 19), A6, OV6 and EPCAM (epithelial cell adhesion molecule); and hepatocyte markers, such as CK8 and CK18 (cytokeratin 8 and 18), ALB (Albumin), c-Met (hepatocyte growth factor receptor), and CYP450 (Yin *et al.* 2002). Furthermore, they express high levels of some hepatoblasts markers, such as *α*-fetoprotein (AFP) and Delta-like protein 1 (Dlk1). Of particular interest is the finding that oval cells express markers associated with hematopoietic lineages such as Thy-1 (thymocyte differentiation antigen, also known as CD90), CD34 (Cluster of Differentiation 34), Sca-1 (Stem cell antigen 1) and c-kit (Omori *et al.* 1997). In addition, in their gene expression profile these cells show expression of CD44 (Yovchev *et al.* 2007) and CD133 (encoded by the Prom 1 gene) (Dorrell *et al.* 2011), which are stem cell markers that have been identified as putative markers for cancer stem cells (Ma *et al.* 2007; Yin *et al.* 2007); as well as markers of different lineages, as N-CAM (neural cell adhesion molecule) and chromogranin, typical of neuroepithelial cell (Libbrecht and Roskams 2002).

Oval cell differentiation towards hepatocytes or cholangiocytes is associated with a progressive loss of oval cell markers and acquisition of a more mature phenotype (Gaudio *et al.* 2009).

A major problem in characterizing oval cells is the lack of a specific cell surface marker to identify and isolate them . Therefore, despite a large number of studies, the exact nature of oval cells – including their origin, stemness and bi-directional differentiation – is still poorly understood (Okabe *et al.* 2009).

3.2 How to study the oval cell response

Several groups have developed protocols in rodents for the enrichment and expansion of HPC (Monga *et al.* 2001). In mouse, various treatments have been successfully used to induce proliferation of oval cells, in some cases even in the absence of impaired hepatocytes proliferation. Among these, we find choline-deficient, ethionine-supplemented diet (CDE) (Tirnitz-Parker *et al.* 2007), methionine choline-deficient diet supplemented with ethionine (MCDA) (Malato *et al.* 2011), bile duct ligation (BDL) (Sackett *et al.* 2009), and DDC (Preisegger *et al.* 1999).

In rat, treatment with CCl_4 preferentially induces proliferation of hepatocytes, and the activation, proliferation, and differentiation of progenitor cells into hepatocytes requires simultaneous administration of D-galactosamine, which blocks RNA and protein synthesis in hepatocytes (Dabeva and Shafritz 1993). Moreover, in rats that had undergone PH, the expansion of oval cells is not observed unless 2-acetaminofluorene (2-AAF) is supplied; this compound is metabolized by cytochrome P450 into hydroxylated metabolites that bind to DNA, hereby blocking cell cycle progression before S phase and impairing cell function specifically in hepatocytes and not in HPC that lack such enzymes (Dusabineza *et al.* 2012).

Ductular reaction in humans is morphologically and immunohistochemically analogous to the rodent oval cell response in the periportal regions. Ductular reaction involves not only HPC but also mesenchymal, vascular hematopoietic and inflammatory cells, together with ECM modifications. As a consequence, it could result in inflammatory infiltration, rich in neutrophil infiltration (cholangiolitis), periportal fibrosis and neovascularization. The magnitude of HPC activation corresponds to the severity of liver fibrosis and inflammation. Ductular reaction has been described in severe acute liver disease, such as fulminant hepatic failure, and many forms of chronic human liver disease such as focal nodular hyperplasia, primary biliary cirrhosis, primary sclerosing cholangitis, chronic hepatitis C, alcoholic and non-alcoholic steatohepatitis and cancer (Clouston *et al.* 2005; Richardson *et al.* 2007; Bird *et al.* 2008; Gouw *et al.* 2011; Ye *et al.* 2014).

3.3 The oval cell response

Upon activation, oval cells proliferate, invade and expand into liver parenchyma from the periportal region. The oval cell response can be divided into 4 phases: activation, proliferation, migration, and differentiation (**FIGURE 4**).

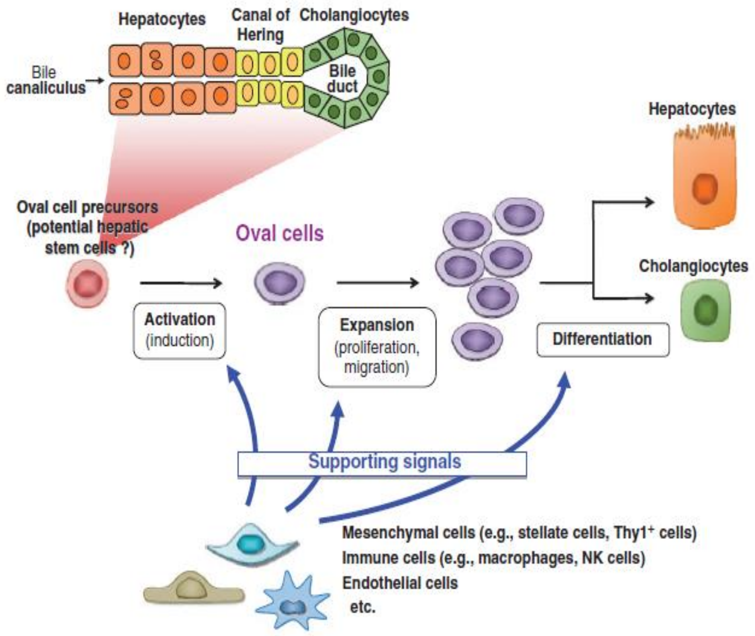


FIGURE 4. Oval cell response

Oval cells are induced in severe liver damage conditions. The origin of oval cells is still under debate. Chronic injury conditions in the liver are usually associated with inflammation, and the roles of lymphocytes and inflammatory responses in oval cell regulation have also been suggested. Moreover, mesenchymal cells such as stellate cells have long been suggested to physically interact with oval cells and thus considered to induce some signals in them.

(Modified from Tanaka et al., 2011)

The regulation of these different phases of the oval cell response seems to be a very complex process involving a number of cytokines and growth factors. These actors that impact the oval cell response can do so either directly (acting on the proliferating cells) or indirectly by signaling to a non–epithelial cell type, which might, in turn, then act on the oval cells. Some of these factors are summarized in the following table (**TABLE 2.**) and include cytokines, like IL-6 or TNF-α, and growth factors such as HGF (hepatocyte growth factor), EGF, TGF-β, among others. Hormones (such as insulin, somatostatin), adipokines (cytokines released by adipocytes, such as leptin) and neurotransmitters (such as serotonin, epinephrine or norepinephrine) have also been reported to regulate HPC response or growth. Finally, there is increasing evidence to suggest that morphogens (factors playing a critical role during embryonic development) such as Wnt and Notch signaling pathways are key regulators of oval cell fate determination in rodents and humans (Hu *et al.* 2007; Apte *et al.* 2008; Boulter *et al.* 2012).

The interactions and signaling crosstalk between these factors and signaling pathways are complex and remain poorly understood (Campbell *et al.* 2001; Nobili *et al.* 2012; Best *et al.* 2013).

TABLA 2. The factors that are involved in each stage of the OCs response

ACTIVATION	PROLIFERATION	MIGRATION	DIFFERENTIATION
IL-6 TNF SCF IL-18 IFN-γ TWEAK	HGF FGF-1 TGF-α TGF-β EGF	uPA tPA HGF	LIF OSM/OSM-R Dlk/Pref-1

SCF: Stem cell factor; IFN-γ: Interferon-γ; TWEAK: Tumor necrosis factor-like weak inducer of apoptosis; FGF-1: Fibroblast growth factor 1; TGF-α: Transforming growth factor alpha; uPA: Urokinase-type plasminogen activator; tPA: tissue-type plasminogen activator; LIF: Leukemia inhibitory factor; OSM: Oncostatin M and OSMR: OSM Receptor.

Hepatocyte injury triggers the oval cell activation, but the mechanism by which the damage is communicated to the oval cell precursors is unknown. Furthermore, the specific roles of various hepatic cell types in the signaling process are unclear (Erker and Grompe 2007). Therefore, several hypotheses exist regarding the source/s of the signal/s that result in the activation of the oval cell precursor:

I. Direct signaling from damaged hepatocytes;

II. Indirect signals from hepatic stellate cells in response to hepatocyte injury;

III. Damage-induced cytokines released from inflammatory cells;

IV. Endothelial cells as a source of the primary signal;

V. Multiple cell types may be required and each may contribute to the activation process.

A particularly important role has been given to inflammatory signals. As just mentioned, different cytokines involved in the inflammatory response appear to play important roles in the oval cell response: IL-6 promotes the phosphorylation of STATs proteins that in turn translocate to the nucleus to act as transcription factors, ultimately leading to the proliferation of oval cells. Thus, STAT3 is highly active in oval cells expanding in the liver after AAF/PH-induced damage (Sanchez *et al.* 2004) and knock-in mouse models have demonstrated that hyperactive STAT3 signaling results in enhanced oval cell numbers (Yeoh *et al.* 2007).

TNF-α is released at the site of liver injury mainly by KCs. TNF-α activates the tumor necrosis factor receptor 1 (TNFR1) on inflammatory cells to release several cytokines. Once oval cells have received this signal, they produce TNF-α themselves, generating an autocrine loop. Further evidence that TNF-α is involved in oval cell activation is that TNFR1 knockout mice have a clearly impaired oval cell response (Knight *et al.* 2000). Other inflammatory cytokines that play a role in this step are the tumor necrosis factor-like weak inducer of apoptosis (TWEAK) (Jakubowski *et al.* 2005); IL-18 response that results in interferon-γ (IFN-γ) secretion and IFN-γ itself (Akhurst *et al.* 2005).

In addition to the inflammatory cytokines, a combination of genetic studies in rodents and *in vitro* studies has allowed identifying HGF, EGF and TGF-β as key factors in the regulation of the oval cell response. Regarding EGF, early gene expression studies showed that EGF is overexpressed during the period of active proliferation and differentiation of progenitor cells in rat liver following the protocol 2-AAF/PH and this increase seems to drive the early proliferation of the progenitor cell compartment. Moreover, *in vivo* infusion of EGF or HGF decreases apoptosis (Nagy *et al.* 1996). Consistently, we have demonstrated that the EGFR signaling promotes oval cells proliferation and survival *in vitro* (Martinez-Palacian *et al.* 2012).

On the other hand, Kitade et al. have demonstrated that c-Met signaling is a strong inducer of hepatocyte differentiation via activation of AKT and STAT3, whereas EGFR is required for NOTCH1-controlled expression of cholangiocyte-specific genes and ductular morphogenesis. Both stimulation of NOTCH1 expression and biliary epithelial differentiation were reduced during progenitor cell-mediated liver regeneration after DDC treatment in EGFR conditional knockout mice, supporting a role of the EGFR/NOTCH1 positive feedback loop for commitment of adult HPCs toward biliary epithelial cell lineage (Kitade *et al.* 2013). The effects of HGF on oval cells will be discussed in detail in another section of the introduction.

Contrarily, suppressor effects for TGF-β in the oval cell response have been described. Specifically, data from literature show that TGF-β overexpression *in vivo* in hepatocytes and oval cells inhibited the oval cell response following DDC treatment in mice (Preisegger *et al.* 1999). We have also demonstrated proliferation inhibitory and pro-apoptotic activities of TGF-β in oval cells in vitro (del Castillo *et al.* 2008).

Oval cells must move from the periportal zone into the liver parenchyma and differentiate. This migratory response involves extensive tissue remodeling. Indeed, a number of studies show that the expansion of the oval cell compartment occurs in association with ECM remodeling, which involves the participation of many proteins, including urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), the uPA receptor (uPAR), and plasminogen activator inhibitor 1 (PAI-1) (Erker and Grompe 2007; Lorenzini *et al.* 2010; Lozoya *et al.* 2011).

3.4 The contribution of oval cell to liver regeneration

The role of oval cells in regeneration of the damaged liver has been tested in transplantation experiments. Thus, transplanted oval cells derived from rat liver or pancreas have proved to proliferate and differentiate into mature hepatocytes, even under non-selective conditions (Dabeva *et al.* 1997). Additionally, repopulation studies utilizing an *in vivo* selection model indicated that mouse oval cells (induced by DDC) have an extensive liver repopulation capacity (Wang *et al.* 2003). These and other studies using similar approaches (Suzuki *et al.* 2008) have convincingly proved their regenerative potential. It is also important to mention that using experimental models of oval cell induction it has been demonstrated that repeated oval cell reactions facilitates their expansion and liver regeneration without increasing the risk of tumorigenesis (Papp *et al.* 2014).

However, other studies using cell tracing technologies in murine models of experimental injuries have revealed that HPC contribution to generate adult parenchymal cells during liver regeneration is minor (Malato *et al.* 2011; Espanol-Suner *et al.* 2012; Rodrigo-Torres *et al.* 2014; Yanger *et al.* 2014), so the actual implication of oval cell expansion in the regenerative response is nowadays a matter of debate (Riehle *et al.* 2011; Shin and Kaestner 2014).

One possibility is that oval cell contribution to regeneration depended on the extent of damage and/or the specific tissue context. In support of this, using different models it has been demonstrated that severe hepatocyte function impairment is necessary to elicit a HPC-mediated-regenerative response in rodents (Choi *et al.* 2014; Lu *et al.* 2015). However, this might not faithfully reflect what occurs in humans, where HPCs are activated in the majority of liver diseases, even in minimal degrees of liver damage (Kohn-Gaone *et al.* 2016).

In spite of the controversy, human HPC have been proposed for cellular based therapy in decompensated liver cirrhosis, since they are less immunogenic and present a higher propagative capacity than adult hepatocytes (Habeeb *et al.* 2015).

3.5 The other side of the coin: oval cells and hepatic fibrosis, cirrhosis and HCC

As it was stated before, during chronic injury the HPC response is activated (Pritchard and Nagy 2010; Williams *et al.* 2014). Indeed, a HPC response has been described in many forms of chronic human liver disease and in severe acute liver disease. The fact that there is an apparent direct correlation between the degree of fibrosis and the extent of HPC expansion (Williams *et al.* 2014) has raised the question of whether fibrosis is beneficial for the HPC-triggered regenerative response, which in turn is beneficial for resolving the fibrotic process or whether fibrosis is unintentionally exacerbated by the progenitor reaction. Clearly, this issue has major implications for the development of either anti-fibrotic or pro-regenerative therapies.

The functional requirement of a HPC response for fibrogenesis has not been established to date. Nevertheless, some researchers have suggested that oval cells drive the fibrotic reaction through the increase of collagen deposition (Jakubowski *et al.* 2005) or the overexpression of integrin alphavbeta6 (αvβ6), which correlates with fibrosis progression in human and experimental liver disease (Patsenker *et al.* 2008). In accordance with this, Kuramitsu et al. propose a vicious circle model in which failure of hepatocyte replication and death during fibrotic liver regeneration activates the reserve progenitor cell compartment, which in turn elicits a severe fibrogenic response and further compromises hepatocyte-mediated liver regeneration in the fibrotic microenvironment (Kuramitsu *et al.* 2013).

In support of the alternative view that considers fibrosis as a promoting factor for oval cell activation, it has been proposed that matrix remodelling could itself drive oval cell expansion and therefore HPC-associated regenerative response (Pi *et al.* 2005; Van Hul *et al.* 2009). These two opposing ideas are not mutually exclusive, and this paradox can be resolved by recognizing heterogeneity in the ductular reaction activation and evolution (Clouston *et al.* 2009).

Besides this, evidence suggest that deregulation of the oval cell activation/differentiation pathway can result in malignant transformation of these cells that subsequently become tumor-initiating cells and drive hepatocarcinogenesis. Several studies based on immunohistochemical analysis of HCCs have concluded that about 28–50% of HCCs express markers of progenitor cells (Roskams 2006; Caldwell and Park 2009). Interestingly, these tumors carry a significantly poorer prognosis and higher recurrence after surgical resection and liver transplantation (Uenishi *et al.* 2003; Lee *et al.* 2006; Yang *et al.* 2010; Kohn-Gaone *et al.* 2016).

Nevertheless, the question is whether this immature intermediate phenotype represents progenitor cell differentiation arrest or dedifferentiation of mature hepatocytes. Histological and immunophenotyping studies favour the progenitor cell differentiation arrest model, that implies a situation in which HPC subjected to certain pathological conditions would evolve to tumor initiating cells, therefore neoplastic transformation of HPC should be a critical molecular event during hepatocarcinogenesis (Kitisin *et al.* 2007). Consistently, exposure of oval cells to carcinogens results in HCC formation and certain carcinogenic regimes are accompanied by oval cells accumulation (Steinberg *et al.* 1994; Sell and Leffert 2008); furthermore, oval cells can become transformed in culture (Dumble *et al.* 2002; Tang *et al.* 2008; Wu *et al.* 2012).

Certainly, HPC constitute a very interesting but still poorly understood cell population. It seems evident the need to better understand the mechanisms of regulation and transformation of HPC during CLD and the implication of different growth factors in this process. Dissecting these pathways is critical for development of drugs and therapies. The use of oval cells for cell therapy is an attractive alternative to organ transplantation for the treatment of liver disease. One of the main advantages of this type of therapy is that it would allow isolation of oval cells from a patient who has a liver disease, further expansion in culture and/or manipulation to increase their regenerative potential, and autologous transplantion to the patient in order to restore liver function.

4. HGF/c-Met SIGNALING PATHWAYS

4.1 HGF: structure, synthesis, expression and function

HGF was originally identified as a strong mitogen for mature hepatocytes *in vitro* in the 1980s (Nakamura 1989). It is also known as Scatter factor (SF), as it was found, independently, as a product of secretion of fibroblasts and smooth muscle cells able to induce epithelial cell motility (Stoker *et al.* 1987); only later it was discovered that the two molecules were actually the same (Naldini *et al.* 1991).

70 kb human HGF gene is located on chromosome 7q21.1 and is composed by 18 exons separated by 17 introns.

HGF is a heterodimeric glycoprotein consisting of a heavy (α) and a light (β) chain with approximate molecular weights of 64,000 and 32,000 daltons, respectively. HGF is structurally related to the blood proteinase precursor plasminogen. The primary structure of HGF is more than 90% homologous in humans and rodents (Nakamura 1989; Zarnegar *et al.* 1992). Like plasminogen, HGF is produced as an inactive protein (pro-HGF), which is subsequently cleaved. This 728 amino acid precursor form binds to the cell surface or the extracellular matrix, presumably via its affinity for heparin-like glycosaminoglycans. Limited proteolysis of pro-HGF at the Arg494 - Val495 bond yields the active disulfide-linked heterodimer (αβ-HGF): the α chain consists of a putative hairpin loop and four kringle domains, each defined by three disulfide bonds, while the β chain has homology to the catalytic domain of serine proteases but lacks enzymatic activity (Patthy *et al.* 1984) (**FIGURE 5**). αβ-HGF binds the c-Met receptor with high affinity.

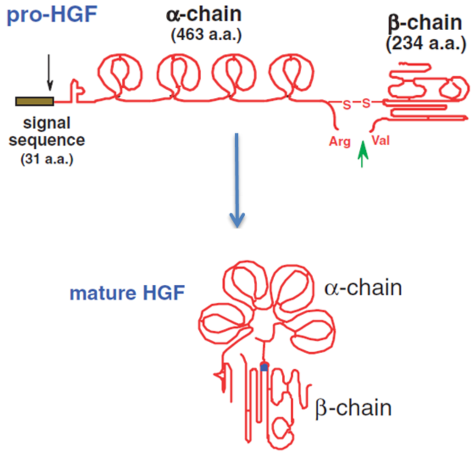


FIGURE 5. Synthesis, structure and maturation of HGF

HGF is produced and secreted as a pro-HGF, which is cleaved at the Arg494 - Val495 bond by HGF-activators to form mature HGF.

(Modified from Nakamura and Mizuno, 2010)

Maturation of secreted pro-HGF into the bioactive form takes place in the extracellular environment through a proteolytic cleavage. Different serine proteases have been implicated in this proteolytic conversion including HGF activator (HGFA), uPA, tPA, blood coagulation factors XIIa and XI, matriptase, hepsin and plasma kallikrein (Naldini *et al.* 1995; Nakamura *et al.* 2011).

In general, HGF is produced and secreted by stroma cells or cells of mesenchymal origin (such as fibroblasts, macrophages, and smooth muscle cells among others) of various organs such as liver, lung, kidney, placenta and brain, both during development and the adult stage (Nakamura and Mizuno 2010). Specifically, in normal liver, the major cellular source of HGF is the HSC (Maher 1993). HGF acts in a paracrine manner, as HGF receptor c-Met is mainly expressed in epithelial cells, although some cellular types express both HGF and c-Met showing therefore an autocrine signaling (Sonnenberg *et al.* 1993; del Castillo *et al.* 2008).

HGF gene transcription is upregulated by inflammatory modulators such as TNF-α, IL-1, IL-6, TGF-β and VEGF (Broten *et al.* 1999). Other molecules that induce the production of HGF are norepinephrine, prostaglandins and also growth factors such as platelet-derived growth factor (PDGF) and FGF (Matsumoto *et al.* 1995; Boccaccio *et al.* 1998).

HGF has been shown to be a mitogen that stimulates growth of various epithelial cells; it also enhances motility of epithelial cells; and acts as a morphogen that induces epithelial tubule formation. Among other processes in which it is involved, we can cite the development of skeletal muscle (Bladt *et al.* 1995), the development of nervous system, bone remodeling and angiogenesis (Birchmeier *et al.* 2003; Nakamura *et al.* 2011). Moreover, HGF stimulates cell invasion and polarization, and inhibits apoptosis (Comoglio and Trusolino 2002; Zhang and Vande Woude 2003; Tulasne and Foveau 2008). Importantly, HGF plays a crucial role in liver, which will be described in detail in a subsequent section (see HGF/c-Met signaling pathway in the liver).

4.2 c-Met: structure, synthesis, expression and function

The MET proto-oncogene located on chromosome 7q21-31 was first isolated from a human osteosarcoma-derived cell line on the basis of its transforming activity *in vitro* (Cooper *et al.* 1984). A few years later, c-Met was identified as the receptor of HGF (Bottaro *et al.* 1991).

The c-Met receptor is formed by proteolytic processing of a common precursor into a single-pass, disulphide-linked α/β heterodimer (50 kD α-chain and a 145 kD β-chain).

The entire extracellular α-chain forms a semaphorin (Sema) domain and is linked to the extracellular portion of the β-chain, which is composed of three types of domain: the N-terminal 500 residues form the Sema domain together with the α-subunit; the plexin–semaphorin–integrin (PSI) domain follows the Sema domain, spans approximately 50 residues and includes four disulphide bonds. This domain is connected to the transmembrane helix *via* four immunoglobulin–plexin–transcription (IPT) domains, which are related to immunoglobulin-like domains. Intracellularly, the c-Met receptor contains a tyrosine kinase catalytic domain flanked by distinctive juxtamembrane and carboxy-terminal sequences (**FIGURE 6**).

The catalytic region contains the catalytic tyrosines Y1234 and Y1235, which upon phosphorylation positively modulate the enzymatic activity, while the juxtamembrane tyrosine 1003 negatively regulates c-Met by recruiting the ubiquitin ligase casitas B-lineage lymphoma (c-CBL). The multifunctional docking site in the C-terminal tail contains tyrosines Y1349 and Y1356, which recruit several transducers and adaptors when c-Met is active (Benvenuti and Comoglio 2007; Organ and Tsao 2011). For its particular structure, c-Met belongs to a family of heterodimeric tyrosine kinases, which also includes Ron, Ryk and Sea receptors (Huff *et al.* 1993; Ronsin *et al.* 1993; Gaudino *et al.* 1994; Maestrini *et al.* 1996).

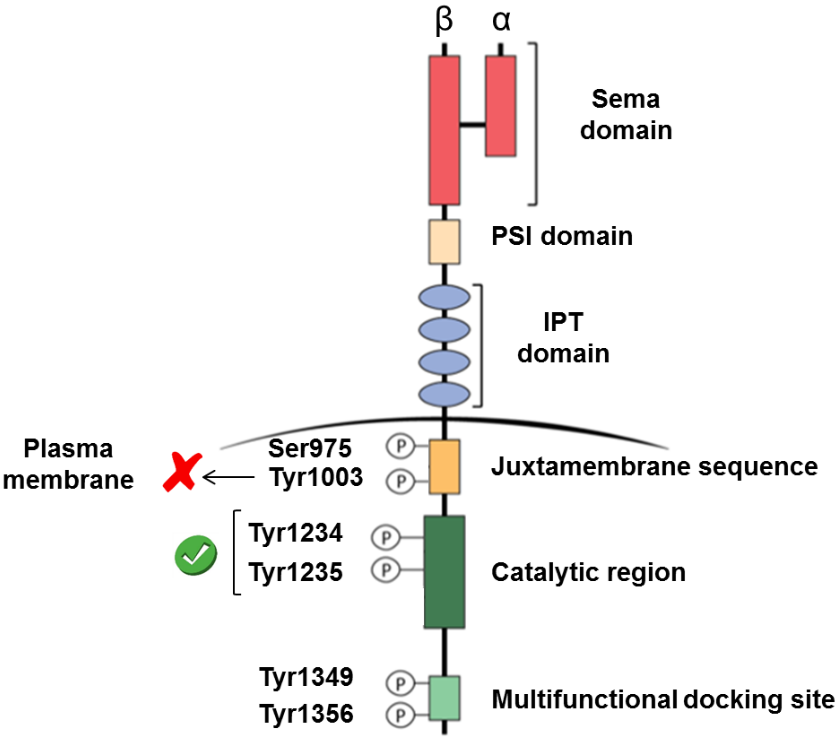


FIGURE 6. c-Met structure

The extracellular region of c-Met is composed of three domains: the Sema domain that includes the entire α-chain and part of the β-chain; the PSI domain and four IPT domains. The intracellular region of c-Met consists of three portions: a juxtamembrane sequence; a catalytic region; and a carboxy-terminal multifunctional docking site.

(Modified from Faria et al., 2011)

HGF ligand contains two c-Met binding sites: a high affinity site is located in the α-chain and recognizes IPT domains of c-Met independently of the HGF maturation. The low affinity site is located in the β-chain, requires HGF processing and interacts with the Sema domain (Stamos *et al.* 2004; Basilico *et al.* 2008).

c-Met is expressed in epithelial cells of many organs, including liver, pancreas, prostate, kidney, muscle and bone marrow, during both embryogenesis and adulthood (Comoglio *et al.* 2008). Its expression is induced by multiple factors including HGF, EGF, IL-1, IL-6, TNF-α, estrogen, progesterone and dexamethasone (Moghul *et al.* 1994; Zarnegar 1995).

4.3 HGF/c-Met signaling pathway

When HGF binds to c-Met, the receptor undergoes autophosphorylation of the Y1234 and Y1235 residues in the kinase domain; subsequently, tyrosine residues in the docking site (Y1349 and Y1356) become phosphorylated and recruit signalling effectors and adaptor proteins such as Growth factor receptor-bound protein 2 (GRB2), PI3K, STAT-3, Src homology-2-containing (SHC) and v-crk sarcoma virus CT10 oncogene homolog (CRK), CRK-like (CRKL) and Src homology domain-containing 5' inositol phosphatase (SHIP-2) (Trusolino *et al.* 2010; Organ and Tsao 2011; Gherardi *et al.* 2012; Granito 2014). These molecules facilitate downstream signalling through several pathways. Moreover, unique to c-Met is its association with the adaptor protein GRB2-associated binding protein 1 (GAB1), a multi-adaptor protein that, once bound to and phosphorylated by c-Met, creates more binding sites for downstream adaptors (Weidner *et al.* 1996).

c-Met engagement results in the activation of multiple signal transduction pathways including the MAPKs cascade (Mitogen Activated Protein Kinases), ERK1 and ERK2, JNK (c-Jun N-terminal Kinases) and p38 MAPK; the PI3K-AKT and its downstream regulator nuclear factor kappaB (NF-κB); and the activation of STAT-3 pathway (Sipeki *et al.* 1999; Maroun *et al.* 2000; Birchmeier *et al.* 2003). All these signaling pathways transmit the information from the cellular membrane to the nucleus to modulate gene expression participating in the control of several cellular processes such as proliferation, survival, invasion and migration (**FIGURE 7)**.

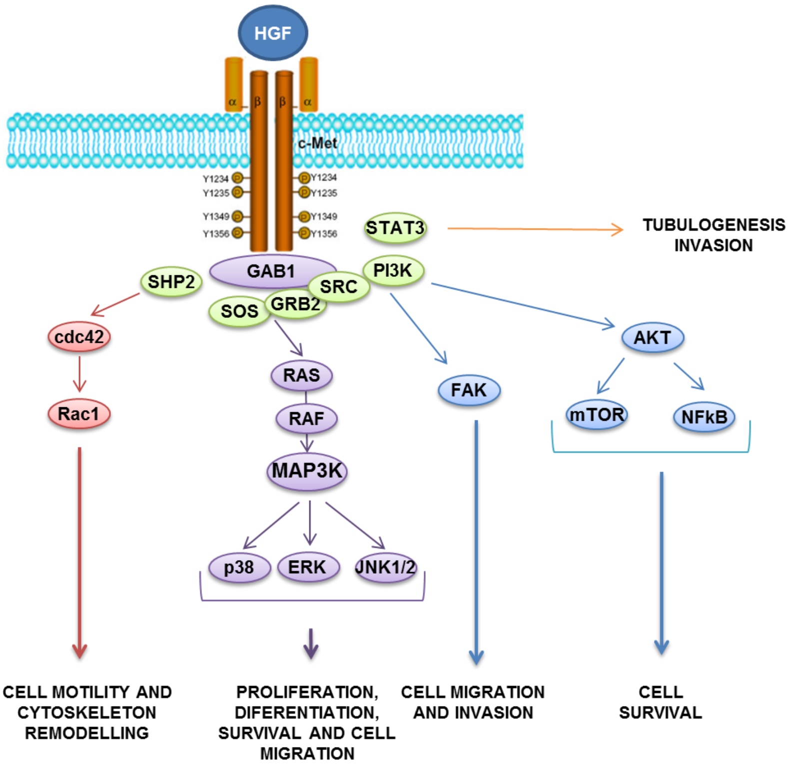


FIGURE 7. The HGF/c-Met signaling pathway

Following HGF binding, the kinase activity of c-Met is switched on. This leads to the activation of downstream signaling pathways that include MAPK, PI3K/AKT and STAT pathways, which mediate c-Met-dependent cell functions.

(Modified from Granito et al., 2014)

Activation of MAPK cascades by c-Met implies the activation of RAS protein through the guanine nucleotide exchanger Son of Sevenless (SOS) *via* binding with SHC and GRB2. This leads to the indirect activation of v-raf murine sarcoma viral oncogene homolog B1 (RAF) kinases, which can subsequently activate the MAPK effector kinase MEK and finally MAPK that then translocate to the nucleus to activate transcription factors responsible for regulating the expression of a large number of genes. In the context of c-Met signaling, MAPK signalling drives cell cycle progression,proliferation and cell motility (Organ and Tsao 2011).

The other major arm of c-Met signalling is the PI3K/Akt signalling axis. The p85 subunit of PI3K can bind either directly to c-Met or indirectly through GAB1, which then signals through AKT/protein kinase B. This axis is primarily responsible for the cell survival response to c-Met signalling (Organ and Tsao 2011).

Furthermore, the direct binding of STAT3 to c-Met results in STAT3 phosphorylation, dimerization and its translocation to the nucleus where it regulates gene expression. This has been shown to result in tubulogenesis (Boccaccio *et al.* 1998) and invasion (Syed *et al.* 2011). However, other reports found that, although it is required for c-Met-mediated tumorigenesis, it has no effect on proliferation, invasion or branching morphogenesis (Zhang *et al.* 2002). Thus, the role of STAT3 in c-Met signaling is probably context- and tissue-dependent.

Cellular migration is also mediated downstream of c-Met by focal adhesion kinase (FAK), which is localized to cellular adhesion complexes. FAK is activated through phosphorylation by SRC family kinases, which have been shown to associate directly with c-Met (Chen and Chen 2006).

c-Met activity is also subjected to inhibitory regulation. Several protein phosphatases have been shown to dephosphorylate either the catalytic tyrosines (PTP1B, protein tyrosine phosphatase 1B) or the docking tyrosines (PIP density enhanced phosphatase, DEP1) (Gandino *et al.* 1994; Xu *et al.* 2012).

Additionally, c-Met signaling can be modulated by cross-talk between Met and different membrane receptors, acting in complex interacting networks (Giordano and Columbano 2014). Thus, c-Met interacts with adhesive receptors, such as CD44 (Orian-Rousseau *et al.* 2002) and the α6β4 integrin (Trusolino *et al.* 2001), with semaphorin receptors (Giordano *et al.* 2002), receptor tyrosine kinases (RTKs), such as members of the EGF receptor Family (EGFR and HER2) (Jo *et al.* 2000) and the VEGF receptor (VEGFR) (Sulpice *et al.* 2009; Lu *et al.* 2012), among others.

4.4 HGF/c-Met signaling pathway in the liver

In the liver, HGF/c-Met pathway comprises a complex and unique signaling network that plays a pivotal role in liver development, stimulating cell growth, motility, differentiation and morphogenesis of hepatocytes (Stoker *et al.* 1987; Hilberg *et al.* 1993; Fausto *et al.* 1995; Nakamura *et al.* 2011). This signaling cascade is not only crucial in hepatic tissue remodeling during fetal development, but also during liver regeneration and the resolution of fibrotic processes. Moreover, it has been demonstrated that the constitutive or prolonged activation of c-Met is involved in tumor growth and cancer progression.

Regarding the essential role of the HGF/c-Met axis in liver development, animal studies have shown that HGF and c-Met provide essential signals for survival and proliferation of hepatocytes during embryogenesis, since *HGF* or c-*Met* knockout mice display profound alterations in the embryonic liver (Bladt *et al.* 1995; Schmidt *et al.* 1995; Uehara *et al.* 1995).

While homozygous null mice for either HGF or c-Met die in utero at day 13 and 16, respectively, due to impaired organogenesis (Uehara *et al.* 1995) when HGF or c-Met are knocked down at later phases during the development, the livers of these mice are reduced in size as a result of decreased hepatocyte proliferation and increased susceptibility to apoptosis.

It is known that in normal liver, the major cellular source of HGF is the HSCbut after liver injury, HGF expression has been thought to increase markedly in proliferating liver sinusoidal endothelial cells (LSECs) (Kinoshita *et al.* 1989; Maher 1993) and the degree of induction of HGF mRNA correlates well to the degree of liver damage; thus, these findings suggest that HGF is produced by the non-parenchymal liver cells after an injury and HGF, acting in a paracrine manner, stimulates the growth of hepatocytes to regenerate the liver.

In the adult animals, loss of c-Met is not critical for hepatocyte function under physiological conditions, however, c-Met becomes critical in response to hepatic injuries. In fact, using MetLivKO mouse models, several studies demonstrated that c-Met activation in hepatocytes promotes mitogenic and antiapoptotic responses for organ repair. Indeed, selective ablation of the c-Met gene in adult mouse hepatocytes dramatically affects the reparative responses of the liver to acute injury *in vivo*. Furthermore, c-Met-deleted hepatocytes contribute to the disruption of tissue remodeling and are responsible for the conversion of a normally acute injury into a chronic lesion. In the same line of evidence, transgenic overexpression of HGF in mice results in enhanced regeneration and increased liver size (Shiota *et al.* 1994). Thus, these evidence demonstrate that HGF is key for a successful regenerative response after acute and chronic liver damage (Borowiak *et al.* 2004; Huh *et al.* 2004; Phaneuf *et al.* 2004).

In addition, an essential anti-fibrogenic role for HGF/c-Met has also been demonstrated (Xia *et al.* 2006; Marquardt *et al.* 2012). Thus, HGF/c-Met signaling has a hepatoprotective effect attenuating liver fibrosis by inhibiting TGF-β pro-fibrotic responses and promoting ECM resolution (Ueki *et al.* 1999; Inagaki *et al.* 2008; Kanemura *et al.* 2008; Giebeler *et al.* 2009). Furthermore, HGF induces apoptosis in myofibroblast, a cell type that as mentioned before plays a central role during the fibrotic process. The work done by Marquardt et al. showed that genetic loss of c-Met in hepatocytes induces a state of “organ stress” characterized by multi-factorial changes both in parenchymal and non-parenchymal cell compartments in the liver. These include reduced hepatocyte proliferation, excessive activation of HSC and increased expression of pro-fibrotic molecules, such as TGF-β, thereby aggravating liver fibrosis.

In conclusion, the pro-regenerative role of HGF/c-Met signaling pathway is the consequence of its powerful anti-fibrotic and anti-apoptotic activities. Although more work is required to determine the exact contribution of HGF to each of these processes, these studies strongly support current efforts directed to the modulation of the HGF/c-Met signaling pathway in CLD in therapeutic settings.

Finally, the HGF/c-Met axis has been also implicated in hepatocarcinogenesis through multiple mechanisms, many of which are still being elucidated (Goyal *et al.* 2013). Although gene amplification is found only at a very low frequency, overexpression of HGF and c-Met is observed in 20-50% of human HCC (Boix *et al.* 1994; Kiss *et al.* 1997), although the prognostic utility of these markers is uncertain. Thus, c-Met overexpression was found to be associated with poorly differentiated HCC and a c-Met regulated gene expression signature was found to define a subset of human HCC with poor prognosis and aggressive phenotype (Daveau *et al.* 2003; Kaposi-Novak *et al.* 2006). Further insights have led to propose that HGF/c-Met activation is important for the acquisition and maintenance of a mesenchymal phenotype and cancer stem cell features (You *et al.* 2011). Importantly, crosstalk between c-Met and other RTKs, EGFR and VEGF, is also implicated in promoting tumor survival (Venepalli and Goff 2013). Nevertheless, although the tumor promoting effects are the most prominent, we must keep in mind that specific growth inhibitory and pro-apoptotic effects have been described for this signaling axis in some tumor cells, including HCC cells (Tulasne and Foveau 2008).

4.5 HGF/c-Met signaling pathway in oval cells

Regarding the role of HGF as a regulator of liver oval cells, it is known that HGF is expressed by HSC that are proliferating in close proximity to oval cells during liver regeneration and it was also reported that HGF accelerates oval cell proliferation after 2-AAF/PH. In fact, *in vivo* activation of oval cell compartment in the AAF/PH liver injury model in rat is accompanied by an increased expression of HGF. Furthermore, HGF infusion during liver regeneration in this model increases oval cell proliferation (Shiota *et al.* 2000). Using a conditional c-Met knock-out mouse model specific for the liver, it was demonstrated that loss of c-Met completely abolishes the oval cell-mediated regenerative response. Interestingly, absence of c-Met targeted multiple cellular processes required for regeneration, including oval cell proliferation, survival, differentiation and migration (Ishikawa *et al.* 2012).

In addition, HGF induces mitogenic and morphogenic responses in oval cells *in vitro.* We have shown that HGF/c-Met signaling promotes oval cells survival via autocrine and paracrine mechanisms involving PI3K activation. Thus, HGF/c-Met/PI3K signaling axis plays a central role in protecting oval cells against TGF-β-induced oxidative stress and apoptosis by acting as an antioxidant signal (Martinez-Palacian *et al.* 2013). Furthermore, our group has recently demonstrated that HGF promotes a morphogenetic response in oval cells *in vitro* that includes a migratory and invasive response through a mechanism involving PI3K activation and MMPs expression and activation (Suarez-Causado *et al.* 2015).

5. BONE MORPHOGENETIC PROTEINS (BMPs)

5.1 BMPs: generalities

BMPs (Bone Morphogenetic Proteins) are multifunctional cytokines that belong to the TGF-β superfamily, being in fact the largest TGF-β subfamily comprising more than 15 ligands in mammals (Herrera *et al.* 2014).

BMP ligands have been classified into 4 different subgroups according to their sequence similarity and functions: (i) BMP2 and 4; (ii) BMP5, 6, 7, 8a and 8b; (iii) BMP9 and BMP10. All BMPs belonging to these three subgroups show osteogenic properties. The other proteins, BMP3, 3b, 11, 12, 13, -14, 15, and 16, that belong to the last group (iv), do not possess osteogenic properties and, in some cases, can even inhibit BMP function. Finally, BMP1 is actually a metalloprotease and it is not considered a member of the family (Lissenberg-Thunnissen *et al.* 2011). The nomenclature of BMPs is complex including BMPs, Growth and Differentiation Factors (GDFs), and other names depending on the method used to identify them.

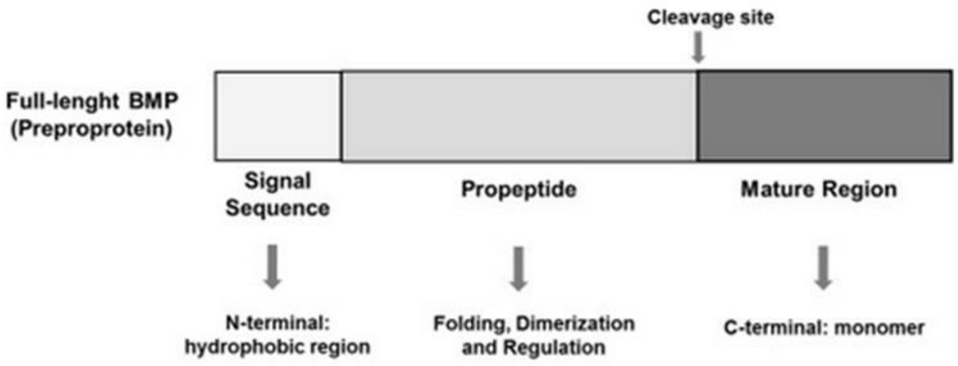


FIGURE 8. General structure of BMPs.

Full-length BMP consists of a signal sequence, a pro-peptide, and the mature region.

(*modified from Carreira et al., 2014*)

Members of the BMP family are synthesized as large dimeric pro-proteins containing a signal peptide for secretion. The pro-domain is cleaved at a consensus Arg-X-X-Arg site by serine endoproteases within the trans-Golgi network to produce the mature protein, which is subsequently secreted. It has been recently shown that the downstream sequence adjacent to the cleavage site determines the cleavage efficiency, while the N-terminal region controls the stability of the processed mature protein (Ducy and Karsenty 2000). Cleavage of the pro-protein does not occur in all BMP ligands: exceptions are BMP9 and GDF8 (Myostatin), in which the pro-region remains attached to the mature protein, even after secretion. Therefore it is known that BMP9 circulates in plasma either as an unprocessed inactive form unable to bind its receptor that can be further activated by furin, a serine endoprotease, or as a mature and fully active form (composed of the mature form associated with its pro-domain) (Sieber *et al.* 2009; Bidart *et al.* 2012). BMPs form homodimers or heterodimers to become active molecules. Strikingly, both *in vitro* and *in vivo* evidence demonstrate that several BMP heterodimers present an increased potency as compared to BMP homodimers, as shown for BMP4/7, BMP2/6, BMP2/7, BMP15/GDF9 and BMP2b/7 (Little and Mullins 2009; Carreira *et al.* 2014; Sanchez-Duffhues *et al.* 2015).

BMPs were originally discovered for their capacity to induce bone and cartilage formation and fracture repair and regulate growth and differentiation of chondroblast and osteoblast cells *in vitro* (Urist 1965). Their role in early development is also well documented, including dorsal-ventral patterning, organogenesis and cell differentiation, being BMPs therefore considered as potent morphogens (Wagner *et al.* 2010). More recently, a role for BMPs in adult tissue homeostasis have started being revealed. Studies from transgenic and knockout mice and from animals and humans with naturally occurring mutations in BMPs and related genes have shown that BMP signaling plays critical roles in heart, neural and cartilage development. BMPs also play an important role in postnatal bone formation.

To date it is known that BMPs regulate several cellular processes, including cell proliferation, differentiation, chemotaxis, apoptosis and migration in many different cell types. Recent evidence indicates that the liver is also an important target of BMPs.

A well-studied aspect about the BMPs effect in liver physiology is undoubtedly their key role in the control of iron homeostasis, where BMP6 binds to its receptors and co-receptor (hemojuvenin) in hepatocytes to regulate hepcidin expression through a Smad dependent mechanism (Finberg 2013). Additionally, an implication of BMPs in CLD has been suggested, although their physiopathological role and importance remain to be further explored. Thus, the role of BMP7 in CLD is somehow controversial. Some studies suggest that BMP7 has a pro-fibrogenic role in liver. Particularly, it has been shown that systemic and hepatic BMP7 is elevated in patients with CLD, which may contribute to progression of liver fibrogenesis *in vivo* since it is able to stimulate HSC proliferation and ECM production *in vitro* (Tacke *et al.* 2007). Contrarily, an anti-fibrogenic effect of BMP7 in liver has also been suggested in agreement with its action in renal fibrosis; in fact, BMP7 through its TGF-beta antagonizing activity significantly inhibits progression of liver fibrosis in the CCl_4-model (Zeisberg *et al.* 2003). Beneficial effects of BMP7 have been also proposed in the liver regenerative process, since BMP7 has been shown to enhance liver regeneration after PH (Sugimoto *et al.* 2007). On the other hand, another member of this family, BMP4 has been recently described as an inhibitor of this process (Do *et al.* 2012). Although more work is needed to clarify the exact contribution of BMPs to the fibrogenic and regenerative processes in the liver, evidence support the concept that this organ is indeed a target of BMP action, and therefore dysregulation of BMP signaling most likely has pathological consequences in the liver (Herrera *et al.* 2014).

5.2 BMP9: structure, synthesis, expression and function

In this very complex context, we are particularly interested in one of the members of this family, the bone morphogenetic protein-9 (BMP9), also known as Growth Differentiation Factor 2 (GDF2), the most recently discovered member of the BMPs family.

BMP9 contains an N-terminal TGF-β-like pro-peptide (pro-domain) (residues 56–257) and a C-terminal TGF-β superfamily domain (325–428). It is synthesized as a precursor protein (Pre-pro-BMP9) composed of 429 amino acids that include a 22 amino acids signal peptide, a 297 amino acids pro-domain (33 kDa) and a 110 amino acids mature protein (12.5 kDa). After secretion, pro-BMP9 homodimerizes and is subsequently cleaved by serine endoproteases. This generates two active forms: the short mature form (25 kDa) and the complexed form (100 kDa) in which the pro-domain remains associated with the mature form in an open-armed conformation (pro-domain (2 X 35 kDa) + mature peptide (2 X 12.5 kDa) (Bidart *et al.* 2011) (**FIGURE 9**).

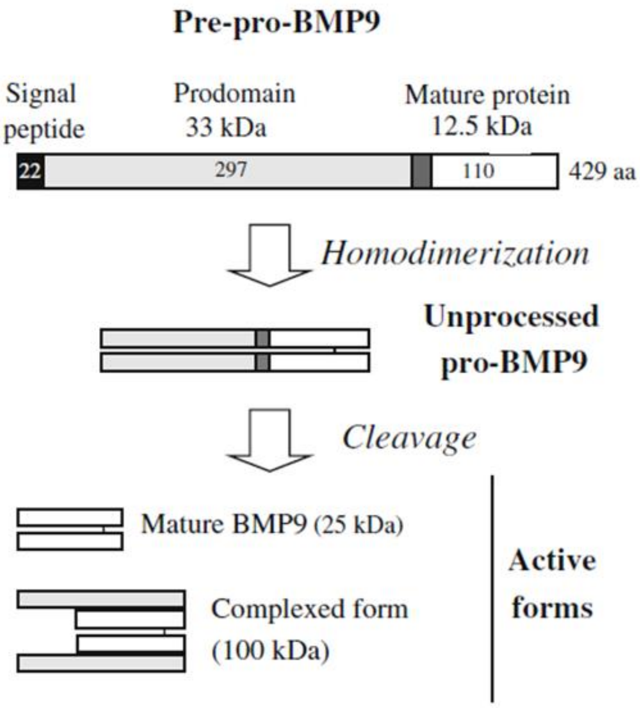


FIGURE 9. Structure of BMP9

BMP9 is synthesized as a precursor protein (Pre-pro-BMP9). After secretion, pro-BMP9 homodimerizes and is subsequently cleaved by serine endoproteases. This generates two active forms: the short mature form (25 kDa) and the complexed form (100 kDa) in which the prodomain remains associated with the mature form.

(Modified from Bidart et al., 2011)

It is well known that BMP9 is highly expressed in the liver and at much lower levels in the brain and lung (more than 100-fold less). Initial studies reported that BMP9 is specifically expressed in NPC (i.e., endothelial, Kupffer and HSC) in rat liver (Miller *et al.* 2000). In contrast, in recent studies performed in mouse and human liver, BMP9 mRNA was not detected in liver endothelial cells, nor in HSC, but rather in hepatocytes and biliary epithelial cells (Bidart *et al.* 2012), and considering the relative proportion of these two populations authors suggest that hepatocytes are the major cellular source of circulating BMP9. The reasons for these discrepancies are not clear, but it is plausible to think that BMP9 expression differs across species.

BMP9 has been shown to be involved in several biological functions, including the induction of the cholinergic phenotype of embryonic basal forebrain cholinergic neurons, the inhibition of the hepatic glucose production and the induction of the expression of key enzymes of lipid metabolism (Chen *et al.* 2003; Lopez-Coviella *et al.*

2005; Truksa *et al.* 2006). Moreover, BMP9 is one of the most potent BMPs able to induce orthotopic bone formation *in vivo* (Xiang *et al.* 2012). But by far, the most studied BMP9 role is that exerted in endothelial cells (Scharpfenecker *et al.* 2007; David *et al.* 2008). Nevertheless, its effects on angiogenesis remain controversial. It was shown that BMP9 inhibited neo-angiogenesis *in vivo* in the mouse sponge assay and inhibited blood circulation in the Chorioallantoic Membrane (CAM)-assay, a system used to study the effect of BMP9 on vascularization (David *et al.* 2008). In contrast with this, Suzuki *et al.* found that BMP9 enhances proliferation of three other kinds of endothelial cells: mouse embryonic-stem-cell-derived endothelial cells (MESECs); mouse normal endothelial cells and tumor associated endothelial cells (TECs), proposing BMP9 as pro-angiogenic factor (Suzuki *et al.* 2010). In spite of this, the most accepted hypothesis is that BMP9 is a pro-angiogenic factor, in fact, inhibiting the ALK1/BMP9 signaling pathway seems to be a useful alternative antiangiogenic therapy in the treatment of inflammation, cancer and vascular dysfunction (Cunha and Pietras 2011).

5.3 BMP9 signaling pathway in the liver

BMP9 binds to heterotetrameric complex receptors located in the cell membrane and are composed of two different subtypes of transmembrane serine-threonine kinase receptors: type I (also known as Activin like kinase receptor, ALK) and type II receptors. Among type I receptors, BMP9 can bind ALK1 (ACVRL1) and ALK2 (ACVRIA); furthermore, BMP9 can also bind three type II receptors, namely BMP type II receptor (BMPRII), activin type II receptor A (ActRIIA) and activin type II receptor B (ActRIIB) (Herrera *et al.* 2012).

Although the high affinity type I receptor for BMP9 is ALK1, receptor mainly expressed in endothelial cells, it has been demonstrated that BMP9 can also bind ALK2 in non-ECs (endothelial cell), such as myoblasts and breast tumor cells (Scharpfenecker *et al.* 2007). Interestingly, recent data support the hypothesis that BMP9 can bind to both type I and type II receptors with rather equally high affinity (Townson *et al.* 2012).

The expression of the BMP9 receptors ALK1, ALK2, BMPRII, ActRIIA and ActRIIB is observed in liver tissue indicating that BMP9 may play biological roles in liver cells through these receptors (Herrera *et al.* 2014).

Like other BMPs, BMP9 binding to its receptors triggers the phosphorylation of Smad1,5,8, which subsequently bind to the Co-Smad, Smad4. The resulting R-Smads/Co-Smad complex translocates to the nucleus, where it recruits specific transcriptional coactivators or corepressor factors to regulate transcription of BMP-responsive genes. This pathway is known as canonical pathway or Smad-dependent signaling and its activation by BMP9 has been documented in all cellular types analyzed up to date, including hepatocytes and HCC cells regardless of the receptors utilized by BMP9 (Herrera *et al.* 2013; Li *et al.* 2013).

Recently, some authors have described that BMP9 can also trigger Smad2/3 phosphorylation, which is classically associated with TGF-β signalling, in different endothelial cells (Sieber *et al.* 2009). No similar observations have been reported in liver cells (Herrera *et al.* 2014).

Additionally, in certain cellular models, BMP9 can also activate non-canonical pathways, referred to as non-Smad pathways that include MAPK (p38, ERK and JNK), PI3K/AKT, and Wnt, and regulates several microRNAs. The canonical and non-canonical signaling pathways have been shown to be induced simultaneously and/or sequentially, which enables them to modulate different biological functions (Sieber *et al.* 2009; Herrera *et al.* 2012) (**FIGURE 10**).

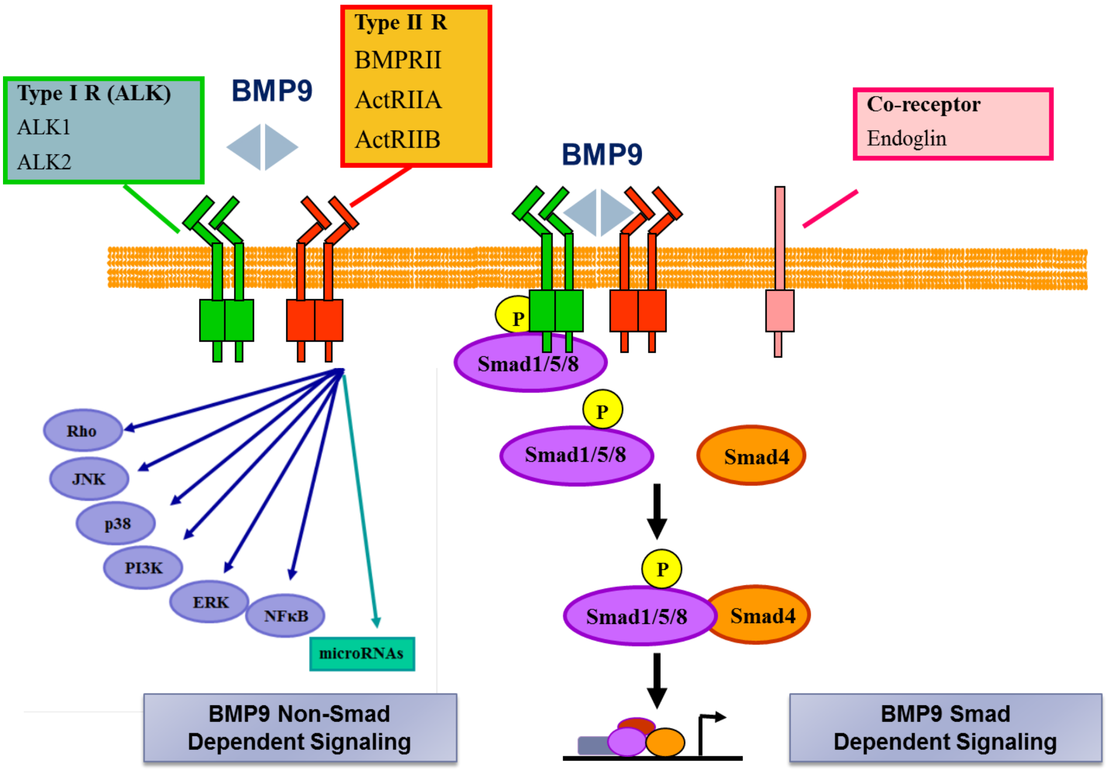


FIGURE 10. Canonical and non-canonical BMP9 signaling pathways

Upon ligand binding, receptor complexes activate the canonical Smad pathway and several non-canonical signaling pathways, among these p38, ERK, JNK MAPKs, as well as PI3K, NF-kB, and Wnt pathways.

(Modified from Herrera, Curr Pharm Des. 2012)

BMP9 signaling, like other BMPs, is modulated by the presence of co-receptors and extracellular regulators. Among the co-receptors, endoglin seems to be of special relevance. Endoglin is a transmembrane protein with large extracellular domains and serine/threonine-rich cytoplasmic regions. It cannot bind ligand on its own but does bind BMP9 in the presence of type I or type II signaling receptors (Scharpfenecker *et al.* 2007; Castonguay *et al.* 2011). It has been postulated that endoglin inhibits BMP9 interaction with type II receptor, thereby blocking BMP9 signaling (Castonguay *et al.* 2011). However, most of the data in the literature indicate that endoglin enhances BMP9 signaling, being therefore required for maximal phosphorylation of Smad1. Other regulator is crossveinless2 (CV2), a member of the chordin family, which binds to BMP9 and inhibits its activity (Yao *et al.* 2012). However, interestingly, noggin is unable to bind BMP9 and to inhibit BMP9 osteogenic activity in mesenchymal cells.

We have mentioned before that BMP9 is produced by the liver although the specific cellular source is not clear.

The first work on the role of BMP9 in hepatocytes was published in 1995 (Song *et al.* 1995). This study described that high concentration of BMP9 may slightly promote proliferation of both rat hepatocytes in primary culture and human tumor cells (HepG2 cell line). Subsequently, our group has shown that BMP9 triggers phosphorylation of Smad1,5,8 and decreases cell viability of normal non-transformed adult mouse hepatocytes in primary culture whereas it did not have any effect in non-tumoral immortalized human hepatocytes. In addition, we have also demonstrated that BMP9 is a proliferative and survival factor for human HCC cells (Herrera *et al.* 2013; Garcia-Alvaro *et al.* 2015). In regard to its role in HCC cells, our collaborators have demonstrated that the BMP9/Smad1 signaling pathway is involved in the induction of EMT, a key process in tumor invasiveness (Li *et al.* 2013).

Data in the literature suggest also an involvement of BMP9 in the regulation of glucose homeostasis. In a screening for insulin like activity compounds, BMP9 was found to exert an anti-diabetic effect both *in vitro* and *in vivo* (Chen *et al.* 2003). In this work authors found that recombinant BMP-9 injection decreases glycemic levels in normal and diabetic mice, with slower kinetic than insulin. The ability of BMP-9 to lower glucose levels seems to be associated with its ability to promote insulin release in pancreatic β-cells, to regulate metabolic signaling through glycogen synthase kinase (GSK) in muscle, and to inhibit hepatic glucose production. Moreover, it has been demonstrated that BMP9 mRNA and protein content were regulated by oral glucose administration, probably as a consequence of the direct and combined actions of insulin and glucose in the liver (Caperuto *et al.* 2008).

Interestingly, unpublished data from our group show that BMP9 not only interferes with the liver regenerative response but it is directly involved in liver fibrosis, therefore acting as a pro-fibrogenic factor (Katja Breitkopf-Heinlein et al., manuscript under revision).

To date nothing is known about the possible role of BMP9 on other liver cell types, in particular HPCs and its contribution to the HPC-mediated regenerative process. This thesis aims to elucidate these aspects for the first time.

BACKGROUND

BACKGROUND

1. ROLE OF BMP9 IN HEPATOCARCINOGENESIS

The study of BMP´s role in tumorigenic processes, and specifically in the liver, has gathered importance in the last few years.

It is known that BMP9 regulates the cell growth of immortalised ovarian surface epithelial cells (IOSE) and epithelial ovarian cancer (EOC) cells (Herrera *et al.* 2009). In this paper, published by Dr. Herrera in 2009, they demonstrated that BMP9 functions as a proliferative factor for these two cellular types via an ALK2/Smad1/Smad4 pathway. Furthermore, immunohistochemistry analysis of human ovarian cancer tissues indicated that 25% of EOC samples express BMP9 *in vivo*. These findings indicate that BMP9 acts as a positive regulator of IOSE and EOC cells proliferation and suggests that BMP9 represents a novel target for therapeutic intervention in EOC. However, the role played by BMP9 in hepatic cells was completely unknown.

Three years ago, it was shown that BMP9 is overexpressed in about 40% of HCC patients (Li *et al.* 2013). In this line, our lab also demonstrated the role of BMP9 in the promotion of HCC cell growth, effect that was not observed in non-transformed hepatocytes (Herrera *et al.* 2013) (**Figure 11**).

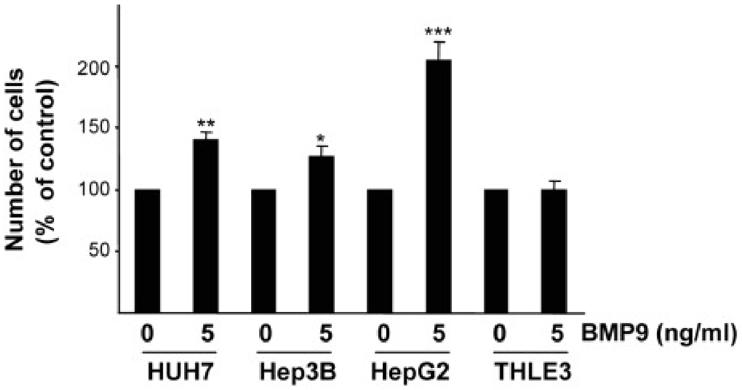


FIGURE 11. BMP9 increases cell number of HCC cell lines but not of immortalized human hepatocytes.

Huh7, Hep3B and HepG2 liver cancer cells and immortalized human hepatocytes (THLE3) were incubated in the absence or in the presence of 5 ng/ml BMP9 in 0.1% FBS media and counted at day 4. Data from 3 independent experiments performed in triplicate (mean ± S.E.M.) are shown. Statistical analysis was carried out using the paired *t*-test and data were compared to untreated samples, *=P<0.05, **=P<0. 01, ***=P<0.001 (Herrera *et al.* 2013).

It was also demonstrated that BMP9 impairs low serum-triggered apoptosis in the liver cancer cell line HepG2. Importantly, using different approaches we also showed that in addition to the paracrine signaling, HepG2 cells presented an autocrine BMP9 production. Altogether, our findings provided new clues for a better understanding of BMP9 contribution in HCC pathogenesis.

In an attempt to search for the signaling mechanisms driving BMP9 effects, we have recently found that BMP9 induces canonical and non-canonical signaling pathways in HepG2, specifically PI3K/AKT and p38MAPK, but only the p38MAPK pathway contributes to the growth-promoting activity of BMP9 (Garcia-Alvaro *et al.* 2015) (**Figure 12**).

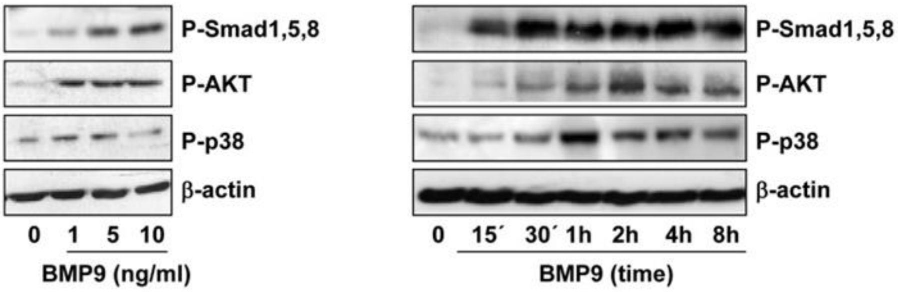


FIGURE 12. BMP9 activates both canonical and non-canonical pathways in HepG2 cells.

HepG2 cells were incubated for one hour with different concentrations of BMP9 (1–10 ng/mL) in 0% FBS media or for different periods of time −/+ BMP9 (5 ng/mL) in 0% FBS media. Western blots were performed with antibodies that recognize phospho-Smad1,5,8, phospho-AKT, phospho-p38 and β-actin (loading control). A representative experiment of two is shown (Garcia-Alvaro *et al.* 2015).

Furthermore, using genetic and pharmacological approaches, we have demonstrated that p38MAPK activation, although dispensable for the BMP9 proliferative activity, is required for the BMP9 pro-survival effect in HepG2 cells (**Figure 13**):

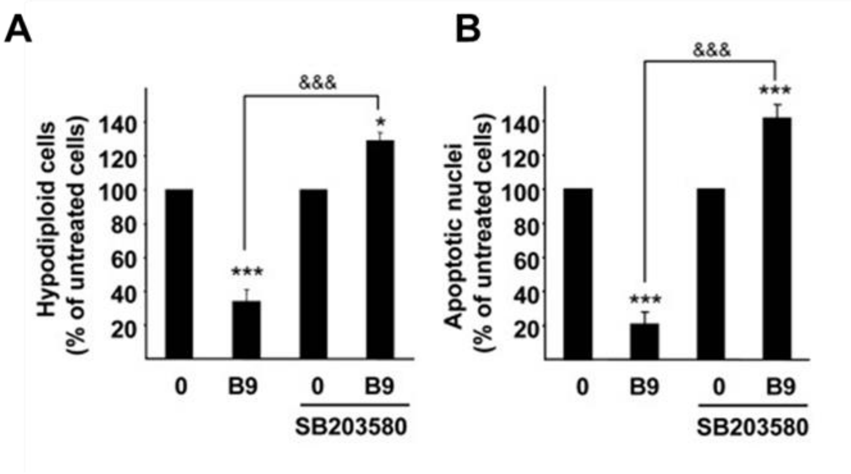


FIGURE 13. p38MAPK is involved in the BMP9 survival effect on serum starvation-triggered apoptosis in HepG2 cells.

HepG2 cells were treated with SB203580 (10 µM) (one hour pre-incubation) and −/+ BMP9 (5 ng/mL) in 0% FBS media for 72 h. **A.** Nuclear DNA content was analyzed by flow cytometry, and the percentages of hypodiploid (apoptotic) cells were obtained. Data are from three independent experiments performed in triplicate (mean ± S.E.M.), displayed as the percentage of untreated cells. **B.** Apoptotic nuclei were visualized and counted after PI staining under a fluorescence microscope. A minimum of 1000 nuclei were counted per condition. Data from two independent experiments performed in triplicate (mean ± S.E.M.) displayed as the percentage of untreated cells. Statistical analysis was carried out using the paired *t*-test, and data were compared to 0% FBS samples, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or as indicated, $^{\&\&\&}$ $p < 0.001$ (Garcia-Alvaro *et al.* 2015).

2. REGULATORY EFFECTS OF RTKs (HGF AND EGF) AND TGF-β ON OVAL CELLS

One of the main objectives of the research conducted in Dr. Sánchez laboratory in the last ten years has been to study the regulation of the multifaceted behavior of oval cells, focusing on characterizing some of the signals and mechanisms regulating their biology and function. Particularly, major efforts have been put forth in characterizing the relevance of the HGF/c-Met pathway and its interaction with other signaling pathways, specifically the EGF/EGFR and TGF-β pathways.

Using c-Met$^{flx/flx}$ mice generated in Dr. S.S.Thorgeirsson´s lab (Huh *et al.* 2004) oval cell lines expressing a functional or non-functional c-Met receptor that lacks tyrosine kinase activity, the Met$^{flx/flx}$ and Met$^{-/-}$ oval cells respectively, were established. It was shown that oval cells harbouring a genetically inactivated Met tyrosine kinase (Met$^{-/-}$oval cells) are more sensitive to both serum-starvation and TGF-β-induced apoptosis than cells expressing a functional Met (Met$^{flx/flx}$), demonstrating that the HGF/c-Met axis plays a pivotal role in oval cell survival (del Castillo *et al.* 2008) (**FIGURE 14 A**). Importantly, Met antiapoptotic activity involves both paracrine and autocrine signaling, since autocrine production of HGF and activation of Met kinase is detected in oval cells (**FIGURE 14 B and C**).

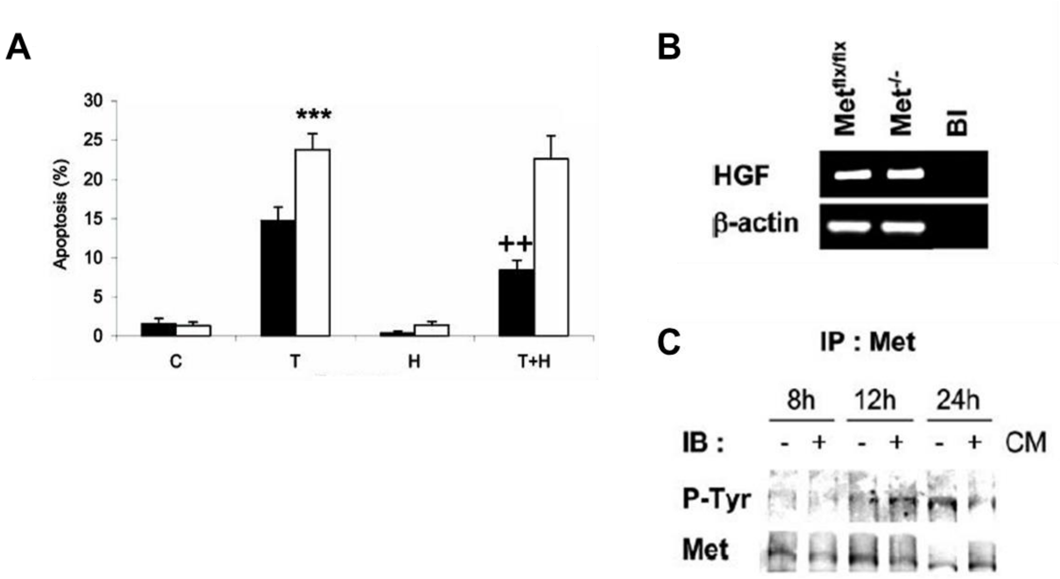


FIGURE 14. HGF reduces the TGF-β-induced apoptosis in Met$^{flx/flx}$ but not in Met$^{-/-}$ oval cells with both paracrine and autocrine signaling

A. Apoptotic index in oval cells treated with TGF-β (T; 1 ng/ml) for 48 hours in the absence or presence of HGF (H; 20 ng/ml). A total of 1000 to 2000 cells were counted per dish after PI staining under a fluorescence microscope in a blinded manner. **Black bars**, Met$^{flx/flx}$ cells. **White bars**, Met$^{-/-}$ cells. Data are mean ± S.E.M. of three experiments. ***$P < 0.001$ (*versus* T, Met$^{flx/flx}$) ++$P < 0.01$ (*versus* T, Met$^{flx/flx}$). **B.** Basal expression of the hgf mRNA as detected by RT-PCR analysis in Met$^{flx/flx}$ and Met $^{-/-}$ oval cells. Bl, Blank, no reverse transcription. β-actin was used for normalization. **C.** Kinetics of Met activation in Met$^{flx/flx}$ cells cultured in the serum-free medium or conditioned serum-free medium (CM). Whole protein extracts were used for immunoprecipitation of Met protein. Phosphorylation was detected by immunoblotting with anti-P-tyrosine antibody using Met as a loading control (del Castillo *et al.* 2008).

Then, the apoptotic response elicited by TGF-β in oval cell and the mechanisms mediating the c-Met-dependent anti-apoptotic activity were characterized. Our results demonstrated that TGF-β induces a mitochondrial apoptotic cell death in oval cells, with up-regulation of BIM and BMF proteins, two BH3-only members of the Bcl-2 family, but only BMF is essential for TGF-β-induced apoptosis. Furthermore, we revealed a signaling pathway in which TGF-β induces oxidative stress associated with an up-regulation of Nox4 and down-regulation of the intracellular antioxidant defenses, which leads to Bmf up-regulation and subsequent cell apoptosis. In addition, the pharmacological inhibition of PI3K did increase sensitivity of Met$^{flx/flx}$ oval cells to TGF-β by enhancing oxidative stress, mimicking Met$^{-/-}$ oval cells and impaired HGF-driven protection against TGF-β-induced apoptosis. These results revealed that HGF/c-Met signaling requires PI3K to promote oval cell survival against TGF-β-induced oxidative stress and apoptosis (Martinez-Palacian *et al.* 2013).

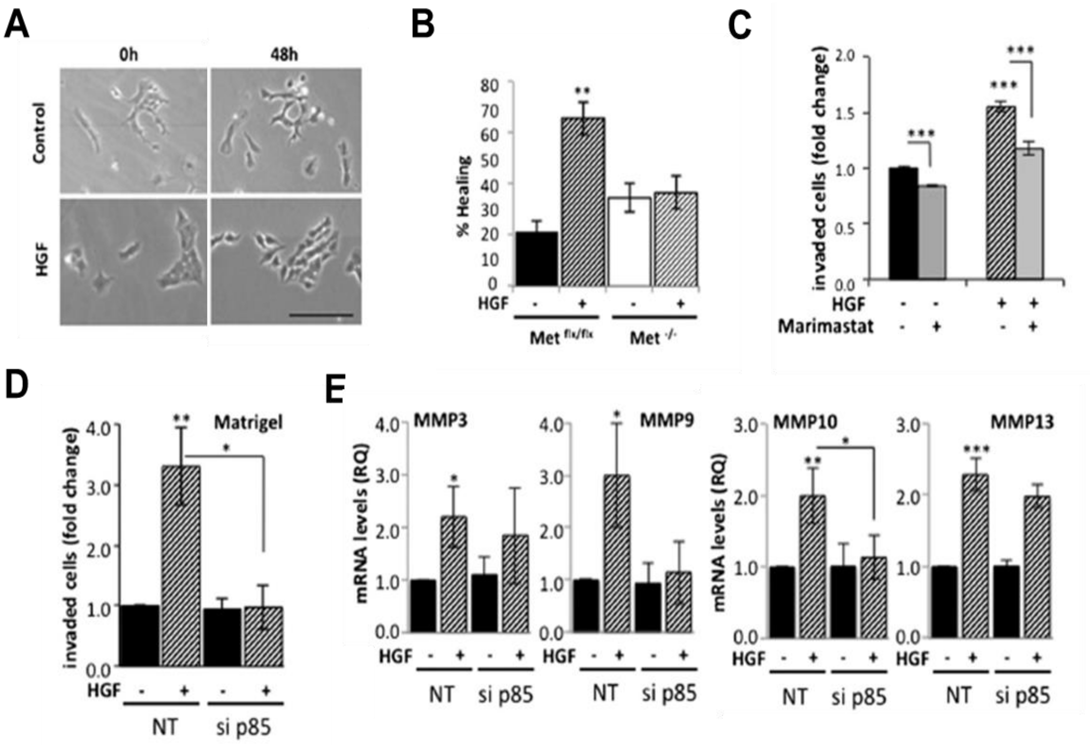


FIGURE 15. PI3K activation is involved in the migratory and invasive effect of HGF in oval cells

A. Scatter assays. Metf$^{lx/flx}$ oval cells were cultured at low density on plastic dishes with or without HGF treatment (40 ng/ml). Representative phase contrast images of a randomly selected area of the dish are shown. Scale bar=100 µm. **B.** Quantitative analysis of wound closure after 48 h. Data are expressed as % of closure and are the mean ± S.E.M. of four independent experiments. **P < 0.01 HGF-treated vs untreated Met$^{flx/flx}$. **C.** Effect of MMP inhibition on oval cell invasion. Invasion assays in the absence or presence of HGF (40 ng/ml) and/or marimastat (10 µM) were conducted. Data represent number of invaded cells expressed as fold change with respect to untreated cells and are mean ± S.E.M. of 2 independent experiments run in duplicate-triplicate. ***P < 0.001. **D.** Effect of p85 silencing on HGF-induced oval cell invasion. 24 h after transfection with non-targeting siRNAs (NT) or p85 specific siRNAs (si p85) cells were trypsinized and seeded on transwell chambers coated with matrigel and cultured for 24 h with or without HGF (40 ng/ml). Data are expressed as fold change with respect to control and are mean ± S.E.M. of 5 independent experiments run in duplicate–triplicate. *P < 0.05; **P < 0.01. E. Effect of p85 silencing on HGF-induced MMPs expression. 24 h after transfection with non-targeting siRNAs (NT) or p85 specific siRNAs (si p85) cells were serum-starved and treated or not with HGF (40 ng/ml) for 24 h. Total RNA was isolated and used for qRT-PCR analysis. Data represent RQ values and are mean ± S.E.M. of 2 experiments. P < 0.05; **P < 0.01; ***P < 0.001 (Suarez-Causado *et al.* 2015).

However, ongoing studies in our lab evidence that oval cells are not only targets of the suppressor effects of TGF-β, but also suffer EMT in response to either acute or chronic treatment with this factor that leads to profound changes in oval cell properties. More importantly, we have data in support of a functional crosstalk between TGF-β and c-Met after EMT since Met kinase activity appears to be critical to allow cell expansion post-EMT (unpublished results). Further research is being conducted in the lab to better characterize these processes.

On the other hand, we have also contributed to show that HGF/c-Met signaling in oval cells does much more than just protecting against TGF-β apoptotic effects. Indeed, we have demonstrated that HGF promotes a migratory and invasive response in oval cells that ultimately leads to cell rearrangement rather than to cell scatter and emulates the morphogenic response occurring *in vivo* during oval cell-mediated regeneration. Additionally, we have explored the signaling mechanisms mediating HGF invasive response and have proved an essential role for PI3K activation and MMPs expression and activation to achieve such response (**FIGURE 15**) (Suarez-Causado *et al.* 2015). The signaling mechanisms mediating MMPs regulation by HGF remain elusive.

OBJECTIVES

Given that BMP9 has shown to be an important regulator of hepatocytes and also of other liver cells and the interesting emergent role of BMP9 as a regulator of liver regeneration and fibrogenesis, the general objective of this work was to characterize the role played by BMP9 in oval cell biology and to elucidate its contribution to the liver regenerative response involving these cells.

To achieve this general aim, we proposed the following specific objectives:

SPECIFIC OBJECTIVES:

1. To analyze the effect of BMP9 deletion in the expansion of oval cells *in vivo* after induction of liver damage and the overall effect on the outcome of the regenerative response.

2. To characterize the signaling pathways and biological activities triggered by BMP9 in oval cells and the potential crosstalk with the HGF/c-Met pathway using an *in vitro* model of oval cell lines.

- **2.1** To analyze the effect of BMP9 on oval cell proliferation, survival and migration/invasion.
- **2.2** To investigate the potential functional crosstalk between BMP-9 and HGF/c-Met signaling pathways and to try to identify the mechanisms involved in this interaction.
- **2.3** To analyze the effect of chronic exposure to BMP9 in oval cells phenotype and properties.

MATHERIALS AND METHODS

1. CELL CULTURE

1.1 Cell models

Met$^{flx/flx}$ and Met$^{-/-}$ oval cell lines were generated from a conditional knockout mouse for c-Met by using Cre-loxP-mediated gene targeting (Huh *et al.* 2004). Inactivation of the mouse c-Met gene was accomplished by a conditional deletion of exon 16 containing a critical ATP-binding site in the intracellular TK domain, essential for the activation of c-Met signaling. The removal of this region leads to the expression of a c-Met receptor lacking its tyrosine kinase activity.

To induce oval cell expansion, nine-week-old male Met$^{flx/flx}$ mice were maintained on 0.1% DDC-supplemented diet for 13 days (Preisegger *et al.* 1999) and then the oval cell-enriched non-parenchymal cell fraction was isolated (del Castillo *et al.* 2008). In order to generate Met$^{-/-}$ oval cells, *in vitro* inactivation of c-Met, was achieved by infecting the parental oval cell lines with adenovirus expressing the Cre recombinase under the control of the cytomegalovirus promoter (Ad-CMV-Cre). Once isolated, these cells were phenotypically and functionally characterized and validated (del Castillo *et al.* 2008).

HepG2, a human HCC epithelial cell line, was obtained from the European Collection of Cell Cultures (ECACC).

1.2 Cell culture conditions and cryopreservation

All cell lines were grown in DMEM (Dulbecco's Modified Eagle Medium) (4.5 g/l glucose) medium (BioWhittaker, BE12-604F) supplemented with 10% fetal bovine serum (FBS) (Life Techologies, 10270106), 20 mM Hepes (pH 7.4) (Sigma, H3375) and antibiotics: penicillin (120µg/ml) (Sigma, P3032), streptomycin (100µg/ml) (Sigma, S9137) and amphotericin B (2,5µg/ml) (Gibco, 15290-026).

Cells were maintained in a humidified incubator at 37ºC and 5% CO_2 atmosphere. Medium was replaced every three days and cells passaged at 90% confluency. In the case of oval cell lines, only early passage cells (passages 1 to 10) were used for the experiments. For HepG2, cells were not maintained more than 20 passages.

Cells were stored in 10% (v/v) DMSO (Dimethyl sulfoxide)/FBS in liquid nitrogen (storage below -170ºC), using cryotubes (Nunc). They were frozen down slowly using the Mr. Frosty freezing container (Thermo Scientific); first, vials containing cells in cold freezing medium were transferred to the freezing container filled with isopropanol, then moved to a -80°C freezer for one day and finally transferred to the liquid nitrogen tank.

Thawing was performed quickly by immersion of cryotubes in a water bath at 37ºC and cells were rapidly diluted into culture medium in tissue culture dishes. When cells were attached, medium was replaced by fresh medium to completely remove the DMSO.

The growth factors used in this work are listed in the table below (**TABLE 3**):

TABLE 3. Growth factors

Growth factors	Concentration	Manufacturer
BMP9	1-5 ng/ml	R&D Systems
HGF	40 ng/ml	R&D Systems
TGF-β	2 ng/ml	Calbiochem

Subconfluent (90%) cells were serum starved for 3-15 hours before treatment. Cells were treated with the following growth factors:

- HGF: 40 ng/ml. When combined with BMP9, HGF was added 15 hours before BMP9 treatment.
- BMP9: different concentrations of BMP9 have been used (1 ng/ml BMP9 for B9T-OC maintaining and 2 ng/ml BMP9 for experiments, except for cell death assays in which 5 ng/ml BMP9 was used).
- TGFβ1: 2 ng/ml.

For long-term treatment, growth factors were re-added every 48 hours.

2. BMP-9 KO MICE

2.1 Generation and genotyping of BMP9-KO mice

The BMP9 knockout mouse model (BMP9-KO) was supplied by Dr. Se-Jin Lee (Johns Hopkins University School of Medicine, Baltimore, MD). BMP9-KO mice were obtained by homologous recombination in R1 embryonic stem cells such that exon 2 encoding the entire mature C-terminal region was replaced by a neomycin resistance cassette. Heterozygous offspring of chimeras were mated out 9 generations to C57BL6/J. BMP9-KO adult mice are viable and fertile, and pups are grossly normal, although arteriovenous malformations and skeletal abnormalities have been described (Ricard *et al.* 2012).

Mice were genotyped by polymerase chain reaction (PCR) of genomic tail DNA using specific primers that specifically detect the presence or absence of wild type (228 bp) or knockout (440 bp) alleles (**FIGURE 16**).

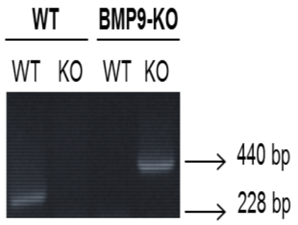


FIGURE 16. Genotyping of BMP9-KO mice

The presence or absence of wild type (228 bp) or knockout (440 bp) alleles was detected by PCR.

Primer sequences used for the amplification were (**TABLE 4**):

TABLE 4. Primer sequences used for BMP-9KO genotype

Allele	Forward primer (5'-3')	Reverse primer (5'-3')
WT	GAA GTA TCG AGT GCC GTG AAG CGG T	CTG CTG CTC ATG GCC GAT CAT CTC C
KO	GAA GTA TCG AGT GCC GTG AAG CGG TG	CGC CTT CTT GAC GAG TTC TTC TGA GGG

The use of animals complied with the institutional and European legislation concerning vivisection, the use of genetically modified organisms, animal care and welfare (European Directive 2010/63/UE adopted by the European Parliament and the Council of the EU on September 22, 2010). Animals were housed in a temperature-controlled environment, with 12 hours light/dark cycles and with access to standard chow and water *ad libitum*. The experimental protocols were approved by the Institutional and regional Committee for animal care and use (For CCl_4 protocol the reference is PROEX 120/14 and for DDC treatment is PROEX 129/16).

2.2 DDC feeding-induced liver injury mouse model

8-12 weeks mice from each genotype (BMP9-KO and WT) were randomly divided into experimental groups (5-10 animals per group) and fed with either a normal diet (control group) or the diet supplemented with 0.1% DDC (Cymit Quimica). The diet was produced by Indigo Laboratories and properly preserved in our laboratory. A scheme of the experimental protocol is shown (**FIGURE 17**):

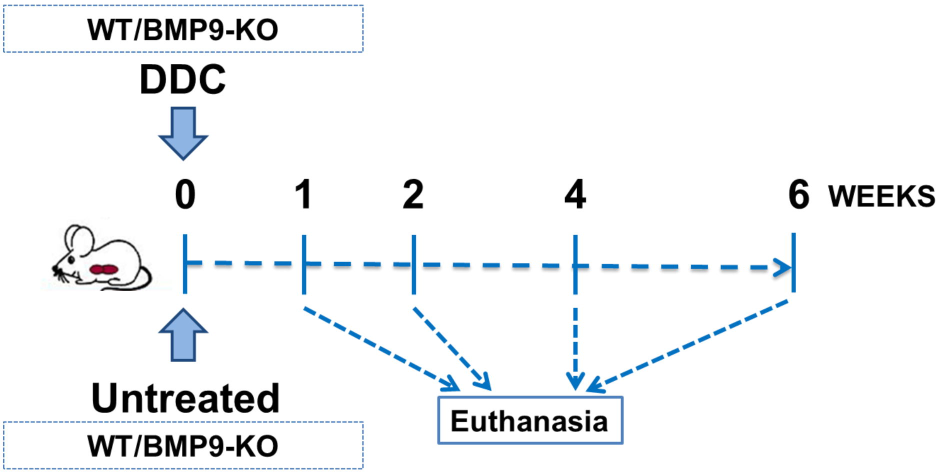


FIGURE 17. General scheme of the DDC protocol

Blood and livers samples were collected (see subsection 4.4 of "Material and methods") after 1, 2, 4 and 6 weeks of DDC or control diet feeding for further analysis.

2.3 CCl_4-induced liver fibrosis mouse model

8-12 weeks BMP9-KO and WT male mice were randomly assigned to two experimental groups (5-10 animals per group): a *control group*, injected only with the vehicle (mineral oil, Sigma) and a *CCl_4 group*, injected with the CCl_4 diluted in mineral oil. CCl_4-treated mice received 0.48 gr/kg body weight of CCl_4 by intraperitoneal injection twice per week for 8 weeks. Control animals were injected with the same volume of mineral oil. Mice were sacrificed 72 hours after last injection to eliminate acute effects of CCl_4.

2.4 Sample collection process

The thoracic cavity of an anesthetized mouse was opened to expose the heart. Blood was obtained by cardiac puncture using a 1 ml hypodermic syringe and needle and placed in ice-cold heparinized tubes. Blood samples were allowed to clot at room temperature and centrifuged at 1800 rpm for 10 min at room temperature for serum separation. Serum was immediately frozen at -80°C for further analysis.

The liver was removed and weighted. Part of the liver lobes were thinly sectioned and fixed in 10% formalin solution (Sigma) overnight. The remaining portions of the liver were collected in RNase-free tubes and immediately snap-frozen in liquid nitrogen for RNA and protein extraction.

2.5 The liver to body weight ratio

The liver to body weight ratio was estimated based on the following formula: $\%=100 \times Ml/Mb$, where Ml is the liver weight and Mb is the total animal weight, at the time of sacrifice.

2.6 Histological Analyses

10% buffered formalin fixed liver tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E). The preparation of these tissue samples in paraffin was performed by Patricia Saperas (Hospital Clínico San Carlos (HCSC) of Madrid.

Liver sections were evaluated and scored according to the Knodell Histological Index (Knodell HAI) by Dr. Julian Sanz Ortega, Head of molecular pathology and anatomical pathology department of HCSC. The Knodell Histological Index (Knodell HAI), the first of this kind and the most widely used system, was used to provide a numerical score to both staging and grading of liver injury, thus providing a semi-quantitative assessment of the observed histological features in this animal model. Briefly, this was based on the assessment of portal/periportal activity with or without bridging necrosis, intralobular necrosis, portal inflammation and fibrosis. The table below shows the Knodell Histology Activity Index (HAI) (Knodell *et al.* 1981) (**TABLE 5**).

TABLE 5. HAI for Numerical Scoring of Liver Biopsy Specimens

Periportal Necrosis	Score	Intralobular Degeneration[*] and Inflammation	Score	Portal Inflammation	Score	Portal Fibrosis	Score
None	**0**	None	**0**	None	**0**	None	**0**
Mild	**1**	Mild (acidophilic bodies, ballooning degeneration and/or scattered foci of hepatocellular necrosis in <1/3 of lobules or nodulles)	**1**	Mild (sprinkling of inflammatory cells in < 1/3 of portal tracts)	**1**	Fibrous portal expansion	**1**
Moderate (involves less than 50% of the circumference of most portal tracts)	**3**	Moderate (involvement of 1/3-2/3 of lobules or nodules)	**3**	Moderate (increased inflammatory cells in 1/3-2/3 of portal tracts)	**3**	Bridging fibrosis	**3**
Marked (involves more than 50% of the circumference of most portal tracts)	**4**	Marked (involvement of >2/3 of lobules or nodules)	**4**	Marked (dense packing of inflammatory cells in > 2/3 of portal tracts)	**4**	Cirrhosis[$]	**4**
Moderate piecemeal necrosis plus bridging necrosis[+]	**5**						
Marked piecemeal necrosis plus bridging necrosis[+]	**6**						

NOTE: HAI score is the combined scores for necrosis, inflammation, and fibrosis.

[*] Degeneration: acidophil bodies, ballooning; focal necrosis-scattered foci of hepatocellular necrosis.
[$] Loss of normal hepatic lobular architecture with fibrous septae separating and surrounding nodules.
[+] Bridging is defined as >= 2 bridges in the liver biopsy specimen.

(Modified by Knodell RG et al., 1981)

2.7 Quantitative morphometric analysis of oval cells expansion

Image J was used to quantify oval cell expansion from H&E stained tissue sections. Areas of oval cell expansion from 12 periportal non-overlapping fields of each animal (at least five animals per group) were outlined and measured at original magnification 100X. Data are expressed as the average percentage of expansion area respect to the untreated group.

2.8 Analysis of serum parameters

Total bilirubin, L-aspartate aminotransferase (L-AST) and L-alanine aminotransferase (L-ALT) activities were measured in a Clinical Analysis Laboratory (UCM) using gold-standard methods and a Cobas Integra 400 Plus Chemistry Analyzer (Roche).

3. DNA ANALYSIS BY PCR

3.1 DNA isolation

Genomic DNA was extracted from tail using a specific lysis buffer: 100 mM Tris-HCl (pH 8.5), 200 mM NaCl, 5 mM EDTA, 0.2% SDS and 100 µg/ml Proteinase K. Briefly, we cut about 1-1.5 cm tail and place it in an eppendorf tube with lysis buffer. Tubes are introduced in a hybridization tube and incubated overnight in an oven at 55°C with agitation. DNA was precipitated by isopropanol. Finally, samples were resuspended in TE buffer pH=8 (10 mM Tris-HCl and 1 mM EDTA). DNA was stored at -20°C until it was processed. DNA concentration was determined spectrophotometrically at 260 nm. An absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per ml.

Genotyping was assessed by PCR using specific primers (see Generation and genotyping of BMP9-KO mice). An amount of DNA of approximately 1 µg was used.

3.2 PCR conditions

PCR analysis was used to evaluate the mice's genotype. PCR reactions were carried out using 0.4 µM specific primers (**TABLE 4**), dNTPs at 200 µM each, 2 mM MgCl2 and 1 unit of Taq Polymerase (BIOLINE, BIO-21040). PCR conditions were: 94°C, 2 min; 94°C, 30 sec and 68°C, 3 min for 35 cycles; 68°C, 3 min and 4°C, hold.

The obtained PCR products were analyzed in 1% agarose gels containing Gel Red Nucleic Acid Stain (Biotium, 41003).

4. mRNA EXPRESION ANALYSIS BY RT-PCR

4.1 Total RNA extraction

Cells were washed twice with cold PBS and total RNA was isolated using RNeasy Mini Kit (Quiagen) following manufacturer instructions. DNase (Quiagen, 79254) treatment was included to avoid possible genomic DNA contamination. RNA was eluted with ultrapure water and stored at -80ºC until use. RNA concentration was determined by spectrophotometry absorbance measurement at 260 nm (1 unit of absorbance at 260 nm corresponds to 40 µg of RNA per ml). To estimate RNA purity, the ratio between the absorbance at 260 and 280 nm was calculated. A ratio A260/A280 of 1.8-2 was accepted as pure RNA.

4.2 cDNA synthesis

Total RNA (1 µg) was reverse-transcribed to generate the cDNA using the Super Script III RT kit (Invitrogen, 18080-040) following instructions from the manufacturer.

Essentially, RNA was incubated with oligo (dT) (0.5 mM) and random primers (2.5 µM) at 65 ºC for 5 min to ensure RNA denaturation and then, placed on ice for at least one min. cDNA synthesis mix (5X RT buffer, 20 Units of RNase inhibitor, 5 mM dithiothreitol (DTT) and 200 Units of SuperScript III) was added to each sample followed by incubation at 50ºC for 1 hour.

The reaction was stopped by heating at 70 ºC for 15 min to inactivate the enzyme and the cDNA was stored at -20 ºC.

4.3 Quantitative PCR (qPCR)

Real time PCR or quantitative PCR (qPCR) was performed using specific primers (**TABLE 6.)** and Fast Start Universal SYBR Green Master (Rox) (Roche, 04913850001) to detect DNA in the 7900 Fast Real Time System (Life Technologies). PCR reactions were done in triplicate. Gusb was used as a housekeeping gene; different negative controls were prepared using RNA or water instead of cDNA.

During the exponential phase of real-time PCR, a fluorescence signal threshold was determined, so that it was significantly greater than background fluorescence. The fractional number of PCR cycles required to reach this threshold is defined as the cycle threshold, or Ct. Based on this, quantification of RNA levels was performed through calculation of RQ (2-ΔΔCt). First, ΔCt value for each sample and gene is obtained as follows: Ct for a gene minus -Ct for GusB=ΔCt under a particular experimental condition and then, this is referred to control ΔCt values (sample ΔCt-control ΔCt= ΔΔCt) to calculate the RQ value.

5.

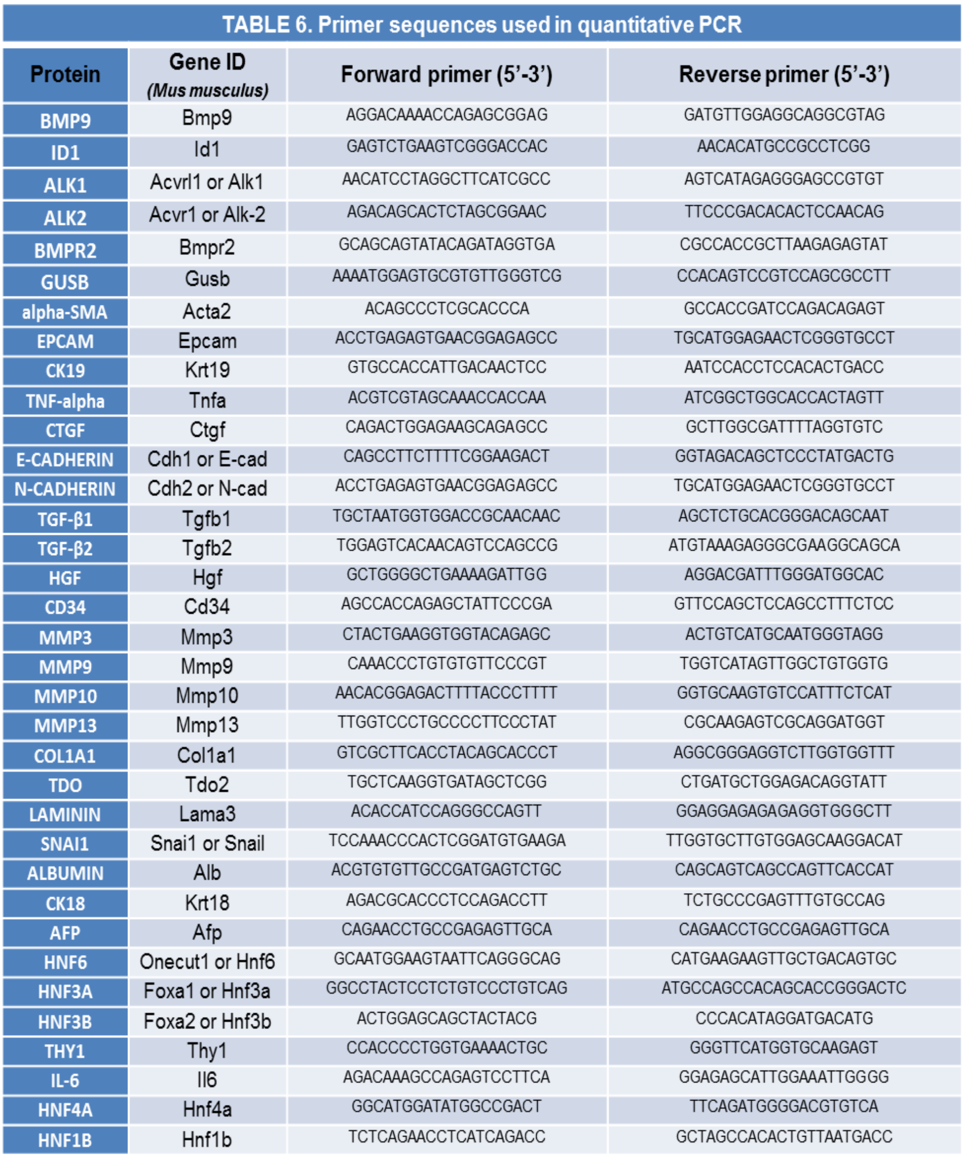


TABLE 6. Primer sequences used in quantitative PCR

Protein	Gene ID *(Mus musculus)*	Forward primer (5’-3’)	Reverse primer (5’-3’)
BMP9	Bmp9	AGGACAAAACCAGAGCGGAG	GATGTTGGAGGCAGGCGTAG
ID1	Id1	GAGTCTGAAGTCGGGACCAC	AACACATGCCGCCTCGG
ALK1	Acvrl1 or Alk1	AACATCCTAGGCTTCATCGCC	AGTCATAGAGGGAGCCGTGT
ALK2	Acvr1 or Alk-2	AGACAGCACTCTAGCGGAAC	TTCCCGACACACTCCAACAG
BMPR2	Bmpr2	GCAGCAGTATACAGATAGGTGA	CGCCACCGCTTAAGAGAGTAT
GUSB	Gusb	AAAATGGAGTGCGTGTTGGGTCG	CCACAGTCCGTCCAGCGCCTT
alpha-SMA	Acta2	ACAGCCCTCGCACCCA	GCCACCGATCCAGACAGAGT
EPCAM	Epcam	ACCTGAGAGTGAACGGAGAGCC	TGCATGGAGAACTCGGGTGCCT
CK19	Krt19	GTGCCACCATTGACAACTCC	AATCCACCTCCACACTGACC
TNF-alpha	Tnfa	ACGTCGTAGCAAACCACCAA	ATCGGCTGGCACCACTAGTT
CTGF	Ctgf	CAGACTGGAGAAGCAGAGCC	GCTTGGCGATTTTAGGTGTC
E-CADHERIN	Cdh1 or E-cad	CAGCCTTCTTTTCGGAAGACT	GGTAGACAGCTCCCTATGACTG
N-CADHERIN	Cdh2 or N-cad	ACCTGAGAGTGAACGGAGAGCC	TGCATGGAGAACTCGGGTGCCT
TGF-β1	Tgfb1	TGCTAATGGTGGACCGCAACAAC	AGCTCTGCACGGGACAGCAAT
TGF-β2	Tgfb2	TGGAGTCACAACAGTCCAGCCG	ATGTAAAGAGGGCGAAGGCAGCA
HGF	Hgf	GCTGGGGCTGAAAAGATTGG	AGGACGATTTGGGATGGCAC
CD34	Cd34	AGCCACCAGAGCTATTCCCGA	GTTCCAGCTCCAGCCTTTCTCC
MMP3	Mmp3	CTACTGAAGGTGGTACAGAGC	ACTGTCATGCAATGGGTAGG
MMP9	Mmp9	CAAACCCTGTGTGTTCCCGT	TGGTCATAGTTGGCTGTGGTG
MMP10	Mmp10	AACACGGAGACTTTTACCCTTTT	GGTGCAAGTGTCCATTTCTCAT
MMP13	Mmp13	TTGGTCCCTGCCCCTTCCCTAT	CGCAAGAGTCGCAGGATGGT
COL1A1	Col1a1	GTCGCTTCACCTACAGCACCCT	AGGCGGGAGGTCTTGGTGGTTT
TDO	Tdo2	TGCTCAAGGTGATAGCTCGG	CTGATGCTGGAGACAGGTATT
LAMININ	Lama3	ACACCATCCAGGGCCAGTT	GGAGGAGAGAGAGGTGGGCTT
SNAI1	Snai1 or Snail	TCCAAACCCACTCGGATGTGAAGA	TTGGTGCTTGTGGAGCAAGGACAT
ALBUMIN	Alb	ACGTGTGTTGCCGATGAGTCTGC	CAGCAGTCAGCCAGTTCACCAT
CK18	Krt18	AGACGCACCCTCCAGACCTT	TCTGCCCGAGTTTGTGCCAG
AFP	Afp	CAGAACCTGCCGAGAGTTGCA	CAGAACCTGCCGAGAGTTGCA
HNF6	Onecut1 or Hnf6	GCAATGGAAGTAATTCAGGGCAG	CATGAAGAAGTTGCTGACAGTGC
HNF3A	Foxa1 or Hnf3a	GGCCTACTCCTCTGTCCCTGTCAG	ATGCCAGCCACAGCACCGGGACTC
HNF3B	Foxa2 or Hnf3b	ACTGGAGCAGCTACTACG	CCCACATAGGATGACATG
THY1	Thy1	CCACCCCTGGTGAAAACTGC	GGGTTCATGGTGCAAGAGT
IL-6	Il6	AGACAAAGCCAGAGTCCTTCA	GGAGAGCATTGGAAATTGGGG
HNF4A	Hnf4a	GGCATGGATATGGCCGACT	TTCAGATGGGGACGTGTCA
HNF1B	Hnf1b	TCTCAGAACCTCATCAGACC	GCTAGCCACACTGTTAATGACC

PROTEIN EXPRESSION ANALYSIS BY WESTERN BLOT

5.1 Cell extract preparation

Cells were washed twice with cold PBS and lysed with the IP buffer (**TABLE 7**).

TABLE 7.

IP Buffer composition

Tris-HCl (pH 7.5)	50 mM
NaCl	150 mM
NP40	5 mM
EGTA	5 mM
EDTA	5 mM
PMSF (*)	1 mM
Aprotinin (*)	10 µg/ml
Leupeptin (*)	10 µg/ml
$NaVO_3$ (*)	1 mM
NaF (*)	20 mM

(*) Protease inhibitors are added immediately before use.

Cells were detached from the plate by scraping, collected in an eppendorf tube and maintained on ice for 20 min. Every 5 min, tubes were vigorously shaken using a vortex. Then, cell lysates were centrifuged at 13000 rpm for 10 min at 4°C. The supernatant (total protein extracts) was transferred to a new tube and stored at -80°C.

5.2 Tissues extracts preparation

25-30 mg of frozen mouse liver tissue were disrupted in a RIPA buffer (**TABLE 8**), with a mortar and pestle.

TABLE 8.

RIPA Buffer composition

Tris-HCl (pH 7.4)	50 mM
NaCl	150 mM
NP40	1 %
Na-deoxycholate	0.25 %
SDS	0.1 %
PMSF (*)	1 mM
Aprotinin (*)	10 µg/ml
Leupeptin (*)	10 µg/ml
$NaVO_3$ (*)	1 mM
NaF (*)	20 mM

(*) Protease inhibitors are added immediately before use.

Every 5 min, tubes were vigorously shaken using a vortex; the digestion take 30 minutes. Then, tissue lysates were centrifuged at 13000 rpm for 10 min at 4°C. The supernatant (total protein extracts) was transferred to a new tube and stored at -80°C.

5.3 Protein quantification

Protein quantification was performed using the method described by Bradford in 1976. After addition of Bradford reagent (diluted 1:5 in ultrapure water) (Bio-Rad) to the samples, absorbance at 595 nm was measured using a plate reader (Powerwave XS, Biotek). Bovine serum albumin (BSA) (0 to 5 µg/µl) was used as a protein standard to generate a standard curve. 60-100 µg of proteins were loaded in each gel.

5.4 Protein electrophoresis

Protein electrophoresis was performed using SDS-polyacrylamide (SDS-PAGE) gels with different acrylamide concentration (6-15%) depending on the size of the protein to be analyzed (**TABLE 9.**).

TABLE 9. SDS-PAGE gels composition

Composition	Separating Gel 8%	Separating Gel 10%	Separating Gel 15%	Stacking Gel (Vf: 5mL)
30% Acrylamide/Bisacrylamide	2.3 mL	3.3 mL	5 mL	0.83 mL
H_2O	5 mL	4 mL	2.3 mL	3.4 mL
1.5 M Tris pH 8.8	2.5 mL	2.5 mL	2.5 mL	-
1 M Tris pH 6.8	-	-	-	0.63 mL
10% SDS (w/v)	100 µl	100 µl	100 µl	50 µl
10% APS	100 µl	100 µl	100 µl	50 µl
TEMED	4 µl	4 µl	4 µl	5 µl

APS: Ammonium Persulfate; TEMED: Tetramethylethylenediamine.

Protein samples were prepared by adding Laemmli buffer 2X (Tris-HCl 125 mM pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, 0.004% (w/v) bromophenol blue and 10% β-mercaptoethanol) to the protein extracts. Then, they were heated at 95ºC and loaded into the gel, as well as the molecular weight markers (SeeBlue Plus2, Invitrogen).

Electrophoresis was developed at constant voltage (80-120 V) using the following running buffer: 25 mM Tris-HCl (pH 8.3), 200 mM Glycine and 0.1% SDS.

5.5 Protein transfer

Proteins from the gels were transferred to a nitrocellulose membrane using a wet transfer apparatus. Then, an electric current of 0.3 A for 90-110 minutes (depending on the number/size of gels) was applied in a transfer buffer (20% methanol, 25 mM Tris, 190 mM glycine). To confirm the efficiency of protein transfer, membrane was stained with a Ponceau S solution (0.5% in 1% acetic acid glacial).

5.6 Immunodetection

After transfer, membranes were washed with distilled water, and then with TTBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS) with 0.05% Tween-20).

Then, they were incubated in the blocking solution (either 5% non-fat dry milk or 5% BSA in TTBS) for 1 hour at room temperature (RT). After blocking, membranes were rinsed with TTBS and incubated with the primary antibody overnight at 4ºC in 5% milk-TTBS or BSA-TTBS at the dilution indicated in **TABLE 10**. Then, membranes were washed 3 times with TTBS (5 min/each) and incubated with the secondary antibody at a dilution of 1:5000 in 5% milk TTBS for 1 hour at RT. Finally, they were washed 3 times with TTBS and incubated with a chemiluminescent solution (ECL from Amersham Biosciences) to visualize proteins in an X-ray film.

The following antibodies were used (**TABLE 10**):

TABLE 10. Primary and secondary antibodies used for Western-Blot.

Primary Antibody	Laboratory	Primary Antibody Dilution	Secondary Antibody	Secondary Antibody Dilution
Phospho-ERK1/2	Cell Signaling Technology 9101	1:1000	RABBIT	1:5000
Phospho-Ser473-AKT	Cell Signaling Technology 9271	1:1000	RABBIT	1:5000
Phospho-SMAD1,5,8	Cell Signaling Technology 9511	1:1000	RABBIT	1:5000
BMP9	Abcam (ab35088)	1:1000	RABBIT	1:5000
EPCAM	Santa Cruz Biotechnology (sc66020)	1:1000	MOUSE	1:5000
Phospho-MET	Cell Signaling Technology 3077S	1:1000	RABBIT	1:5000
Phospho-STAT3	Cell Signaling Technology 9131	1:1000	RABBIT	1:5000
STAT3	Cell Signaling Technology 9132	1:1000	RABBIT	1:5000
SMAD1	Cell Signaling Technology 9743	1:1000	RABBIT	1:5000
E-CADHERIN	BD Transduction Laboratories 610181	1:1000	MOUSE	1:5000
ALBUMIN	Nordic immunological Laboratories	1:1000	RABBIT	1:5000
Phospho-SMAD2	Cell Signaling Technology 3101	1:1000	RABBIT	1:5000
β-ACTIN	Sigma 4-5441	1:1000	MOUSE	1:5000

6. TRANSCRIPTIONAL REPORTER ASSAY

Cells (12500 cells/sq cm) were seeded in 6-well plates. The next day, cells were transiently transfected using the plasmid pGL3 BRE Luciferase (pGL3(BRE)-luc; Promega). After 36 hours, cells were serum starved overnight and treated with BMP9 (2 ng/ml). Luciferase activity was determined after 6 hours of stimulation. Specifically, luciferase activity was quantified using the luciferase assay (Promega) with a Victor luminometer (Wallac), as previously described by Dr. Peter ten Dijke's group (Korchynskyi and ten Dijke 2002). In all transfections, protein quantification served as internal control to normalize the luciferase activity.

7. GENE SILENCING BY shRNA AND siRNA

Stable ALK1 and ALK2 knockdown in oval cells was achieved by infection with mouse ALK1 and ALK2 shRNA Lentiviral Particles. Briefly, cells were seeded in a 6-well plate one day prior to the viral infection at a density that guaranteed 50-60% confluence the next day. Cells were incubated with the virus for 24 hours, and then cells were maintained with puromycin (2 µg/ml) to select clones expressing the shRNA. Clones were picked, expanded and analyzed for ALK1 and ALK2 expression. Cells transfected with the empty vector (plKO) were also generated and used as a control.

For ALK1 knockdown three different target-specific constructs were used (Sigma, #1 TRCN0000022540-553, #2 TRCN0000231253-8405 and #3 TRCN0000231254-8403), inserted in the pLKO.1-puro plasmid. For ALK2 knockdown, only one construct was used (Sigma, TRCN0000000441). All of them were kindly supplied by Dr. Peter ten Dijke's group.

Transient Smad1 knockdown was performed by transfection with a siRNA targeting mouse Smad1 (Dharmacon, L-055765-00). siControl non targeting (NT) siRNA pool (Dharmacon, D-001210-03-05) was used as negative control. For siRNA transfection we used TransITsiQuest reagent (Mirus) according to the manufacturer´s recommendation and a final siRNA concentration of 100 nM. After transfection, cells were incubated for 24 hours in complete medium to allow efficient target down-regulation prior to protein harvesting or cell replating for further experiments (48 hours post transfection).

8. [^{125}I] BMP-9 binding assay

[^{125}I] BMP-9 binding assay was performed in the laboratory of Dr. Peter ten Dijke. Iodination of BMP-9 was performed according to the chloramine T method and cells were subsequently affinity-labeled with the radioactive ligand as described by Dr. Peter ten Dijke's group (van Meeteren *et al.* 2012). In brief, cells were incubated on ice for 2 hours with the radioactive ligand. After incubation, cells were washed and crosslinking was performed using 54 mM disuccinimidyl suberate (DSS) and 3 mM bis(sulfosuccinimidyl)suberate (BS3, Pierce) for 15 min. Cells were washed, scraped and lysed. Lysates were incubated with the respective antisera for 2.5 hours and immune complexes were precipitated by adding protein A Sepharose (Amersham).

Samples were washed, boiled in sample buffer and subjected to SDS-PAGE. Gels were dried and scanned with the STORM imaging system (Amersham).

9. ANALYSIS OF PROLIFERATION, CELL VIABILITY AND APOPTOSIS

9.1 Thymidine Incorporation Assay

Cells were plated at a density of 17,500 cells/sq cm in DMEM with 10% FBS. The following day, cells were serum starved and incubated for 48 hours with or without BMP9 (2 ng/ml). After 6-8 hours of treatment, we added to the plates a mixture of radioactive thymidine (0.5 µCi/ml [^{3}H]-thymidine, GE Healthcare) and cold thymidine (1 µM, Sigma). After 24 hours of incubation with this mixture, we removed the radioactive medium and washed the plates with cold PBS. Then, we covered cells with TCA (trichloroacetic acid) 10% solution (w/v) for 20 minutes at 4°C, to precipitate protein and nucleic acids and we washed the plates twice with ethanol 70% (v/v), allowing them to dry completely. Finally, the precipitate was collected using a solution 0.1 N NaOH, 2% Na_2CO_3 and 0.5% SDS. An aliquot was transferred to scintillation vials containing scintillation liquid (Perkin Elmer) and radioactive nucleotide incorporation was read in a liquid scintillation counter (LKB Wallac, model 1209 Rackbeta). The results are expressed as counts per minute (cpm) in each experimental condition.

9.2 Analysis of cell number

Oval cells (15000 cells/sq cm) were seeded in 12 multiwell plates in triplicate. The following day cells were serum deprived and treated with the stimulus. Cells were counted daily for 4 days. For that, medium was removed, cells were washed twice with PBS, detached using trypsin and finally counted after staining with Trypan Blue using a Neubauer chamber.

9.3 Analysis of apoptosis by propidium iodide staining

Oval cells (about 30000 cells/sq cm) were seeded in 35 mm plates in triplicate. After treatment, they were washed twice with cold PBS and fixed using a mixture of methanol and acetic acid (3:1) for 30 minutes at room temperature. Then, they were washed twice with PBS and stained for 20 minutes at 37°C with a solution of propidium iodide (PI) (Sigma) containing PI 5 µg/ml, 0,1% Triton X-100, EDTA 0,1 M and 25 U/ml RNAsa (Sigma). Finally, dishes were washed with PBS and coverslipped using Mowiol mounting medium. Cells undergoing apoptosis were scored under inverted fluorescence microscope (Eclipse TE300, Nikon) at high magnification (x60) following standard morphological criteria. Apoptotic indices were calculated after counting a minimum of 1,000 cells per treatment in a blinded manner

9.4 Analysis of caspase -3 activity

Cell were scrapped in PBS, collected by centrifugation at 5,000 rpm for 5 minutes at 4ºC and lysed in 5 mM Tris-HCl (pH 8), 20 mM EDTA, 0.5% Triton X-100 for 30 minutes on ice vortexing every 5 minutes. Lysates were centrifuged at 13,000 rpm for 10 minutes to remove residual membrane and stored at -80°C, previously separating aliquots to assess the protein concentration. The protein concentration was measured using the Bradford method, explained above. Then, 20 µg protein were incubated for 2 hours at 37°C in the dark in the presence of the synthetic substrate of caspase-3 Ac-DEVD-AMC (BD Pharmingen) at a 20 µM concentration in a reaction buffer whose composition is: 20 mM HEPES (pH 7.5), 10% Glicerol and 2mM DTT. Proteolysis of the synthetic substrate by active caspase 3 present in the lysates liberates the fluorogenic compound AMC, whose fluorescence was measured in a fluorimeter (Perkin Elmer LS-50, Excitation/Emission (nm): 380/440). A unit of caspase activity is the amount of enzyme that will lead to a one unit increase in the fluorescence intensity. Results are expressed as units of activity per microgram of protein.

10. CLONOGENIC ASSAY

To measure the colony-forming and self-renewal capacity, cells (100) were seeded in a 6 cm dishes. After 8-10 days, the medium was removed and cell colonies were fixed and stained with a 0.2% crystal violet w/v in 2% ethanol solution. The total number of clones was quantified using Image J program and their size using Open CFU program.

11. MIGRATION AND INVASION ASSAYS

11.1 Wound healing assay

Cells (34000 cells/sq cm) were seeded in 35mm-cell culture dishes and grown to confluence in complete medium. A sterile 10 µl pipette tip was used to make a wound across a cell culture monolayer. Cell debris was removed by discarding the medium and washing three times with PBS. Cells were then incubated in DMEM-0% for 24 hours. Multiple photographs of the wound were taken immediately after wounding (0 hours) and 4, 8, 12 and 24 hours under a phase-contrast microscopy using Eclipse TE300 Nikon microscope coupled to a digital sight DS-U2 camera. The efficiency of the wound healing process was determined by calculating the area of the cell gap using TScratch software. Three images were used for each wound at each experimental point.

11.2 Invasion assay

In vitro invasion assays were performed using transwell inserts (24-well plate inserts; 8 µm pore size; cell growth area 0.3 sq cm, BD Bioscience) coated with type IV collagen (7.5 µg/ml) according to the manufacturer's recommendation. 67000 cells/sq cm were added to the upper chamber and incubated in the presence of the different treatments in DMEM-0% FBS. After 24 hours in culture, cells in the upper chamber were carefully removed with a cotton swab and the cells that had invaded the membrane were fixed with paraformaldehyde (PFA) 4% (20 minutes) and stained with crystal violet 0.2% (Sigma Ref C-0775) in water for 20 minutes. Several representative photographs of each well were taken. Quantification of the invasion process was done by counting stained invaded cells in at least 10 fields/insert using phase-contrast microscopy (Eclipse TE300, Nikon) and a 10X objective. Each experiment was run in triplicates to confirm reproducibility of data.

12. QUANTITATIVE DETERMINATION OF UREA

Quantitative determination of urea concentrations in cell culture medium was done using the SPINREACT kit, according to the manufacturer´s recommendation.

Briefly, cells (1 × 10^6 cells) were seeded on 100mm plate. The following day, the cells were left with a minimal amount of medium (3 ml) overnight. The next day, culture media were collected and measured. To perform the assay, 10 μL of sample were transferred into 1cm cuvette with the working reagent (WR), mixed and incubated 10 minutes at room temperature. Then, sodium hypochlorite (NACIO) was added to each cuvette and mixed quickly. The reaction was incubated for 10 minutes at room temperature. Optical density (OD) at 580 nm was measured using a plate reader (Powerwave XS, Biotek). The concentration of a standard urea sample containing 50 mg/dL urea was also measured.

The urea concentration in the sample was calculated from the OD values:

$$\frac{mg}{dl}[Urea] = \frac{\text{OD Sample} - \text{OD Blank}}{\text{OD Standard} - \text{OD Blank}} X\ [Standard]\frac{mg}{dl}$$

where OD Sample, OD Standard, and OD Blank are OD580 nm values of the sample, standard, and water blank, respectively. [Standard] is the concentration of the urea standard (50 mg/dL or 0.1665 mmol/L). The values obtained are expressed as mg/dl of urea in the sample respect to the cell number.

13. STATISTICAL ANALYSIS

Data have been represented as the mean value of 3-10 independent experiments ± S.E.M. When only one experiment was shown, data have been represented as the mean value ± S.D. (standard deviation). When comparisons were made between two experimental groups, unpaired Student's t-test was used. To compare more than two groups, analysis of variance (ANOVA) was performed.

RESULTS

1. BMP9 REGULATES OVAL CELL EXPANSION *IN VIVO*

Previous experiments performed in our laboratory and studies from other groups described that BMP9 regulates the function of different liver cell populations. As indicated in the "group background" section, BMP9 is a proliferative and survival factor for human HCC cells (Herrera *et al.* 2013). Moreover, it has been reported to be produced by the healthy liver (Miller *et al.* 2000; Bidart *et al.* 2012) and to be overexpressed in liver tumors (Herrera *et al.* 2013; Li *et al.* 2013). Its role in other pathological conditions is a matter of study.

However, nothing is known about its regulatory role in another hepatic cell population, the oval cells. As mentioned before, expansion of hepatic progenitors in the liver has been observed in chronic liver injury (Lowes *et al.* 1999; Fausto and Campbell 2003; Duncan *et al.* 2009; Riehle *et al.* 2011) and this process is considered as the second line of defense against liver injury. Taking into consideration the emergent role of BMP9 in liver pathophysiology, we decided to investigate the role of BMP9 in oval cells in chronic liver injury and the potential implications in the context of liver regeneration.

1.1 Lack of BMP9 leads to an amplified ductular response after DDC treatment

To analyze whether genetic deletion of BMP9 has any effect on oval cell expansion, we used a model of liver injury induced by a diet containing the porphyrogenic agent DDC, that has been shown to activate oval cells (Preisegger et al. 1999; Wang et al. 2003) (Yamazaki et al. 2011). BMP9-KO mice were generated in Dr. Lee laboratory and their phenotype is described in "Material and methods" section. Thus, WT and BMP9-KO mice were fed a 0.1% DDC supplemented diet for 1, 2, 4 and 6 weeks.

Histological analysis, in particular H&E staining, showed the typical periportal ductular reactions with oval cells, identified as small cells with an oval nucleus and scant cytoplasm, expanding from the periportal regions in the liver of WT and BMP9-KO treated mice (**FIGURE 18**). Interestingly, H&E staining showed an increase in oval cell number in the BMP9-KO liver when compared to WT at 6 weeks of DDC feeding (**FIGURE 18 A**). To confirm the visual observation and quantify the oval cell expansion, periportal ductular reaction areas were measured from H&E stained sections as described in "Material and methods" section. Quantitative analysis demonstrated that the ductular reaction after 6 weeks of DDC feeding was more pronounced in BMP9-KO than in WT mice (**FIGURE 18 B**). We also analyzed the expression of two well-established oval cells markers, Epcam and cytokeratin 19 (Krt19), in livers of untreated and DDC-treated WT and BMP9-KO mice. In order to do this, total RNA from liver tissues was obtained followed by qRT-PCR analysis of Epcam and Krt19 mRNA levels. We observed an increase in the expression of both oval cell markers in WT and BMP9-KO mice submitted to DDC diet (**FIGURE 18 C**). Importantly, the up-regulation of Epcam and Krt19 expression was more pronounced in BMP9-KO compared to WT mice at 6 weeks. The differential expression of Epcam observed in BMP9-KO was confirmed at the protein level, by western blot analysis performed with liver protein extracts obtained in the same conditions (**FIGURE 18 D, right and left panels**).

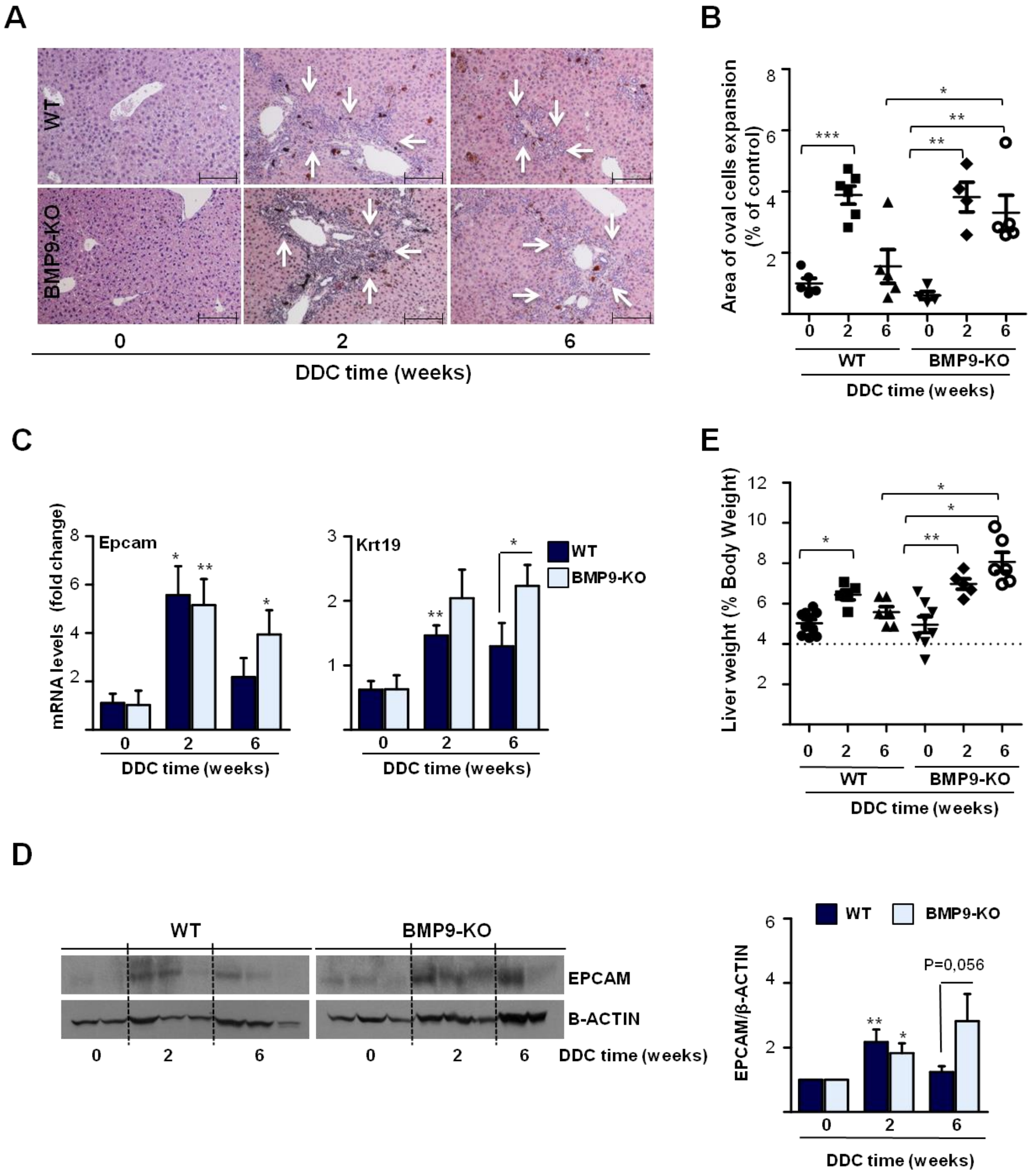


FIGURE 18. More pronounced ductular response in BMP9-KO compared to WT mice after DDC feeding

A. Representative images of H&E staining in liver tissues of WT and BMP9-KO livers untreated or after 2 and 6 weeks of DDC diet. Images are shown at 100x magnification. **B.** Quantitative morphometric analysis of oval cells expansion using ImageJ software. Areas from 12 periportal regions of each animal (at least five animals per group) were measured, and the percentage respect the untreated group was calculated. **C.** Total mRNA was isolated from the livers of WT or BMP9-KO mice untreated or after DDC treatment for 2 or 6 weeks. Epcam and Krt19 mRNA levels were determined by quantitative RT-PCR and normalized to Gusb. Data are expressed relative to untreated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 4 animals. **D.** Protein was isolated from the livers of WT or BMP9-KO mice untreated or after DDC treatment for 2 or 6 weeks. Western blot analysis was performed using an antibody that recognizes EPCAM and β-ACTIN (used as loading control). A representative experiment of 9 is shown (left panel). Optical density values relative to loading control were calculated. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 4 animals (right panel). **E.** The liver to body weight ratio was estimated based on the following formula: %=100 × Ml/Mb, where Ml is the liver weight and Mb is the total animal weight. Data are expressed as the mean ± SEM of at least 5 animals for group. *p<0.05, **p<0.01 and ***p<0.001.

The evaluation of liver-to-body weight ratio is used to examine the degree of liver damage (Best *et al.* 2013) but it is as well predictive of an enhanced liver regeneration. In fact, the liver-to-body weight ratio is significantly lower in rats with a deficient liver function (Dusabineza *et al.* 2012). Consistently, the impairment of liver regeneration and function observed in DDC-treated c-Met deficient mice was associated with a lack of increase in liver-to-body weight ratio along the DDC treatment (Ishikawa *et al.* 2012). Taking this into consideration, we measured this parameter in both WT and BMP9-KO mice at the different time points of treatment. Consistent with a damage-induced compensatory regenerative response, we saw an increase in the liver-to-body weight ratio in those mice submitted to the diet, but this increase was higher in BMP9-KO as compared to WT mice after 6 weeks (**FIGURE 18 E**), further supporting a greater expansion of the oval cell population in the absence of BMP9.

All these data together evidence that BMP9-KO present a greater expansion of the oval cell population than WT in response to a DDC diet, which suggest a negative regulatory role for BMP9 on the oval cell expansion.

1.2 BMP9 signaling is decreased in DDC fed mice during oval cell expansion

To further explore the potential role of BMP9 as a negative regulator in oval cell expansion, we next analyzed BMP9 expression levels during this process in WT mice fed with a DDC diet. qRT-PCR analysis revealed a significant decrease of Bmp9 mRNA levels during the *in vivo* expansion of oval cells. This decrease was observed as early as one week after initiation of DDC diet and was sustained up to 6 weeks of treatment (**FIGURE 19 A**). Analysis of BMP9 protein levels by western blot gave similar results (**FIGURE 19 B**). In addition to BMP9, we analyzed the activation of SMAD1,5,8 and we found a significant decrease of SMAD1,5,8 phosphorylation after DDC treatment compared to the untreated animals. Moreover, we also detected a significant downregulation of important BMP9 type I (Alk1 and Alk-2) and type II (Bmpr2) receptors during the DDC treatment (**FIGURE 19 C**).

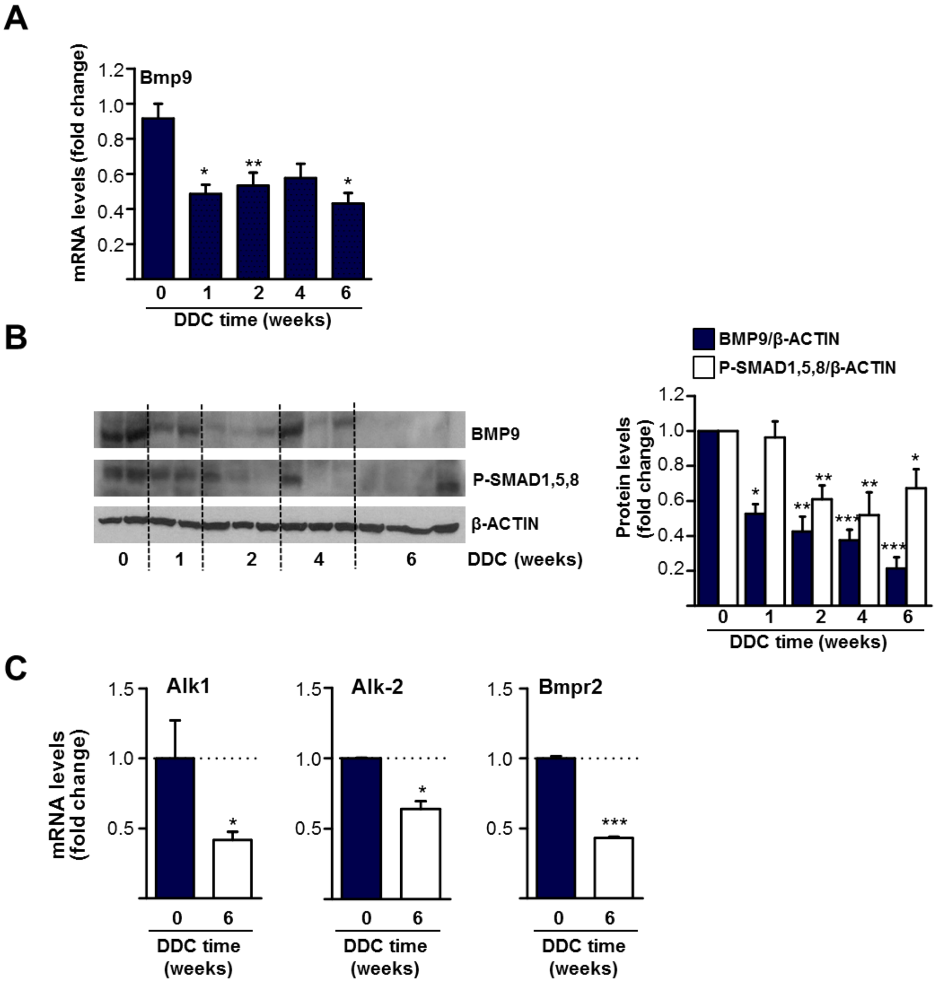


FIGURE 19. Decreased expression of BMP9 and its signalling components in DDC-treated mice

A. Total mRNA was isolated from the livers of WT mice untreated or after DDC treatment for 1, 2, 4 or 6 weeks. Bmp9 mRNA levels were determined by quantitative RT-PCR and normalized to Gusb. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 5 animals. **B.** Protein was isolated from the livers of WT mice after DDC treatment for 1, 2, 4 or 6 weeks. Western blot analysis was performed using an antibody that recognizes BMP9 or phosphorylated SMAD1,5,8 (P-SMAD1,5,8). β-ACTIN was analyzed as loading control. A representative experiment of 2 is shown (left panel). Optical density values relative to loading control were calculated. Data are expressed relative to non- treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 4 animals (right panel). **C.** Total mRNA was isolated from the livers of WT mice after 6 weeks of DDC treatment. Alk1, Alk-2 and Bmpr2 mRNA levels were determined by quantitative RT-PCR and normalized to Gusb. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 5 animals. Data were compared with the untreated condition. **p*<0.05, ***p*<0.01 and ****p*<0.001.

Collectively, these findings indicate that BMP9 and its major signaling components are downregulated during the ductular reaction in the DDC model and suggest that BMP9 signaling downregulation might be critical to allow *in vivo* expansion of oval cells.

1.3 Increased activation of AKT and ERK1/2-MAPKs in BMP9-KO livers during oval cell expansion. Regulation of HGF/c-Met pathway.

Next, we wanted to explore the mechanisms responsible for the amplified oval cell expansion observed in BMP9-KO mice. Since AKT and ERK1/2-MAPKs have been extendedly involved in the regulation of cell proliferation, survival and motility in hepatocytes and oval cells (Michalopoulos and DeFrances 1997; Coutant *et al.* 2002; Okano *et al.* 2003; Fremin *et al.* 2007; Martinez-Palacian *et al.* 2013; Utley *et al.* 2014; Suarez-Causado *et al.* 2015), we performed a comparative analysis of the activation of these two pathways in WT and BMP9-KO liver obtained from mice submitted to the DDC diet. Interestingly, BMP9-KO animals showed a significant increase in the levels of both activated AKT and ERK1/2-MAPKs along the DDC treatment (**FIGURE 20**) suggesting a role for these kinases in promoting expansion of oval cells in these mice.

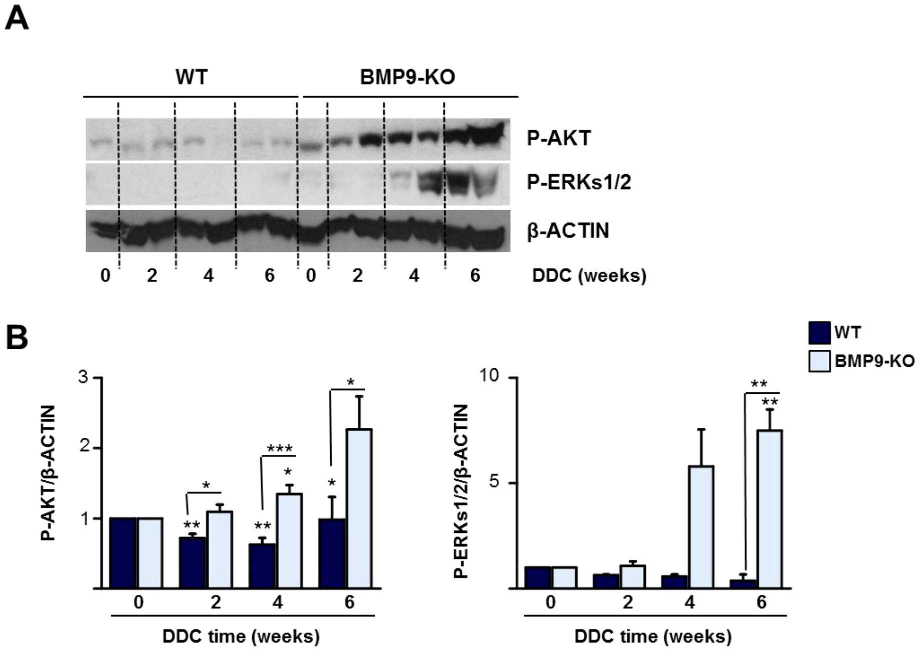


FIGURE 20. Increased activation of AKT and ERK1/2-MAPKs in BMP9-KO after DDC treatment

A. Protein was isolated from the livers of WT and BMP9-KO mice untreated or after DDC treatment for 2, 4 or 6 weeks. Western blot analysis was performed using an antibody that recognizes phosphorylated AKT (P-AKT) and phosphorylated ERK1/2-MAPKs (P-ERKs1/2). β-ACTIN was analyzed as loading control. A representative experiment of 3 is shown. **B.** Optical density values relative to loading control were calculated. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 4 animals. Data were compared with the untreated condition or as indicated. **$p<0.05$, **$p<0.01$ and ***$p<0.001$.

It is well known that HGF/c-Met signaling pathway plays a critical role in liver regeneration including the regenerative response mediated by oval cells (Borowiak *et al.* 2004; Huh *et al.* 2004; Michalopoulos 2010; Riehle *et al.* 2011; Ishikawa *et al.* 2012). Indeed, using the DDC model, it has been shown that loss of c-Met decreases oval cell pool, migration and differentiation (Ishikawa *et al.* 2012). Interestingly, c-Met mutant livers failed to activate AKT and ERK1/2-MAPKs after DDC diet. This prompted us to check whether the amplified oval cell expansion seen in BMP9-KO mice submitted to the DDC diet was somehow associated with alterations in the activation of HGF/c-Met pathway (**FIGURE 21**).

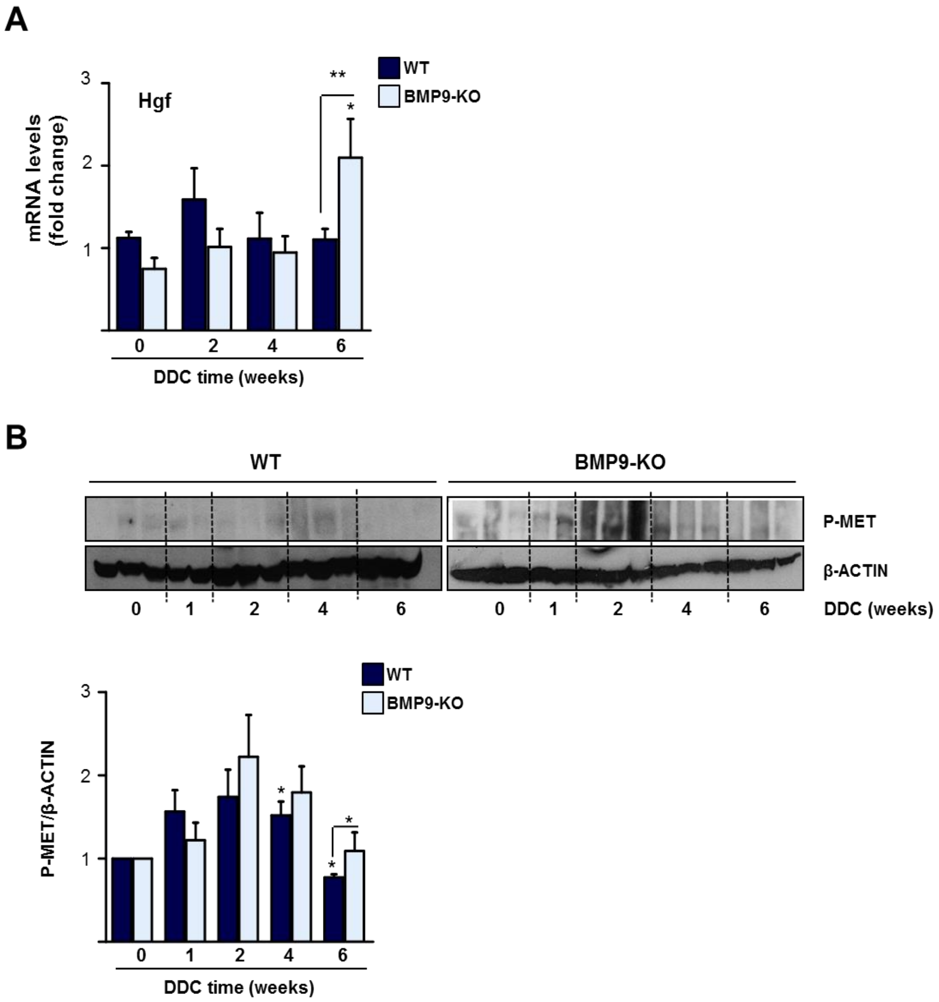


FIGURE 21. Increased expression of Hgf and activation of c-Met in BMP9-KO mice under DDC diet

A. Total mRNA was isolated from the livers of WT and BMP9 KO mice untreated or after DDC treatment for 2, 4 or 6 weeks. Hgf mRNA levels were determined by quantitative RT-PCR and normalized to Gusb. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 5 animals. **B.** Protein was isolated from the livers of WT and BMP9 KO mice after DDC treatment for 1, 2, 4 or 6 weeks. Western blot analysis was performed using an antibody that recognizes phosphorylated-MET (P-MET). β-ACTIN was analyzed as loading control. A representative experiment of 3 is shown (upper panel). Optical density values relative to loading control were calculated. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 5 animals (lower panel). Data were compared with the untreated condition or as indicated, *p<0.05 and **p<0.01.

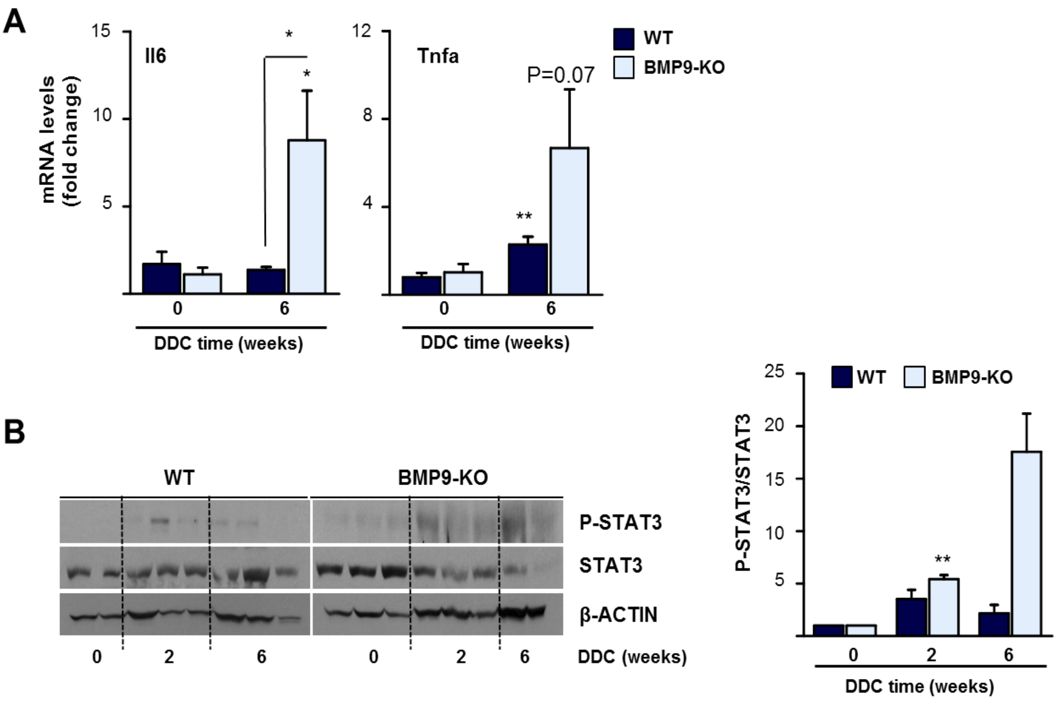
As shown in **FIGURE 21 A**, BMP9-KO mice expressed higher levels of hepatic Hgf compared to WT at 6 weeks of DDC treatment, coinciding with the increase in oval cell expansion. To study whether this increase in Hgf was associated with an increased activation of the pathway, we analyzed the phosphorylation levels of c-MET using a phospho-MET (Tyr1234/1235) antibody. c-MET phosphorylation was much higher and more prolonged in BMP9-KO mice than in WT mice (from 2 weeks and up to 6 weeks of treatment) (**FIGURE 21 B**). These data suggest that the absence of BMP9 results in an increased activation of the HGF/c-Met pathway and lead us to speculate on a possible functional interaction between these two signaling pathways operating to adequately regulate oval cell expansion in mice.

1.4 Enhanced hepatic inflammatory response in DDC fed BMP9-KO mice

Different evidence have shown that oval cell proliferation *in vivo* is associated with inflammation (Libbrecht *et al.* 2000; Knight *et al.* 2005; Viebahn and Yeoh 2008). Indeed, there are *in vitro* and *in vivo* data in the literature indicating that inflammatory cytokines, including IL-6, IFN-γ, TNF-α and TWEAK, induce oval cell proliferation (Brooling *et al.* 2005; Jakubowski *et al.* 2005; Knight *et al.* 2005; Nguyen *et al.* 2007; Viebahn and Yeoh 2008; Kitade *et al.* 2016). On this basis, to further investigate the mechanisms behind the amplified oval cell response in BMP9-KO mice, we next analyzed the inflammatory response in WT and BMP9-KO mice (**FIGURE 22**).

First, we checked the hepatic expression of two pro-inflammatory cytokines, Il6 and Tnfa in WT and BMP9-KO mice submitted to the DDC diet. We found that the DDC treatment did not result in changes in Il-6 expression levels in WT animals, whereas Tnfa was upregulated. In contrast, in BMP9-KO mice both Il6 and Tnfa were upregulated in response to the DDC diet and in the case of Il6, this increase was statistically significant when compared with WT (**FIGURE 22 A**). In addition, we analyzed STAT3 activation as a way to measure cytokine-driven signaling. The levels of the phosphorylated form of STAT3 were higher in BMP9-KO mice than in WT in response to liver injury (**FIGURE 22 B**). These results, all together, suggest that there is an enhanced hepatic inflammatory response in BMP9-KO mice, which could contribute to the oval cell expansion and proliferation.

To further analyze the inflammatory status, coded histological liver sections of WT and BMP9-KO animals fed with the DDC diet were scored independently by a single pathologist using the Knodell HAI (Knodell *et al.* 1981). As described in “Material and methods”, this system evaluates different parameters: periportal bridging necrosis, intralobular inflammation, portal inflammation and bridging fibrosis portal. The results of Knodell scoring system are presented in the table (**FIGURE 22 C**). According to the total score, the cases were divided into five groups (Mushtaq *et al.* 1997). According to the Knodell scoring system, no differences were observed in untreated WT and BMP9-KO (animals included in groups 0 and I). DDC-fed BMP9-KO mice fell into the category III, scoring 9-12, with severe portal inflammation and moderate intralobular inflammation (three of the four animals analyzed have score 3 for the intralobular inflammation parameter). On the contrary, DDC-fed WT analyzed showed the same degree of portal inflammation but a mild degree of intralobular inflammation (indeed, in this case three of the four animals analyzed have score 1 for the intralobular inflammation parameter). No differences emerged in terms of periportal necrosis and fibrosis.



C

Knodell Histology Activity Index (HAI)

HAI	Number				Percentage	
	WT		BMP9-KO		WT	BMP9-KO
DDC time (weeks)	0	6	0	6		
0 (Group 0)	3	-	3	-	37.5	37.5
1-3 (Group I)	1	-	1	-	12.5	12.5
4-9 (Group II)	-	2	-	-	25	0
10-12 (Group III)	-	2	-	4	25	50
13-18 (Group VI)	-	-	-	-	0	0

FIGURE 22. Enhanced hepatic inflammatory response in BMP9-KO livers submitted to DDC diet

A. Total mRNA was isolated from the livers of WT and BMP9 KO mice untreated or after DDC treatment for 6 weeks. Il6 and Tnfa mRNA levels were determined by quantitative RT-PCR and normalized to Gusb. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 5 animals. **B**. Protein was isolated from the livers of WT and BMP9 KO mice after DDC treatment for 2 or 6 weeks. Western blot analysis was performed using an antibody that recognizes phosphorylated-STAT3 (P-STAT3) and total STAT3. β-ACTIN was analyzed as loading control. One experiment is shown (left panel). Optical density values relative to total STAT3 normalized levels were calculated. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. (right panel). Data were compared with the untreated condition or as indicated, **p*<0.05 and ***p*<0.01. **C**. The table showed the results of Knodell scoring system for the samples analyzed; 4 animals for each condition have been used.

From the analysis of these data, we concluded that the intralobular inflammation is increased in BMP9-KO mice compared to WT in response to DDC treatment.

In summary, using different approaches, we have shown that BMP9-KO mice display a greater inflammation concomitant with an amplified oval cell expansion compared to WT in response to DDC diet, further supporting the hypothesis that the inflammatory process could have a role in the promotion of oval cell expansion.

1.5 Reduced liver damage in BMP9-KO mice under DDC diet

So far, our results show a greater oval cell expansion in mice lacking BMP9, but we do not know the consequences of this phenomenon for the progression and outcome of the liver injury process. As mentioned in previous sections, recent evidence in the literature suggest that a sustained oval cell response may aggravate fibrosis (Pritchard and Nagy 2010; Chobert *et al.* 2012; Kuramitsu *et al.* 2013).

Thus, authors postulate that activation of oval cell-mediated regeneration in fibrotic livers is associated with a more pronounced fibrogenic response. This prompted us to study the degree of fibrosis in our mouse model after DDC treatment by analyzing the expression levels of a panel of fibrogenic markers such as Tgfb1, Tgfb2, procollagen α 1(I) (Col1a1) and α-smooth muscle actin (Acta2).

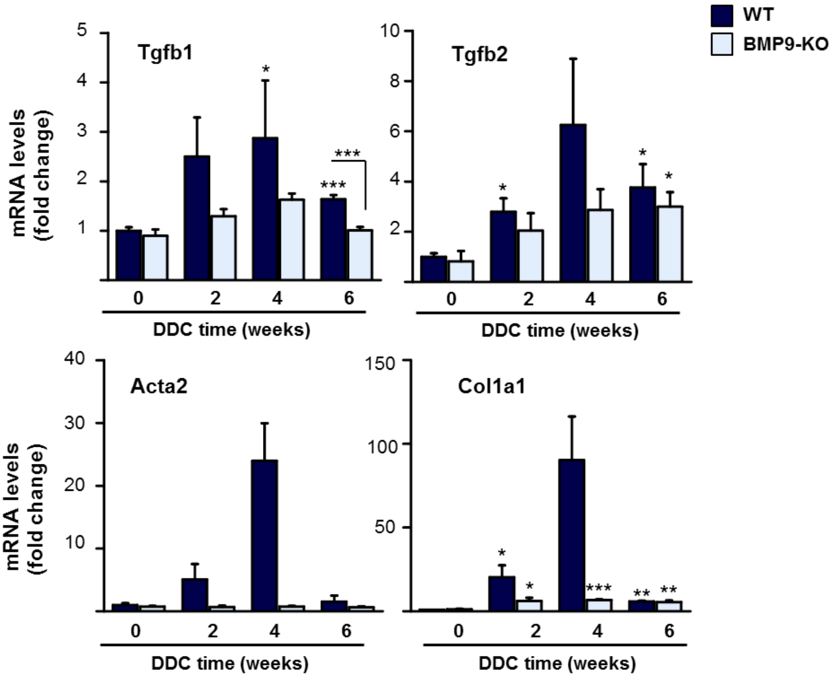


FIGURE 23. Reduced hepatic fibrosis markers in BMP9-KO mice under a DDC diet.

Total mRNA was isolated from the livers of WT and BMP9 KO mice untreated or after DDC treatment for 2, 4 and 6 weeks. Tgfb1, Tgfb2, Col1a1 and Acta2 mRNA levels were determined by quantitative RT-PCR and normalized to Gusb. Data are expressed relative to non-treated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 4 animals. Data were compared with the untreated condition or as indicated, **p*<0.05, ***p*<0.01 and ****p*<0.001.

Consistent with an induction of hepatic damage, all the markers analyzed were increased in WT mice after DDC treatment, showing a peak of induction at 4 weeks, whereas in BMP9-KO mice this increase in pro-fibrogenic markers was very mild or even absent as in the case of Acta2 (**FIGURE 23**).

Although these data suggested that liver injury triggered by DDC treatment was reduced in BMP9-KO as compared to WT mice, to unequivocally evaluate liver damage, we measured serum blood liver function parameters, in particular alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin total levels, in serum of DDC fed mice (**FIGURE 24**). These parameters were elevated in response to DDC, with a peak at 2 weeks of treatment and decline over time, which is likely related to the oval cell mediated regenerative response that contribute to restore liver function. The profile was similar in BMP9-KO mice but clearly the increase in ALT, AST and bilirubin was attenuated, being these values statistically different from those obtained in WT mice at 6 weeks of DDC treatment, providing strong evidence that liver damage is attenuated in BMP9-KO mice.

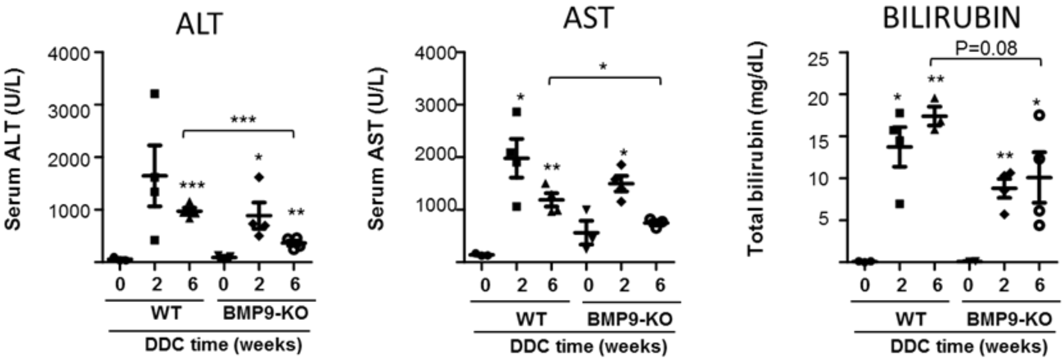


FIGURE 24. Reduced serum ALT, AST and total bilirubin levels in BMP9-KO mice under a DDC diet

ALT, AST and bilirubin serum levels of WT and BMP9 KO mice untreated or after DDC treatment for 2 and 6 weeks. Data are mean ± S.E.M. of at least 3 animals per group. Data were compared with the untreated condition or as indicated, *p<0.05, **p<0.01 and ***p<0.001.

Collectively, our data indicate that the absence of BMP9 *in vivo* not only results in a greater expansion of the oval cell compartment and an accentuation of inflammation but also in a significant attenuation of liver fibrosis and damage, evidencing a more efficient regeneration in BMP9-KO livers in response to DDC-induced damage. Hence, our data suggest that BMP9 may act as a negative regulator of the oval cell mediated liver regeneration.

2. RELEVANCE OF BMP9-MEDIATED SIGNALING IN OVAL CELL FUNCTION. CROSSTALK WITH THE HGF/c-Met PATHWAY

To further study this novel potential role of BMP9 as a suppressor factor for oval cell expansion and to characterize the molecular mechanisms mediating such effect, we performed additional experiments using an *in vitro* model of oval cells previously established and characterized in our laboratory.

2.1 BMP9 induces the canonical signaling pathway in oval cells *in vitro*

First, we checked if oval cells respond to BMP9 with an activation of the canonical SMAD pathway (**FIGURE 25**). We found that BMP9 induced SMAD1,5,8 phosphorylation in a dose dependent manner in oval cells. When we performed a time course analysis of SMAD1,5,8 phosphorylation, we found that activation occurred after 30 minutes of BMP9 treatment, with a maximum at 2 hours. To test the SMAD-dependent transcriptional response to BMP9, we transfected the cells with the plasmid pGL3 BRE Luciferase (pGL3(BRE)-luc) containing a reporter construct consisting of a BMP-response element (BRE) from the Id1 promoter fused to a luciferase reporter gene. Indeed, BMP9 increased BRE-luciferase activity in oval cells (**FIGURE 25 B**) and consistently, it also enhanced Id1 mRNA expression (**FIGURE 25 C**), a well-studied target gene of the BMP/SMAD signaling pathway.

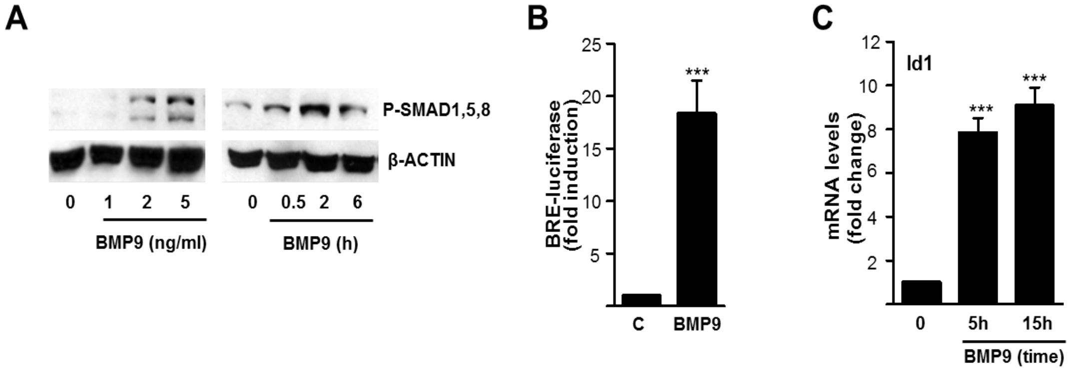


FIGURE 25. BMP9 induces the SMAD1,5,8 signaling pathway in oval cells

A. Oval cells were incubated with different concentrations of BMP9 for 1 hour (*left panel)* or for different periods of time with BMP9 (2 ng/ml) (*right panel*) in 0% FBS media. Western blots were performed with antibodies that recognized P-SMAD1,5,8 and β-ACTIN as loading control. A representative experiment of 3 is shown. **B.** Oval cells were transfected with a pGL3(BRE)-luciferase reporter construct and then were serum starved and treated for 15 hours with BMP9 (2 ng/ml). Luciferase activity was normalized to cell number. Data are shown as fold change (relative to untreated cells) and are pooled from three experiments (n=6) (mean ± S.E.M). **C.** Oval cells were incubated ± BMP9 (2 ng/ml) for different periods of time in 0% FBS media and Id1 levels were analyzed by qRT-PCR and normalized to Gusb. Data are shown as fold induction (relative to untreated cells) and correspond to one representative experiment (n=3) out of 3 performed (mean ± S.D.). Data are expressed as fold induction (relative to untreated cells) and are pooled from 4 independent experiments performed in triplicate (mean ± S.E.M). $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

2.1 BMP9 reduces oval cell number and increases invasion

As BMP9 has been shown before to control hepatic cell growth (Herrera *et al.* 2013), we analyzed the response of oval cells to BMP9 in terms of cell growth.

We incubated oval cells with BMP9 for different periods of time in the absence of serum, and we found that BMP9 moderately but significantly decreases oval cell number (**FIGURE 26 A**). The effect of BMP9 in oval cell growth seems to be due to combination of two effects, a cell proliferation inhibitory effect, measured by [^{3}H] thymidine incorporation assay (**FIGURE 26 B**) and a pro-apoptotic effect. In fact, we measured the caspase-3 activity and the apoptotic index in cells treated with BMP9 and our data reveal that BMP9 is capable of inducing a mild but significant apoptotic cell death (**FIGURE 26 C and D**). Altogether, these results confirm that BMP9 has suppressor effects in oval cells.

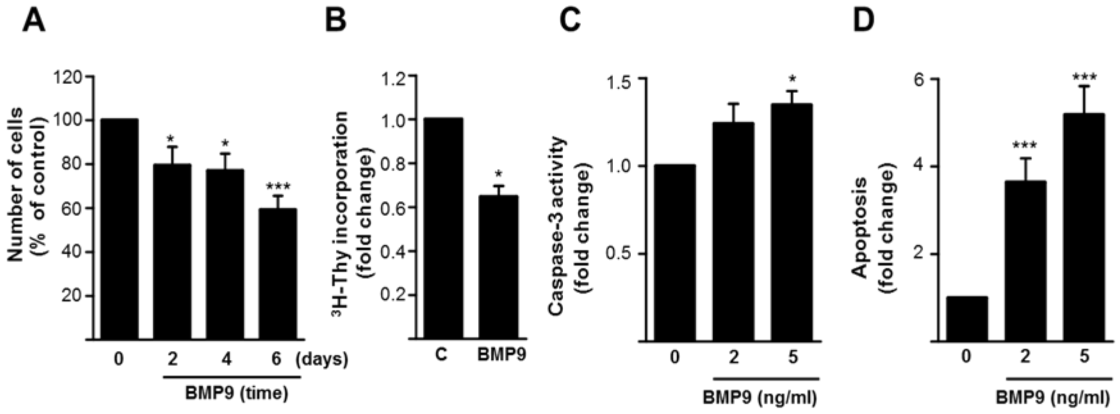


FIGURE 26. BMP9 reduces oval cell proliferation and induces apoptosis *in vitro*

A. Oval cells were incubated with 2 ng/ml BMP9 in 0% FBS media and counted at day 2, 4 and 6. Data from 5 independent experiments with n=3 displayed as percentage of untreated cells (mean ± S.E.M.). **B.** DNA synthesis as determined by thymidine incorporation in oval cells cultured for 24 hours with BMP9 (2 ng/ml). Data are mean ± S.E.M. of 3 independent experiments and are displayed as fold induction (relative to untreated cells). **C-D.** Oval cells were serum-starved and incubated in the absence or presence of BMP9 (2 ng/ml) for 24 hours. **C.** Cells were lysed and caspase-3 activity was measured. Data are expressed as fold induction (relative to untreated cells) and are pooled from 2 independent experiments performed in duplicate (mean ± S.E.M). **D.** Apoptotic nuclei were visualized and counted after PI staining under a fluorescence microscope. A minimum of 1000 nuclei was counted per condition. Data are expressed as fold induction (relative to untreated cells) and are pooled from 4 independent experiments performed in triplicate (mean ± S.E.M). *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Oval cell expansion in the injured liver not involves only cell proliferation and survival but also other biological processes, such as migration and invasion through liver parenchyma. Data from the literature indicated that other BMP members regulate cell invasion (Maegdefrau and Bosserhoff 2012; Chen *et al.* 2014; Haasters *et al.* 2014; Kim *et al.* 2014). Also BMP9 has been recently reported to induce EMT and increase cell migration in transformed hepatic cells (Li *et al.* 2013).

Based on this evidence, we questioned whether BMP9 could also regulate oval cell migration and invasion. To address this question, we assayed the migratory/invasive capacity of oval cells through extracellular matrix substrates in response to BMP9 using transwells coated with collagen IV (**FIGURE 27**).

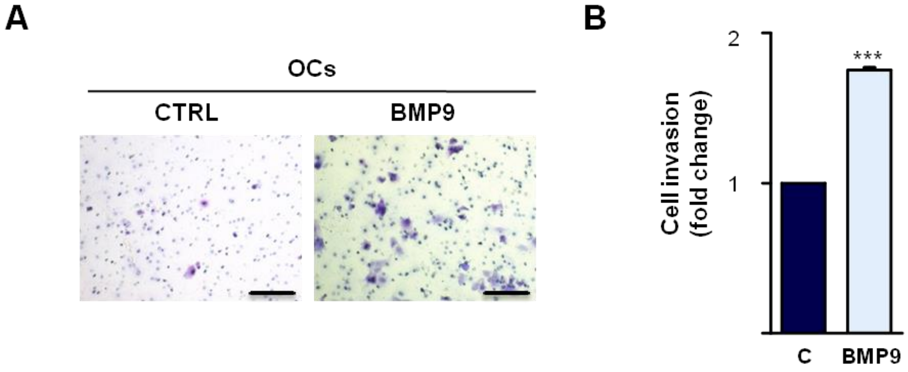


FIGURE 27. BMP9 increases oval cell invasion

Oval cells were plated in the upper chamber of transwell-24 well plate covered with collagen in serum-free medium ± BMP9 (2 ng/ml). Each well contained approximately 2 × 10^4 cells. Medium without FBS (0%) was placed in the lower chamber. Cells were allowed to pass through collagen for 24 hours and then were fixed in PFA and stained with crystal violet. **A.** Representative images of invading cells after staining with crystal violet (phase contrast microscope) of the different culture conditions are shown. Scale bar= 100 µm. **B.** Histograms show the mean value ± S.D. of the number of invading cells respect to control from one representative experiment (n=3) out of 3 performed. ****p*<0.001.

Results demonstrated a significant increase in the number of oval cells that migrated and invaded to the bottom side of the filters upon BMP9 treatment as evidenced by the microcopy images and quantitative analysis.

2.2 ALK2 mediates BMP9-triggered signaling and suppressor effects in oval cells

We have shown before that BMP9 triggers the canonical pathway in oval cells (**FIGURE 25**), as it has been described in other liver cell types (Herrera *et al.* 2013; Li *et al.* 2013). In an effort to further elucidate BMP9 signaling pathway in oval cells, we next studied the receptors involved. As other members of the BMP family, BMP9 binds to a heterotetrameric transmembrane receptor complex comprised by type I and type II serine threonine kinase receptors. ALK1 is the high affinity receptor for BMP9, but ALK2 has been described to mediate BMP9 signaling in some cell types (Herrera *et al.* 2009; Luo *et al.* 2010).

To define which specific receptors were required for BMP9 signaling in oval cells, cells were incubated with ^{125}I_BMP9 and crosslinked receptor complexes were immunoprecipitated with ALK1, ALK2 and BMPR2 specific antibodies. Results show that BMP9 binds to ALK2 and BMPRII but not to ALK1 in oval cells (**FIGURE 28 A**). We also checked the relative expression of ALK1 and ALK2 in oval cells. To do this, we compared the dCt values, the normalized Ct value of the target gene. Specifically, we calculated the dCt for each gene (Alk1 and Alk-2), and then we calculated the relative gene expression using this formula: %RG (Relative gene) expression for Gene1= 100 x 2^(-dCt gene1). Results show that Alk-2 is expressed at higher levels than Alk1 in oval cells (**FIGURE 28 B**).

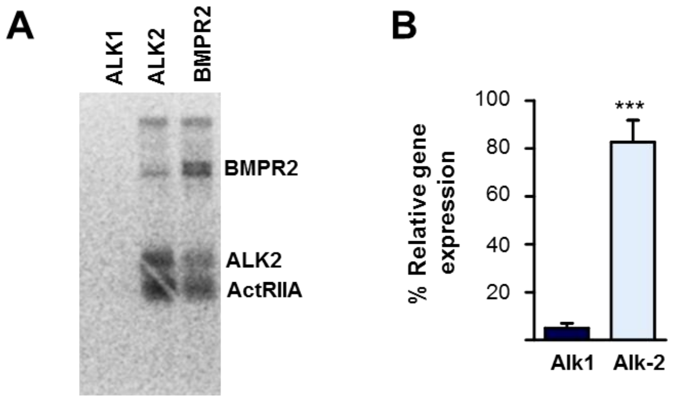


FIGURE 28. BMP9 binds to ALK2 and BMPRII in oval cells

A. Oval cells were affinity-labelled with ^{125}I_BMP9 and crosslinked ligand-receptors complexes were immunoprecipitated with specific antisera as indicated and subjected to SDS-PAGE and autoradiography. **B.** RNA from oval cells was isolated and Alk1 and Alk-2 levels were analyzed by qRT-PCR and normalized to Gusb. Data are expressed as relative gene expression and are from 8 independent experiments (n=3) (mean ± S.E.M.). ***p<0.001.

As an additional approach to further confirm that ALK2 is the main type I receptor for BMP9 in oval cells, we perform stable knockdown experiments; Alk-2 shRNA lentiviral vector was used to generate oval cells with reduced levels of this receptor. Using this strategy we obtained cells with a 60% reduction of Alk-2 expression levels (**FIGURE 29 A**). shALK2 cells failed to phosphorylate SMAD1,5,8 in response to BMP9 and also showed a complete inhibition of SMAD-dependent transcriptional activity (**FIGURE 29 B and C**). Moreover, in shALK2 cells the suppressor effect of BMP9 was completely abolished as evidenced by the fact that oval cells with reduced levels of Alk-2 did not present a decrease in cell number after BMP9 treatment (**FIGURE 29 D**). Altogether these data indicate that ALK2 mediates BMP9 suppressor effects in oval cells.

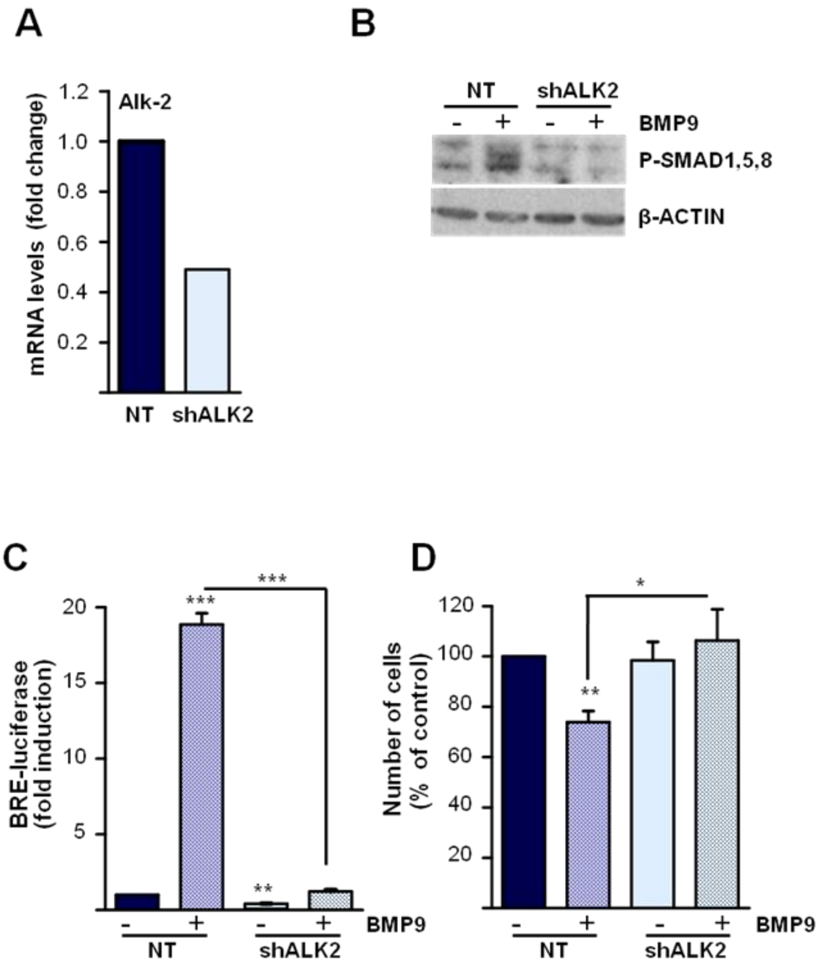


FIGURE 29. ALK2 mediates BMP9-triggered signaling and suppressor effect in oval cells

A-D. Alk-2 knockdown oval cells were generated by stable infection with lentiviral vectors expressing an ALK2 targeting short hairpin (shALK2) or a non-targeting short hairpin (NT). **A.** Alk-2 mRNA levels were determined by quantitative RT-PCR. Data expressed relative to NT cells (assigned an arbitrary value of 1). **B.** NT and shALK2 stable cells were treated with BMP9 (2 ng/ml) for 30 minutes in 0% FBS media. Western blots were performed with antibodies that recognized P-SMAD1,5,8 and β-ACTIN as loading control. A representative experiment of 2 is shown. **C.** NT and shALK2 stable oval cells were transfected with pGL3(BRE)-luciferase reporter construct and then were serum starved and treated for 15 hours with BMP9 (2 ng/ml). Luciferase activity was normalized to cell number. Data are shown as fold change (relative to NT untreated cells) and are from 1 representative experiment (n=6) (mean ± S.D.). **D.** NT and shALK2 stable oval cells were treated with BMP9 (2 ng/ml) in 0% FBS and counted at day 2. Data are from 3 independent experiments performed in triplicate, displayed as percentage of NT untreated cells (mean ± S.E.M). Data were compared with the untreated condition or as indicated. *p<0.05, **p<0.01 and ***p<0.001.

2.3 HGF/c-Met signaling inhibits BMP9-triggered suppressor effects in oval cells *in vitro*

Our *in vivo* data have revealed an over-activation of the HGF/c-Met pathway in BMP9-KO livers as compared to WT upon DDC feeding (**FIGURE 21**) providing the first evidence of a potential functional crosstalk between BMP9 and HGF/c-Met pathways in the regulation of oval cells. Interestingly, signaling crosstalk between HGF and other BMPs has been already reported in other cell types (Imai *et al.* 2005; Ye *et al.* 2007; Ye *et al.* 2008; Tsai *et al.* 2012; Shibasaki *et al.* 2015). Based on all this, we then aimed to deepen in the analysis of this potential crosstalk.

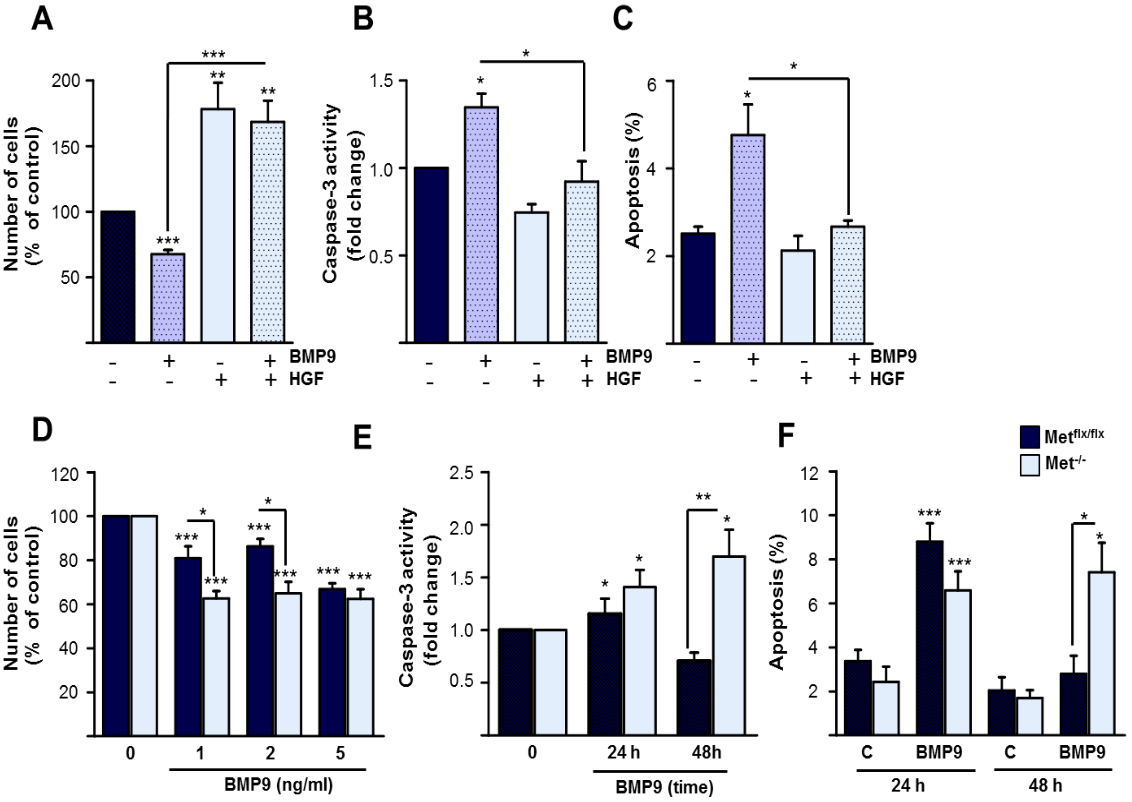


FIGURE 30. HGF/c-Met signaling inhibits BMP9-triggered suppressor effects in oval cells *in vitro*

A. Met$^{flx/flx}$ oval cells were incubated with BMP9 (5 ng/ml) in the absence or presence of HGF (40 ng/ml) in 0% FBS media and counted at day 4. Data from 4 independent experiments with n=3 displayed as percentage of untreated cells (mean ± S.E.M.). **B-C.** Met$^{flx/flx}$ oval cells were incubated with BMP9 (5 ng/ml) in absence or presence of HGF (40 ng/ml) in 0% FBS media for 24 hours. **B.** Caspase-3 activity was measured. Data are expressed as fold induction (relative to untreated cells) and are from 4 independent experiments (mean ± S.E.M). **C.** Apoptotic index was calculated by counting apoptotic nuclei after PI staining under a fluorescence microscope. A minimum of 1000 nuclei was counted per condition. Data from 2 independent experiments performed in triplicate (mean ± S.E.M.) are shown. **D.** Met$^{flx/flx}$ and Met$^{-/-}$ oval cells were incubated with different concentrations of BMP9 in 0% FBS media and counted at day 4. Data from 7 independent experiments with n=3 displayed as percentage of untreated cells (mean ± S.E.M.). **E-F.** Met$^{flx/flx}$ and Met$^{-/-}$ oval cells were incubated with BMP9 (2 ng/ml) in 0% FBS media for 24 and 48 hours. **E.** Caspase-3 activity.. Data are expressed as fold induction (relative to untreated cells) and are from 3 independent experiments performed in duplicate (mean ± S.E.M). **F.** Apoptotic nuclei.. Data are from 3 independent experiments performed in triplicate (mean ± S.E.M.). Data were compared with the untreated condition or as indicated. *p<0.05, **p<0.01 and ***p<0.001.

In a first step, we treated oval cells with BMP9 in the presence of HGF and check the effects in cell growth. We found that HGF is able to prevent the BMP9 inhibitory effects, both in terms of cell loss (**FIGURE 30 A**) and apoptotic effect (**FIGURE 30 B and C**). As a complementary approach, we took advantage of an *in vitro* model of oval cells that express a mutated inactive form of c-Met receptor lacking tyrosine kinase activity (Met$^{-/-}$), previously developed in our laboratory (del Castillo *et al.* 2008). We evaluated if the absence of a functional c-Met had any effect on the BMP9 inhibitory activity in oval cells. As shown in **FIGURE 30 D**, Met$^{-/-}$ oval cells were more sensitive to BMP9 suppressor actions as evidenced by an increase in cell loss and apoptotic index in Met$^{-/-}$ oval cells compared to their normal counterparts (**FIGURE 30 E and F)**. Jointly, these results further support a potential functional crosstalk between BMP9 and HGF/c-Met signaling pathways in oval cells.

In a next step, we investigated whether this functional crosstalk could reflect a signaling interaction between BMP9 and HGF/c-Met in oval cells. In order to solve this question, we compared the BMP9-triggered signaling response in Met$^{flx/flx}$ and Met$^{-/-}$ oval cells.

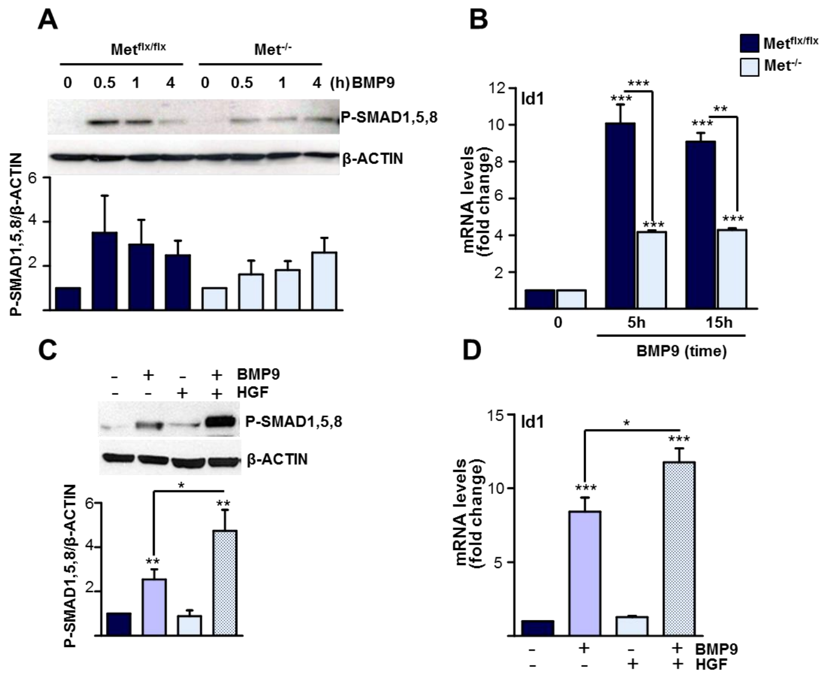


FIGURE 31. BMP9 and HGF/c-Met signaling crosstalk in oval cells

A. Met$^{flx/flx}$ and Met$^{-/-}$ oval cells were incubated with BMP9 (2 ng/ml) in 0% FBS media for different periods of time. Western blots were performed with antibodies that recognized P-SMAD1,5,8 and β-ACTIN as loading control. A representative experiment is shown (upper panel). Optical density values relative to loading control were calculated. Untreated samples were assigned an arbitrary value of 1. Data are mean ± S.E.M of 4 different experiments (bottom panel). **B.** Met$^{flx/flx}$ and Met$^{-/-}$ oval cells were incubated with BMP9 (2 ng/ml) in 0% FBS media for different periods of time and Id1 levels were analyzed by qRT-PCR and normalized to Gusb. Data are shown as fold induction (relative to untreated cells) and are from 2 independent experiments (mean ± S.E.M). **C.** Met$^{flx/flx}$ oval cells were incubated with BMP9 (2 ng/ml) in absence or presence of HGF (40 ng/ml) in 0% FBS media for 30 minutes. Western blots were performed with antibodies that recognized P-SMAD1,5,8 and β-ACTIN as loading control. A representative experiment is shown (upper panel). Optical density values relative to loading control were calculated. Untreated samples were assigned an arbitrary value of 1. Data are mean ± S.E.M of 8 different experiments (bottom panel). **D.** Met$^{flx/flx}$ oval cells were incubated with BMP9 (2 ng/ml) in absence or presence of HGF (40 ng/ml) in 0% FBS media for 15 hours and Id1 levels were analyzed by qRT-PCR and normalized to Gusb. Data are shown as fold induction (relative to untreated cells) and are from 4 independent experiments (mean ± S.E.M). Data were compared with the untreated condition or as indicated. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Interestingly, our result show that BMP9-induced phosphorylation of SMAD1,5,8 was reduced in Met$^{-/-}$ oval cells compared to Met$^{flx/flx}$ oval cells (**FIGURE 31 A**), effect that was consistent with a significant attenuation of Id1 up-regulation (**FIGURE 31 B**). Conversely, using a complementary approach, we found that HGF potentiates BMP9-triggered SMAD1,5,8 signaling in Met$^{flx/flx}$ cells (**FIGURE 31 C**), which is associated with a stronger Id1 up-regulation (**FIGURE 31 D**). These data suggest that HGF/c-Met positively modulates BMP9-triggered SMAD1,5,8 signaling in oval cells.

2.4 BMP9 and HGF/c-Met signaling crosstalk in oval cells is dependent on ALK1/SMAD1

Once we had evidenced that a functional signaling crosstalk between BMP9 and HGF/c-Met pathways was operating in oval cells, we aimed at characterizing the mechanism mediating this interaction. We hypothesized that the increased phosphorylation of SMAD1,5,8 observed upon combined treatment of HGF and BMP9, could be due to changes in the expression of the BMP9 receptors in oval cells. In order to check this possibility, we analyzed the expression of Alk1, Alk-2 and Bmpr2 in Met$^{flx/flx}$ oval cells in the absence and presence of HGF and BMP9.

Neither Alk-2 nor Bmpr2 expression were modulated by BMP9 or HGF or the combination of both. However, interestingly, Alk1 expression levels were increased in response to BMP9 and HGF and the up-regulation was further enhanced under the combined treatment (**FIGURE 32**).

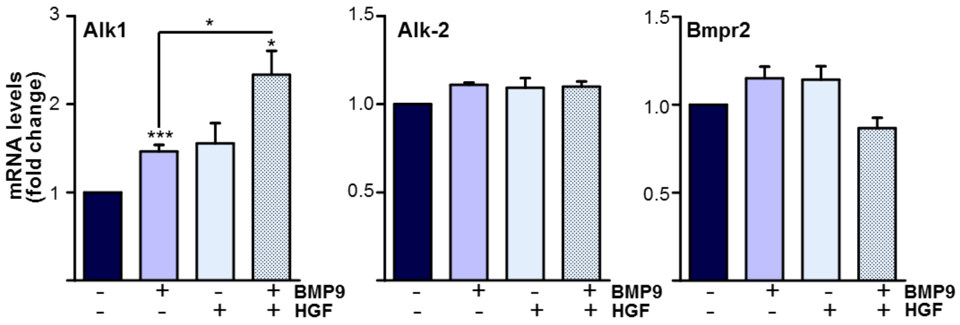


FIGURE 32. Effect of HGF and/or BMP9 treatment on BMP9 receptors expression in oval cells

Metf$^{lx/flx}$ oval cells were incubated with BMP9 (2 ng/ml) in absence or presence of HGF (40 ng/ml) in 0% FBS media for 1 hour and Alk1, Alk-2 and Bmpr2 levels were analyzed by qRT-PCR and normalized to Gusb. Data are shown as fold induction (relative to untreated cells) and are from 2 independent experiments (mean ± S.E.M). Data were compared with the untreated condition or as indicated. **p*<0.05, ***p*<0.01 and ****p*<0.001.

Based on this result, we wondered if ALK1/SMAD1 could have a role in the crosstalk between BMP9 and HGF/c-Met in oval cells. To answer this question, we performed stable knockdown experiments using an Alk-1 shRNA lentiviral vector to generate cells with reduced levels of this receptor. Using this strategy we obtained an 80% reduction of Alk-1 expression levels in oval cells (**FIGURE 33 A**). Alk1 knockdown did not affect BMP9-induced SMAD activation or decrease in oval cell number (**FIGURE 33 B, C and D**). However, HGF-mediated amplification of BMP9-triggered SMAD1,5,8 phosphorylation and subsequent SMAD-dependent transcriptional activity were lost

(**FIGURE 33 B and C**). Moreover, in the absence of Alk1, HGF was not able to prevent BMP9 suppressor effect in oval cells (**FIGURE 33 D**). Hence, we next decided to perform transient Smad1 knock-down using a Smad1 siRNA in oval cells and check its consequences in oval cell number.We reached up to 70% reduction of total Smad1 expression levels in oval cell upon siRNA-mediated Smad1 silencing (**FIGURE 33 E**). In the absence of Smad1, BMP9 was still able to decrease cell number but the protective effect of HGF against BMP9 was blocked (**FIGURE 33 F**).

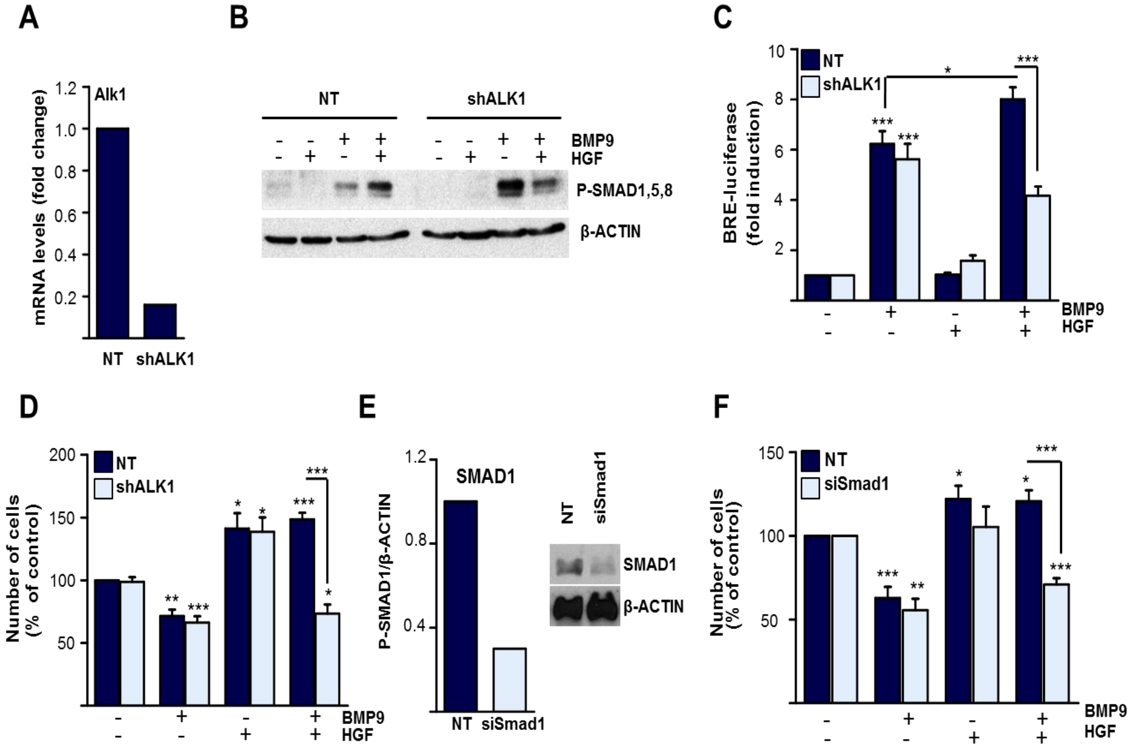


FIGURE 33. ALK1/SMAD1 drives the BMP9/c-Met signaling crosstalk and the protective effect of HGF in oval cell

A-D. Alk1 knockdown oval cells were generated by stable infection with lentiviral vectors expressing an Alk1 targeting short hairpin (shALK1) or a non-targeting short hairpin (NT). **A.** Alk-1 mRNA levels were determined by quantitative RT-PCR. Data expressed relative to NT cells (assigned an arbitrary value of 1). **B.** NT and shALK1 stable cells were treated with BMP9 (2 ng/ml) in absence or presence of HGF (40 ng/ml) in 0% FBS media for 30 minutes. Western blots were performed with antibodies that recognized P-SMAD1,5,8 and β-ACTIN as loading control. A representative experiment of 2 is shown. **C.** NT and shALK1 stable oval cells were transfected with pGL3(BRE)-luciferase reporter construct and then were serum starved and treated for 15 hours with BMP9 (2 ng/ml) in absence or presence of HGF (40 ng/ml). Luciferase activity was normalized to cell number. Data are shown as fold change (relative to untreated cells) and are from 2 independent experiments (n=6) (mean ± S.E.M). **D.** NT and shALK1 stable oval cells were treated with BMP9 (2 ng/ml) in absence or presence of HGF (40 ng/ml) in 0% FBS media and counted at day 2. Data from 2 independent experiments performed in triplicate, displayed as percentage of NT untreated cells (mean ± S.E.M). Data were compared with the untreated condition or as indicated. **E-F.** Smad1 knockdown oval cells were generated by transient infection with siRNA (siSmad1) or a non-targeting siRNA (NT). **E.** SMAD1 protein levels were determined by western blot analysis in oval cells with (siSmad1) or without (NT) Smad1 knockdown. A representative experiment of 2 is shown. The proteins levels were normalized with β-ACTIN and western blots quantification is shown. **F.** NT and siSmad1 oval cellss were incubated with BMP9 (5 ng/ml) in the absence or presence of HGF (40 ng/ml) in 0% FBS media and counted at day 4. Data from 2 independent experiments with n=3-6 displayed as percentage of untreated cells (mean ± S.E.M.). Data were compared with the untreated condition or as indicated. *p<0.05, **p<0.01 and ***p<0.001.

These results suggest that ALK1/SMAD1 is required for HGF-mediated potentiation of BMP9 signaling as well as for HGF protective effects on BMP9-suppressor action in oval cells.

2.5 HGF/c-Met signaling potentiates the pro-invasive activity of BMP9 in oval cells

Since both BMP9 (**FIGURE 27**) and HGF (Suarez-Causado *et al.* 2015) regulate oval cell invasion and our data indicate that HGF potentiates BMP9-triggered SMAD1,5,8 signaling, we wonder whether the BMP9/HGF crosstalk could also participate in the regulatory control of the invasive properties of oval cells.

Confirming our previous results, data in **FIGURE 34** show an increased invasive capacity of oval cells in the presence of HGF or BMP9 but more importantly, cell invasion was significantly increased with respect to individual treatments in response to the combined treatment (HGF + BMP9).

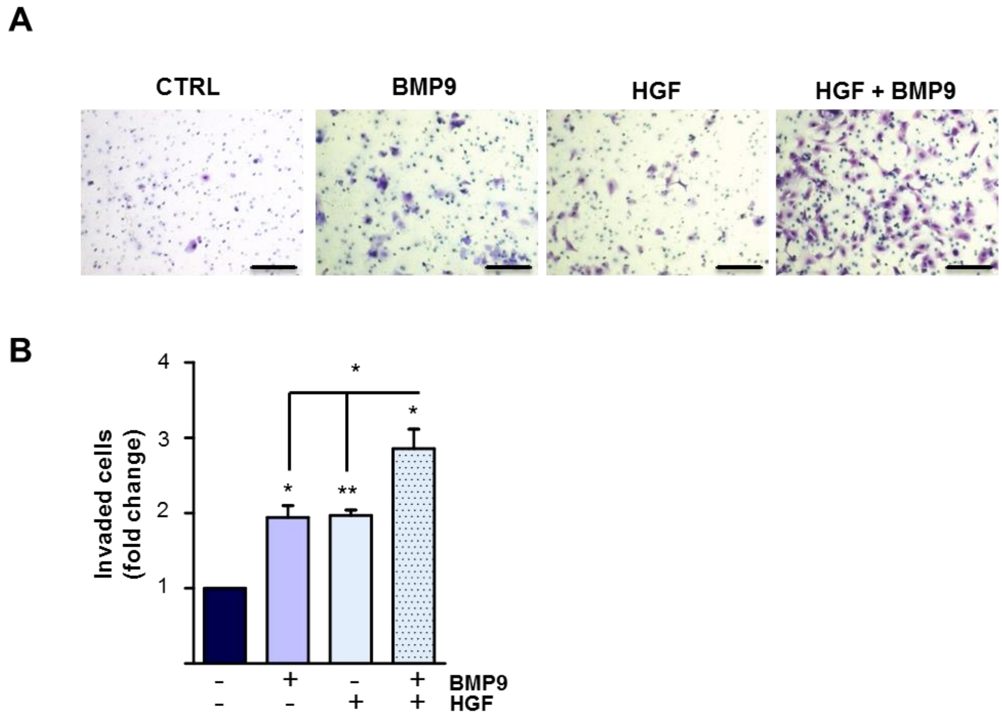


FIGURE 34. Effect of HGF and/or BMP9 treatment in oval cell invasion

Metf$^{lx/flx}$ oval cells were plated in the upper 24-well transwell units with 8-µm chamber covered with collagen in serum-free medium. Each well contained approximately 2 × 10^4 cells. Medium without FBS (0%), was placed in the lower chamber. Cells were allowed to pass through collagen for 24 hours and then were fixed in PFA and stained with crystal violet. **A.** Representative images of invading cells after staining with crystal violet (phase contrast microscope) of the different culture conditions are shown. Scale bar= 100 µm. **B.** Histograms show the mean value ± S.E.M. of the number of invading cells respect to control (n=3). Data were compared with the untreated condition or as indicated, *p<0.05 and **p<0.01.

Cell invasion is a complex response that involves many different cellular proteins. Among them, the relevance of MMPs for extracellular matrix degradation during cell migration/invasion has been extensively demonstrated (Bourboulia and Stetler-

Stevenson 2010; Kessenbrock *et al.* 2010). Their expression and activity can be modulated by growth factors and cytokines. In fact, HGF has been reported to increase the expression of several members of the MMP family to mediate tubulogenesis or to increase tumor cell invasiveness (Kadono *et al.* 1998; Monvoisin *et al.* 2002). In particular, our group have shown that HGF increases oval cell invasion through extracellular matrix, process that is at least partly mediated by expression and activation of some MMPs (Suarez-Causado *et al.* 2015).

This prompted us to analyze the expression of various MMPs, including MMP3, 9, 10, 13 and other molecules involved in migration and invasion, CTGF and LAMININ (Pyke *et al.* 1995; Liu and Korc 2012; Xiu *et al.* 2012).

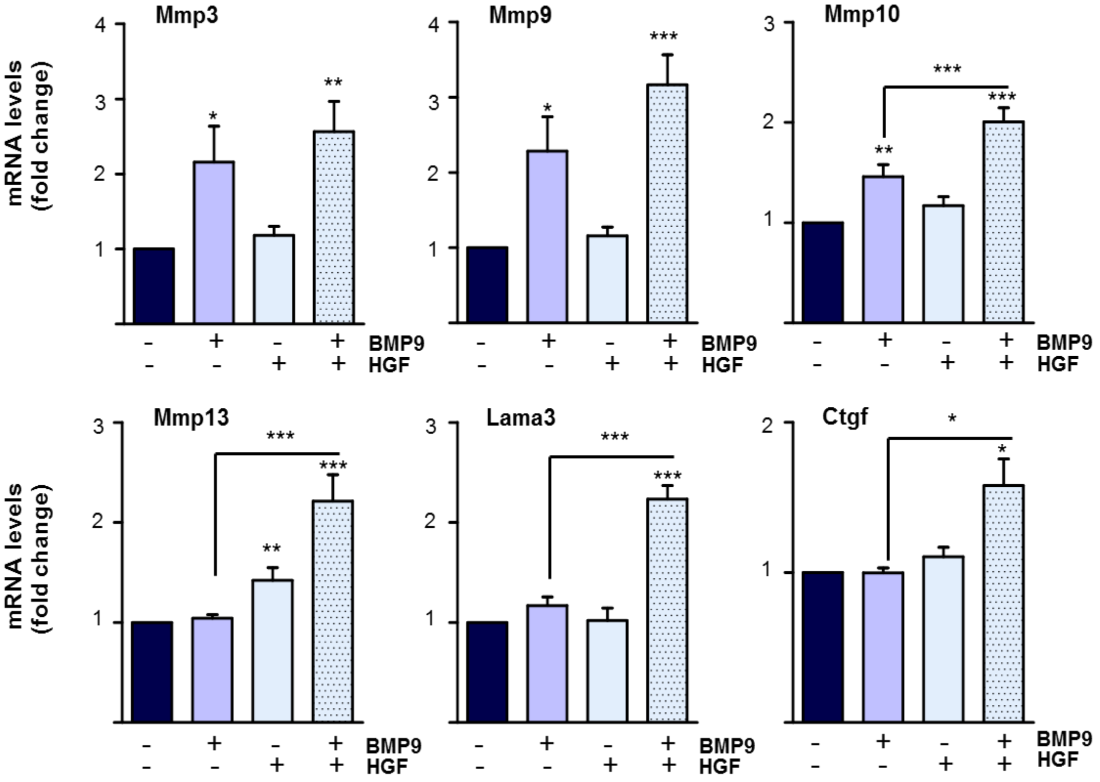


FIGURE 35. Analysis of the expression of different MMPs in response to HGF and/or BMP9 in oval cell

Met$^{flx/flx}$ oval cells were incubated with BMP9 (2 ng/ml) in absence or presence of HGF (40 ng/ml) in 0% FBS media for 1 hour and Mmp3, 9, 10, 13, Ctgf and Lama3 levels were analyzed by qRT-PCR and normalized to Gusb. Data are shown as fold induction (relative to untreated cells) and are from at least 3 independent experiments performed in triplicate (mean ± S.E.M). Data were compared with the untreated condition or as indicated, *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

As shown in **FIGURE 35**, BMP9 and HGF alone were able to induce the expression of some of these genes but combined treatment resulted in maximal induction, particularly Mmp10, 13, Lama3 and Ctgf mRNAs, which showed significant changes with respect to single treatment. These results suggest a role for these genes in the increased invasive response observed in oval cells upon combined treatment.

2.6 Smad1 is required for the migratory response of oval cells to BMP9 alone or with HGF

As it is well known, SMADs proteins are the major mediators of BMPs/TGF-β ligands actions. Previously, we observed that BMP9-triggered SMAD1,5,8 activation is amplified by HGF, in parallel to increased cell invasion. These results opened the question of which is the precise role of SMAD1,5,8 in the oval cell invasive response to BMP9 and HGF. Hence, we next decided to perform transient Smad1 knock-down using a Smad1 siRNA in oval cells and to study its effects on oval cell invasion mediated by BMP9 and/or HGF.

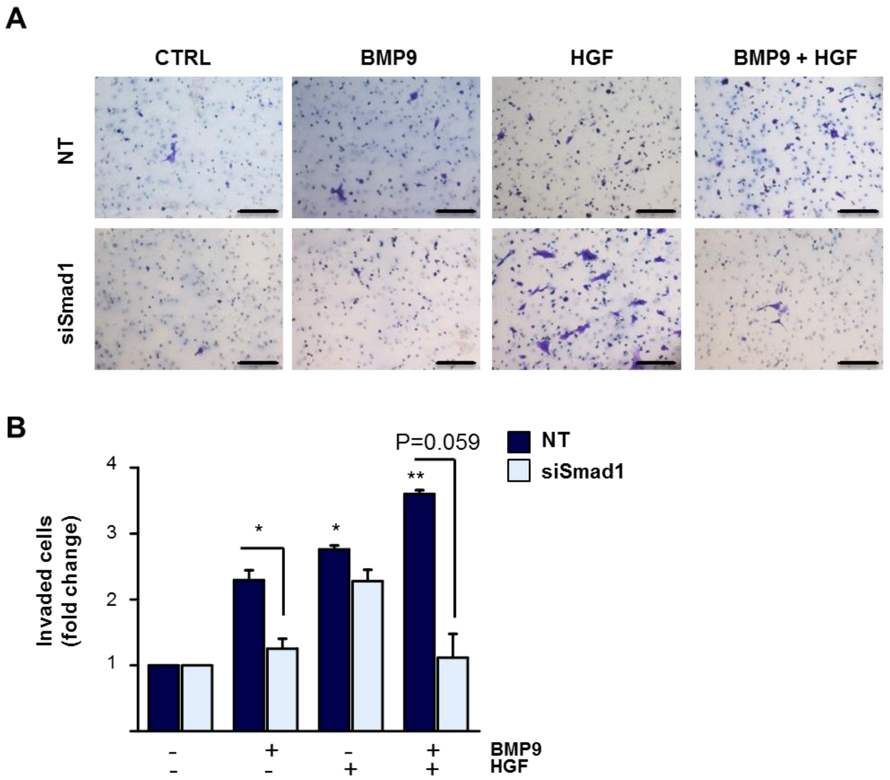


FIGURE 36. Smad1 is required for the migratory response of oval cells to BMP9 alone or with HGF

A-B. NT and siSmad1 oval cells were incubated with BMP9 (5 ng/ml) in the absence or presence of HGF (40 ng/ml) in 0% FBS media and plated in the upper 24-well transwell units coated with collagen I in serum-free medium. Each well contained approximately 2 × 10^4 cells. Medium without FBS (0%), was placed in the lower chamber. Cells were allowed to pass through collagen for 24 hours and then were fixed in PFA and stained with crystal violet. **A.** Representative images of invading cells after staining with crystal violet (phase contrast microscope) of the different culture conditions are shown. Scale bar= 100 µm. **B.** Histograms show the mean value ± S.E.M. of the number of invading cells respect to control. Data from two independent experiments performed in triplicate. Data were compared with the untreated condition or as indicated. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Smad1 silencing blocked BMP9-induced oval cell invasion and also abolished the enhancement in cell invasion induced by combined treatment; however, the pro-invasive effect of HGF alone on oval cell migration was not affected (**FIGURE 36 A and B)**. Thus, Smad1 appears to play an essential role in the pro-invasive response triggered in oval cells by BMP9 alone or in combination of HGF.

3. EFFECT OF BMP9 CHRONIC TREATMENT ON OVAL CELL PHENOTYPE AND FUNCTION

The role of BMP9 in liver fibrosis is currently poorly understood, although there are some indirect evidences that have linked BMP9 with this process (Li and Liu 2007; Bi and Ge 2014; Munoz-Felix *et al.* 2016). Consistent with this, results from our laboratory indicate that BMP9 and Id1 mRNA levels are increased in the CCl_4-induced liver fibrosis experimental model (**FIGURE 37**), that suggest a correlation between BMP9 and liver fibrosis.

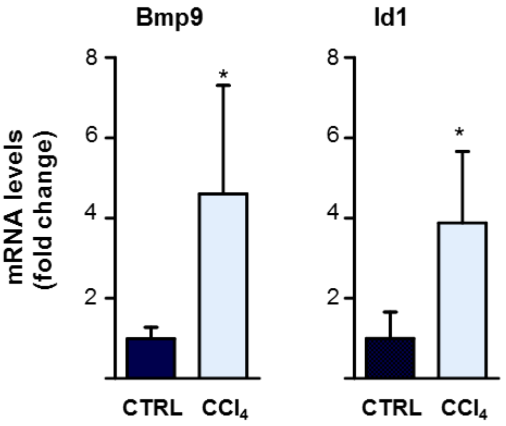


FIGURE 37. Bmp9 and Id1 hepatic expression in the CCl_4-induced chronic liver injury mouse model

Total mRNA was isolated from the livers of WT mice untreated or after 8 weeks of CCl_4 treatment. BMP9 and Id1 mRNA levels were determined by quantitative RT-PCR and normalized to Gusb. Data are expressed relative to untreated samples (assigned an arbitrary value of 1) and are mean ± S.E.M. of at least 4 animals. Data were compared with the untreated condition. *$p<0.05$.

Given that chronic liver disease is also associated with oval cell expansion (see introduction) we wanted to study the effect of a chronic exposure of oval cells to BMP9, as an *in vitro* approach to resemble the *in vivo* liver fibrosis context. In fact, we and others have previously used this approach in the laboratory to evaluate the effects of the chronic exposure to TGF-β in different types of liver cells, such as rat fetal hepatocytes (Valdes *et al.* 2002; Kaimori *et al.* 2007), human HCC cells (Bertran *et al.* 2009; Caja *et al.* 2011) and oval cells (unpublished data). Hence, following a modified version of a protocol established in our laboratory, we generated an oval cell line chronically treated with BMP9 (thereafter referred to as B9T-OCs that stands for BMP9-treated oval cells) and then we characterized them phenotypically and functionally in order to clarify the implications for liver fibrosis.

3.1 Generation of B9T-OCs

Oval cells were first treated for 96 hours (4 days) with a high concentration of BMP9 (5 ng/ml) capable of inducing a maximum apoptotic response in the absence of serum (as described in **FIGURE 26**). After that, remaining cells were cultured in medium supplemented with serum in the presence of BMP9 until they reached confluency (passage 0 or p0), which was considered the starting point for subculture. Then, cells were submitted to subsequent passages. After four passages (approximately 2 weeks after beginning of the generation process) it was already considered a stable and chronically treated line (B9T-OCs). B9T-OC population was maintained and sub-cultured in complete DMEM supplemented with a BMP9 maintenance dose (1 ng/ml) (**FIGURE 38**).

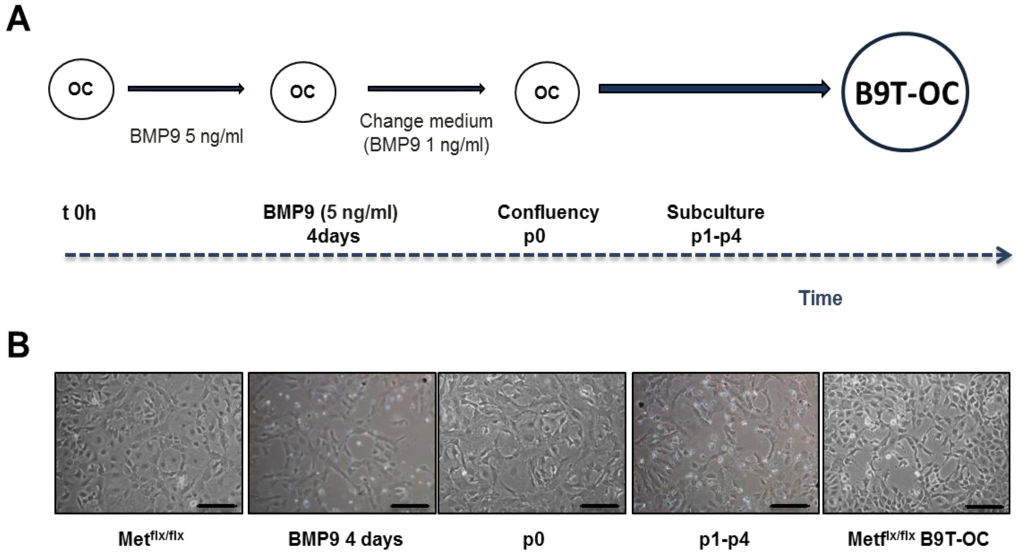


FIGURE 38. Generation of BMP9-treated oval cells (B9T-OC)

A. Scheme of the protocol for the generation of B9T-OCs. **B.** Contrast microscopy images corresponding to Met$^{flx/flx}$ B9T-OCs and the parental line (Met$^{flx/flx}$) during the generation process. Scale bar= 100 µm.

3.2 Phenotypic characterization of B9T-OCs

Once the B9T-OC were established, we aimed to perform a detailed phenotypic characterization of these cells. For that, we isolated mRNA from cells at different passages and analyze the expression of an array of phenotypic markers in comparison with the parental (normal) oval cells. Among the analyzed markers we included EMT markers, such as Snail, E-cadherin and N-cadherin, since previous observations had shown that BMP9 is capable of inducing an EMT process in liver cells (Li *et al.* 2013). We also included hematopoietic stem cell markers expressed by oval cells, Cd34 and Thy-1; and other hepatic lineage and differentiation markers, such as albumin (Alb) and α-fetoprotein (Afp), two plasma proteins secreted by hepatocytes and liver progenitors; hepatic transcription factors such as forkhead box protein A2 (Foxa2 or Hnf-3B), hepatocyte nuclear factor 4 alpha (Hnf4A), hepatocyte nuclear factor-1-beta (Hnf1B), onecut transcription factor (Hnf6), forkhead box protein A1 (Foxa1 or Hnf-3A), tryptophan 2,3-dioxygenase (Tdo), a key enzyme in the physiological regulation of tryptophan metabolism, which is commonly used as a marker of functionally mature hepatocytes; and cytokeratin 18 (Krt18), a cytoskeleton protein characteristic of epithelial cells that is present in hepatocytes and liver progenitors. We found a significant decrease in the mRNA levels of Snail and the mesenchymal marker N-cadherin and increase in E-cadherin levels after chronic treatment with BMP9. In addition to this, the expression of Cd34 and Thy1 was profoundly downregulated whereas most of the hepatic lineage markers, except for Foxa1, as well as the epithelial marker Krt18 were significantly induced (**FIGURE 39**).

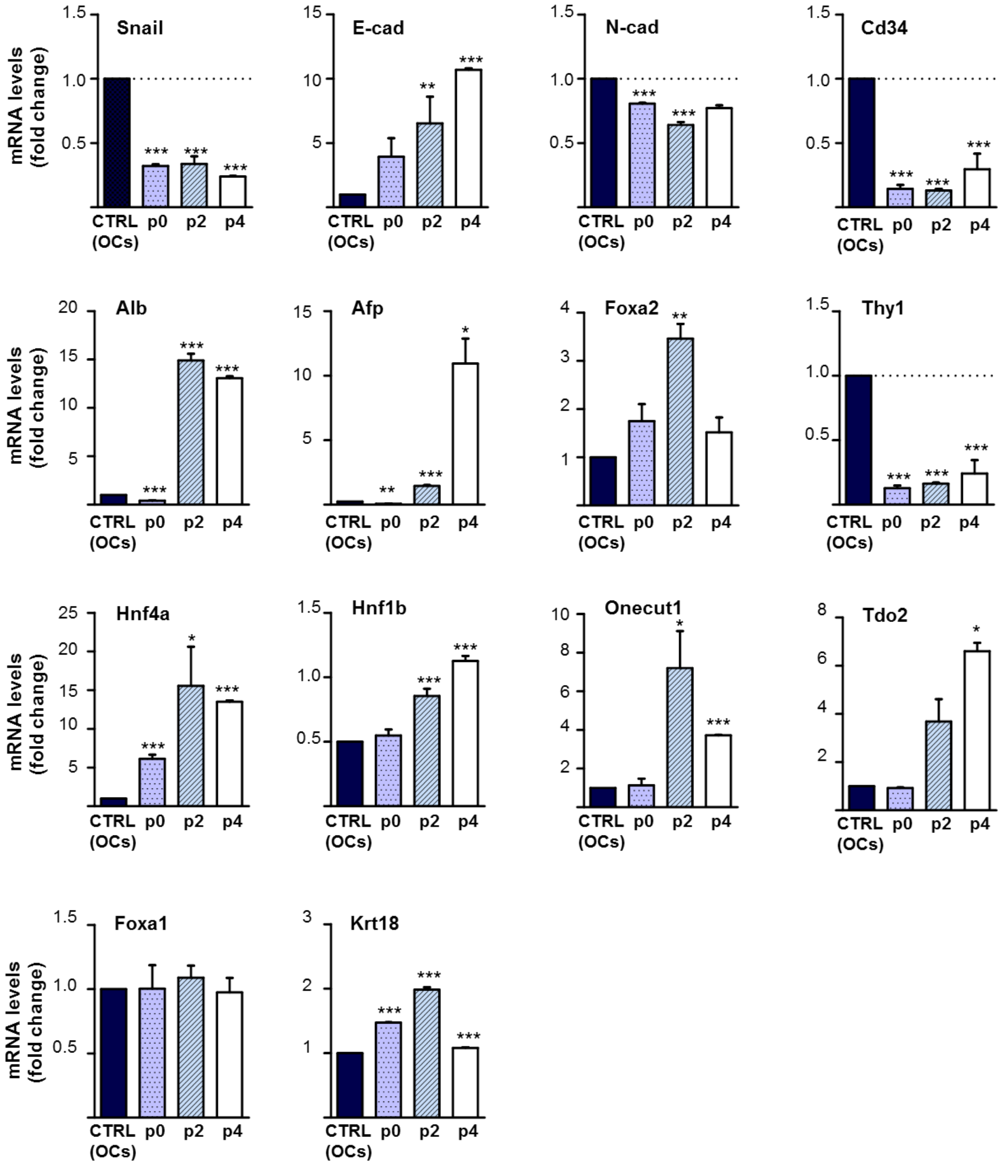


FIGURE 39. Gene expression profile of B9T-OC

RNA from parental oval cells (CTRLs) and from B9T-OC at different passages was obtained (passage 0, 2 and 4) and qRT-PCR was performed to analyse gene expression. Gusb was used for normalization. Data are expressed relative to oval cell (CTRL) (assigned an arbitrary value of 1) and are mean ± S.E.M of 2 independent experiments performed in triplicate. Data were compared with the untreated condition. *p<0.05, **p<0.01 and ***p<0.001.

The up-regulation of albumin and E-cadherin expression in B9T-OCs was also confirmed at the protein levels by western blot analysis (**FIGURE 40**).

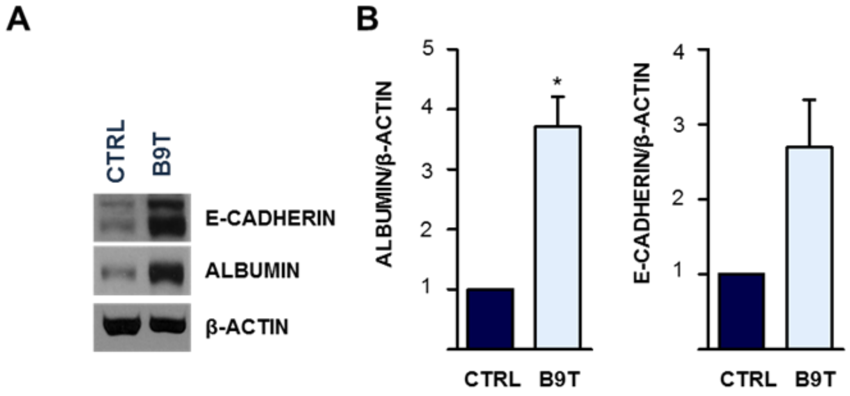


FIGURE 40. ALBUMIN and E-CADHERIN expression are increased in B9T-OCs

Parental oval cells (CTRL) and B9T-OCs (B9T) were maintained in the absence of serum for 15 hours and then, total proteins were isolated. Western blot analysis was performed using antibodies that recognize E-CADHERIN, ALBUMIN and β-ACTIN analyzed as loading control. **A.** A representative experiment is shown. **B.** Optical density values relative to loading control were calculated. Data are expressed relative to CTRL samples (assigned an arbitrary value of 1) and are mean ± S.E.M.of 3 independent experiments. Data were compared with the untreated condition. $^*p<0.05$.

Altogether, these results discarded the acquisition of a mesenchymal and a stem-like phenotype in chronically BMP9-treated oval cells and suggest oval cells suffer a switch towards a more epithelial and mature hepatic phenotype.

As a functional test for oval cell differentiation toward hepatocyte lineage in B9T-OCs we evaluated urea production, since it is one of the most commonly used test to measure liver specific functions in cells *in vitro*.

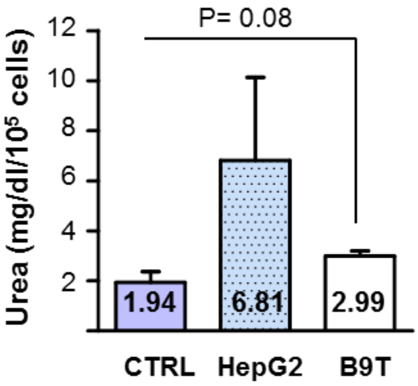


FIGURE 41. Production of urea in parental oval cells and B9T-OCs

Urea levels in parental oval cells (CTRL) and B9T-OCs supernatants were measured after an overnight culture with a minimal amount of medium. HepG2 cells were used as positive control. The concentration of urea was expressed as mg/dl/10^5cells plated. Data represent the mean ± SEM of 3 independent experiments performed in duplicate. Statistical analysis was carried out using the paired t-test and data were compared to normal oval cells (CTRL).

The level of urea produced by normal oval cells was very low as expected for undifferentiated hepatic progenitor cells. After BMP9 chronic treatment, urea production was significantly increased and reached levels of 3 mg/dl/10^5cells. We included HepG2 cells as a reference of a differentiated hepatic cell line, because it is widely used for liver functionality/cytotoxicity tests. Consistent with their differentiated phenotype, these cells reached the highest levels of urea synthesis *in vitro* under the same culture conditions (**FIGURE 41**).

These data provided additional evidence supporting that oval cells chronically exposed to BMP9 acquire hepatocyte-like specific functions.

3.3 Effect of BMP9 chronic treatment on oval cells growth capacity

In order to deepen our understanding on the effects of chronic treatment with BMP9 in oval cell properties and behavior, we next evaluated the cell growth capacity in the absence or in the presence of serum (**FIGURE 42**).

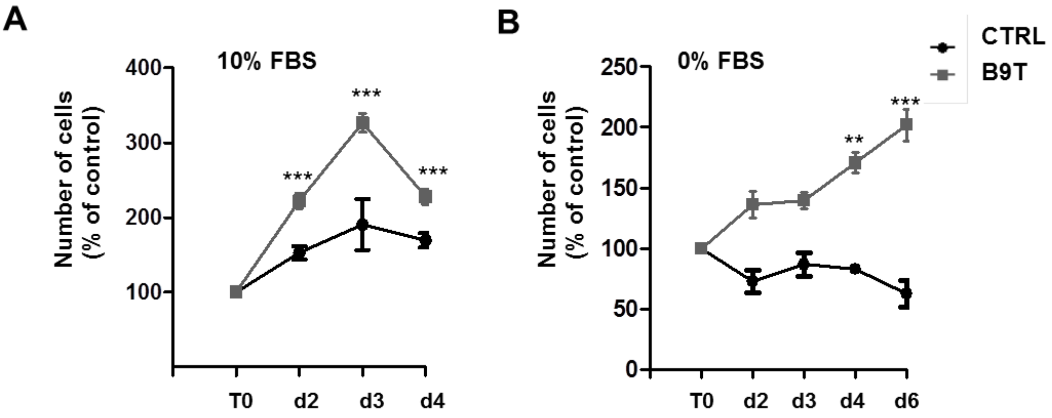


FIGURE 42. B9T-OC growth capacity in the absence and presence of serum

Parental oval cells (CTRL) and B9T-OC (B9T) were cultured in the presence (**A**) or absence (**B**) of 10% FBS for different periods of time and then the number of cells was counted. Data are expressed respect to control (mean ± S.E.M) and are from 2 independent experiments (n= 6-10). Data were compared with the untreated condition or as indicated. $*p<0.05$, $**p<0.01$ and $***p<0.001$.

The growth curve in the presence of 10% FBS highlights that B9T-OCs have acquired growth advantage over their normal counterparts in response to the mitogenic signals present in the serum. Even more interesting is the profile obtained in the absence of serum. In fact, while parental oval cells display a decrease in cell number, which is a consequence of the previously shown apoptotic response elicited by serum withdrawal (del Castillo *et al.* 2008), B9T-OC show a progressive increase in cell number along time in culture that could be associated to an intrinsic greater capacity to proliferate and perhaps also acquisition of apoptosis resistance. This process remains to be further analyzed.

In addition to the standard growth curves, we performed clonogenic assays to test the clonal growth capacity of B9T-OC in comparison to normal parental oval cells (**FIGURE 43**).

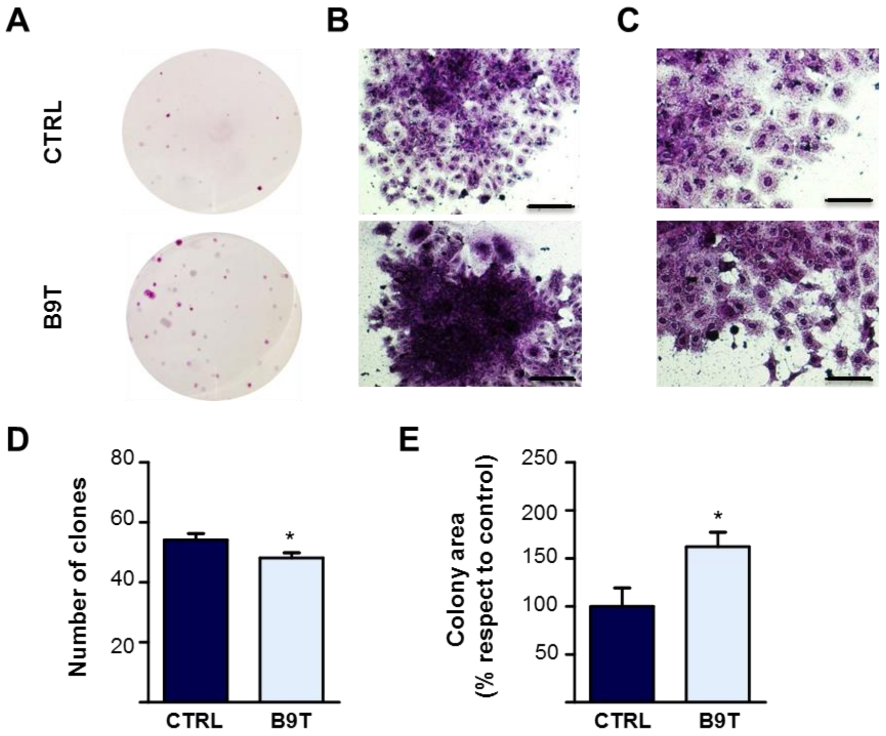


FIGURE 43. Clonogenic growth of B9T-OC

Clonogenic assays of parental oval cells (CTRL) and B9T-OCs (B9T). **A.** Representative images of clones from a plate and representative phase contrast microscope images of **B.** clones from a plate and **C.** individual foci. Scale bar= 100 µm (second column) and scale bar= 50 µm (third column). **D.** The number of clones. Data are expressed respect to CTRL (mean ± S.E.M) and are from 1 experiment (n= 6). **E**. Area of clones. The areas of 10 different clones were measured. Data were compared with the CTRL (normal OCs). *p<0.05.

As shown in **FIGURE 43 D**, chronic treatment with BMP9 results in a slight decrease in clonogenic growth. In addition to colony number counting, we measured the diameter of the colonies using Image J software. This type of analysis demonstrated that the size of B9T-OC-formed colonies was bigger that the colonies formed by parental oval cells (**FIGURE 43 E**). Further analysis is necessary to determine whether differences in size are due to differences in clonal cell growth or it is the consequence of alterations in cell morphology.

3.4 BMP9 chronic treatment promotes cell migration and invasion

As our data indicated that BMP9 regulates oval cell invasion in acute treatment (**FIGURE 27**), we wanted to investigate whether BMP9 chronic treatment could have any effect on oval cell migratory and invasive capacity. To examine cell migration, wound-healing assays were carried out in parental oval cells and B9T-OC. These assays showed that oval cells have an intrinsic capacity to migrate towards the wound (as previously reported by (Suarez-Causado *et al.* 2015) but this capacity was increased in B9T-OC, as evidenced by a faster wound closure (**FIGURE 44**).

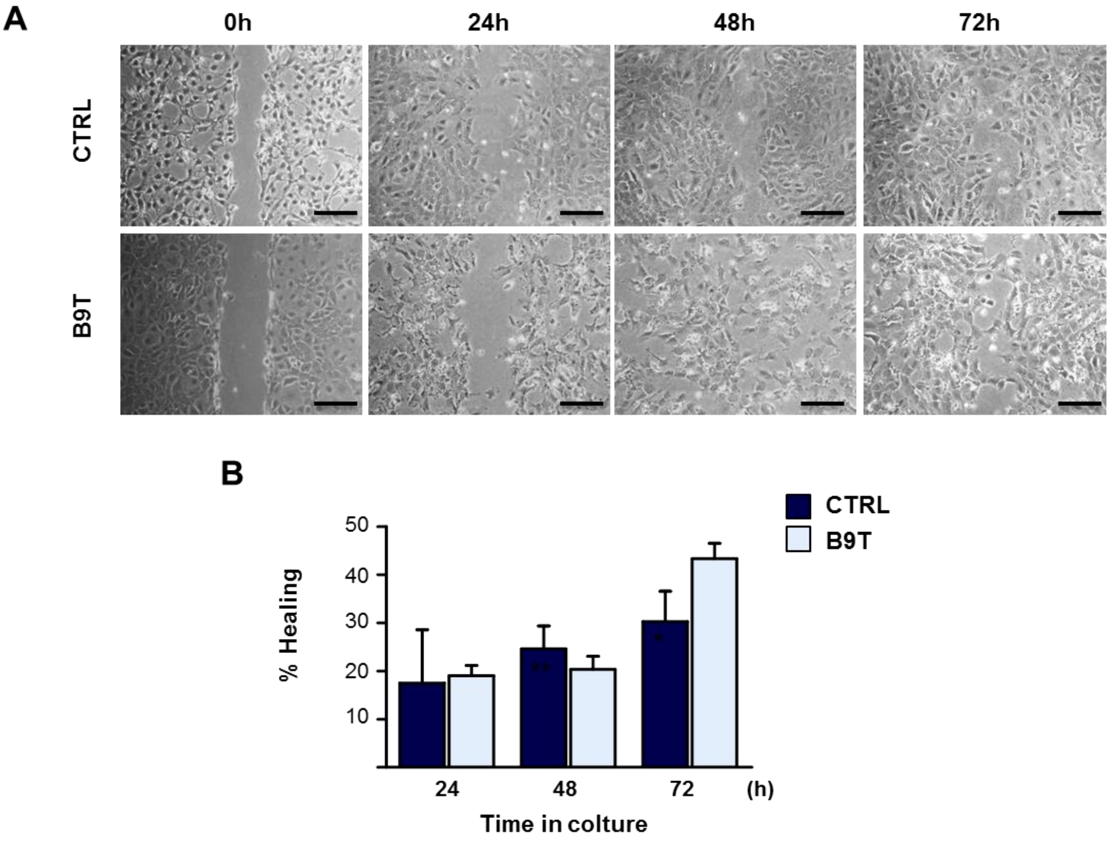


FIGURE 44. BMP9 chronic treatment increases oval cell migration

Wound healing assay. A straight scratch was performed and cells were allowed to migrate in the presence of serum free medium. **A.** Representative phase contrast microscopy images at 0, 24, 48 and 72 hours of migration. Scale bar= 50 µm. **B.** Histograms show the quantitative analysis of wound closure after 24, 48 and 72 hours. Data are expressed as % of closure and are the mean ± S.E.M. of 2 independent experiments performed in duplicate.

However, considering that these cells have the ability to grow in the absence of serum (see **FIGURE 42 B**) and that we could not use mitomycin in the assay due to its toxic effects in oval cells, we cannot exclude that this phenomenon may be due at least partially to cell proliferation. Other approaches should be performed to further analyze B9T-OC migratory capacity.

We next examined the invasiveness capacity of B9T-OCs. In agreement with migration data, we found a significant increase in the number of cells that invade through collagen in the BMP9T-OC as compared with parental oval cells (**FIGURE 45**).

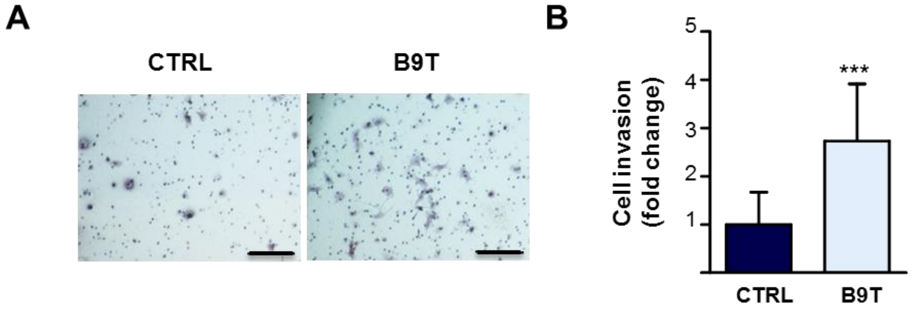


FIGURE 45. BMP9 chronic treatment increases oval cell invasion

Parental oval cells (CTRL) and B9T-OCs were plated in serum-free medium in the upper 24-well transwell units coated with collagen. Each well contained approximately 2×10^4 cells. Medium without FBS (0%) was placed in the lower chamber. Cells were allowed to pass through collagen-coated filters for 24 hours and then were fixed in PFA and stained with crystal violet. **A.** Representative images of invading cells after staining with crystal violet (phase contrast microscope). Scale bar= 100 µm. **B.** Histograms show the mean value ± S.D of the number of invading cells respect to control (n=3). Data were compared with the parental oval cells, ****p*<0.001.

These results demonstrate that BMP9 chronic treatment promote cell migration and invasion in oval cells through a mechanism that remains to be characterized.

3.5 B9T-OCs ARE MORE RESISTENT TO SUPPRESSOR FACTORS (TGF-β AND BMP9)

Results in **FIGURE 42 B** have shown that B9T-OCs do not die when cultured in the absence of serum but rather grow along time in culture. These results prompted us to perform additional studies to analyze cell behavior in the presence of different cytostatic and/or pro-apoptotic stimuli. As previous work from our group have already shown that TGF-β induces an apoptotic cell death in oval cells (Del Castillo *et al.* 2006), we studied the effect of TGF-β treatment on B9T-OCs population, in terms of cell number and apoptotic index.

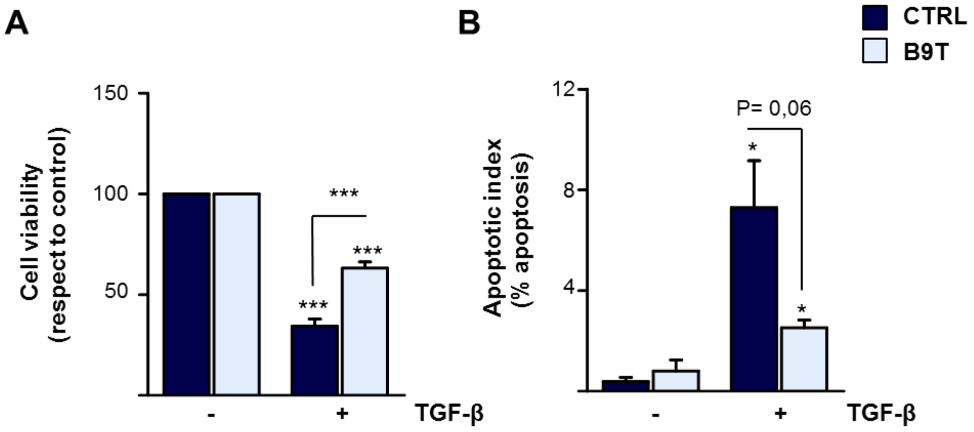


FIGURE 46. B9T-OCs are more resistant to TGF-β-induced apoptosis than parental oval cells

Parental oval cells (CTRL) and B9T-OCs were serum-starved and incubated in the presence of TGF-β (2 ng/ml) for 48 hours. **A.** Cells were counted. Data from 2 independent experiments (n= 4-6) are shown and expressed as percentage of untreated cells (mean ± S.E.M). **B.** Apoptotic index was calculated by counting apoptotic nuclei after PI staining under a fluorescence microscope. A minimum of 1000 nuclei was counted per condition. Data from 2 independent experiments performed in triplicate (mean ± S.E.M.) are shown. Data were compared with the normal oval cellss, **p*<0.05, ***p*<0.01 and ****p*<0.001.

As shown in **FIGURE 46**, chronic treatment with BMP9 significantly decreased both the percentage of cell loss and the apoptotic index upon TGF-β treatment compared to parental oval cells, clearly evidencing that chronic exposure to BMP9 make oval cells more resistant to the cytostatic and pro-apoptotic effects of TGF-β.

To clarify if the higher resistance to TGF-β actions of B9T-OCs could be related to alterations in TGF-β-induced signaling, B9T-OC and normal oval cells were incubated in the presence of TGF-β and western blot was performed using an anti-phospho-SMAD2 (Ser645/467) antibody.

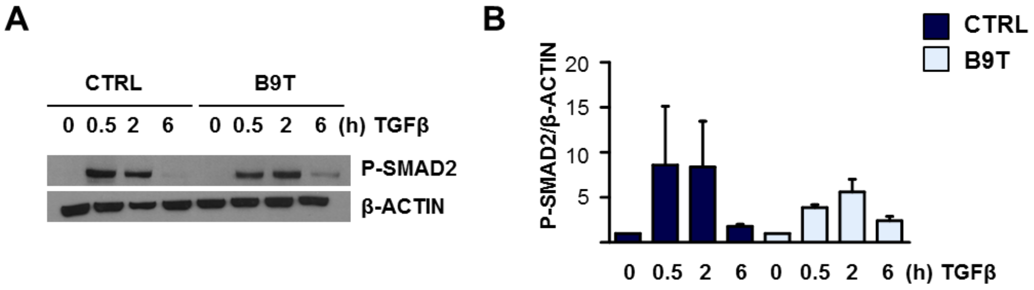


FIGURE 47. No differences in TGF-β-triggered SMAD2 phosphorylation in B9T-OC and normal oval cells

Parental oval cells (CTRL) and B9T-OCs were serum starved for 16 hours. Then, cells were treated with TGF-β (2 ng/ml) for 0.5, 2 and 6 hours. Western blot analysis was performed using antibodies that recognize P-SMAD2 and β-ACTIN was analyzed as loading control. **A.** A representative experiment is shown. **B.** Optical density values relative to loading control were calculated. Data are expressed relative to untreated samples (assigned an arbitrary value of 1) and are mean ± S.E.M.of 3 independent experiments.

As shown in **FIGURE 47**, no significant differences in the levels of phospho-SMAD2 were observed upon TGF-β treatment between the two cell lines. These data discarded an association between a decreased apoptotic response to TGF-β and a decreased activation of the SMAD signaling pathway.

We also tested the cell response to an acute treatment with BMP9, again in terms of cell number and apoptotic index.

Our data show that BMP9 effects on oval cell viability and apoptosis are practically abolished in B9T-OCs (**FIGURE 48**), demonstrating that these cells present a higher capacity to resist to the BMP9-suppressor effects.

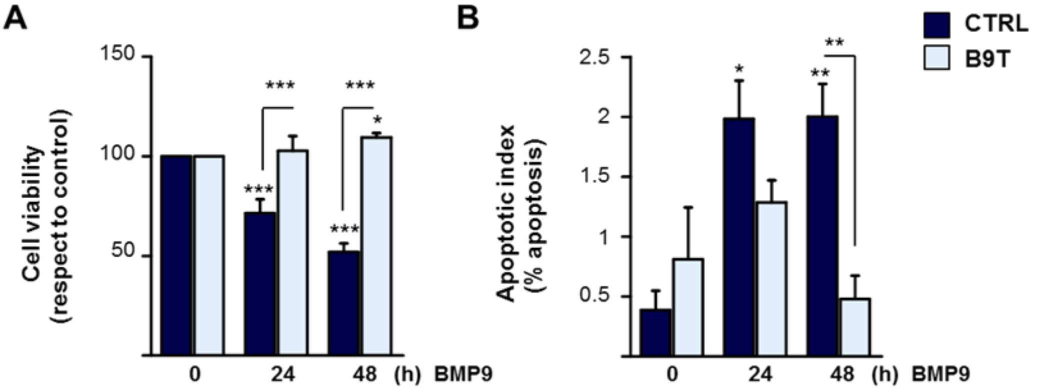


FIGURE 48. B9T-OCs are more resistant to suppressor actions of BMP9 than parental oval cells

A. Parental oval cells (CTRL) and B9T-OCs were serum-starved and incubated in the presence of BMP9 (2 ng/ml) for 24 and 48 hours. Cells were counted. Data from 2 independent experiments (n= 4-6) are shown and expressed as percentage of untreated cells (mean ± S.E.M). **B.** Apoptotic index was calculated by counting apoptotic nuclei after PI staining under a fluorescence microscope. A minimum of 1000 nuclei was counted per condition. Data from 2 independent experiments performed in triplicate (mean ± S.E.M.) are shown. Data were compared with the untreated condition or as indicated. **p*<0.05, ***p*<0.01 and ****p*<0.001.

When we analyzed SMAD signaling in B9T-OC after acute treatment with BMP9 no significant differences in the levels of phospho-SMAD1,5,8 were observed (**FIGURE 49**).

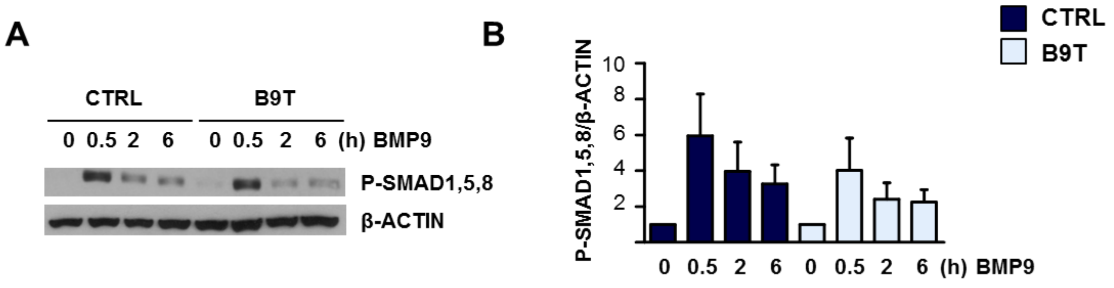


FIGURE 49. No differences on BMP9 triggered-SMAD1,5,8 phosphorylation in B9T-OC and parental oval cells

Parental oval cells (CTRL) and B9T-OCs were serum starved for 16 hours. Then, cells were treated with BMP9 (2 ng/ml) for 0.5, 2 and 6 hours. Western blot analysis was performed using antibodies that recognize P-SMAD1,5,8 and β-ACTIN was analyzed as loading control. **A.** A representative experiment is shown. **B.** Optical density values relative to loading control were calculated. Data are expressed relative to untreated samples (assigned an arbitrary value of 1) and are mean ± S.E.M.of 3 independent experiments.

Nevertheless, we cannot rule out that the altered cell response to both TGF-β and BMP9 was correlated with changes in activation of other non-SMAD pathways. Future experiments will clarify this issue.

In summary, our data evidence that oval cell chronically exposed to BMP9 have a more differentiated hepatocyte-like phenotype, higher proliferative, migratory and invasive capacities and are more resistant to pro-apoptotic stimuli, such as serum withdrawal and treatment with BMP9 or TGF-β. Further experiments are required to better elucidate the importance of these changes in oval cells properties in an *in vivo* physiopathological context.

DISCUSSION

1. BMP9 REGULATES OVAL CELL EXPANSION *IN VIVO* AND *IN VITRO*.

The purpose of this study was to elucidate the role of BMP9 during liver regeneration mediated by oval cells in the DDC-induced cholestatic liver disease model and to examine the effects of BMP9 on oval cell biology.

Understanding the signaling and molecular regulation of liver regeneration is of high interest because it gives us the basis to improve or modulate the liver regenerative capacity, thus opening new avenues in therapeutic intervention in CLDs aside from orthotopic liver transplantation, which could potentially help to decrease mortality. In the last ten years pieces of evidence have accumulated linking BMP signaling to the regenerative process in the liver. For instance, both BMP2 and BMP4 are downregulated following PH in rats and mice, respectively, which suggests that reduced levels of these factors may be necessary for hepatocyte proliferation. In fact, maintenance of BMP4 expression after PH inhibits hepatocyte proliferation (Xu *et al.* 2006; Do *et al.* 2012). Contrariwise, BMP7 has been reported to function as a hormone that facilitate liver regeneration enhancing hepatocyte proliferation (Sugimoto *et al.* 2007). Less data are available about the role of BMPs in oval cell-mediated liver regeneration. Nakasutka et al have reported a transient expression of BMP2 in oval like-cells after acute treatment with CCl_4 (Nakatsuka *et al.* 2007), although it should be noted that this is not a good model for oval cell-mediated regeneration. On the other hand, BMP4 alone is able to induce differentiation of WB-F344 rat liver epithelial stem like cells into hepatocyte-like cells (Wang *et al.* 2015). More interestingly, BMP7 seems to be a key molecule in the butylidenephthalide-promoted reduction of liver fibrosis induced by thioacetamide (Chuang *et al.* 2016). This BMP7 anti-fibrotic effect is associated with inhibition of TGF-β and EMT and an increase in proliferation of oval cells. Nevertheless, whether or not BMP7 has direct effects on the oval cell population is not clarified.

But, what about BMP9? BMP9 was first isolated in developing mouse liver (Celeste 1994). Nowadays, we know that BMP9 is primarily expressed in the liver in physiological conditions although the hepatic cell population responsible for its production is still a matter of debate (Miller *et al.* 2000; Bidart *et al.* 2012). Importantly, BMP9 expression appears to be modulated in different pathological contexts: (i) BMP9 is differentially expressed in liver tissues from HCC patients (Herrera *et al.* 2013; Li *et al.* 2013); (ii) it has been recently speculated that BMP9 could have a regenerative role in the human liver based on the presence of BMP9's receptors on the surface of human hepatocytes and on BMP9 expression in Dissè's spaces, which might serve as an important hepatic stem cell niche (Sosa *et al.* 2011); (iii) finally, we and others have demonstrated that BMP9 is upregulated in experimental models of liver fibrosis (Breitkopf-Heinlein et al., Gut, under revision). These data provide evidence of a link between BMP9 and liver regeneration after various insults, but a potential link with oval cell biology and function in the regenerating liver is absent. Here we found that the expression of BMP9 and its receptors are decreased upon cholestatic injury (**FIGURE 19**) and more importantly, we demonstrate that the absence of BMP9 in the liver results in an amplified oval cell expansion from the periportal regions (**FIGURE 18 A and B**) followed by an improved liver regeneration in DDC-treated mice, further confirmed by the increase in liver-to-body mass ratio along DDC treatment (**FIGURE 18 E**). Histologically, the most striking difference between WT and BMP9-KO livers was that

BMP9-KO livers developed a more extensive network of branching oval cell ducts radiating from the periportal areas toward the parenchyma. This increased oval cell expansion seen in histological sections is demonstrated by the increased expression of oval cells markers (Krt19 and Epcan) (**FIGURE 18 C**). Altogether, these data constitute the first evidence supporting a role for BMP9 as a negative regulator of oval cell expansion and oval cell-mediated liver regeneration.

Supporting the beneficial effects of BMP9 deletion on the regenerative process, we show a strong activation of PI3K/AKT and ERK1/2 signalling after DDC diet in BMP9-KO mice (**FIGURE 20**). Strikingly, we do not see activation of ERKs nor PI3K/AKT in WT mice during the DDC treatment at the time points analyzed. Most likely, it is due to different kinetics since a clear regenerative response is taking place in these mice. A more detailed time course analysis of the activation of these signals will clarify this issue. In any event, our data seem consistent with a major role of these kinases in promoting oval cell expansion during liver regeneration. Indeed, Ishikawa et al. (Ishikawa *et al.* 2012) using the same model of chronic liver injury showed that c-Met mutant livers, which showed impaired oval cell expansion and liver regeneration, were unable to activate AKT and ERK MAPKs signaling pathways. Interestingly, some authors have pointing out differential roles for these kinases in HPCs, such that ERK1/2 activation increases cell self-renewal while AKT together with STAT3 drive HPC differentiation toward hepatocytes (Kitade *et al.* 2013). However, ERK signaling pathway is also critical during differentiation of ESC-derived hepatic progenitors into hepatocytes (Gao *et al.* 2014). Our data show a delayed activation of ERKs as compared to AKT in BMP9-KO mice, which could be consistent with a differential role for them during the oval cell response but this issue remains open for further investigation.

Interestingly, concomitant with ERK and PI3K/AKT overactivation, we also found an up-regulation of Hgf levels and an increase in the levels of active phosphorylated c-Met in DDC-treated BMP9-KO mice (**FIGURE 21**). The overactivation of c-Met in BMP9-KO livers suggests that this signaling pathway plays a role in the amplification of oval cell expansion and oval cell-mediated regeneration observed in the absence of BMP9. A key role of c-Met in stem-cell-mediated liver regeneration was elegantly demonstrated by utilizing mice harboring c-Met floxed alleles and Alb-Cre or Mx1-Cre transgenes. Indeed, the absence of c-Met signaling severely altered stem cell/oval cell micro-environment and impaired stem cell-mediated liver regeneration, finally leading to the death of mice (Ishikawa *et al.* 2012).

To the best of our knowledge, a thorough time-course analysis of phosphorylation of c-Met during the DDC-induced experimental chronic injury model has not been previously performed. Here we show that the activation of c-Met takes place coinciding with the peak of oval cell expansion (around two weeks of treatment), with a similar kinetics to the AKT activation. It is well established that one of the major arms of c-Met signaling is the PI3K/AKT signaling axis, and that this axis is primarily responsible for the cell survival response to c-Met signaling in hepatocytes and in oval cells (Schulze-Bergkamen *et al.* 2004; Moumen *et al.* 2007; Martinez-Palacian *et al.* 2013). Although we and others have also reported AKT-dependent proliferative and migratory/invasive activities in oval cells (Okano *et al.* 2003; Suarez-Causado *et al.* 2015). On the other hand, Factor et al. in a very interesting work demonstrated that c-Met signaling in hepatocytes is essential for sustaining long-term ERK1/2 activation throughout liver regeneration, and alternative pathways may account for the early ERK1/2 activation (Factor *et al.* 2010). Based on all this, it is tempting to speculate that HGF/c-Met signaling is responsible for the strong activation of PI3K/AKT and ERKs. However, the contributions made by other growth factors, such us the EGF/EGFR axis, remains to be investigated, especially in light of the fact that EGF and HGF trigger common but independent signaling pathways in mouse oval cells, through their respective receptors, EGFR and c-Met, and both RTKs have shown to be important to drive oval cell proliferation and survival (Martinez-Palacian *et al.* 2012). Regardless of the identity of the growth factor/s contributing to over-activation of these signals, our data clearly show that deletion of BMP9 signaling results in amplification of the HGF signaling pathways, which suggest for the first time a potential negative crosstalk between these pathways during oval cell expansion *in vivo*.

Our data show that DDC treatment results in an inflammatory response, based on the increase in the expression of inflammatory markers (TNF-α and IL-6) and identification of infiltrating inflammatory cells by histological examination of WT and BMP9-KO livers (**FIGURE 22**). IL-6 and TNF-α upregulation has been already described in this model of chronic cholestatic liver injury. Thus, upregulation of these cytokines together with an increase in STAT3 phosphorylation was observed at 2 and 12 weeks of DDC treatment (Plum *et al.* 2010). Here, we confirm the enhanced expression of inflammatory cytokines although we analyzed a different time point (6 weeks of DDC diet). At this time point, WT animals display an increased expression of TNF-α, but not IL-6, whereas in BMP9-KO mice both cytokines are strongly elevated. Expression of inflammatory cytokines is associated with STAT3 phosphorylation in both WT and BMP9-KO mice but once again it was higher in BMP9-KO mice. Furthermore, using the Knodell Histological Index (Knodell HAI), a system to grade and stage chronic liver damage, we have been able to corroborate that BMP9-KO livers show a greater inflammatory response than the WT. These data provide solid evidence of an enhanced hepatic inflammatory response in DDC-fed BMP9-KO mice.

Although it is clear that inflammation accompanies the oval cell response during chronic liver injury, how exactly these inflammatory cytokines influence oval cell activation, expansion and fate is not well known. Based on the fact that the increased inflammation is concomitant with an ameliorated liver damage, as evidenced by decreased expression of fibrosis markers and decreased serum levels of biochemical markers of liver damage (**FIGURE 24**), our data suggest that the inflammatory response could have a positive role in oval cell-mediated liver regeneration, as it has been previously proposed by other authors (Evarts *et al.* 1992; Knight *et al.* 2005; Xiang *et al.* 2012). Thus, Xiang et al., in the 2-AAF/PH model showed that macrophage-depletion significantly inhibited hepatic expression of IL-6 and TNF-α, along with a lack of STAT3 phosphorylation during the early stages following PH, seriously compromising the oval cell expansion and impairing the process of liver regeneration.

We have not addressed the question of whether, and how, BMP9 could modulate the production of IL-6 and TNF-α and no information is available in the literature in this respect. However, recent analyses of BMP9-dependent responses in endothelial cells implicate BMP9 in the regulation of chemokine signaling pathways. In particular, BMP9 stimulation is able to repress the inflammatory chemokine axis CCL2/CCR2, effect that is mediated by both endoglin and ALK1 (Young *et al.* 2015). The CCL2/CCR2 axis plays a critical role in hepatic recruitment of monocytes during injury (Marra and Tacke 2014). Additionally, combination of microarray and system biology approaches has recently allowed to show that Ccl2, a key gene in the chemokine branch of the NF-kB signaling pathway, is up-regulated during rat liver regeneration and ultimately results in enhanced oval cell proliferation by promoting the expression of cell proliferation stimulatory gene CCND1, and restraining the expression of cell proliferation inhibitory gene DUSP1 (Zhao *et al.* 2016). All these data stimulate further research to clarify the role of BMP9 in regulation of inflammatory chemokines and cytokines during liver regeneration and how this might be determinant for the regeneration outcome.

In addition to changes in the inflammatory response, BMP9-KO mice also show evidence of alterations in the degree of fibrosis based on the decreased expression of fibrogenic markers (**FIGURE 23**), which suggests a pro-fibrogenic role for BMP9 in the liver. Evidences in the literature supporting a connection between BMP9 and fibrosis are scarce and indirect. Recently, it has been published that BMP9 induces an increase in collagen, fibronectin and CTGF expression in cultured mice fibroblasts (Munoz-Felix *et al.* 2016). Moreover, BMP9-KO mice present a defect in matrix deposition (Levet *et al.* 2015), which is a key process in fibrogenesis, serving therefore as an indirect evidence of a role for BMP9 in this process. Although additional approaches are being carrying out to fully demonstrate the pro-fibrogenic role of BMP9 in the DDC model, results presented in this thesis are consistent with a work done in collaboration between our lab and others revealing a direct connection between BMP9 and liver fibrosis. BMP9 is upregulated in experimental models of liver fibrosis (we also show here increase of BMP9 after CCl_4 treatment) but more importantly, loss of BMP9 signaling (BMP9-KO and ALK1-Fc mice) results in a significant decrease in the fibrotic process, as evidenced by lower Col1a1 expression and decreased collagen fibers in the tissue, and lower SMA protein levels in the liver (Breitkopf-Heinlein et al., Gut, under revision). Our results also bring up an important issue of discussion in the liver regeneration field nowadays, which refers to the exact role played by oval cells during the regenerative response. It is out of the question that the ductular reaction constitutes the basic regenerative response to chronic liver injury and that liver fibrosis occurs when repairs become deregulated. The pro-regenerative potential of oval cells has been demonstrated (Wang *et al.* 2003; Strick-Marchand *et al.* 2004; Papp *et al.* 2014). However, a direct correlation between fibrosis stage and oval cell number was also observed (Roskams *et al.* 2003). At that time, it was not determined whether it was a cause or consequence relationship, but recently, a number of studies have suggested that oval cells actually drive the fibrotic reaction (Jakubowski *et al.* 2005). In our experimental model, we observe that an amplified ductular reaction (in BMP9-KO mice) correlates with reduced expression of fibrogenic markers and reduced levels of liver damage parameters in serum, further supporting a beneficial, rather than detrimental, role of oval cell expansion in the liver regeneration process. Question remains unanswered, but it is plausible to think that the specific microenvironment surrounding oval cells in different liver injuries might determine cell fate and the outcome of the regenerative response.

Based on all these results, it is clear that BMP9 has a regulatory role on oval cell biology *in vivo*. Using the DDC liver injury model we have shown that BMP9 is a major determinant of adult hepatic stem cell biology. Lack of BMP9 promotes the expansion of oval cells *in vivo*, probably supported by increased inflammation, and reduces liver fibrosis and the overall degree of liver damage. Altogether, data support a more efficient regenerative response in BMP9-KO mice during cholestatic liver injury, highlighting a direct relationship between stronger oval cell expansion and a better regeneration outcome in terms of recovering the hepatic function (**FIGURE 50**).

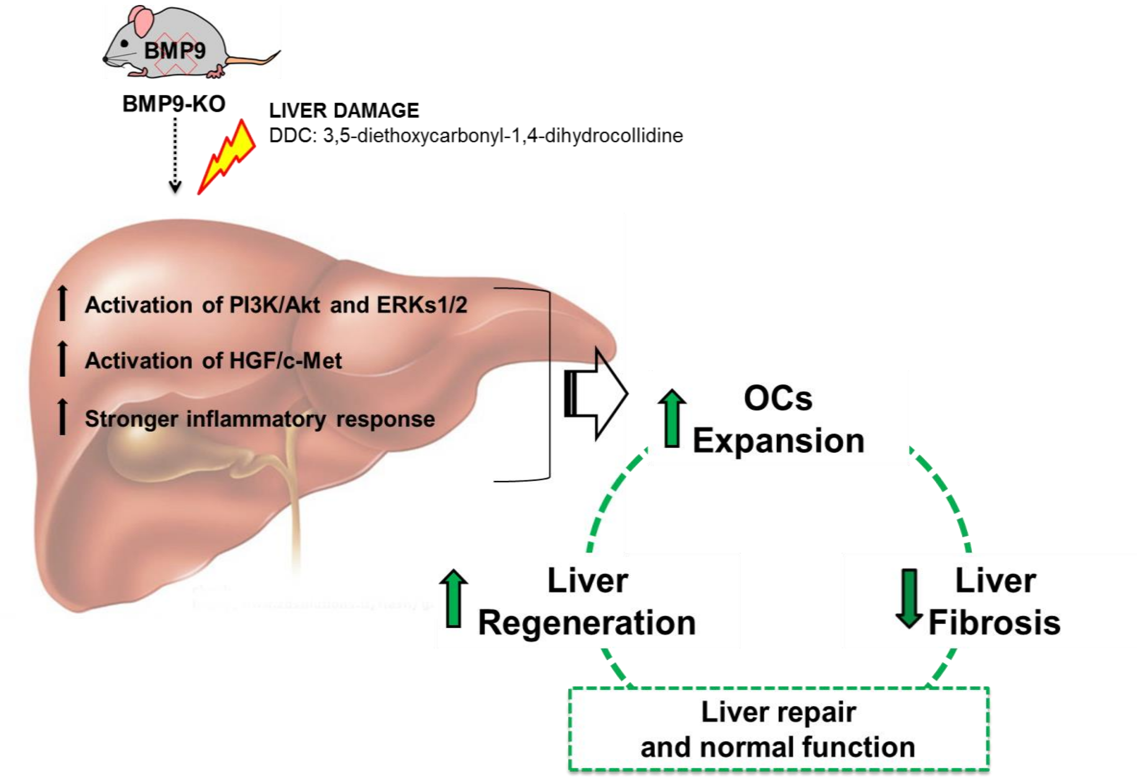


FIGURE 50. Role of BMP9 on oval cell-mediated liver regeneration

The increased expansion of oval cells observed in DDC-fed BMP9-KO mice is accompanied by a stronger inflammatory response but attenuated liver fibrosis, suggesting a more efficient liver regeneration.

Further studies will be required to elucidate the dynamic interaction of oval cells with the liver microenvironment in this regenerative context. We believe this could be critical not only in this particular pathophysiological context but others as well and would open the door to future therapeutic approaches based on inhibiting BMP9 pathway in order to strengthen the regenerative response in different acute and chronic liver diseases.

The *in vivo* observations evidencing a negative effect of BMP9 on oval cell expansion have been complemented by *in vitro* studies that allowed us to confirm such effect while deepening in its overall regulatory role on these cells and the underlying molecular mechanisms. By using oval cell lines we have shown that oval cells indeed respond to BMP9, and in agreement with *in vivo* observations, BMP9 exerts a suppressor action on these cells.

We have shown for the first time that oval cells respond to BMP9 treatment with an activation of SMAD1,5,8 at different doses and time points (**FIGURE 25 A**). SMAD activation is followed by a rapid and robust up-regulation of the SMAD-dependent gene Id1 (**FIGURE 25 C**). Since BMP9 is capable of inducing both SMAD1,5,8 and SMAD2,3 phosphorylation in other cell types (Sieber *et al.* 2009; van Caam *et al.* 2015) we checked the activation of this pathway, but no response was observed (data not shown), therefore discarding the implication of SMAD2/3 in mediating BMP9-triggered responses in oval cells.

The suppressor effects of BMP9 in oval cell were demonstrated by a decrease in oval cell number and an increase in apoptosis (**FIGURE 26**). The effects of BMP9 are cell type specific, as both pro- and anti-proliferative effects have been described. Thus, BMP9 inhibits proliferation in lymphatic endothelial cells (LEC) (Yoshimatsu *et al.* 2013) and osteosarcoma cells (Li *et al.* 2012) while enhances proliferation in multiple types of ovarian cancer cells and endothelial cells (Herrera *et al.* 2009; Suzuki *et al.* 2010). As to the hepatic cells, we have previously described that hepatocytes and HCC cells respond to BMP9 with a decrease and an increase in cell proliferation, respectively (Herrera *et al.* 2013) further highlighting interesting opposite actions in normal and transformed cells.

The apoptotic effect of BMP9 appears to be in dispute as well. Recently, Long et al. have proposed BMP9 as the preferred ligand for preventing apoptosis and enhancing monolayer integrity in both pulmonary arterial endothelial cells (PAECs) and blood outgrowth endothelial cells from subjects with pulmonary artery hypertension via BMPR-II (Long *et al.* 2015). In HepG2 cells BMP9 exerts a strong survival effect against serum deprivation-induced apoptosis (Herrera *et al.* 2013). However, several studies have also reported BMP9-mediated pro-apoptotic effects in different cell types, including prostate cancer cells (Ye *et al.* 2008) and INA-6 and IH-1 cells, two different types of human multiple myeloma cell lines (Olsen *et al.* 2014).

Oval cells now join the group of BMP9 responsive hepatic cell types, showing both growth inhibition and induction of apoptosis. Why BMP9 effects are so heterogeneous is not clear at all. Extensive research is needed to clarify whether is a matter of activation of different receptors and signaling pathways, balance between canonical and non-canonical signaling, crosstalk with other growth factors and/or cytokines, among other potential explanations.

Here, we have been able to demonstrate that ALK2 mediates BMP9-triggered signaling and suppressor effects in oval cells. Although oval cells express the stated BMP9-high affinity receptor ALK1, its levels are much lower than those of ALK2 (**FIGURE 28**). Crosslinking assays confirmed that ALK2 is the main receptor binding BMP9 in these cells but the unequivocal demonstration that ALK2 is mediating BMP9-suppressor effects came by *in vitro* knockdown approaches showing that ALK2 deletion completely abolishes BMP9-induced oval cell loss (**FIGURE 29 D**) while ALK1 deletion has no effect (**FIGURE 33 D**). This is not the only cell system where ALK2 has been shown to mediate BMP9 signaling. In fact, although ALK1 is the main signaling receptor for BMP9 in endothelial cells (David *et al.* 2007; Scharpfenecker *et al.* 2007), ALK2 has been shown to be critical, either alone or together with ALK1, to promote ovarian surface epithelial and ovarian cancer cells proliferation, BMP9-induced osteogenic signaling in mesenchymal stem cells or BMP9-induced growth suppression in myeloma cells (Herrera *et al.* 2009; Luo *et al.* 2010; Olsen *et al.* 2014).

Intriguingly, our data strongly suggest that BMP9 requires binding to ALK2 but not Smad1 signaling to act as a suppressor factor in oval cells, since siRNA-mediated knockdown of Smad1 did not impair the BMP9 negative effect on oval cell number (**FIGURE 33 F**). Data in the literature support the concept that BMPs effects could rely not only on the SMAD canonical pathway, but also on non-canonical signaling pathways. For example, BMP9-induced apoptosis in different cell types can be both SMAD-dependent and independent. In human colon cancer cells, Yuan et al. demonstrated that the anti-proliferative activity of resveratrol is due to the activation of the BMP9/p38 MAPK signaling pathway in a BMPR-dependent manner (Yuan *et al.* 2016). Both canonical and non-canonical BMP signaling are important for dorsal otocyst patterning; specifically, the canonical SMAD pathway regulates Dlx5 expression, whereas a non-canonical pathway via PKA regulates Hmx3 (Ohta *et al.* 2016). In addition, BMP9-triggered p38 and ERK1/2 MAPKs have opposite effects during osteogenic differentiation of mesenchymal progenitor cell (MPCs) (Zhao *et al.* 2012). Finally, our group has previously demonstrated that in HepG2 cells BMP9 induces SMAD signaling but also p38 and AKT/PI3K activation, and only p38 is required for its survival activity (Garcia-Alvaro *et al.* 2015). Future studies will be conducted in the lab to analyze if BMP9 triggers non-canonical signaling pathways in oval cells and if so, its potential relevance for BMP9 suppressor action. Nevertheless, we cannot rule out the possibility that activation of SMAD 5 or 8 could be compensating for the lack of Smad1 signaling and be sufficient to cause oval cell loss upon BMP9 treatment in Smad1-knockdown oval cells. Characterization of single and double knockouts for Smad1 and Smad5 suggest that they share interchangeable roles as transcriptional modulators of BMP target genes (Conidi *et al.* 2011). Nevertheless, differential regulatory roles for Smad 1 and 5 during embryonic hematopoiesis have also been proposed (McReynolds *et al.* 2007). Smad1 and Smad5 double knockdown experiments would be very useful to clarify this issue.

Strikingly, our *in vitro* studies indicate that BMP9 is more than just a suppressor factor in oval cells. Thus, BMP9 also induces invasion in these cells (**FIGURE 27**), and this is in line with recent studies showing that BMP9 can act as a critical modulator of cell invasion and migration, at least in certain cell types. Once again, the scenario is not at all clear. Although some researchers have shown that BMP9 can inhibit the invasion and migration of different cancer cells *in vitro* and *in vivo* (Wang *et al.* 2011; Lv *et al.* 2013; Ren *et al.* 2014; Wang *et al.* 2016), in the liver the expression levels of BMP9 have been positively correlated with invasion through a mechanism associated with EMT induction (Li *et al.* 2013). This might be relevant for different physio-pathological processes such as cell migration occurring during embryonic development or tissue regeneration. We have not yet approached the relevance of each of the BMP9-mediated activities in oval cells (i.e. inhibition of proliferation, induction of apoptosis, promotion of migration and ECM invasion) during oval cell-associated liver regeneration *in vivo*. Particularly interesting is the pro-migratory/pro-invasive activity. Although BMP9-KO mice do not seem to show any impairment in oval cell migration into liver parenchyma, certainly it is a process that merits a deeper analysis prompting us to continue this line of research.

As to the signals mediating the pro-migratory/pro-invasive activity, experiments are now being conducted to confirm whether ALK2 is also the type I receptor triggering this response, although presumably it is what one would expect based on the expression data and the crosslinking results. But, differently from the suppressor activities, BMP9-mediated oval cell migration/invasion requires Smad1 activation (**FIGURE 33 F**), evidencing the activation of specific signaling branches to trigger the multiple BMP9 responses in oval cells.

Certainly, there is much work to do but altogether, our results provide novel evidence supporting a role for BMP9 as a master regulator of liver pathophysiology. In particular, our work emphasizes its implication in liver regenerative responses and its multi-target actions, regulating different hepatic cell populations, among which oval cells would hold an important position.

2. A SIGNALING CROSSTALK BETWEEN BMP9 AND HGF/c-MET REGULATES OVAL CELLS FUNCTION

One of the most interesting findings of this work is the demonstration of a functional crosstalk between the BMP9 and the HGF/c-Met signaling pathways. The same as any other signaling molecule, BMP9 may perform its biological functions by cross-talking with other pathways. In this work, we have studied the crosstalk at the signaling level and also its biological consequences. Using two different and complementary approaches, we have been able to show that c-Met signaling increases the canonical signaling pathway triggered by BMP9 by increasing phosphorylation of SMAD1,5,8 that resulted in an augmented Id1 up-regulation (**FIGURE 31**). Although this is the first evidence of a signaling and functional crosstalk between BMP9 and HGF/c-Met, these results are in line with other works describing crosstalk between HGF/c-Met and other BMPs. For instance, a bidirectional regulation of expression appears to exist between HGF and BMP2, a crucial mediator in bone formation during fracture healing. Thus, in human osteoblasts HGF increases BMP2 production (Tsai *et al.* 2012), while blocking c-Met signaling inhibits BMP2-induced HGF production and enhances BMP2-induced osteoblast differentiation (Shibasaki *et al.* 2015). Furthermore, HGF induces BMPR2 expression in mouse fibroblasts and muscle-derived mesenchymal cells, thus contributing to fracture repair (Imai *et al.* 2005). BMP7 expression is also up-regulated by HGF in prostate cancer cells although the precise mechanism that control this upregulation is not known (Ye *et al.* 2008). Interestingly, using a sophisticated culture system in which primary rat hepatocytes are cultured on printed arrays of collagen alone or with HGF and/or BMP7, Jones et al. have demonstrated that combination of HGF and BMP7 reached the highest anti-apoptotic effect during exposure to ethanol while maintaining the epithelial phenotype (Jones *et al.* 2010). However, authors did not address whether these effects involve direct or indirect crosstalk between these signals or are driven by cooperative but independent pathways.

To investigate the consequences of the BMP9/HGF signaling crosstalk in oval cells, we have studied the potential modulation by HGF/c-Met of BMP9-induced suppressor and migratory effects. On one side, we have found that cell invasion was significantly increased in response to the combined treatment with respect to HGF or BMP9 treatments alone (**FIGURE 34**). The enhanced invasive response induced by BMP9-HGF treatment in oval cells correlates with an increased expression of various MMPs, including MMP3, 9, 10, 13 (**FIGURE 35**), although other proteases might be also involved. Previous results from our group indicate that HGF/c-Met pathway acting through PI3K and p38MAPK induces a complex and specific migratory response in oval cells that involves induction and activation of MMPs (Suarez-Causado *et al.* 2015). BMP signaling also regulates matrix synthesis and degradation having being described collagen10a1, MMP2 and MMP9 as some of its targets, thus affecting cellular differentiation and migration (Bergeron *et al.* 2007; Li *et al.* 2012; van Caam *et al.* 2015). Based on all this, and awaiting for further experiments to directly address the relevance of these molecules in the pro-invasive response elicited by BMP9 alone or in combination with HGF in oval cells, our data suggest that MMPs might contribute to such response.

Co-treatment with BMP9 and HGF also regulates CTGF and Laminin-a3 expression (**FIGURE 35**), other molecules with a known role in cell invasion and migration (Kessenbrock *et al.* 2010). Laminin subunit alpha-3, encoded by the LAMA3 gene, is one of the three subunits of laminin 5, a major component of basement membranes and tumor ECM (Ryan *et al.* 1994). It has been demonstrated that up-regulation of LAMA-3 promotes invasion and metastasis of several cellular types (Tani *et al.* 1997; Kinoshita *et al.* 2012; Liu and Korc 2012); particularly, up-regulation of LAMA3 in HCC patients led to propose it as a molecular target for HCC therapy (Midorikawa *et al.* 2002; Zekri *et al.* 2008). However, laminin-a3 is not only important in carcinogenesis. We have previously described that laminin-a3 is upregulated by HGF in oval cells (Suarez-Causado *et al.* 2015) concomitant with induction of a migratory/invasive response that seems to recapitulate the epithelial morphogenic program induced by HGF during the liver regenerative response involving oval cells. Consistently, the typical regenerating ductules structures formed by oval cells in the parenchyma are always outlined by laminin-positive basement membranes (Dezso *et al.* 2012), so it is tempting to speculate that up-regulation of laminin by HGF and BMP9 could be an important part of the mechanism contributing to the oval cell-mediated regenerative response.

As to the potential relevance of CTGF upregulation in oval cells, it is more difficult to speculate. CTGF is known to be upregulated by BMP9 during osteoblast differentiation of mesenchymal stem cell (Luo *et al.* 2004) but no further data are available regarding regulation by BMP9 in liver cells. On the other hand, HGF-mediated decrease in CTGF expression has been related to HGF inhibitory effects against TGF-β pro-fibrotic activity (Inoue *et al.* 2003; Sherriff-Tadano *et al.* 2006; Bogatkevich *et al.* 2007). However, in co-cultures of proximal tubular epithelial cells (PTEC) and renal tubulo-interstitial fibroblasts (TFB) co-treatment of HGF and TGF-β results in a bi-phasic regulation (early increase followed by later decrease) in CTGF that seems to be important for the coordinated regulation of epithelial-mesenchymal interactions (Inoue *et al.* 2002). CTGF is also a target for HGF in the liver, having an important role on cell migration, invasion and cell cycle progression in human HCC, pointing out once again its association with EMT, specifically the downregulation of E-cadherin and upregulation of FSP-1 (Xiu *et al.* 2012). Neither HGF nor BMP9 induce EMT in oval cells *in vitro* (Suarez-Causado *et al.*, and data not shown), so a link with this process is not obvious in our experimental model. Nevertheless, further investigation will help us to clarify the role played by these molecules in the regulation of oval cell function *in vitro* and *in vivo* and the signaling cascades involved in the regulation of MMPs, CTGF and Laminin by BMP9-HGF signaling crosstalk in oval cells.

The BMP9-HGF/c-Met signaling crosstalk in oval cells is far from simple. While HGF/c-Met signaling amplifies BMP9-triggered SMAD activation and oval cell migration and invasion, which evidences a positive signaling and functional interaction between these two signaling pathways, HGF/c-Met signaling also blocks the inhibitory effects of BMP9 on oval cells, evidencing a negative functional crosstalk. Besides demonstrating the existence of this versatile crosstalk, we show that HGF/c-Met-mediated induction of ALK1 is required for counteracting BMP9 suppressor actions in oval cells and that

Smad1 activation is required for both the HGF/c-Met mediated protective effects against BMP9 and the pro-migratory/invasive activity of BMP9 alone or with HGF cells (**FIGURE 33**). These results reveal a striking inverse correlation between the levels of SMAD1,5,8 activation and the cytostatic and apoptotic response of BMP9 but a direct correlation with the pro-invasive activity. Regarding the role of Smad1 in cell migration/invasion, results are not so surprising. In fact, previous work has revealed that BMPs signaling through SMADs enhances invasion and bone metastasis of breast cancer cells (Katsuno *et al.* 2008). There are also data in the literature in favour of both a pro-survival and a pro-apoptotic function of SMAD1,5 that might depend on the stimuli, dose and cell type among other factors. Sustained activation of SMADs by TGF-β or other BMPs has been implicated in apoptosis induction (Kiyono and Shibuya 2003; Daly *et al.* 2008; Holien *et al.* 2012). According to this, it has been demonstrated that BMP9 induces apoptosis in myeloma cells *in vitro* by signaling through ALK2 and that this effect is SMAD-dependent (Olsen *et al.* 2014). In contrast, Ueki et al. demonstrated that the activation of BMP-Smad1/5/8 promotes survival of retinal ganglion cells after damage *in vivo* (Ueki and Reh 2012), which agrees with data from other groups in different contexts (Xu *et al.* 2015). In our system, SMAD1 activation seems to promote oval cell survival and migration/invasion. It might be also possible that SMAD signaling triggers other biological activities not yet defined and that this functional crosstalk might be even more complex than expected, but the most important question here, still unanswered, is how HGF/c-Met signaling is driving ALK1/SMAD1 activation.

A schematic diagram depicting the BMP9-triggered signalling and response in oval cells is shown (**FIGURE 51**).

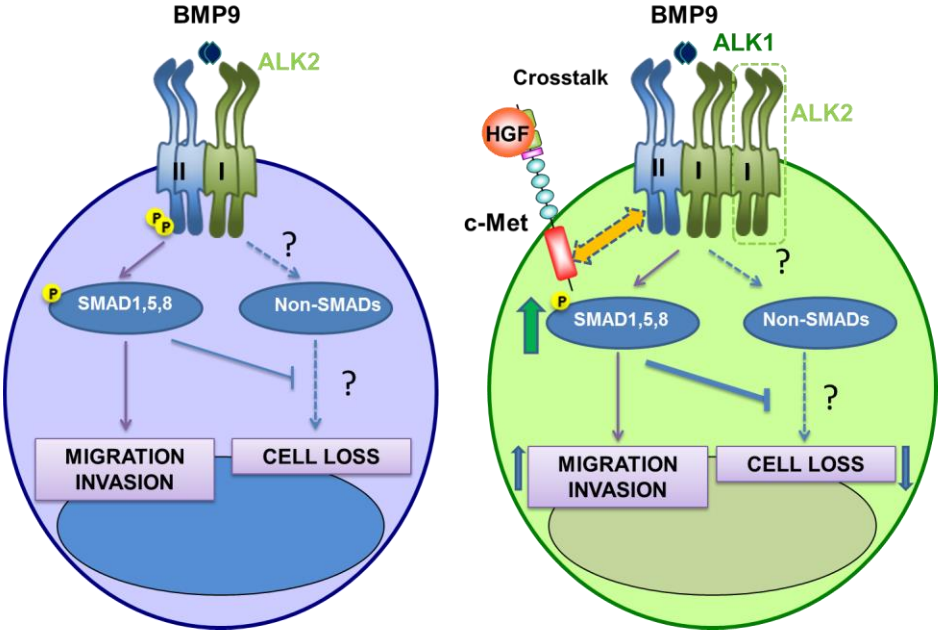


FIGURE 51. BMP9-triggered signalling and response in oval cells

BMP9 raises as a novel regulator of oval cells biology, eliciting both suppressor and pro-migratory/invasive activities. BMP9 requires binding to ALK2 but not SMAD1 signaling to act as a suppressor factor. However, Smad activation drives the pro-migratory/invasive response. HGF/c-Met signaling amplifies BMP9-triggered SMAD activation and oval cell migration and invasion while counteracting BMP9 suppressor actions via ALK1/SMAD1 signaling.

Some of the obvious questions that arise are: is this a c-Met-dependent mechanism? Does it involve physical interaction between c-Met and ALK1 or is it just a matter of switching the balance from ALK2 to ALK1 receptor? Further studies are needed to clarify these and other questions regarding the mechanisms involved in this novel BMP9-HGF/c-Met crosstalk operating in oval cells.

3. CHRONIC TREATMENT OF OVAL CELLs WITH BMP9 PROMOTES DIFFERENTIATION AND ENHANCES SURVIVAL

In this last part of the thesis we have studied the effect of a chronic exposure of oval cells to BMP9, resembling the *in vivo* fibrosis situation.

The critical role of TGF-β in organ fibrosis is well known. However, the possible involvement of BMPs in this process has been suggested recently. Most of the knowledge we have about the role of BMPs during the fibrogenetic process comes from kidney fibrosis studies, which have shown a protective anti-fibrotic role for BMP7 (Weiskirchen *et al.* 2009). BMP7 inhibits or reverses fibrosis in experimental models of chronic kidney disease, mainly by antagonizing TGF-β–induced EMT (Zeisberg and Kalluri 2008; Meng *et al.* 2013; Li *et al.* 2015). Something similar seems to occur in the liver, where overexpression of BMP7 suppresses the development of liver fibrosis in rats, reducing the expression of α-SMA and type I collagen and hydroxyproline content (Kinoshita *et al.* 2007). Using the CCl_4-induced mouse liver fibrosis model, Zeisberg et al. demonstrated that BMP7 significantly inhibits progression of liver fibrosis by inhibiting the accumulation of hepatocyte-derived fibroblasts via EMT (Zeisberg *et al.* 2007). Despite these observations, the anti-fibrotic effect of BMP7 is still controversial and some studies even propose that BMP7 is a promoter of fibrosis in kidney and liver (Tacke *et al.* 2007; Dudas *et al.* 2009). Other BMP ligands have been also involved in organ fibrosis, although their role is again controversial. Both BMP6 and BMP2 have been shown to participate in renal fibrosis (Simone *et al.* 2012; Yano *et al.* 2015). BMP4 pro-fibrotic role has been described in liver during bile duct ligation-induced liver fibrosis in rats (Fan *et al.* 2006). On the other hand, BMP2 has the potential to attenuate TGF-β1-induced renal interstitial fibrosis and pressure overload-induced cardiac fibrosis (Yang *et al.* 2011; Wang *et al.* 2012) and BMP6 inhibits hepatic fibrosis in non-alcoholic fatty liver disease (Arndt *et al.* 2015).

We have already mentioned in the first part of the discussion that an association between BMP9 and liver fibrosis has been proposed, although to date, direct evidences supporting this association are missing. Probably, the most stimulating data in this respect are those published by Li *et al*. proposing that BMP9 via ALK1/2 and Smad1 activation triggers EMT in liver cancer cells and acts as a HCC promoter (Li *et al.* 2013). Considering the tight connection between EMT and liver fibrosis and its relevance for HCC development, it urges to speculate on the role of BMP9 as a pro-fibrotic factor during the pre-neoplastic stages of liver cancer. Our *in vivo* observations showing an improvement in fibrosis during DDC-induced liver injury in the absence of BMP9 (**FIGURE 23**) constitutes additional evidence in support of a pro-fibrotic role for BMP9 in the liver. This, together with the increased expression of BMP-9 and Id-1 in the fibrotic livers of mice that received repeated injections of CCl_4 (**FIGURE 37**) and the fact that both BMP9 KO mice and ALK1-Fc mice, a different model of loss of BMP9 signaling, display a significant decrease in the CCl_4-induced fibrotic process (Breitkopf-Heinlein et al., Gut, under revision) strongly suggest that BMP9 is a pro-fibrotic factor in the liver. With the aim of further exploring this hypothesis and analyzing the oval cells as a target of BMP9 in the fibrotic liver, we tried to establish an *in vitro* context somehow mimicking the *in vivo* situation by means of chronic treatment of oval cells with BMP9. We reasoned that a detailed phenotypic and functional characterization of this population (B9T-OCs) would allow us to predict the *in vivo* fate of these cells in the context of chronic liver damage and help us to better understand their potential relationship with liver fibrosis development and progression.

Strikingly, gene expression analysis of a selected set of phenotypic markers revealed that long exposure to BMP9 in oval cells results on one side in a decrease in Snail, the major master regulator and inducer of EMT (Kalluri and Weinberg 2009), together with an increase in E-cadherin and a decrease in N-cadherin, an epithelial and mesenchymal marker, respectively (**FIGURE 39**). These results pointed to an acquisition of a more epithelial, rather than mesenchymal phenotype. On the other side, based on the expression profile of hepatic and stem/progenitor cell markers in oval cells and B9T-OC, BMP9 seems to promote a step-forward in the hepatic differentiation process of mouse oval cells toward hepatocytes.

Hepatocyte-lineage marker genes can be classified into different groups, representing stages in the sequence of molecular events of hepatocyte differentiation. Among the earliest markers are α-fetoprotein (AFP) and hepatocyte nuclear factor-3β (HNF-3β), which are expressed in endodermal cells, the precursor of all hepatocyte-lineage cells. Next group would include fetal hepatocyte markers such as albumin (Tilghman and Belayew 1982; Zaret 2002); followed by perinatal hepatocyte markers such as glucose-6-phosphatase (G6Pase) and tyrosine aminotransferase (TAT) (Haber *et al.* 1995). The last group includes postnatal (mature) hepatocyte markers such as cytochrome P450-3a (Cyp3a), phosphoenolpyruvate carboxykinase (Pepck) and tryptophan 2,3-dioxygenase (TDO) (Nagao *et al.* 1986; Noda *et al.* 1994). Our analysis shows that BMP9 induces the expression of markers belonging to all different groups, from early to late stage differentiation markers. Furthermore, we believe that the following observations can be considered as unequivocal evidences of a differentiation process into hepatocytes: (i) the increase in HNF-4α, which is considered to be not only a key member of the complex regulatory network that defines and maintains the hepatocyte phenotype but an absolute requirement for hepatocyte differentiation and epithelial morphology (Spath and Weiss 1998; Li *et al.* 2000); (ii) the up-regulation of Tdo2, which encodes for a well-known adult liver-specific enzyme, whose induction is universally accepted as a proof of acquisition of a mature phenotype; and (iii) the notably increased capacity of B9T-OC to produce urea, a specific function of mature hepatocytes.

Further evidence supporting this idea is the profound down-regulation of cd34 and Thy-1 expression, which are hematopoietic markers typically expressed in stem/progenitor cells and cancer stem cells, also expressed in both fetal and adult liver progenitors (Petersen *et al.* 2003; Masson *et al.* 2006). A recent study in a different model of progenitor cells (mesenchymal stromal cells) has proposed that Thy-1 might work as an obstacle in the pathway of differentiation commitment, since a reduction in its expression enhances the osteogenic and adipogenic differentiation of these cells (Moraes *et al.* 2016). It is worth mentioning that Thy-1 is quite an interesting marker not exempt of controversy. Some authors have described that Thy-1 is expressed in hepatic myofibroblast, cells in tight contact with oval cells, rather than in oval cells themselves (Dezso *et al.* 2007) while others came to the conclusion that Thy-1 positive cells are activated mesenchymal-epithelial cells distinct from resident stellate cells, myofibroblast and oval cells (Yovchev *et al.* 2009). The phenotypic heterogeneity of the oval cell population is well known, as it is known their mixed mesenchymal and epithelial phenotype and their phenotypic plasticity (Yovchev *et al.* 2008; Deng *et al.* 2011). Despite the fact that the exact nature of the Thy-1 positive cells in the oval cell population is still obscure, the most consistent trait is their mesenchymal phenotype. Based on all this, the strong reduction in the expression of Thy-1 by chronic treatment with BMP9 might be reflecting not just the differentiation of oval cells into hepatocytes but also the switch towards a more epithelial phenotype.

The importance of BMP signals during liver specification in embryogenesis has been demonstrated. Indeed, BMP2 and BMP4 have shown to be strongly expressed during hepatic induction in the fetus (Zaret 2002). Furthermore, although no alteration in liver gene induction is observed in BMP4 homozygous mutant embryos, noggin-promoted inhibition of BMP signaling blocks the initial induction of hepatic genes in ventral foregut explants (Rossi *et al.* 2001), which is restored by adding BMP2 or BMP4. Besides this, no published data is available concerning a specific role for BMP9 in hepatocyte differentiation. Therefore, our data in oval cells constitute the first evidence in that direction, demonstrating a role for BMP9 as a critical regulator of oval cell differentiation into hepatocytes. It is important to point out that these data are in agreement with additional data from our lab and others indicating that BMP9 helps maintain hepatocyte phenotype in culture preventing EMT and loss of metabolic activities. Consistent with this effect of BMP9, BMP9 KO hepatocytes show evidences of an altered phenotype, such as decreased expression of albumin, HNF-4a and E-cadherin and increased expression of fibronectin, a mesenchymal marker (Breitkopf-Heinlein et al., Gut, under revision). Based on all these observations, we propose a novel double function for BMP9 as a differentiation factor and promoter/stabilizer of epithelial phenotype in hepatocyte and hepatic progenitors. This opens up new perspectives in the research field of oval cells, in particular, regarding the signaling factors regulating the phenotype and differentiation of these cells, which until now were pretty much restricted to some of the classical well-known liver regulatory signals, namely EGFR ligands and HGF (Kitade *et al.* 2013). How BMP9 triggers these effects is totally unknown, opening new paths of research in this direction as well.

It is somehow surprising that while BMP9 promotes a more mature and epithelial phenotype in oval cells, at the same time it confers clear advantages in cell proliferation, survival, and migration/invasion, which certainly evidence that these cells could have acquired a more aggressive phenotype (**FIGURES from 42 to 45**). These properties could be easily associated with an EMT process, but our data show that this is not the case based on the pattern of expression of Snail, E-cadherin and N-cadherin. With this profile, we find a pro-fibrotic role of BMP9-treated oval cells harder to sustain, which is not incompatible with the pro-fibrotic role proposed for BMP9, but it would suggest that oval cells are not a target population of its pro-fibrotic activity. On the other side, the ability of B9T-OC to form colonies in soft-agar or to form tumors in xenografts assays has not yet been tested, therefore it is not possible to assert whether or not long-term exposure to BMP9 is sufficient to promote malignant transformation in oval cells. Considering that Thy-1 has been proposed as biomarker of several tumors including HCC and cancer stem cells (Yang *et al.* 2008; Yang *et al.* 2008; Sukowati *et al.* 2013), loss of Thy-1 in B9T-OC may point to the opposite direction. Additional studies are required to clarify this issue but we should keep in mind that the acquisition of a more differentiated, proliferative, migratory, invasive and resistant to apoptosis phenotype could be consistent with an augmentation of the regenerative potential of oval cells. These cellular features could certainly improve and/or accelerate the regenerative response facilitating the restoration of liver function upon injury.

Another issue, still unresolved, is the mechanism promoting resistance of B9T-OC to TGF-β-induced apoptosis. The fact that no significant differences in kinetics or intensity of SMAD2,3 phosphorylation were observed in these cells in response to TGF-β (**FIGURE 47**) make us speculate the potential involvement of non-canonical pathways although we cannot completely discard a role for the SMAD signaling pathway in this cellular response. On the other hand, since we have previously found that TGF-β induces a mitochondria-dependent apoptotic cell death in oval cells that is dependent on oxidative stress (Martinez-Palacian *et al.* 2013), it would be very interesting to test if B9T-OC show a greater antioxidant capacity that protects them against the pro-apoptotic effects of TGF-β. These hypotheses will be tested in the next months.

Similarly, loss of suppressor effects of BMP9 in B9T-OCs is apparently not associated to changes in the levels of phospho-SMAD1,5,8 (**FIGURE 49**). This would be in agreement with our results showing that Smad1 is not required for the suppressor effect of BMP9 in oval cells (**FIGURE 33**). The possible involvement of non-SMAD pathways is currently being investigated.

In summary, our work shows for the first time that BMP9 could push oval cells to differentiate into hepatocytes. BMP9-promoted differentiation is associated with important phenotypic and functional changes that may confer an advantage to this population during the liver regeneration process in response to chronic injury (**FIGURE 52**). Although we think that our results do not support a pro-fibrotic role of these cells, this hypothesis still needs to be proved. Future studies will clarify whether BMP9 may or not significantly contribute, in the long term, to the pro-regenerative function of the oval cells.

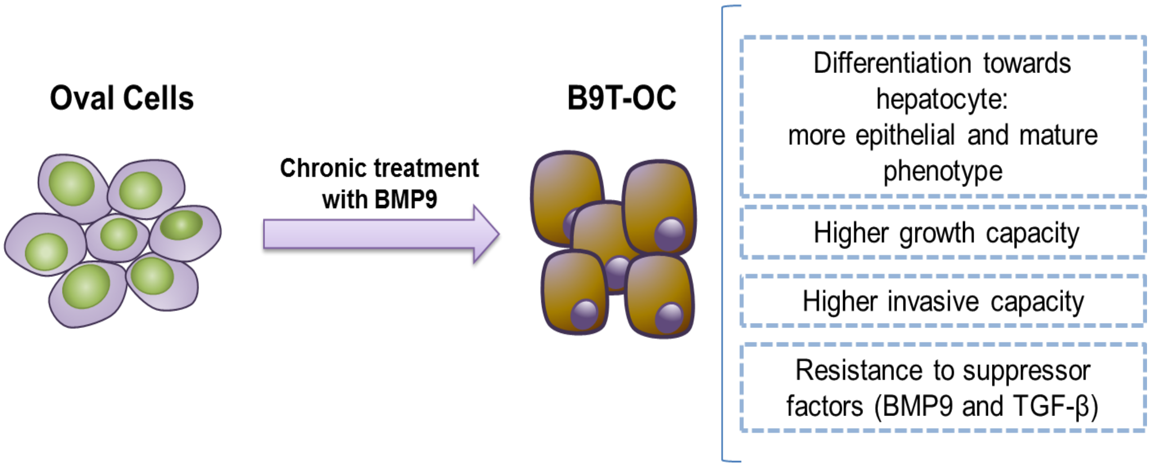


FIGURE 52. Effect of chronic treatment of oval cells with BMP9

GENERAL DISCUSSION

Analysing the behaviour of oval cells in response to regulatory factors is key to expand our knowledge and depth of understanding of some of the most important liver pathophysiological processes, namely liver regeneration and hepatocarcinogenesis. This could be of fundamental importance to fully understand the relevance of these cells in liver and potentially in the future to manipulate them for therapeutic purposes

In the present study we have demonstrated for the first time that BMP9 is an important regulator of oval cell biology. Acute treatment of oval cells with BMP9 is associated with a significant reduction of cell viability and a mild increase in apoptosis but also an increased cell invasive capacity. Our results have evidenced that the suppressor effect of BMP9 in oval cells requires its binding to ALK2, but it does not appear to require the activation of the canonical pathway. However, the apoptotic pathway triggered by BMP9 and its major mediators have not been characterized yet. Although the receptor mediating the pro-invasive activity of BMP9 is awaiting characterization we demonstrate that Smad1 activation is required for such activity highlighting the activation of multiple signaling pathways in oval cells to trigger different responses. The growth inhibitory and apoptotic effects of BMP9 *in vitro* are reflected in a general suppressor effect *in vivo*, where the absence of BMP9 results in a greater expansion of the oval cell population likely contributing to a faster and more efficient recovery from DDC toxicity. Based on these findings we could speculate that the suppressor effect of BMP9 is predominant over the pro-invasive effect, but we certainly need to expand our *in vivo* studies to clarify these and other questions.

Furthermore, we have described a novel functional crosstalk between BMP9 and HGF/c-Met signaling pathway, in which HGF protects against the suppressor effect of BMP9 while potentiating the pro-invasive activity. This crosstalk is far from a complete characterization but we provide clear evidence that it involves overexpression of ALK1 and overactivation of SMAD and we believe that may have significant implications in liver regeneration that deserve further attention.

Interestingly, chronic treatment of oval cells with BMP9 results in a completely different outcome. Its suppressor effect is counteracted by still unknown mechanisms resulting in proliferative and survival advantages while cells move further in their differentiation process toward hepatocyte and acquire a more epithelial phenotype. These observations lead us to propose a role for BMP9 as a differentiation factor and promoter/stabilizer of epithelial phenotype in hepatic progenitors. In spite of the fact that the pro-fibrotic role of BMP9 in liver is becoming clear, we hypothesize that the long-term effects of BMP9 could contribute to increase the regenerative potential of oval cells rather than increase their pro-fibrotic potential, but certainly, the *in vivo* implications of such effects are still unknown. It would be very interesting to address whether there might be some alterations in oval cell differentiation in BMP9-KO mice. Isolation and characterization of BMP9-KO oval cells is on the way and we hope it might also help us to answer some of these questions. The consequences of chronic treatment with BMP9 on the fate of oval cells upon transplantation in the damaged liver, together with additional *in vivo* models where oval cells are locally exposed to BMP9 should also be analyzed.

All these studies could be helpful for a correct interpretation of the oval cells-mediated regenerative process and its subsequent outcome. It is clear that BMP9 could have high potential as a target in the therapy of several CLDs, and further exploration may lead to novel therapeutic approaches for these important diseases.

CONCLUSIONS

CONCLUSIONS

1. The absence of BMP9 results in an increased oval cell expansion *in vivo* in the DDC-induced cholestatic liver disease model, concomitant with an increased activation of AKT and ERK1/2 MAPKs and the HGF/c-MET pathway.

2. The amplified oval cell reaction observed in BMP9 KO mice correlates with an enhanced hepatic inflammatory response, reduced expression of fibrogenic markers, and decreased serum levels of biochemical markers of liver damage, which overall evidence an ameliorated liver damage and a more efficient recovery from DDC toxicity.

3. BMP9 induces the canonical signaling pathway (SMAD1,5,8) in oval cells *in vitro* through ALK2 type I receptor.

4. BMP9 acts as a suppressor factor in oval cells by reducing proliferation and increasing apoptosis, effect that requires binding to ALK2 but not Smad1 activation.

5. BMP9 also increases the invasive capacity of oval cells by a Smad1-dependent mechanism.

6. A functional crosstalk between BMP9 and HGF/c-Met signaling pathway operates in oval cells: HGF/c-Met signaling enhances BMP9-triggered SMAD1,5,8 activation and protects against the suppressor effect of BMP9 while potentiating its pro-invasive activity.

7. BMP9 and HGF/c-Met signalling crosstalk in oval cells involves overexpression of ALK1 and overactivation of SMADs.

8. Chronic treatment of oval cells with BMP9 provides proliferative, survival and invasive advantages while cells move further in their differentiation process toward hepatocyte and acquire a more epithelial phenotype.

CONCLUDING REMARKS

This study constitutes the first evidence supporting a role for BMP9 as an important regulator of oval cell biology. Although in vivo and in vitro approaches demonstrate that BMP9 act as a suppressor signal in oval cells, the response to BMP9 in these cells is far more complex than expected. Together with the inhibitory action we also reveal a pro-invasive activity for BMP9/Smad1 signaling. Furthermore, its effects are modulated by signalling crosstalk with the HGF/c-Met pathway and depend on the time of exposure to BMP9. Chronic treatment with BMP9 confers advantages to the cells that reverse the suppressor response and promote differentiation into hepatocytes. Altogether, our results suggest that BMP9 could significantly contribute to modulate the liver regenerative capacity by regulating oval cells. Full characterization of the mechanisms mediating these responses is needed to prove this hypothesis and may lead to novel therapeutic approaches in chronic liver disease.

REFERENCES

Akhurst, B., V. Matthews, K. Husk, M. J. Smyth, L. J. Abraham and G. C. Yeoh (2005). "Differential lymphotoxin-beta and interferon gamma signaling during mouse liver regeneration induced by chronic and acute injury." Hepatology **41**(2): 327-335.

Alison, M. R., M. H. Golding and C. E. Sarraf (1996). "Pluripotential liver stem cells: facultative stem cells located in the biliary tree." Cell Prolif **29**(7): 373-402.

Apte, U., M. D. Thompson, S. Cui, B. Liu, B. Cieply and S. P. Monga (2008). "Wnt/beta-catenin signaling mediates oval cell response in rodents." Hepatology **47**(1): 288-295.

Arndt, S., E. Wacker, C. Dorn, A. Koch, M. Saugspier, W. E. Thasler, A. Hartmann, A. K. Bosserhoff and C. Hellerbrand (2015). "Enhanced expression of BMP6 inhibits hepatic fibrosis in non-alcoholic fatty liver disease." Gut **64**(6): 973-981.

Arriazu, E., M. Ruiz de Galarreta, F. J. Cubero, M. Varela-Rey, M. P. Perez de Obanos, T. M. Leung, A. Lopategi, A. Benedicto, I. Abraham-Enachescu and N. Nieto (2014). "Extracellular matrix and liver disease." Antioxid Redox Signal **21**(7): 1078-1097.

Basilico, C., A. Arnesano, M. Galluzzo, P. M. Comoglio and P. Michieli (2008). "A high affinity hepatocyte growth factor-binding site in the immunoglobulin-like region of Met." J Biol Chem **283**(30): 21267-21277.

Benvenuti, S. and P. M. Comoglio (2007). "The MET receptor tyrosine kinase in invasion and metastasis." J Cell Physiol **213**(2): 316-325.

Berasain, C., A. Nicou, O. Garcia-Irigoyen, M. U. Latasa, R. Urtasun, M. Elizalde, F. Salis, M. J. Perugorria, J. Prieto, J. A. Recio, F. J. Corrales and M. A. Avila (2012). "Epidermal growth factor receptor signaling in hepatocellular carcinoma: inflammatory activation and a new intracellular regulatory mechanism." Dig Dis **30**(5): 524-531.

Berasain, C., M. J. Perugorria, M. U. Latasa, J. Castillo, S. Goni, M. Santamaria, J. Prieto and M. A. Avila (2009). "The epidermal growth factor receptor: a link between inflammation and liver cancer." Exp Biol Med (Maywood) **234**(7): 713-725.

Bergeron, E., M. E. Marquis, I. Chretien and N. Faucheux (2007). "Differentiation of preosteoblasts using a delivery system with BMPs and bioactive glass microspheres." J Mater Sci Mater Med **18**(2): 255-263.

Bertran, E., L. Caja, E. Navarro, P. Sancho, J. Mainez, M. M. Murillo, A. Vinyals, A. Fabra and I. Fabregat (2009). "Role of CXCR4/SDF-1 alpha in the migratory phenotype of hepatoma cells that have undergone epithelial-mesenchymal transition in response to the transforming growth factor-beta." Cell Signal **21**(11): 1595-1606.

Best, J., L. Dolle, P. Manka, J. Coombes, L. A. van Grunsven and W. K. Syn (2013). "Role of liver progenitors in acute liver injury." Front Physiol **4**: 258.

Bi, J. and S. Ge (2014). "Potential roles of BMP9 in liver fibrosis." Int J Mol Sci **15**(11): 20656-20667.

Bidart, M., N. Ricard, S. Levet, M. Samson, C. Mallet, L. David, M. Subileau, E. Tillet, J. J. Feige and S. Bailly (2011). "BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain." Cell Mol Life Sci **69**(3): 313-324.

Bidart, M., N. Ricard, S. Levet, M. Samson, C. Mallet, L. David, M. Subileau, E. Tillet, J. J. Feige and S. Bailly (2012). "BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain." Cell Mol Life Sci **69**(2): 313-324.

Birchmeier, C., W. Birchmeier, E. Gherardi and G. F. Vande Woude (2003). "Met, metastasis, motility and more." Nat Rev Mol Cell Biol **4**(12): 915-925.

Bird, T. G., S. Lorenzini and S. J. Forbes (2008). "Activation of stem cells in hepatic diseases." Cell Tissue Res **331**(1): 283-300.

Blachier, M., H. Leleu, M. Peck-Radosavljevic, D. C. Valla and F. Roudot-Thoraval (2013). "The burden of liver disease in Europe: a review of available epidemiological data." J Hepatol **58**(3): 593-608.

Bladt, F., D. Riethmacher, S. Isenmann, A. Aguzzi and C. Birchmeier (1995). "Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud." Nature **376**(6543): 768-771.

Boccaccio, C., M. Ando, L. Tamagnone, A. Bardelli, P. Michieli, C. Battistini and P. M. Comoglio (1998). "Induction of epithelial tubules by growth factor HGF depends on the STAT pathway." Nature **391**(6664): 285-288.

Bogatkevich, G. S., A. Ludwicka-Bradley, K. B. Highland, F. Hant, P. J. Nietert, C. B. Singleton, C. A. Feghali-Bostwick and R. M. Silver (2007). "Impairment of the antifibrotic effect of hepatocyte growth factor in lung fibroblasts from African Americans: possible role in systemic sclerosis." Arthritis Rheum **56**(7): 2432-2442.

Boix, L., J. L. Rosa, F. Ventura, A. Castells, J. Bruix, J. Rodes and R. Bartrons (1994). "c-met mRNA overexpression in human hepatocellular carcinoma." Hepatology **19**(1): 88-91.

Boll, M., L. W. Weber, E. Becker and A. Stampfl (2001). "Mechanism of carbon tetrachloride-induced hepatotoxicity. Hepatocellular damage by reactive carbon tetrachloride metabolites." Z Naturforsch C **56**(7-8): 649-659.

Borowiak, M., A. N. Garratt, T. Wustefeld, M. Strehle, C. Trautwein and C. Birchmeier (2004). "Met provides essential signals for liver regeneration." Proc Natl Acad Sci U S A **101**(29): 10608-10613.

Bottaro, D. P., J. S. Rubin, D. L. Faletto, A. M. Chan, T. E. Kmiecik, G. F. Vande Woude and S. A. Aaronson (1991). "Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product." Science **251**(4995): 802-804.

Boulter, L., O. Govaere, T. G. Bird, S. Radulescu, P. Ramachandran, A. Pellicoro, R. A. Ridgway, S. S. Seo, B. Spee, N. Van Rooijen, O. J. Sansom, J. P. Iredale, S. Lowell, T. Roskams and S. J. Forbes (2012). "Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease." Nat Med **18**(4): 572-579.

Bourboulia, D. and W. G. Stetler-Stevenson (2010). "Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion." Semin Cancer Biol **20**(3): 161-168.

Brady, A. M. and E. A. Lock (1992). "Inhibition of ferrochelatase and accumulation of porphyrins in mouse hepatocyte cultures exposed to porphyrinogenic chemicals." Arch Toxicol **66**(3): 175-181.

Brooling, J. T., J. S. Campbell, C. Mitchell, G. C. Yeoh and N. Fausto (2005). "Differential regulation of rodent hepatocyte and oval cell proliferation by interferon gamma." Hepatology **41**(4): 906-915.

Broten, J., G. Michalopoulos, B. Petersen and J. Cruise (1999). "Adrenergic stimulation of hepatocyte growth factor expression." Biochem Biophys Res Commun **262**(1): 76-79.

Caja, L., E. Bertran, J. Campbell, N. Fausto and I. Fabregat (2011). "The transforming growth factor-beta (TGF-beta) mediates acquisition of a mesenchymal stem cell-like phenotype in human liver cells." J Cell Physiol **226**(5): 1214-1223.

Caldwell, S. and S. H. Park (2009). "The epidemiology of hepatocellular cancer: from the perspectives of public health problem to tumor biology." J Gastroenterol **44 Suppl 19**: 96-101.

Calvisi, D. F., R. M. Pascale and F. Feo (2007). "Dissection of signal transduction pathways as a tool for the development of targeted therapies of hepatocellular carcinoma." Rev Recent Clin Trials **2**(3): 217-236.

Campbell, J. S., L. Prichard, F. Schaper, J. Schmitz, A. Stephenson-Famy, M. E. Rosenfeld, G. M. Argast, P. C. Heinrich and N. Fausto (2001). "Expression of suppressors of cytokine signaling during liver regeneration." J Clin Invest **107**(10): 1285-1292.

Caperuto, L. C., G. F. Anhe, T. D. Cambiaghi, E. H. Akamine, D. do Carmo Buonfiglio, J. Cipolla-Neto, R. Curi and S. Bordin (2008). "Modulation of bone morphogenetic protein-9 expression and processing by insulin, glucose, and glucocorticoids: possible candidate for hepatic insulin-sensitizing substance." Endocrinology **149**(12): 6326-6335.

Cardinale, V., Y. Wang, G. Carpino, C. B. Cui, M. Gatto, M. Rossi, P. B. Berloco, A. Cantafora, E. Wauthier, M. E. Furth, L. Inverardi, J. Dominguez-Bendala, C. Ricordi, D. Gerber, E. Gaudio, D. Alvaro and L. Reid (2011). "Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets." Hepatology **54**(6): 2159-2172.

Carreira, A. C., G. G. Alves, W. F. Zambuzzi, M. C. Sogayar and J. M. Granjeiro (2014). "Bone Morphogenetic Proteins: structure, biological function and therapeutic applications." Arch Biochem Biophys **561**: 64-73.

Castonguay, R., E. D. Werner, R. G. Matthews, E. Presman, A. W. Mulivor, N. Solban, D. Sako, R. S. Pearsall, K. W. Underwood, J. Seehra, R. Kumar and A. V. Grinberg (2011). "Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth." J Biol Chem **286**(34): 30034-30046.

Celeste, A. J., Song JJ, Cox K, Rosen V, Wozney JM Suppl 1: 136, 1994. (1994). "Bone morphogenetic protein-9, a new member of the TGF-beta superfamily." J Bone Min Res, **Supp 1**: 136.

Chatterjee, R. and A. Mitra (2015). "An overview of effective therapies and recent advances in biomarkers for chronic liver diseases and associated liver cancer." Int Immunopharmacol **24**(2): 335-345.

Chen, C., K. J. Grzegorzewski, S. Barash, Q. Zhao, H. Schneider, Q. Wang, M. Singh, L. Pukac, A. C. Bell, R. Duan, T. Coleman, A. Duttaroy, S. Cheng, J. Hirsch, L. Zhang, Y. Lazard, C. Fischer, M. C. Barber, Z. D. Ma, Y. Q. Zhang, P. Reavey, L. Zhong, B. Teng, I. Sanyal, S. M. Ruben, O. Blondel and C. E. Birse (2003). "An integrated functional genomics screening program reveals a role for BMP-9 in glucose homeostasis." Nat Biotechnol **21**(3): 294-301.

Chen, J. C., S. T. Yang, C. Y. Lin, C. J. Hsu, C. H. Tsai, J. L. Su and C. H. Tang (2014). "BMP-7 enhances cell migration and alphavbeta3 integrin expression via a c-Src-dependent pathway in human chondrosarcoma cells." PLoS One **9**(11): e112636.

Chen, S. Y. and H. C. Chen (2006). "Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion." Mol Cell Biol **26**(13): 5155-5167.

Chobert, M. N., D. Couchie, A. Fourcot, E. S. Zafrani, Y. Laperche, P. Mavier and A. Brouillet (2012). "Liver precursor cells increase hepatic fibrosis induced by chronic carbon tetrachloride intoxication in rats." Lab Invest **92**(1): 135-150.

Choi, T. Y., N. Ninov, D. Y. Stainier and D. Shin (2014). "Extensive conversion of hepatic biliary epithelial cells to hepatocytes after near total loss of hepatocytes in zebrafish." Gastroenterology **146**(3): 776-788.

Chou, W. Y., C. N. Lu, T. H. Lee, C. L. Wu, K. S. Hung, A. M. Concejero, B. Jawan and C. H. Wang (2006). "Electroporative interleukin-10 gene transfer ameliorates carbon tetrachloride-induced murine liver fibrosis by MMP and TIMP modulation." Acta Pharmacol Sin **27**(4): 469-476.

Chuang, H. M., H. L. Su, C. Li, S. Z. Lin, S. Y. Yen, M. H. Huang, L. I. Ho, T. W. Chiou and H. J. Harn (2016). "The Role of Butylidenephthalide in Targeting the Microenvironment Which Contributes to Liver Fibrosis Amelioration." Front Pharmacol **7**: 112.

Clouston, A. D., J. R. Jonsson and E. E. Powell (2009). "Hepatic progenitor cell-mediated regeneration and fibrosis: chicken or egg?" Hepatology **49**(5): 1424-1426.

Clouston, A. D., E. E. Powell, M. J. Walsh, M. M. Richardson, A. J. Demetris and J. R. Jonsson (2005). "Fibrosis correlates with a ductular reaction in hepatitis C: roles of impaired replication, progenitor cells and steatosis." Hepatology **41**(4): 809-818.

Cohen-Naftaly, M. and S. L. Friedman (2011). "Current status of novel antifibrotic therapies in patients with chronic liver disease." Therap Adv Gastroenterol **4**(6): 391-417.

Comoglio, P. M., S. Giordano and L. Trusolino (2008). "Drug development of MET inhibitors: targeting oncogene addiction and expedience." Nat Rev Drug Discov **7**(6): 504-516.

Comoglio, P. M. and L. Trusolino (2002). "Invasive growth: from development to metastasis." J Clin Invest **109**(7): 857-862.

Conidi, A., S. Cazzola, K. Beets, K. Coddens, C. Collart, F. Cornelis, L. Cox, D. Joke, M. P. Dobreva, R. Dries, C. Esguerra, A. Francis, A. Ibrahimi, R. Kroes, F. Lesage, E. Maas, I. Moya, P. N. Pereira, E. Stappers, A. Stryjewska, V. van den Berghe, L. Vermeire, G. Verstappen, E. Seuntjens, L. Umans, A. Zwijsen and D. Huylebroeck (2011). "Few Smad proteins and many Smad-interacting proteins yield multiple functions and action modes in TGFbeta/BMP signaling in vivo." Cytokine Growth Factor Rev **22**(5-6): 287-300.

Connolly, M. K., A. S. Bedrosian, J. Mallen-St Clair, A. P. Mitchell, J. Ibrahim, A. Stroud, H. L. Pachter, D. Bar-Sagi, A. B. Frey and G. Miller (2009). "In liver fibrosis, dendritic cells govern hepatic inflammation in mice via TNF-alpha." J Clin Invest **119**(11): 3213-3225.

Cooper, C. S., M. Park, D. G. Blair, M. A. Tainsky, K. Huebner, C. M. Croce and G. F. Vande Woude (1984). "Molecular cloning of a new transforming gene from a chemically transformed human cell line." Nature **311**(5981): 29-33.

Coutant, A., C. Rescan, D. Gilot, P. Loyer, C. Guguen-Guillouzo and G. Baffet (2002). "PI3K-FRAP/mTOR pathway is critical for hepatocyte proliferation whereas MEK/ERK supports both proliferation and survival." Hepatology **36**(5): 1079-1088.

Cunha, S. and K. Pietras (2011). "ALK1 as an emerging target for anti-angiogenic therapy of cancer." Blood.

Dabeva, M. D., S. G. Hwang, S. R. Vasa, E. Hurston, P. M. Novikoff, D. C. Hixson, S. Gupta and D. A. Shafritz (1997). "Differentiation of pancreatic epithelial progenitor cells into hepatocytes following transplantation into rat liver." Proc Natl Acad Sci U S A **94**(14): 7356-7361.

Dabeva, M. D. and D. A. Shafritz (1993). "Activation, proliferation, and differentiation of progenitor cells into hepatocytes in the D-galactosamine model of liver regeneration." Am J Pathol **143**(6): 1606-1620.

Daly, A. C., R. A. Randall and C. S. Hill (2008). "Transforming growth factor beta-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth." Mol Cell Biol **28**(22): 6889-6902.

Daveau, M., M. Scotte, A. Francois, C. Coulouarn, G. Ros, Y. Tallet, M. Hiron, M. F. Hellot and J. P. Salier (2003). "Hepatocyte growth factor, transforming growth factor alpha, and their receptors as combined markers of prognosis in hepatocellular carcinoma." Mol Carcinog **36**(3): 130-141.

David, L., C. Mallet, M. Keramidas, N. Lamande, J. M. Gasc, S. Dupuis-Girod, H. Plauchu, J. J. Feige and S. Bailly (2008). "Bone morphogenetic protein-9 is a circulating vascular quiescence factor." Circ Res **102**(8): 914-922.

David, L., C. Mallet, S. Mazerbourg, J. J. Feige and S. Bailly (2007). "Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells." Blood **109**(5): 1953-1961.

del Castillo, G., V. M. Factor, M. Fernandez, A. Alvarez-Barrientos, I. Fabregat, S. S. Thorgeirsson and A. Sanchez (2008). "Deletion of the Met tyrosine kinase in liver progenitor oval cells increases sensitivity to apoptosis in vitro." Am J Pathol **172**(5): 1238-1247.

Del Castillo, G., M. M. Murillo, A. Alvarez-Barrientos, E. Bertran, M. Fernandez, A. Sanchez and I. Fabregat (2006). "Autocrine production of TGF-beta confers resistance to apoptosis after an epithelial-mesenchymal transition process in hepatocytes: Role of EGF receptor ligands." Exp Cell Res **312**(15): 2860-2871.

Deng, H., H. F. Wang, Y. B. Gao, X. L. Jin and J. C. Xiao (2011). "Hepatic progenitor cell represents a transitioning cell population between liver epithelium and stroma." Med Hypotheses **76**(6): 809-812.

Dezso, K., P. Jelnes, V. Laszlo, K. Baghy, C. Bodor, S. Paku, N. Tygstrup, H. C. Bisgaard and P. Nagy (2007). "Thy-1 is expressed in hepatic myofibroblasts and not oval cells in stem cell-mediated liver regeneration." Am J Pathol **171**(5): 1529-1537.

Dezso, K., V. Papp, E. Bugyik, H. Hegyesi, G. Safrany, C. Bodor, P. Nagy and S. Paku (2012). "Structural analysis of oval-cell-mediated liver regeneration in rats." Hepatology **56**(4): 1457-1467.

Do, N., R. Zhao, K. Ray, K. Ho, M. Dib, X. Ren, P. Kuzontkoski, E. Terwilliger and S. J. Karp (2012). "BMP4 is a novel paracrine inhibitor of liver regeneration." Am J Physiol Gastrointest Liver Physiol **303**(11): G1220-1227.

Dorrell, C., L. Erker, J. Schug, J. L. Kopp, P. S. Canaday, A. J. Fox, O. Smirnova, A. W. Duncan, M. J. Finegold, M. Sander, K. H. Kaestner and M. Grompe (2011). "Prospective isolation of a bipotential clonogenic liver progenitor cell in adult mice." Genes Dev **25**(11): 1193-1203.

Du, W. J., J. H. Zhen, Z. Q. Zeng, Z. M. Zheng, Y. Xu, L. Y. Qin and S. J. Chen (2013). "Expression of interleukin-17 associated with disease progression and liver fibrosis with hepatitis B virus infection: IL-17 in HBV infection." Diagn Pathol **8**: 40.

Duarte, S., J. Baber, T. Fujii and A. J. Coito (2015). "Matrix metalloproteinases in liver injury, repair and fibrosis." Matrix Biol **44-46**: 147-156.

Ducy, P. and G. Karsenty (2000). "The family of bone morphogenetic proteins." Kidney Int **57**(6): 2207-2214.

Dudas, P. L., R. L. Argentieri and F. X. Farrell (2009). "BMP-7 fails to attenuate TGF-beta1-induced epithelial-to-mesenchymal transition in human proximal tubule epithelial cells." Nephrol Dial Transplant **24**(5): 1406-1416.

Dumble, M. L., E. J. Croager, G. C. Yeoh and E. A. Quail (2002). "Generation and characterization of p53 null transformed hepatic progenitor cells: oval cells give rise to hepatocellular carcinoma." Carcinogenesis **23**(3): 435-445.

Duncan, A. W., C. Dorrell and M. Grompe (2009). "Stem cells and liver regeneration." Gastroenterology **137**(2): 466-481.

Dusabineza, A. C., N. K. Van Hul, J. Abarca-Quinones, P. Starkel, M. Najimi and I. A. Leclercq (2012). "Participation of liver progenitor cells in liver regeneration: lack of evidence in the AAF/PH rat model." Lab Invest **92**(1): 72-81.

Erker, L. and M. Grompe (2007). "Signaling networks in hepatic oval cell activation." Stem Cell Res **1**(2): 90-102.

Espanol-Suner, R., R. Carpentier, N. Van Hul, V. Legry, Y. Achouri, S. Cordi, P. Jacquemin, F. Lemaigre and I. A. Leclercq (2012). "Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice." Gastroenterology **143**(6): 1564-1575 e1567.

Esrefoglu, M. (2013). "Role of stem cells in repair of liver injury: experimental and clinical benefit of transferred stem cells on liver failure." World J Gastroenterol **19**(40): 6757-6773.

Evarts, R. P., H. Nakatsukasa, E. R. Marsden, Z. Hu and S. S. Thorgeirsson (1992). "Expression of transforming growth factor-alpha in regenerating liver and during hepatic differentiation." Mol Carcinog **5**(1): 25-31.

Fabregat, I., J. Moreno-Caceres, A. Sanchez, S. Dooley, B. Dewidar, G. Giannelli, P. Ten Dijke and I.-L. Consortium (2016). "TGF-beta signalling and liver disease." FEBS J **283**(12): 2219-2232.

Factor, V. M., D. Seo, T. Ishikawa, P. Kaposi-Novak, J. U. Marquardt, J. B. Andersen, E. A. Conner and S. S. Thorgeirsson (2010). "Loss of c-Met disrupts gene expression program required for G2/M progression during liver regeneration in mice." PLoS One **5**(9).

Falkowski, O., H. J. An, I. A. Ianus, L. Chiriboga, H. Yee, A. B. West and N. D. Theise (2003). "Regeneration of hepatocyte 'buds' in cirrhosis from intrabiliary stem cells." J Hepatol **39**(3): 357-364.

Fan, J., H. Shen, Y. Sun, P. Li, F. Burczynski, M. Namaka and Y. Gong (2006). "Bone morphogenetic protein 4 mediates bile duct ligation induced liver fibrosis through activation of Smad1 and ERK1/2 in rat hepatic stellate cells." J Cell Physiol **207**(2): 499-505.

Fattovich, G., T. Stroffolini, I. Zagni and F. Donato (2004). "Hepatocellular carcinoma in cirrhosis: incidence and risk factors." Gastroenterology **127**(5 Suppl 1): S35-50.

Fausto, N. and J. S. Campbell (2003). "The role of hepatocytes and oval cells in liver regeneration and repopulation." Mech Dev **120**(1): 117-130.

Fausto, N., A. D. Laird and E. M. Webber (1995). "Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration." FASEB J **9**(15): 1527-1536.

Fickert, P., U. Stoger, A. Fuchsbichler, T. Moustafa, H. U. Marschall, A. H. Weiglein, O. Tsybrovskyy, H. Jaeschke, K. Zatloukal, H. Denk and M. Trauner (2007). "A new xenobiotic-induced mouse model of sclerosing cholangitis and biliary fibrosis." Am J Pathol **171**(2): 525-536.

Fickert, P., M. Trauner, A. Fuchsbichler, C. Stumptner, K. Zatloukal and H. Denk (2002). "Bile acid-induced Mallory body formation in drug-primed mouse liver." Am J Pathol **161**(6): 2019-2026.

Finberg, K. E. (2013). "Regulation of systemic iron homeostasis." Curr Opin Hematol **20**(3): 208-214.

Forbes, S. J. and M. Parola (2011). "Liver fibrogenic cells." Best Pract Res Clin Gastroenterol **25**(2): 207-217.

Frantz, C., K. M. Stewart and V. M. Weaver (2010). "The extracellular matrix at a glance." J Cell Sci **123**(Pt 24): 4195-4200.

Fremin, C., F. Ezan, P. Boisselier, A. Bessard, G. Pages, J. Pouyssegur and G. Baffet (2007). "ERK2 but not ERK1 plays a key role in hepatocyte replication: an RNAi-mediated ERK2 knockdown approach in wild-type and ERK1 null hepatocytes." Hepatology **45**(4): 1035-1045.

Friedman, A. Y. H. a. S. L. (2003). "Sinusoidal events during fibrosing liver injury." Expert Reviews in Molecular Medicine.

Friedman, S. L. (2008). "Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver." Physiol Rev **88**(1): 125-172.

Friedman, S. L. (2008). "Mechanisms of hepatic fibrogenesis." Gastroenterology **134**(6): 1655-1669.

Gandino, L., P. Longati, E. Medico, M. Prat and P. M. Comoglio (1994). "Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase." J Biol Chem **269**(3): 1815-1820.

Gao, W., P. Zhou, X. Ma, B. Tschudy-Seney, J. Chen, N. L. Magner, A. Revzin, J. A. Nolta, M. A. Zern and Y. Duan (2014). "Ethanol negatively regulates hepatic differentiation of hESC by inhibition of the MAPK/ERK signaling pathway in vitro." PLoS One **9**(11): e112698.

Garcia-Alvaro, M., A. Addante, C. Roncero, M. Fernandez, I. Fabregat, A. Sanchez and B. Herrera (2015). "BMP9-Induced Survival Effect in Liver Tumor Cells Requires p38MAPK Activation." Int J Mol Sci **16**(9): 20431-20448.

Gaudino, G., A. Follenzi, L. Naldini, C. Collesi, M. Santoro, K. A. Gallo, P. J. Godowski and P. M. Comoglio (1994). "RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP." EMBO J **13**(15): 3524-3532.

Gaudio, E., G. Carpino, V. Cardinale, A. Franchitto, P. Onori and D. Alvaro (2009). "New insights into liver stem cells." Dig Liver Dis **41**(7): 455-462.

Gherardi, E., W. Birchmeier, C. Birchmeier and G. Vande Woude (2012). "Targeting MET in cancer: rationale and progress." Nat Rev Cancer **12**(2): 89-103.

Giannelli, G., W. Mikulits, S. Dooley, I. Fabregat, A. Moustakas, P. Ten Dijke, P. Portincasa, P. Winter, R. Janssen, S. Leporatti, B. Herrera and A. Sanchez (2016). "The rationale for targeting TGF-beta in chronic liver diseases." Eur J Clin Invest **46**(4): 349-361.

Giebeler, A., M. V. Boekschoten, C. Klein, M. Borowiak, C. Birchmeier, N. Gassler, H. E. Wasmuth, M. Muller, C. Trautwein and K. L. Streetz (2009). "c-Met confers protection against chronic liver tissue damage and fibrosis progression after bile duct ligation in mice." Gastroenterology **137**(1): 297-308, 308 e291-294.

Gieling, R. G., K. Wallace and Y. P. Han (2009). "Interleukin-1 participates in the progression from liver injury to fibrosis." Am J Physiol Gastrointest Liver Physiol **296**(6): G1324-1331.

Gilgenkrantz, H. and A. Collin de l'Hortet (2011). "New insights into liver regeneration." Clin Res Hepatol Gastroenterol **35**(10): 623-629.

Giordano, S. and A. Columbano (2014). "Met as a therapeutic target in HCC: facts and hopes." J Hepatol **60**(2): 442-452.

Giordano, S., S. Corso, P. Conrotto, S. Artigiani, G. Gilestro, D. Barberis, L. Tamagnone and P. M. Comoglio (2002). "The semaphorin 4D receptor controls invasive growth by coupling with Met." Nat Cell Biol **4**(9): 720-724.

Godoy, P., N. J. Hewitt, U. Albrecht, M. E. Andersen, N. Ansari, S. Bhattacharya, J. G. Bode, J. Bolleyn, C. Borner, J. Bottger, A. Braeuning, R. A. Budinsky, B. Burkhardt, N. R. Cameron, G. Camussi, C. S. Cho, Y. J. Choi, J. Craig Rowlands, U. Dahmen, G. Damm, O. Dirsch, M. T. Donato, J. Dong, S. Dooley, D. Drasdo, R. Eakins, K. S. Ferreira, V. Fonsato, J. Fraczek, R. Gebhardt, A. Gibson, M. Glanemann, C. E. Goldring, M. J. Gomez-Lechon, G. M. Groothuis, L. Gustavsson, C. Guyot, D. Hallifax, S. Hammad, A. Hayward, D. Haussinger, C. Hellerbrand, P. Hewitt, S. Hoehme, H. G. Holzhutter, J. B. Houston, J. Hrach, K. Ito, H. Jaeschke, V. Keitel, J. M. Kelm, B. Kevin Park, C. Kordes, G. A. Kullak-Ublick, E. L. LeCluyse, P. Lu, J. Luebke-Wheeler, A. Lutz, D. J. Maltman, M. Matz-Soja, P. McMullen, I. Merfort, S. Messner, C. Meyer, J. Mwinyi, D. J. Naisbitt, A. K. Nussler, P. Olinga, F. Pampaloni, J. Pi, L. Pluta, S. A. Przyborski, A. Ramachandran, V. Rogiers, C. Rowe, C. Schelcher, K. Schmich, M. Schwarz, B. Singh, E. H. Stelzer, B. Stieger, R. Stober, Y. Sugiyama, C. Tetta, W. E. Thasler, T. Vanhaecke, M. Vinken, T. S. Weiss, A. Widera, C. G. Woods, J. J. Xu, K. M. Yarborough and J. G. Hengstler (2013). "Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME." Arch Toxicol **87**(8): 1315-1530.

Gouw, A. S., A. D. Clouston and N. D. Theise (2011). "Ductular reactions in human liver: diversity at the interface." Hepatology **54**(5): 1853-1863.

Goyal, L., M. D. Muzumdar and A. X. Zhu (2013). "Targeting the HGF/c-MET pathway in hepatocellular carcinoma." Clin Cancer Res **19**(9): 2310-2318.

Granito, A. (2014). "c-MET receptor tyrosine kinase as a molecular target in advanced hepatocellular carcinoma." Journal of Hepatocellular Carcinoma **Volume 2015:2** Pages 29-38.

Haasters, F., D. Docheva, C. Gassner, C. Popov, W. Bocker, W. Mutschler, M. Schieker and W. C. Prall (2014). "Mesenchymal stem cells from osteoporotic patients reveal reduced migration and invasion upon stimulation with BMP-2 or BMP-7." Biochem Biophys Res Commun **452**(1): 118-123.

Habeeb, M. A., S. K. Vishwakarma, A. Bardia and A. A. Khan (2015). "Hepatic stem cells: A viable approach for the treatment of liver cirrhosis." World J Stem Cells **7**(5): 859-865.

Haber, B. A., S. Chin, E. Chuang, W. Buikhuisen, A. Naji and R. Taub (1995). "High levels of glucose-6-phosphatase gene and protein expression reflect an adaptive response in proliferating liver and diabetes." J Clin Invest **95**(2): 832-841.

Hata, S., M. Namae and H. Nishina (2007). "Liver development and regeneration: from laboratory study to clinical therapy." Dev Growth Differ **49**(2): 163-170.

Herrera, B., S. Dooley and K. Breitkopf-Heinlein (2014). "Potential roles of bone morphogenetic protein (BMP)-9 in human liver diseases." Int J Mol Sci **15**(4): 5199-5220.

Herrera, B., M. Garcia-Alvaro, S. Cruz, P. Walsh, M. Fernandez, C. Roncero, I. Fabregat, A. Sanchez and G. J. Inman (2013). "BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells." PLoS One **8**(7): e69535.

Herrera, B., A. Sanchez and I. Fabregat (2012). "BMPs And Liver: More Questions Than Answers." Curr Pharm Des **18**(27): 4114-4125.

Herrera, B., M. van Dinther, P. Ten Dijke and G. J. Inman (2009). "Autocrine bone morphogenetic protein-9 signals through activin receptor-like kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation." Cancer Res **69**(24): 9254-9262.

Higgins GM, A. R. (1931). "Experimental pathology of the liver." Arch Pathol Lab Med **12**: 186-202.

Hilberg, F., A. Aguzzi, N. Howells and E. F. Wagner (1993). "c-jun is essential for normal mouse development and hepatogenesis." Nature **365**(6442): 179-181.

Holien, T., T. K. Vatsveen, H. Hella, C. Rampa, G. Brede, L. A. Groseth, M. Rekvig, M. Borset, T. Standal, A. Waage and A. Sundan (2012). "Bone morphogenetic proteins induce apoptosis in multiple myeloma cells by Smad-dependent repression of MYC." Leukemia **26**(5): 1073-1080.

Hsieh, W. C., A. C. Mackinnon, W. Y. Lu, J. Jung, L. Boulter, N. C. Henderson, K. J. Simpson, B. Schotanus, D. Wojtacha, T. G. Bird, C. N. Medine, D. C. Hay, T. Sethi, J. P. Iredale and S. J. Forbes (2015). "Galectin-3 regulates hepatic progenitor cell expansion during liver injury." Gut **64**(2): 312-321.

Hu, M., M. Kurobe, Y. J. Jeong, C. Fuerer, S. Ghole, R. Nusse and K. G. Sylvester (2007). "Wnt/beta-catenin signaling in murine hepatic transit amplifying progenitor cells." Gastroenterology **133**(5): 1579-1591.

Huff, J. L., M. A. Jelinek, C. A. Borgman, T. J. Lansing and J. T. Parsons (1993). "The protooncogene c-sea encodes a transmembrane protein-tyrosine kinase related to the Met/hepatocyte growth factor/scatter factor receptor." Proc Natl Acad Sci U S A **90**(13): 6140-6144.

Huh, C. G., V. M. Factor, A. Sanchez, K. Uchida, E. A. Conner and S. S. Thorgeirsson (2004). "Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair." Proc Natl Acad Sci U S A **101**(13): 4477-4482.

Imai, Y., H. Terai, C. Nomura-Furuwatari, S. Mizuno, K. Matsumoto, T. Nakamura and K. Takaoka (2005). "Hepatocyte growth factor contributes to fracture repair by upregulating the expression of BMP receptors." J Bone Miner Res **20**(10): 1723-1730.

Inagaki, Y., K. Higashi, M. Kushida, Y. Y. Hong, S. Nakao, R. Higashiyama, T. Moro, J. Itoh, T. Mikami, T. Kimura, G. Shiota, I. Kuwabara and I. Okazaki (2008). "Hepatocyte growth factor suppresses profibrogenic signal transduction via nuclear export of Smad3 with galectin-7." Gastroenterology **134**(4): 1180-1190.

Inoue, T., H. Okada, T. Kobayashi, Y. Watanabe, Y. Kanno, J. B. Kopp, T. Nishida, M. Takigawa, M. Ueno, T. Nakamura and H. Suzuki (2003). "Hepatocyte growth factor counteracts transforming growth factor-beta1, through attenuation of connective tissue growth factor induction, and prevents renal fibrogenesis in 5/6 nephrectomized mice." FASEB J **17**(2): 268-270.

Inoue, T., H. Okada, T. Kobayashi, Y. Watanabe, T. Kikuta, Y. Kanno, M. Takigawa and H. Suzuki (2002). "TGF-beta1 and HGF coordinately facilitate collagen turnover in subepithelial mesenchyme." Biochem Biophys Res Commun **297**(2): 255-260.

Ishikawa, T., V. M. Factor, J. U. Marquardt, C. Raggi, D. Seo, M. Kitade, E. A. Conner and S. S. Thorgeirsson (2012). "Hepatocyte growth factor/c-met signaling is required for stem-cell-mediated liver regeneration in mice." Hepatology **55**(4): 1215-1226.

Jakubowski, A., C. Ambrose, M. Parr, J. M. Lincecum, M. Z. Wang, T. S. Zheng, B. Browning, J. S. Michaelson, M. Baetscher, B. Wang, D. M. Bissell and L. C. Burkly (2005). "TWEAK induces liver progenitor cell proliferation." J Clin Invest **115**(9): 2330-2340.

Jo, M., D. B. Stolz, J. E. Esplen, K. Dorko, G. K. Michalopoulos and S. C. Strom (2000). "Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells." J Biol Chem **275**(12): 8806-8811.

Johnson, P. J. (2005). "Non-surgical treatment of hepatocellular carcinoma." HPB (Oxford) **7**(1): 50-55.

Jones, C. N., N. Tuleuova, J. Y. Lee, E. Ramanculov, A. H. Reddi, M. A. Zern and A. Revzin (2010). "Cultivating hepatocytes on printed arrays of HGF and BMP7 to characterize protective effects of these growth factors during in vitro alcohol injury." Biomaterials **31**(23): 5936-5944.

Kadono, Y., K. Shibahara, M. Namiki, Y. Watanabe, M. Seiki and H. Sato (1998). "Membrane type 1-matrix metalloproteinase is involved in the formation of hepatocyte growth factor/scatter factor-induced branching tubules in madin-darby canine kidney epithelial cells." Biochem Biophys Res Commun **251**(3): 681-687.

Kaimori, A., J. Potter, J. Y. Kaimori, C. Wang, E. Mezey and A. Koteish (2007). "Transforming growth factor-beta1 induces an epithelial-to-mesenchymal transition state in mouse hepatocytes in vitro." J Biol Chem **282**(30): 22089-22101.

Kalluri, R. and E. G. Neilson (2003). "Epithelial-mesenchymal transition and its implications for fibrosis." J Clin Invest **112**(12): 1776-1784.

Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest **119**(6): 1420-1428.

Kanemura, H., Y. Iimuro, M. Takeuchi, T. Ueki, T. Hirano, K. Horiguchi, Y. Asano and J. Fujimoto (2008). "Hepatocyte growth factor gene transfer with naked plasmid DNA ameliorates dimethylnitrosamine-induced liver fibrosis in rats." Hepatol Res **38**(9): 930-939.

Kang, L. I., W. M. Mars and G. K. Michalopoulos (2012). "Signals and cells involved in regulating liver regeneration." Cells **1**(4): 1261-1292.

Kaposi-Novak, P., J. S. Lee, L. Gomez-Quiroz, C. Coulouarn, V. M. Factor and S. S. Thorgeirsson (2006). "Met-regulated expression signature defines a subset of human hepatocellular carcinomas with poor prognosis and aggressive phenotype." J Clin Invest **116**(6): 1582-1595.

Katsuno, Y., A. Hanyu, H. Kanda, Y. Ishikawa, F. Akiyama, T. Iwase, E. Ogata, S. Ehata, K. Miyazono and T. Imamura (2008). "Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway." Oncogene **27**(49): 6322-6333.

Kessenbrock, K., V. Plaks and Z. Werb (2010). "Matrix metalloproteinases: regulators of the tumor microenvironment." Cell **141**(1): 52-67.

Kim, M. J., K. M. Kim, J. Kim and K. N. Kim (2014). "BMP-2 promotes oral squamous carcinoma cell invasion by inducing CCL5 release." PLoS One **9**(10): e108170.

Kim, T. H., W. M. Mars, D. B. Stolz, B. E. Petersen and G. K. Michalopoulos (1997). "Extracellular matrix remodeling at the early stages of liver regeneration in the rat." Hepatology **26**(4): 896-904.

Kinoshita, K., Y. Iimuro, K. Otogawa, S. Saika, Y. Inagaki, Y. Nakajima, N. Kawada, J. Fujimoto, S. L. Friedman and K. Ikeda (2007). "Adenovirus-mediated expression of BMP-7 suppresses the development of liver fibrosis in rats." Gut **56**(5): 706-714.

Kinoshita, T., T. Hanazawa, N. Nohata, N. Kikkawa, H. Enokida, H. Yoshino, T. Yamasaki, H. Hidaka, M. Nakagawa, Y. Okamoto and N. Seki (2012). "Tumor suppressive microRNA-218 inhibits cancer cell migration and invasion through targeting laminin-332 in head and neck squamous cell carcinoma." Oncotarget **3**(11): 1386-1400.

Kinoshita, T., K. Tashiro and T. Nakamura (1989). "Marked increase of HGF mRNA in non-parenchymal liver cells of rats treated with hepatotoxins." Biochem Biophys Res Commun **165**(3): 1229-1234.

Kiss, A., N. J. Wang, J. P. Xie and S. S. Thorgeirsson (1997). "Analysis of transforming growth factor (TGF)-alpha/epidermal growth factor receptor, hepatocyte growth Factor/c-met,TGF-beta receptor type II, and p53 expression in human hepatocellular carcinomas." Clin Cancer Res **3**(7): 1059-1066.

Kitade, M., V. M. Factor, J. B. Andersen, A. Tomokuni, K. Kaji, H. Akita, A. Holczbauer, D. Seo, J. U. Marquardt, E. A. Conner, S. B. Lee, Y. H. Lee and S. S. Thorgeirsson (2013). "Specific fate decisions in adult hepatic progenitor cells driven by MET and EGFR signaling." Genes Dev **27**(15): 1706-1717.

Kitade, M., K. Kaji and H. Yoshiji (2016). "The relationship between hepatic progenitor cell-mediated liver regeneration and non-parenchymal cells." Hepatol Res.

Kitisin, K., M. J. Pishvaian, L. B. Johnson and L. Mishra (2007). "Liver stem cells and molecular signaling pathways in hepatocellular carcinoma." Gastrointest Cancer Res **1**(4 Suppl 2): S13-21.

Kiyono, M. and M. Shibuya (2003). "Bone morphogenetic protein 4 mediates apoptosis of capillary endothelial cells during rat pupillary membrane regression." Mol Cell Biol **23**(13): 4627-4636.

Klaas, M., T. Kangur, J. Viil, K. Maemets-Allas, A. Minajeva, K. Vadi, M. Antsov, N. Lapidus, M. Jarvekulg and V. Jaks (2016). "The alterations in the extracellular matrix composition guide the repair of damaged liver tissue." Sci Rep **6**: 27398.

Kmiec, Z. (2001). "Cooperation of liver cells in health and disease." Adv Anat Embryol Cell Biol **161**: III-XIII, 1-151.

Knight, B., V. B. Matthews, B. Akhurst, E. J. Croager, E. Klinken, L. J. Abraham, J. K. Olynyk and G. Yeoh (2005). "Liver inflammation and cytokine production, but not acute phase protein synthesis, accompany the adult liver progenitor (oval) cell response to chronic liver injury." Immunol Cell Biol **83**(4): 364-374.

Knight, B., V. B. Matthews, J. K. Olynyk and G. C. Yeoh (2005). "Jekyll and Hyde: evolving perspectives on the function and potential of the adult liver progenitor (oval) cell." Bioessays **27**(11): 1192-1202.

Knight, B., G. C. Yeoh, K. L. Husk, T. Ly, L. J. Abraham, C. Yu, J. A. Rhim and N. Fausto (2000). "Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice." J Exp Med **192**(12): 1809-1818.

Knodell, R. G., K. G. Ishak, W. C. Black, T. S. Chen, R. Craig, N. Kaplowitz, T. W. Kiernan and J. Wollman (1981). "Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis." Hepatology **1**(5): 431-435.

Kohn-Gaone, J., J. Gogoi-Tiwari, G. A. Ramm, J. K. Olynyk and J. E. Tirnitz-Parker (2016). "The role of liver progenitor cells during liver regeneration, fibrogenesis, and carcinogenesis." Am J Physiol Gastrointest Liver Physiol **310**(3): G143-154.

Kong, X., D. Feng, H. Wang, F. Hong, A. Bertola, F. S. Wang and B. Gao (2012). "Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice." Hepatology **56**(3): 1150-1159.

Korchynskyi, O. and P. ten Dijke (2002). "Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter." J Biol Chem **277**(7): 4883-4891.

Kovalovich, K., R. A. DeAngelis, W. Li, E. E. Furth, G. Ciliberto and R. Taub (2000). "Increased toxin-induced liver injury and fibrosis in interleukin-6-deficient mice." Hepatology **31**(1): 149-159.

Kuramitsu, K., D. Y. Sverdlov, S. B. Liu, E. Csizmadia, L. Burkly, D. Schuppan, D. W. Hanto, L. E. Otterbein and Y. Popov (2013). "Failure of fibrotic liver regeneration in mice is linked to a severe fibrogenic response driven by hepatic progenitor cell activation." Am J Pathol **183**(1): 182-194.

Lachenmayer, A., C. Alsinet, C. Y. Chang and J. M. Llovet (2010). "Molecular approaches to treatment of hepatocellular carcinoma." Dig Liver Dis **42 Suppl 3**: S264-272.

Lee, J. S., J. Heo, L. Libbrecht, I. S. Chu, P. Kaposi-Novak, D. F. Calvisi, A. Mikaelyan, L. R. Roberts, A. J. Demetris, Z. Sun, F. Nevens, T. Roskams and S. S. Thorgeirsson (2006). "A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells." Nat Med **12**(4): 410-416.

Lee, T. K., A. Castilho, S. Ma and I. O. Ng (2009). "Liver cancer stem cells: implications for a new therapeutic target." Liver Int **29**(7): 955-965.

Levet, S., M. Ouarne, D. Ciais, C. Coutton, M. Subileau, C. Mallet, N. Ricard, M. Bidart, T. Debillon, F. Faravelli, C. Rooryck, J. J. Feige, E. Tillet and S. Bailly (2015). "BMP9 and BMP10 are necessary for proper closure of the ductus arteriosus." Proc Natl Acad Sci U S A **112**(25): E3207-3215.

Li, B., Y. Yang, S. Jiang, B. Ni, K. Chen and L. Jiang (2012). "Adenovirus-mediated overexpression of BMP-9 inhibits human osteosarcoma cell growth and migration through downregulation of the PI3K/AKT pathway." Int J Oncol **41**(5): 1809-1819.

Li, J., G. Ning and S. A. Duncan (2000). "Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha." Genes Dev **14**(4): 464-474.

Li, M. X. and B. C. Liu (2007). "Epithelial to mesenchymal transition in the progression of tubulointerstitial fibrosis." Chin Med J (Engl) **120**(21): 1925-1930.

Li, Q., X. Gu, H. Weng, S. Ghafoory, Y. Liu, T. Feng, J. Dzieran, L. Li, I. Ilkavets, M. Kruithof-de Julio, S. Munker, A. Marx, A. Piiper, E. A. Alonso, N. Gretz, C. Gao, S. Wolfl, S. Dooley and K. Breitkopf-Heinlein (2013). "Bone morphogenetic protein-9 (BMP-9) induces epithelial to mesenchymal transition (EMT) in hepatocellular carcinoma cells." Cancer Sci **104**(3): 398-408.

Li, R. X., W. H. Yiu and S. C. Tang (2015). "Role of bone morphogenetic protein-7 in renal fibrosis." Front Physiol **6**: 114.

Libbrecht, L., V. Desmet, B. Van Damme and T. Roskams (2000). "Deep intralobular extension of human hepatic 'progenitor cells' correlates with parenchymal inflammation in chronic viral hepatitis: can 'progenitor cells' migrate?" J Pathol **192**(3): 373-378.

Libbrecht, L. and T. Roskams (2002). "Hepatic progenitor cells in human liver diseases." Semin Cell Dev Biol **13**(6): 389-396.

Liedtke, C., T. Luedde, T. Sauerbruch, D. Scholten, K. Streetz, F. Tacke, R. Tolba, C. Trautwein, J. Trebicka and R. Weiskirchen (2013). "Experimental liver fibrosis research: update on animal models, legal issues and translational aspects." Fibrogenesis Tissue Repair **6**(1): 19.

Lissenberg-Thunnissen, S. N., D. J. de Gorter, C. F. Sier and I. B. Schipper (2011). "Use and efficacy of bone morphogenetic proteins in fracture healing." Int Orthop **35**(9): 1271-1280.

Little, S. C. and M. C. Mullins (2009). "Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis." Nat Cell Biol **11**(5): 637-643.

Liu, F. and M. Korc (2012). "Cdk4/6 inhibition induces epithelial-mesenchymal transition and enhances invasiveness in pancreatic cancer cells." Mol Cancer Ther **11**(10): 2138-2148.

Liu, Y., C. Meyer, C. Xu, H. Weng, C. Hellerbrand, P. ten Dijke and S. Dooley (2013). "Animal models of chronic liver diseases." Am J Physiol Gastrointest Liver Physiol **304**(5): G449-468.

Long, L., M. L. Ormiston, X. Yang, M. Southwood, S. Graf, R. D. Machado, M. Mueller, B. Kinzel, L. M. Yung, J. M. Wilkinson, S. D. Moore, K. M. Drake, M. A. Aldred, P. B. Yu, P. D. Upton and N. W. Morrell (2015). "Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension." Nat Med **21**(7): 777-785.

Lopez-Coviella, I., M. T. Follettie, T. J. Mellott, V. P. Kovacheva, B. E. Slack, V. Diesl, B. Berse, R. S. Thies and J. K. Blusztajn (2005). "Bone morphogenetic protein 9 induces the transcriptome of basal forebrain cholinergic neurons." Proc Natl Acad Sci U S A **102**(19): 6984-6989.

Lopez-Luque, J., D. Caballero-Diaz, A. Martinez-Palacian, C. Roncero, J. Moreno-Caceres, M. Garcia-Bravo, E. Grueso, A. Fernandez, E. Crosas-Molist, M. Garcia-Alvaro, A. Addante, E. Bertran, A. M. Valverde, A. Gonzalez-Rodriguez, B. Herrera, L. Montoliu, T. Serrano, J. C. Segovia, M. Fernandez, E. Ramos, A. Sanchez and I. Fabregat (2016). "Dissecting the role of epidermal growth factor receptor catalytic activity during liver regeneration and hepatocarcinogenesis." Hepatology **63**(2): 604-619.

Lorenzini, S., T. G. Bird, L. Boulter, C. Bellamy, K. Samuel, R. Aucott, E. Clayton, P. Andreone, M. Bernardi, M. Golding, M. R. Alison, J. P. Iredale and S. J. Forbes (2010). "Characterisation of a stereotypical cellular and extracellular adult liver progenitor cell niche in rodents and diseased human liver." Gut **59**(5): 645-654.

Lowes, K. N., B. A. Brennan, G. C. Yeoh and J. K. Olynyk (1999). "Oval cell numbers in human chronic liver diseases are directly related to disease severity." Am J Pathol **154**(2): 537-541.

Lozoya, O. A., E. Wauthier, R. A. Turner, C. Barbier, G. D. Prestwich, F. Guilak, R. Superfine, S. R. Lubkin and L. M. Reid (2011). "Regulation of hepatic stem/progenitor phenotype by microenvironment stiffness in hydrogel models of the human liver stem cell niche." Biomaterials **32**(30): 7389-7402.

Lu, K. V., J. P. Chang, C. A. Parachoniak, M. M. Pandika, M. K. Aghi, D. Meyronet, N. Isachenko, S. D. Fouse, J. J. Phillips, D. A. Cheresh, M. Park and G. Bergers (2012). "VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex." Cancer Cell **22**(1): 21-35.

Lu, W. Y., T. G. Bird, L. Boulter, A. Tsuchiya, A. M. Cole, T. Hay, R. V. Guest, D. Wojtacha, T. Y. Man, A. Mackinnon, R. A. Ridgway, T. Kendall, M. J. Williams, T. Jamieson, A. Raven, D. C. Hay, J. P. Iredale, A. R. Clarke, O. J. Sansom and S. J. Forbes (2015). "Hepatic progenitor cells of biliary origin with liver repopulation capacity." Nat Cell Biol **17**(8): 971-983.

Luo, J., M. Tang, J. Huang, B. C. He, J. L. Gao, L. Chen, G. W. Zuo, W. Zhang, Q. Luo, Q. Shi, B. Q. Zhang, Y. Bi, X. Luo, W. Jiang, Y. Su, J. Shen, S. H. Kim, E. Huang, Y. Gao, J. Z. Zhou, K. Yang, H. H. Luu, X. Pan, R. C. Haydon, Z. L. Deng and T. C. He (2010). "TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells." J Biol Chem **285**(38): 29588-29598.

Luo, Q., Q. Kang, W. Si, W. Jiang, J. K. Park, Y. Peng, X. Li, H. H. Luu, J. Luo, A. G. Montag, R. C. Haydon and T. C. He (2004). "Connective tissue growth factor (CTGF) is regulated by Wnt and bone morphogenetic proteins signaling in osteoblast differentiation of mesenchymal stem cells." J Biol Chem **279**(53): 55958-55968.

Lv, Z., D. Yang, J. Li, M. Hu, M. Luo, X. Zhan, P. Song, C. Liu, H. Bai, B. Li, Y. Yang, Y. Chen, Q. Shi and Y. Weng (2013). "Bone morphogenetic protein 9 overexpression reduces osteosarcoma cell migration and invasion." Mol Cells **36**(2): 119-126.

Ma, S., K. W. Chan, L. Hu, T. K. Lee, J. Y. Wo, I. O. Ng, B. J. Zheng and X. Y. Guan (2007). "Identification and characterization of tumorigenic liver cancer stem/progenitor cells." Gastroenterology **132**(7): 2542-2556.

Maegdefrau, U. and A. K. Bosserhoff (2012). "BMP activated Smad signaling strongly promotes migration and invasion of hepatocellular carcinoma cells." Exp Mol Pathol **92**(1): 74-81.

Maestrini, E., L. Tamagnone, P. Longati, O. Cremona, M. Gulisano, S. Bione, F. Tamanini, B. G. Neel, D. Toniolo and P. M. Comoglio (1996). "A family of transmembrane proteins with homology to the MET-hepatocyte growth factor receptor." Proc Natl Acad Sci U S A **93**(2): 674-678.

Maher, J. J. (1993). "Cell-specific expression of hepatocyte growth factor in liver. Upregulation in sinusoidal endothelial cells after carbon tetrachloride." J Clin Invest **91**(5): 2244-2252.

Malarkey, D. E., K. Johnson, L. Ryan, G. Boorman and R. R. Maronpot (2005). "New insights into functional aspects of liver morphology." Toxicol Pathol **33**(1): 27-34.

Malato, Y., S. Naqvi, N. Schurmann, R. Ng, B. Wang, J. Zape, M. A. Kay, D. Grimm and H. Willenbring (2011). "Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration." J Clin Invest **121**(12): 4850-4860.

Malik, R., C. Selden and H. Hodgson (2002). "The role of non-parenchymal cells in liver growth." Semin Cell Dev Biol **13**(6): 425-431.

Mao, S. A., J. M. Glorioso and S. L. Nyberg (2014). "Liver regeneration." Transl Res **163**(4): 352-362.

Marcos, A., R. A. Fisher, J. M. Ham, M. L. Shiffman, A. J. Sanyal, V. A. Luketic, R. K. Sterling, A. S. Fulcher and M. P. Posner (2000). "Liver regeneration and function in donor and recipient after right lobe adult to adult living donor liver transplantation." Transplantation **69**(7): 1375-1379.

Maroun, C. R., M. A. Naujokas, M. Holgado-Madruga, A. J. Wong and M. Park (2000). "The tyrosine phosphatase SHP-2 is required for sustained activation of extracellular signal-regulated kinase and epithelial morphogenesis downstream from the met receptor tyrosine kinase." Mol Cell Biol **20**(22): 8513-8525.

Marquardt, J. U., D. Seo, L. E. Gomez-Quiroz, K. Uchida, M. C. Gillen, M. Kitade, P. Kaposi-Novak, E. A. Conner, V. M. Factor and S. S. Thorgeirsson (2012). "Loss of c-Met accelerates development of liver fibrosis in response to CCl(4) exposure through deregulation of multiple molecular pathways." Biochim Biophys Acta **1822**(6): 942-951.

Marra, F. and F. Tacke (2014). "Roles for chemokines in liver disease." Gastroenterology **147**(3): 577-594 e571.

Marshall, A., S. Rushbrook, S. E. Davies, L. S. Morris, I. S. Scott, S. L. Vowler, N. Coleman and G. Alexander (2005). "Relation between hepatocyte G1 arrest, impaired hepatic regeneration, and fibrosis in chronic hepatitis C virus infection." Gastroenterology **128**(1): 33-42.

Martinez-Hernandez, A. and P. S. Amenta (1995). "The extracellular matrix in hepatic regeneration." FASEB J **9**(14): 1401-1410.

Martinez-Palacian, A., G. del Castillo, B. Herrera, M. Fernandez, C. Roncero, I. Fabregat and A. Sanchez (2012). "EGFR is dispensable for c-Met-mediated proliferation and survival activities in mouse adult liver oval cells." Cell Signal **24**(2): 505-513.

Martinez-Palacian, A., G. del Castillo, A. Suarez-Causado, M. Garcia-Alvaro, D. de Morena-Frutos, M. Fernandez, C. Roncero, I. Fabregat, B. Herrera and A. Sanchez (2013). "Mouse hepatic oval cells require Met-dependent PI3K to impair TGF-beta-induced oxidative stress and apoptosis." PLoS One **8**(1): e53108.

Masson, N. M., I. S. Currie, J. D. Terrace, O. J. Garden, R. W. Parks and J. A. Ross (2006). "Hepatic progenitor cells in human fetal liver express the oval cell marker Thy-1." Am J Physiol Gastrointest Liver Physiol **291**(1): G45-54.

Matsumoto, K., H. Okazaki and T. Nakamura (1995). "Novel function of prostaglandins as inducers of gene expression of HGF and putative mediators of tissue regeneration." J Biochem **117**(2): 458-464.

McClelland, R., E. Wauthier, J. Uronis and L. Reid (2008). "Gradients in the liver's extracellular matrix chemistry from periportal to pericentral zones: influence on human hepatic progenitors." Tissue Eng Part A **14**(1): 59-70.

McReynolds, L. J., S. Gupta, M. E. Figueroa, M. C. Mullins and T. Evans (2007). "Smad1 and Smad5 differentially regulate embryonic hematopoiesis." Blood **110**(12): 3881-3890.

Meng, X. M., A. C. Chung and H. Y. Lan (2013). "Role of the TGF-beta/BMP-7/Smad pathways in renal diseases." Clin Sci (Lond) **124**(4): 243-254.

Michalopoulos, G. K. (2007). "Liver regeneration." J Cell Physiol **213**(2): 286-300.

Michalopoulos, G. K. (2010). "Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas." Am J Pathol **176**(1): 2-13.

Michalopoulos, G. K. (2014). "Advances in liver regeneration." Expert Rev Gastroenterol Hepatol **8**(8): 897-907.

Michalopoulos, G. K. and M. C. DeFrances (1997). "Liver regeneration." Science **276**(5309): 60-66.

Midorikawa, Y., S. Tsutsumi, H. Taniguchi, M. Ishii, Y. Kobune, T. Kodama, M. Makuuchi and H. Aburatani (2002). "Identification of genes associated with dedifferentiation of hepatocellular carcinoma with expression profiling analysis." Jpn J Cancer Res **93**(6): 636-643.

Miller, A. F., S. A. Harvey, R. S. Thies and M. S. Olson (2000). "Bone morphogenetic protein-9. An autocrine/paracrine cytokine in the liver." J Biol Chem **275**(24): 17937-17945.

Moghul, A., L. Lin, A. Beedle, A. Kanbour-Shakir, M. C. DeFrances, Y. Liu and R. Zarnegar (1994). "Modulation of c-MET proto-oncogene (HGF receptor) mRNA abundance by cytokines and hormones: evidence for rapid decay of the 8 kb c-MET transcript." Oncogene **9**(7): 2045-2052.

Monga, S. P., Y. Tang, F. Candotti, A. Rashid, O. Wildner, B. Mishra, S. Iqbal and L. Mishra (2001). "Expansion of hepatic and hematopoietic stem cells utilizing mouse embryonic liver explants." Cell Transplant **10**(1): 81-89.

Monvoisin, A., C. Bisson, K. Si-Tayeb, C. Balabaud, A. Desmouliere and J. Rosenbaum (2002). "Involvement of matrix metalloproteinase type-3 in hepatocyte growth factor-induced invasion of human hepatocellular carcinoma cells." Int J Cancer **97**(2): 157-162.

Moraes, D. A., T. T. Sibov, L. F. Pavon, P. Q. Alvim, R. S. Bonadio, J. R. Da Silva, A. Pic-Taylor, O. A. Toledo, L. C. Marti, R. B. Azevedo and D. M. Oliveira (2016). "A reduction in CD90 (THY-1) expression results in increased differentiation of mesenchymal stromal cells." Stem Cell Res Ther **7**(1): 97.

Morten A. Karsdal, T. M.-J., Federica Genovese, Jacob H. Kristensen, Mette J. Nielsen, Jannie Marie B. Sand, Niels-Ulrik B. Hansen, Anne-Christine Bay-Jensen, Cecilie L. Bager, Aleksander Krag, Andy Blanchard, Henrik Krarup, Diana J. Leeming, Detlef Schuppan (2014). "Novel insights into the function and dynamics of extracellular matrix in liver fibrosis." Am J Physiol Gastrointest Liver Physiol. **2015 May 15**: 308(310):G807-330.

Moumen, A., A. Ieraci, S. Patane, C. Sole, J. X. Comella, R. Dono and F. Maina (2007). "Met signals hepatocyte survival by preventing Fas-triggered FLIP degradation in a PI3k-Akt-dependent manner." Hepatology **45**(5): 1210-1217.

Munoz-Felix, J. M., C. Cuesta, N. Perretta-Tejedor, M. Subileau, F. J. Lopez-Hernandez, J. M. Lopez-Novoa and C. Martinez-Salgado (2016). "Identification of bone morphogenetic protein 9 (BMP9) as a novel profibrotic factor in vitro." Cell Signal **28**(9): 1252-1261.

Munoz-Felix, J. M., C. Cuesta, N. Perretta-Tejedor, M. Subileau, F. J. Lopez-Hernandez, J. M. Lopez-Novoa and C. Martinez-Salgado (2016). "Identification of bone morphogenetic protein 9 (BMP9) as a novel profibrotic factor in vitro." Cell Signal **28**(9): 1252-1261.

Mushtaq, S., M. Muzaffar, M. T. Khadim, W. Z. Tariq and N. Mamoon (1997). "A morphological study of histological activity index (HAI) and scoring system in hepatitis-C." J Pak Med Assoc **47**(6): 162-165.

Nagao, M., T. Nakamura and A. Ichihara (1986). "Developmental control of gene expression of tryptophan 2,3-dioxygenase in neonatal rat liver." Biochim Biophys Acta **867**(4): 179-186.

Nagy, P., H. C. Bisgaard, E. Santoni-Rugiu and S. S. Thorgeirsson (1996). "In vivo infusion of growth factors enhances the mitogenic response of rat hepatic ductal (oval) cells after administration of 2-acetylaminofluorene." Hepatology **23**(1): 71-79.

Nakamura, T. (1989). "[Growth factor and growth inhibitor for hepatocyte proliferation]." Gan To Kagaku Ryoho **16**(3 Pt 2): 481-488.

Nakamura, T. and S. Mizuno (2010). "The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine." Proc Jpn Acad Ser B Phys Biol Sci **86**(6): 588-610.

Nakamura, T., K. Sakai, T. Nakamura and K. Matsumoto (2011). "Hepatocyte growth factor twenty years on: Much more than a growth factor." J Gastroenterol Hepatol **26 Suppl 1**: 188-202.

Nakatsuka, R., M. Taniguchi, M. Hirata, G. Shiota and K. Sato (2007). "Transient expression of bone morphogenic protein-2 in acute liver injury by carbon tetrachloride." J Biochem **141**(1): 113-119.

Naldini, L., E. Vigna, A. Bardelli, A. Follenzi, F. Galimi and P. M. Comoglio (1995). "Biological activation of pro-HGF (hepatocyte growth factor) by urokinase is controlled by a stoichiometric reaction." J Biol Chem **270**(2): 603-611.

Naldini, L., K. M. Weidner, E. Vigna, G. Gaudino, A. Bardelli, C. Ponzetto, R. P. Narsimhan, G. Hartmann, R. Zarnegar, G. K. Michalopoulos and et al. (1991). "Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor." EMBO J **10**(10): 2867-2878.

Nasir, G. A., S. Mohsin, M. Khan, S. Shams, G. Ali, S. N. Khan and S. Riazuddin (2013). "Mesenchymal stem cells and Interleukin-6 attenuate liver fibrosis in mice." J Transl Med **11**: 78.

Nguyen, L. N., M. H. Furuya, L. A. Wolfraim, A. P. Nguyen, M. S. Holdren, J. S. Campbell, B. Knight, G. C. Yeoh, N. Fausto and W. T. Parks (2007). "Transforming growth factor-beta differentially regulates oval cell and hepatocyte proliferation." Hepatology **45**(1): 31-41.

Nobili, V., G. Carpino, A. Alisi, A. Franchitto, G. Alpini, R. De Vito, P. Onori, D. Alvaro and E. Gaudio (2012). "Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease." Hepatology **56**(6): 2142-2153.

Noda, C., C. Fukushima, T. Fujiwara, K. Matsuda, Y. Kobune and A. Ichihara (1994). "Developmental regulation of rat serine dehydratase gene expression: evidence for the presence of a repressor in fetal hepatocytes." Biochim Biophys Acta **1217**(2): 163-173.

Novo, E., S. Cannito, E. Morello, C. Paternostro, C. Bocca, A. Miglietta and M. Parola (2015). "Hepatic myofibroblasts and fibrogenic progression of chronic liver diseases." Histol Histopathol **30**(9): 1011-1032.

O'Reilly, S., M. Ciechomska, R. Cant and J. M. van Laar (2014). "Interleukin-6 (IL-6) trans signaling drives a STAT3-dependent pathway that leads to hyperactive transforming growth factor-beta (TGF-beta) signaling promoting SMAD3 activation and fibrosis via Gremlin protein." J Biol Chem **289**(14): 9952-9960.

Ohta, S., B. Wang, S. L. Mansour and G. C. Schoenwolf (2016). "BMP regulates regional gene expression in the dorsal otocyst through canonical and non-canonical intracellular pathways." Development **143**(12): 2228-2237.

Okabe, M., Y. Tsukahara, M. Tanaka, K. Suzuki, S. Saito, Y. Kamiya, T. Tsujimura, K. Nakamura and A. Miyajima (2009). "Potential hepatic stem cells reside in EpCAM+ cells of normal and injured mouse liver." Development **136**(11): 1951-1960.

Okano, J., G. Shiota, K. Matsumoto, S. Yasui, A. Kurimasa, I. Hisatome, P. Steinberg and Y. Murawaki (2003). "Hepatocyte growth factor exerts a proliferative effect on oval cells through the PI3K/AKT signaling pathway." Biochem Biophys Res Commun **309**(2): 298-304.

Olsen, O. E., K. F. Wader, K. Misund, T. K. Vatsveen, T. B. Ro, A. K. Mylin, I. Turesson, B. F. Stordal, S. H. Moen, T. Standal, A. Waage, A. Sundan and T. Holien (2014). "Bone morphogenetic protein-9 suppresses growth of myeloma cells by signaling through ALK2 but is inhibited by endoglin." Blood Cancer J **4**: e196.

Omori, N., M. Omori, R. P. Evarts, T. Teramoto, M. J. Miller, T. N. Hoang and S. S. Thorgeirsson (1997). "Partial cloning of rat CD34 cDNA and expression during stem cell-dependent liver regeneration in the adult rat." Hepatology **26**(3): 720-727.

Organ, S. L. and M. S. Tsao (2011). "An overview of the c-MET signaling pathway." Ther Adv Med Oncol **3**(1 Suppl): S7-S19.

Orian-Rousseau, V., L. Chen, J. P. Sleeman, P. Herrlich and H. Ponta (2002). "CD44 is required for two consecutive steps in HGF/c-Met signaling." Genes Dev **16**(23): 3074-3086.

Papp, V., A. Rokusz, K. Dezso, E. Bugyik, V. Szabo, Z. Pavai, S. Paku and P. Nagy (2014). "Expansion of hepatic stem cell compartment boosts liver regeneration." Stem Cells Dev **23**(1): 56-65.

Park, B. J., Y. J. Lee and H. R. Lee (2014). "Chronic liver inflammation: clinical implications beyond alcoholic liver disease." World J Gastroenterol **20**(9): 2168-2175.

Patsenker, E., Y. Popov, F. Stickel, A. Jonczyk, S. L. Goodman and D. Schuppan (2008). "Inhibition of integrin alphavbeta6 on cholangiocytes blocks transforming growth factor-beta activation and retards biliary fibrosis progression." Gastroenterology **135**(2): 660-670.

Patthy, L., M. Trexler, Z. Vali, L. Banyai and A. Varadi (1984). "Kringles: modules specialized for protein binding. Homology of the gelatin-binding region of fibronectin with the kringle structures of proteases." FEBS Lett **171**(1): 131-136.

Pellicoro, A., P. Ramachandran, J. P. Iredale and J. A. Fallowfield (2014). "Liver fibrosis and repair: immune regulation of wound healing in a solid organ." Nat Rev Immunol **14**(3): 181-194.

Petersen, B. E., B. Grossbard, H. Hatch, L. Pi, J. Deng and E. W. Scott (2003). "Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers." Hepatology **37**(3): 632-640.

Phaneuf, D., A. D. Moscioni, C. LeClair, S. E. Raper and J. M. Wilson (2004). "Generation of a mouse expressing a conditional knockout of the hepatocyte growth factor gene: demonstration of impaired liver regeneration." DNA Cell Biol **23**(9): 592-603.

Pi, L., S. H. Oh, T. Shupe and B. E. Petersen (2005). "Role of connective tissue growth factor in oval cell response during liver regeneration after 2-AAF/PHx in rats." Gastroenterology **128**(7): 2077-2088.

Plum, W., D. F. Tschaharganeh, D. C. Kroy, E. Corsten, S. Erschfeld, U. Dierssen, H. Wasmuth, C. Trautwein and K. L. Streetz (2010). "Lack of glycoprotein 130/signal transducer and activator of transcription 3-mediated signaling in hepatocytes enhances chronic liver injury and fibrosis progression in a model of sclerosing cholangitis." Am J Pathol **176**(5): 2236-2246.

Preisegger, K. H., V. M. Factor, A. Fuchsbichler, C. Stumptner, H. Denk and S. S. Thorgeirsson (1999). "Atypical ductular proliferation and its inhibition by transforming growth factor beta1 in the 3,5-diethoxycarbonyl-1,4-dihydrocollidine mouse model for chronic alcoholic liver disease." Lab Invest **79**(2): 103-109.

Pritchard, M. T. and L. E. Nagy (2010). "Hepatic fibrosis is enhanced and accompanied by robust oval cell activation after chronic carbon tetrachloride administration to Egr-1-deficient mice." Am J Pathol **176**(6): 2743-2752.

Pyke, C., S. Salo, E. Ralfkiaer, J. Romer, K. Dano and K. Tryggvason (1995). "Laminin-5 is a marker of invading cancer cells in some human carcinomas and is coexpressed with the receptor for urokinase plasminogen activator in budding cancer cells in colon adenocarcinomas." Cancer Res **55**(18): 4132-4139.

Racanelli, V. and B. Rehermann (2006). "The liver as an immunological organ." Hepatology **43**(2 Suppl 1): S54-62.

Ren, W., Y. Liu, S. Wan, C. Fei, W. Wang, Y. Chen, Z. Zhang, T. Wang, J. Wang, L. Zhou, Y. Weng, T. He and Y. Zhang (2014). "BMP9 inhibits proliferation and metastasis of HER2-positive SK-BR-3 breast cancer cells through ERK1/2 and PI3K/AKT pathways." PLoS One **9**(5): e96816.

Ricard, N., D. Ciais, S. Levet, M. Subileau, C. Mallet, T. A. Zimmers, S. J. Lee, M. Bidart, J. J. Feige and S. Bailly (2012). "BMP9 and BMP10 are critical for postnatal retinal vascular remodeling." Blood **119**(25): 6162-6171.

Richardson, M. M., J. R. Jonsson, E. E. Powell, E. M. Brunt, B. A. Neuschwander-Tetri, P. S. Bhathal, J. B. Dixon, M. D. Weltman, H. Tilg, A. R. Moschen, D. M. Purdie, A. J. Demetris and A. D. Clouston (2007). "Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction." Gastroenterology **133**(1): 80-90.

Riehle, K. J., Y. Y. Dan, J. S. Campbell and N. Fausto (2011). "New concepts in liver regeneration." J Gastroenterol Hepatol **26 Suppl 1**: 203-212.

Rockey, D. C. (2008). "Current and future anti-fibrotic therapies for chronic liver disease." Clin Liver Dis **12**(4): 939-962, xi.

Rodrigo-Torres, D., S. Affo, M. Coll, O. Morales-Ibanez, C. Millan, D. Blaya, A. Alvarez-Guaita, C. Rentero, J. J. Lozano, M. A. Maestro, M. Solar, V. Arroyo, J. Caballeria, L. A. van Grunsven, C. Enrich, P. Gines, R. Bataller and P. Sancho-Bru (2014). "The biliary epithelium gives rise to liver progenitor cells." Hepatology **60**(4): 1367-1377.

Ronsin, C., F. Muscatelli, M. G. Mattei and R. Breathnach (1993). "A novel putative receptor protein tyrosine kinase of the met family." Oncogene **8**(5): 1195-1202.

Roskams, T. (2006). "Liver stem cells and their implication in hepatocellular and cholangiocarcinoma." Oncogene **25**(27): 3818-3822.

Roskams, T., S. Q. Yang, A. Koteish, A. Durnez, R. DeVos, X. Huang, R. Achten, C. Verslype and A. M. Diehl (2003). "Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease." Am J Pathol **163**(4): 1301-1311.

Roskams, T. A., N. D. Theise, C. Balabaud, G. Bhagat, P. S. Bhathal, P. Bioulac-Sage, E. M. Brunt, J. M. Crawford, H. A. Crosby, V. Desmet, M. J. Finegold, S. A. Geller, A. S. Gouw, P. Hytiroglou, A. S. Knisely, M. Kojiro, J. H. Lefkowitch, Y. Nakanuma, J. K. Olynyk, Y. N. Park, B. Portmann, R. Saxena, P. J. Scheuer, A. J. Strain, S. N. Thung, I. R. Wanless and A. B. West (2004). "Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers." Hepatology **39**(6): 1739-1745.

Rossi, J. M., N. R. Dunn, B. L. Hogan and K. S. Zaret (2001). "Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm." Genes Dev **15**(15): 1998-2009.

Ryan, M. C., R. Tizard, D. R. VanDevanter and W. G. Carter (1994). "Cloning of the LamA3 gene encoding the alpha 3 chain of the adhesive ligand epiligrin. Expression in wound repair." J Biol Chem **269**(36): 22779-22787.

Sackett, S. D., Y. Gao, S. Shin, Y. B. Esterson, A. Tsingalia, R. S. Hurtt, K. Brondell, K. H. Kaestner and L. E. Greenbaum (2009). "Foxl1 promotes liver repair following cholestatic injury in mice." Lab Invest **89**(12): 1387-1396.

Sanchez-Duffhues, G., C. Hiepen, P. Knaus and P. Ten Dijke (2015). "Bone morphogenetic protein signaling in bone homeostasis." Bone **80**: 43-59.

Sanchez, A., V. M. Factor, I. S. Schroeder, P. Nagy and S. S. Thorgeirsson (2004). "Activation of NF-kappaB and STAT3 in rat oval cells during 2-acetylaminofluorene/partial hepatectomy-induced liver regeneration." Hepatology **39**(2): 376-385.

Scharpfenecker, M., M. van Dinther, Z. Liu, R. L. van Bezooijen, Q. Zhao, L. Pukac, C. W. Lowik and P. ten Dijke (2007). "BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis." J Cell Sci **120**(Pt 6): 964-972.

Schmidt, C., F. Bladt, S. Goedecke, V. Brinkmann, W. Zschiesche, M. Sharpe, E. Gherardi and C. Birchmeier (1995). "Scatter factor/hepatocyte growth factor is essential for liver development." Nature **373**(6516): 699-702.

Schulze-Bergkamen, H., D. Brenner, A. Krueger, D. Suess, S. C. Fas, C. R. Frey, A. Dax, D. Zink, P. Buchler, M. Muller and P. H. Krammer (2004). "Hepatocyte growth factor induces Mcl-1 in primary human hepatocytes and inhibits CD95-mediated apoptosis via Akt." Hepatology **39**(3): 645-654.

Schuppan, D. (1990). "Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins." Semin Liver Dis **10**(1): 1-10.

Schuppan, D. and N. H. Afdhal (2008). "Liver cirrhosis." Lancet **371**(9615): 838-851.

Schuppan, D., M. Ruehl, R. Somasundaram and E. G. Hahn (2001). "Matrix as a modulator of hepatic fibrogenesis." Semin Liver Dis **21**(3): 351-372.

Sell, S. and H. L. Leffert (2008). "Liver cancer stem cells." J Clin Oncol **26**(17): 2800-2805.

Sherriff-Tadano, R., A. Ohta, F. Morito, M. Mitamura, Y. Haruta, S. Koarada, Y. Tada, K. Nagasawa and I. Ozaki (2006). "Antifibrotic effects of hepatocyte growth factor on scleroderma fibroblasts and analysis of its mechanism." Mod Rheumatol **16**(6): 364-371.

Shibasaki, S., S. Kitano, M. Karasaki, S. Tsunemi, H. Sano and T. Iwasaki (2015). "Blocking c-Met signaling enhances bone morphogenetic protein-2-induced osteoblast differentiation." FEBS Open Bio **5**: 341-347.

Shin, S. and K. H. Kaestner (2014). "The origin, biology, and therapeutic potential of facultative adult hepatic progenitor cells." Curr Top Dev Biol **107**: 269-292.

Shin, S., G. Walton, R. Aoki, K. Brondell, J. Schug, A. Fox, O. Smirnova, C. Dorrell, L. Erker, A. S. Chu, R. G. Wells, M. Grompe, L. E. Greenbaum and K. H. Kaestner (2011). "Foxl1-Cre-marked adult hepatic progenitors have clonogenic and bilineage differentiation potential." Genes Dev **25**(11): 1185-1192.

Shiota, G., T. Kunisada, K. Oyama, A. Udagawa, T. Nomi, K. Tanaka, A. Tsutsumi, M. Isono, T. Nakamura, H. Hamada, T. Sakatani, S. Sell, K. Sato, H. Ito and H. Kawasaki (2000). "In vivo transfer of hepatocyte growth factor gene accelerates proliferation of hepatic oval cells in a 2-acetylaminofluorene/partial hepatectomy model in rats." FEBS Lett **470**(3): 325-330.

Shiota, G., T. C. Wang, T. Nakamura and E. V. Schmidt (1994). "Hepatocyte growth factor in transgenic mice: effects on hepatocyte growth, liver regeneration and gene expression." Hepatology **19**(4): 962-972.

Sieber, C., J. Kopf, C. Hiepen and P. Knaus (2009). "Recent advances in BMP receptor signaling." Cytokine Growth Factor Rev **20**(5-6): 343-355.

Simone, S., C. Cosola, A. Loverre, M. Cariello, F. Sallustio, F. Rascio, L. Gesualdo, F. P. Schena, G. Grandaliano and G. Pertosa (2012). "BMP-2 induces a profibrotic phenotype in adult renal progenitor cells through Nox4 activation." Am J Physiol Renal Physiol **303**(1): F23-34.

Sipeki, S., E. Bander, L. Buday, G. Farkas, E. Bacsy, D. K. Ways and A. Farago (1999). "Phosphatidylinositol 3-kinase contributes to Erk1/Erk2 MAP kinase activation associated with hepatocyte growth factor-induced cell scattering." Cell Signal **11**(12): 885-890.

Song, J. J., A. J. Celeste, F. M. Kong, R. L. Jirtle, V. Rosen and R. S. Thies (1995). "Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation." Endocrinology **136**(10): 4293-4297.

Sonnenberg, E., D. Meyer, K. M. Weidner and C. Birchmeier (1993). "Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development." J Cell Biol **123**(1): 223-235.

Sosa, I., O. Cvijanovic, T. Celic, D. Cuculic, Z. Crncevic-Orlic, L. Vukelic, S. Zoricic Cvek, L. Dudaric, A. Bosnar and D. Bobinac (2011). "Hepatoregenerative role of bone morphogenetic protein-9." Med Sci Monit **17**(12): HY33-35.

Spath, G. F. and M. C. Weiss (1998). "Hepatocyte nuclear factor 4 provokes expression of epithelial marker genes, acting as a morphogen in dedifferentiated hepatoma cells." J Cell Biol **140**(4): 935-946.

Stamos, J., R. A. Lazarus, X. Yao, D. Kirchhofer and C. Wiesmann (2004). "Crystal structure of the HGF beta-chain in complex with the Sema domain of the Met receptor." EMBO J **23**(12): 2325-2335.

Starkel, P. and I. A. Leclercq (2011). "Animal models for the study of hepatic fibrosis." Best Pract Res Clin Gastroenterol **25**(2): 319-333.

Steinberg, P., R. Steinbrecher, S. Radaeva, P. Schirmacher, H. P. Dienes, F. Oesch and P. Bannasch (1994). "Oval cell lines OC/CDE 6 and OC/CDE 22 give rise to cholangio-cellular and undifferentiated carcinomas after transformation." Lab Invest **71**(5): 700-709.

Stoker, M., E. Gherardi, M. Perryman and J. Gray (1987). "Scatter factor is a fibroblast-derived modulator of epithelial cell mobility." Nature **327**(6119): 239-242.

Strick-Marchand, H., S. Morosan, P. Charneau, D. Kremsdorf and M. C. Weiss (2004). "Bipotential mouse embryonic liver stem cell lines contribute to liver regeneration and differentiate as bile ducts and hepatocytes." Proc Natl Acad Sci U S A **101**(22): 8360-8365.

Stumptner, C., A. Fuchsbichler, M. Lehner, K. Zatloukal and H. Denk (2001). "Sequence of events in the assembly of Mallory body components in mouse liver: clues to the pathogenesis and significance of Mallory body formation." J Hepatol **34**(5): 665-675.

Suarez-Causado, A., D. Caballero-Diaz, E. Bertran, C. Roncero, A. Addante, M. Garcia-Alvaro, M. Fernandez, B. Herrera, A. Porras, I. Fabregat and A. Sanchez (2015). "HGF/c-Met signaling promotes liver progenitor cell migration and invasion by an epithelial-mesenchymal transition-independent, phosphatidyl inositol-3 kinase-dependent pathway in an in vitro model." Biochim Biophys Acta **1853**(10 Pt A): 2453-2463.

Sugimoto, H., C. Yang, V. S. LeBleu, M. A. Soubasakos, M. Giraldo, M. Zeisberg and R. Kalluri (2007). "BMP-7 functions as a novel hormone to facilitate liver regeneration." Faseb J **21**(1): 256-264.

Sukowati, C. H., B. Anfuso, G. Torre, P. Francalanci, L. S. Croce and C. Tiribelli (2013). "The expression of CD90/Thy-1 in hepatocellular carcinoma: an in vivo and in vitro study." PLoS One **8**(10): e76830.

Sulpice, E., S. Ding, B. Muscatelli-Groux, M. Berge, Z. C. Han, J. Plouet, G. Tobelem and T. Merkulova-Rainon (2009). "Cross-talk between the VEGF-A and HGF signalling pathways in endothelial cells." Biol Cell **101**(9): 525-539.

Sun, H. C. and Z. Y. Tang (2004). "Angiogenesis in hepatocellular carcinoma: the retrospectives and perspectives." J Cancer Res Clin Oncol **130**(6): 307-319.

Suzuki, A., S. Sekiya, M. Onishi, N. Oshima, H. Kiyonari, H. Nakauchi and H. Taniguchi (2008). "Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver." Hepatology **48**(6): 1964-1978.

Suzuki, Y., N. Ohga, Y. Morishita, K. Hida, K. Miyazono and T. Watabe (2010). "BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo." J Cell Sci **123**(Pt 10): 1684-1692.

Syed, Z. A., W. Yin, K. Hughes, J. N. Gill, R. Shi and J. L. Clifford (2011). "HGF/c-met/Stat3 signaling during skin tumor cell invasion: indications for a positive feedback loop." BMC Cancer **11**: 180.

Tacke, F., E. Gabele, F. Bataille, R. F. Schwabe, C. Hellerbrand, F. Klebl, R. H. Straub, T. Luedde, M. P. Manns, C. Trautwein, D. A. Brenner, J. Scholmerich and B. Schnabl (2007). "Bone morphogenetic protein 7 is elevated in patients with chronic liver disease and exerts fibrogenic effects on human hepatic stellate cells." Dig Dis Sci **52**(12): 3404-3415.

Takahashi, Y., K. Sugimoto, H. Inui and T. Fukusato (2015). "Current pharmacological therapies for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis." World J Gastroenterol **21**(13): 3777-3785.

Tanaka, M., T. Itoh, N. Tanimizu and A. Miyajima (2011). "Liver stem/progenitor cells: their characteristics and regulatory mechanisms." J Biochem **149**(3): 231-239.

Tang, Y., K. Kitisin, W. Jogunoori, C. Li, C. X. Deng, S. C. Mueller, H. W. Ressom, A. Rashid, A. R. He, J. S. Mendelson, J. M. Jessup, K. Shetty, M. Zasloff, B. Mishra, E. P. Reddy, L. Johnson and L. Mishra (2008). "Progenitor/stem cells give rise to liver cancer due to aberrant TGF-beta and IL-6 signaling." Proc Natl Acad Sci U S A **105**(7): 2445-2450.

Tani, T., A. Lumme, A. Linnala, E. Kivilaakso, T. Kiviluoto, R. E. Burgeson, L. Kangas, I. Leivo and I. Virtanen (1997). "Pancreatic carcinomas deposit laminin-5, preferably adhere to laminin-5, and migrate on the newly deposited basement membrane." Am J Pathol **151**(5): 1289-1302.

Thorgeirsson, S. S. (1996). "Hepatic stem cells in liver regeneration." FASEB J **10**(11): 1249-1256.

Tilghman, S. M. and A. Belayew (1982). "Transcriptional control of the murine albumin/alpha-fetoprotein locus during development." Proc Natl Acad Sci U S A **79**(17): 5254-5257.

Tirnitz-Parker, J. E., J. N. Tonkin, B. Knight, J. K. Olynyk and G. C. Yeoh (2007). "Isolation, culture and immortalisation of hepatic oval cells from adult mice fed a choline-deficient, ethionine-supplemented diet." Int J Biochem Cell Biol **39**(12): 2226-2239.

Torrecilla, S. and J. M. Llovet (2015). "New molecular therapies for hepatocellular carcinoma." Clin Res Hepatol Gastroenterol **39 Suppl 1**: S80-85.

Townson, S. A., E. Martinez-Hackert, C. Greppi, P. Lowden, D. Sako, J. Liu, J. A. Ucran, K. Liharska, K. W. Underwood, J. Seehra, R. Kumar and A. V. Grinberg (2012). "Specificity and structure of a high affinity activin receptor-like kinase 1 (ALK1) signaling complex." J Biol Chem **287**(33): 27313-27325.

Truksa, J., H. Peng, P. Lee and E. Beutler (2006). "Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6." Proc Natl Acad Sci U S A **103**(27): 10289-10293.

Trusolino, L., A. Bertotti and P. M. Comoglio (2001). "A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth." Cell **107**(5): 643-654.

Trusolino, L., A. Bertotti and P. M. Comoglio (2010). "MET signalling: principles and functions in development, organ regeneration and cancer." Nat Rev Mol Cell Biol **11**(12): 834-848.

Tsai, S. Y., Y. L. Huang, W. H. Yang and C. H. Tang (2012). "Hepatocyte growth factor-induced BMP-2 expression is mediated by c-Met receptor, FAK, JNK, Runx2, and p300 pathways in human osteoblasts." Int Immunopharmacol **13**(2): 156-162.

Tulasne, D. and B. Foveau (2008). "The shadow of death on the MET tyrosine kinase receptor." Cell Death Differ **15**(3): 427-434.

Uehara, Y., O. Minowa, C. Mori, K. Shiota, J. Kuno, T. Noda and N. Kitamura (1995). "Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor." Nature **373**(6516): 702-705.

Ueki, T., Y. Kaneda, H. Tsutsui, K. Nakanishi, Y. Sawa, R. Morishita, K. Matsumoto, T. Nakamura, H. Takahashi, E. Okamoto and J. Fujimoto (1999). "Hepatocyte growth factor gene therapy of liver cirrhosis in rats." Nat Med **5**(2): 226-230.

Ueki, Y. and T. A. Reh (2012). "Activation of BMP-Smad1/5/8 signaling promotes survival of retinal ganglion cells after damage in vivo." PLoS One **7**(6): e38690.

Uenishi, T., S. Kubo, T. Yamamoto, T. Shuto, M. Ogawa, H. Tanaka, S. Tanaka, K. Kaneda and K. Hirohashi (2003). "Cytokeratin 19 expression in hepatocellular carcinoma predicts early postoperative recurrence." Cancer Sci **94**(10): 851-857.

Urist, M. R. (1965). "Bone: formation by autoinduction." Science **150**(698): 893-899.

Urtasun, R., M. U. Latasa, M. I. Demartis, S. Balzani, S. Goni, O. Garcia-Irigoyen, M. Elizalde, M. Azcona, R. M. Pascale, F. Feo, P. Bioulac-Sage, C. Balabaud, J. Muntane, J. Prieto, C. Berasain and M. A. Avila (2011). "Connective tissue growth factor autocriny in human hepatocellular carcinoma: oncogenic role and regulation by epidermal growth factor receptor/yes-associated protein-mediated activation." Hepatology **54**(6): 2149-2158.

Usunier, B., M. Benderitter, R. Tamarat and A. Chapel (2014). "Management of fibrosis: the mesenchymal stromal cells breakthrough." Stem Cells Int **2014**: 340257.

Utley, S., D. James, N. Mavila, M. V. Nguyen, C. Vendryes, S. M. Salisbury, J. Phan and K. S. Wang (2014). "Fibroblast growth factor signaling regulates the expansion of A6-expressing hepatocytes in association with AKT-dependent beta-catenin activation." J Hepatol **60**(5): 1002-1009.

Valdes, F., A. M. Alvarez, A. Locascio, S. Vega, B. Herrera, M. Fernandez, M. Benito, M. A. Nieto and I. Fabregat (2002). "The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes." Mol Cancer Res **1**(1): 68-78.

van Caam, A., E. Blaney Davidson, A. Garcia de Vinuesa, E. van Geffen, W. van den Berg, M. J. Goumans, P. ten Dijke and P. van der Kraan (2015). "The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFbeta1." Osteoarthritis Cartilage **23**(6): 985-995.

Van Hul, N. K., J. Abarca-Quinones, C. Sempoux, Y. Horsmans and I. A. Leclercq (2009). "Relation between liver progenitor cell expansion and extracellular matrix deposition in a CDE-induced murine model of chronic liver injury." Hepatology **49**(5): 1625-1635.

van Meeteren, L. A., M. Thorikay, S. Bergqvist, E. Pardali, C. G. Stampino, D. Hu-Lowe, M. J. Goumans and P. ten Dijke (2012). "Anti-human activin receptor-like kinase 1 (ALK1) antibody attenuates bone morphogenetic protein 9 (BMP9)-induced ALK1 signaling and interferes with endothelial cell sprouting." J Biol Chem **287**(22): 18551-18561.

Venepalli, N. K. and L. Goff (2013). "Targeting the HGF-cMET Axis in Hepatocellular Carcinoma." Int J Hepatol **2013**: 341636.

Viebahn, C. S. and G. C. Yeoh (2008). "What fires prometheus? The link between inflammation and regeneration following chronic liver injury." Int J Biochem Cell Biol **40**(5): 855-873.

Wagner, D. O., C. Sieber, R. Bhushan, J. H. Borgermann, D. Graf and P. Knaus (2010). "BMPs: from bone to body morphogenetic proteins." Sci Signal **3**(107): mr1.

Wang, J., Y. Weng, M. Zhang, Y. Li, M. Fan, Y. Guo, Y. Sun, W. Li and Q. Shi (2016). "BMP9 inhibits the growth and migration of lung adenocarcinoma A549 cells in a bone marrow stromal cellderived microenvironment through the MAPK/ERK and NF-kappaB pathways." Oncol Rep **36**(1): 410-418.

Wang, K., H. Feng, W. Ren, X. Sun, J. Luo, M. Tang, L. Zhou, Y. Weng, T. C. He and Y. Zhang (2011). "BMP9 inhibits the proliferation and invasiveness of breast cancer cells MDA-MB-231." J Cancer Res Clin Oncol **137**(11): 1687-1696.

Wang, S., A. Sun, L. Li, G. Zhao, J. Jia, K. Wang, J. Ge and Y. Zou (2012). "Up-regulation of BMP-2 antagonizes TGF-beta1/ROCK-enhanced cardiac fibrotic signalling through activation of Smurf1/Smad6 complex." J Cell Mol Med **16**(10): 2301-2310.

Wang, X., M. Foster, M. Al-Dhalimy, E. Lagasse, M. Finegold and M. Grompe (2003). "The origin and liver repopulating capacity of murine oval cells." Proc Natl Acad Sci U S A **100 Suppl 1**: 11881-11888.

Wang, Z. M., X. H. Yuan and H. Shen (2015). "BMP-4 induced proliferation and oriented differentiation of rat hepatic oval cells into hepatocytes." Asian Pac J Trop Med **8**(5): 412-416.

Weber, L. W., M. Boll and A. Stampfl (2003). "Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model." Crit Rev Toxicol **33**(2): 105-136.

Weidner, K. M., S. Di Cesare, M. Sachs, V. Brinkmann, J. Behrens and W. Birchmeier (1996). "Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis." Nature **384**(6605): 173-176.

Weiskirchen, R., S. K. Meurer, O. A. Gressner, J. Herrmann, E. Borkham-Kamphorst and A. M. Gressner (2009). "BMP-7 as antagonist of organ fibrosis." Front Biosci **14**: 4992-5012.

Wells, R. G. (2008). "Cellular sources of extracellular matrix in hepatic fibrosis." Clin Liver Dis **12**(4): 759-768, viii.

Weng, H. L., D. C. Feng, S. Radaeva, X. N. Kong, L. Wang, Y. Liu, Q. Li, H. Shen, Y. P. Gao, R. Mullenbach, S. Munker, T. Huang, J. L. Chen, V. Zimmer, F. Lammert, P. R. Mertens, W. M. Cai, S. Dooley and B. Gao (2013). "IFN-gamma inhibits liver progenitor cell proliferation in HBV-infected patients and in 3,5-diethoxycarbonyl-1,4-dihydrocollidine diet-fed mice." J Hepatol **59**(4): 738-745.

Williams, J. A., S. Manley and W. X. Ding (2014). "New advances in molecular mechanisms and emerging therapeutic targets in alcoholic liver diseases." World J Gastroenterol **20**(36): 12908-12933.

Williams, M. J., A. D. Clouston and S. J. Forbes (2014). "Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion." Gastroenterology **146**(2): 349-356.

Wu, K., J. Ding, C. Chen, W. Sun, B. F. Ning, W. Wen, L. Huang, T. Han, W. Yang, C. Wang, Z. Li, M. C. Wu, G. S. Feng, W. F. Xie and H. Y. Wang (2012). "Hepatic transforming growth factor beta gives rise to tumor-initiating cells and promotes liver cancer development." Hepatology **56**(6): 2255-2267.

Xia, J. L., C. Dai, G. K. Michalopoulos and Y. Liu (2006). "Hepatocyte growth factor attenuates liver fibrosis induced by bile duct ligation." Am J Pathol **168**(5): 1500-1512.

Xiang, L., C. Liang, K. Zhen-Yong, Y. Liang-Jun and D. Zhong-Liang (2012). "BMP9-induced osteogenetic differentiation and bone formation of muscle-derived stem cells." J Biomed Biotechnol **2012**: 610952.

Xiang, S., H. H. Dong, H. F. Liang, S. Q. He, W. Zhang, C. H. Li, B. X. Zhang, B. H. Zhang, K. Jing, S. Tomlinson, N. van Rooijen, L. Jiang, K. Cianflone and X. P. Chen (2012). "Oval cell response is attenuated by depletion of liver resident macrophages in the 2-AAF/partial hepatectomy rat." PLoS One **7**(4): e35180.

Xiu, M., Y. H. Liu, D. R. Brigstock, F. H. He, R. J. Zhang and R. P. Gao (2012). "Connective tissue growth factor is overexpressed in human hepatocellular carcinoma and promotes cell invasion and growth." World J Gastroenterol **18**(47): 7070-7078.

Xu, C. P., W. M. Ji, G. R. van den Brink and M. P. Peppelenbosch (2006). "Bone morphogenetic protein-2 is a negative regulator of hepatocyte proliferation downregulated in the regenerating liver." World J Gastroenterol **12**(47): 7621-7625.

Xu, L., Y. Liu, Y. Hou, K. Wang, Y. Wong, S. Lin and G. Li (2015). "U0126 promotes osteogenesis of rat bone-marrow-derived mesenchymal stem cells by activating BMP/Smad signaling pathway." Cell Tissue Res **359**(2): 537-545.

Xu, Y., J. Zhou, T. E. Carey, J. B. McHugh, J. J. Voorhees and G. J. Fisher (2012). "Receptor-type Protein tyrosine phosphatase beta regulates met phosphorylation and function in head and neck squamous cell carcinoma." Neoplasia **14**(11): 1015-1022.

Yamazaki, Y., R. Moore and M. Negishi (2011). "Nuclear receptor CAR (NR1I3) is essential for DDC-induced liver injury and oval cell proliferation in mouse liver." Lab Invest **91**(11): 1624-1633.

Yang, A. T., D. D. Hu, P. Wang, M. Cong, T. H. Liu, D. Zhang, Y. M. Sun, W. S. Zhao, J. D. Jia and H. You (2016). "TGF-beta1 Induces the Dual Regulation of Hepatic Progenitor Cells with Both Anti- and Proliver Fibrosis." Stem Cells Int **2016**: 1492694.

Yang, L., S. Li, H. Hatch, K. Ahrens, J. G. Cornelius, B. E. Petersen and A. B. Peck (2002). "In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells." Proc Natl Acad Sci U S A **99**(12): 8078-8083.

Yang, X. R., Y. Xu, B. Yu, J. Zhou, S. J. Qiu, G. M. Shi, B. H. Zhang, W. Z. Wu, Y. H. Shi, B. Wu, G. H. Yang, Y. Ji and J. Fan (2010). "High expression levels of putative hepatic stem/progenitor cell biomarkers related to tumour angiogenesis and poor prognosis of hepatocellular carcinoma." Gut **59**(7): 953-962.

Yang, Y. L., H. Z. Ju, S. F. Liu, T. C. Lee, Y. W. Shih, L. Y. Chuang, J. Y. Guh, Y. Y. Yang, T. N. Liao, T. J. Hung and M. Y. Hung (2011). "BMP-2 suppresses renal interstitial fibrosis by regulating epithelial-mesenchymal transition." J Cell Biochem **112**(9): 2558-2565.

Yang, Z. F., D. W. Ho, M. N. Ng, C. K. Lau, W. C. Yu, P. Ngai, P. W. Chu, C. T. Lam, R. T. Poon and S. T. Fan (2008). "Significance of CD90+ cancer stem cells in human liver cancer." Cancer Cell **13**(2): 153-166.

Yang, Z. F., P. Ngai, D. W. Ho, W. C. Yu, M. N. Ng, C. K. Lau, M. L. Li, K. H. Tam, C. T. Lam, R. T. Poon and S. T. Fan (2008). "Identification of local and circulating cancer stem cells in human liver cancer." Hepatology **47**(3): 919-928.

Yanger, K., D. Knigin, Y. Zong, L. Maggs, G. Gu, H. Akiyama, E. Pikarsky and B. Z. Stanger (2014). "Adult hepatocytes are generated by self-duplication rather than stem cell differentiation." Cell Stem Cell **15**(3): 340-349.

Yano, R., H. M. Golbar, T. Izawa, O. Sawamoto, M. Kuwamura and J. Yamate (2015). "Participation of bone morphogenetic protein (BMP)-6 and osteopontin in cisplatin (CDDP)-induced rat renal fibrosis." Exp Toxicol Pathol **67**(2): 99-107.

Yao, Y., M. Jumabay, A. Ly, M. Radparvar, A. H. Wang, R. Abdmaulen and K. I. Bostrom (2012). "Crossveinless 2 regulates bone morphogenetic protein 9 in human and mouse vascular endothelium." Blood **119**(21): 5037-5047.

Ye, F., Y. Y. Jing, S. W. Guo, G. F. Yu, Q. M. Fan, F. F. Qu, L. Gao, Y. Yang, D. Wu, Y. Meng, F. H. Yu and L. X. Wei (2014). "Proliferative ductular reactions correlate with hepatic progenitor cell and predict recurrence in HCC patients after curative resection." Cell Biosci **4**(1): 50.

Ye, L., H. Kynaston and W. G. Jiang (2008). "Bone morphogenetic protein-9 induces apoptosis in prostate cancer cells, the role of prostate apoptosis response-4." Mol Cancer Res **6**(10): 1594-1606.

Ye, L., J. M. Lewis-Russell, G. Davies, A. J. Sanders, H. Kynaston and W. G. Jiang (2007). "Hepatocyte growth factor up-regulates the expression of the bone morphogenetic protein (BMP) receptors, BMPR-IB and BMPR-II, in human prostate cancer cells." Int J Oncol **30**(2): 521-529.

Ye, L., J. M. Lewis-Russell, A. J. Sanders, H. Kynaston and W. G. Jiang (2008). "HGF/SF up-regulates the expression of bone morphogenetic protein 7 in prostate cancer cells." Urol Oncol **26**(2): 190-197.

Yeoh, G. C., M. Ernst, S. Rose-John, B. Akhurst, C. Payne, S. Long, W. Alexander, B. Croker, D. Grail and V. B. Matthews (2007). "Opposing roles of gp130-mediated STAT-3 and ERK-1/ 2 signaling in liver progenitor cell migration and proliferation." Hepatology **45**(2): 486-494.

Yin, L., M. Sun, Z. Ilic, H. L. Leffert and S. Sell (2002). "Derivation, characterization, and phenotypic variation of hepatic progenitor cell lines isolated from adult rats." Hepatology **35**(2): 315-324.

Yin, S., J. Li, C. Hu, X. Chen, M. Yao, M. Yan, G. Jiang, C. Ge, H. Xie, D. Wan, S. Yang, S. Zheng and J. Gu (2007). "CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity." Int J Cancer **120**(7): 1444-1450.

Yokoo, H., T. R. Harwood, D. Racker and S. Arak (1982). "Experimental production of Mallory bodies in mice by diet containing 3,5-diethoxycarbonyl-1,4-dihydrocollidine." Gastroenterology **83**(1 Pt 1): 109-113.

Yoshimatsu, Y., Y. G. Lee, Y. Akatsu, L. Taguchi, H. I. Suzuki, S. I. Cunha, K. Maruyama, Y. Suzuki, T. Yamazaki, A. Katsura, S. P. Oh, T. A. Zimmers, S. J. Lee, K. Pietras, G. Y. Koh, K. Miyazono and T. Watabe (2013). "Bone morphogenetic protein-9 inhibits lymphatic vessel formation via activin receptor-like kinase 1 during development and cancer progression." Proc Natl Acad Sci U S A **110**(47): 18940-18945.

You, H., W. Ding, H. Dang, Y. Jiang and C. B. Rountree (2011). "c-Met represents a potential therapeutic target for personalized treatment in hepatocellular carcinoma." Hepatology **54**(3): 879-889.

Young, K., E. Tweedie, B. Conley, J. Ames, M. FitzSimons, P. Brooks, L. Liaw and C. P. Vary (2015). "BMP9 Crosstalk with the Hippo Pathway Regulates Endothelial Cell Matricellular and Chemokine Responses." PLoS One **10**(4): e0122892.

Yovchev, M. I., P. N. Grozdanov, B. Joseph, S. Gupta and M. D. Dabeva (2007). "Novel hepatic progenitor cell surface markers in the adult rat liver." Hepatology **45**(1): 139-149.

Yovchev, M. I., P. N. Grozdanov, H. Zhou, H. Racherla, C. Guha and M. D. Dabeva (2008). "Identification of adult hepatic progenitor cells capable of repopulating injured rat liver." Hepatology **47**(2): 636-647.

Yovchev, M. I., J. Zhang, D. S. Neufeld, P. N. Grozdanov and M. D. Dabeva (2009). "Thymus cell antigen-1-expressing cells in the oval cell compartment." Hepatology **50**(2): 601-611.

Yuan, S. X., D. X. Wang, Q. X. Wu, C. M. Ren, Y. Li, Q. Z. Chen, Y. H. Zeng, Y. Shao, J. Q. Yang, Y. Bai, P. Zhang, Y. Yu, K. Wu, W. J. Sun and B. C. He (2016). "BMP9/p38 MAPK is essential for the antiproliferative effect of resveratrol on human colon cancer." Oncol Rep **35**(2): 939-947.

Zaret, K. S. (2002). "Regulatory phases of early liver development: paradigms of organogenesis." Nat Rev Genet **3**(7): 499-512.

Zarnegar, R. (1995). "Regulation of HGF and HGFR gene expression." EXS **74**: 33-49.

Zarnegar, R., B. Petersen, M. C. DeFrances and G. Michalopoulos (1992). "Localization of hepatocyte growth factor (HGF) gene on human chromosome 7." Genomics **12**(1): 147-150.

Zatloukal, K., C. Stumptner, A. Fuchsbichler, P. Fickert, C. Lackner, M. Trauner and H. Denk (2004). "The keratin cytoskeleton in liver diseases." J Pathol **204**(4): 367-376.

Zeisberg, M., J. Hanai, H. Sugimoto, T. Mammoto, D. Charytan, F. Strutz and R. Kalluri (2003). "BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury." Nat Med **9**(7): 964-968.

Zeisberg, M. and R. Kalluri (2008). "Reversal of experimental renal fibrosis by BMP7 provides insights into novel therapeutic strategies for chronic kidney disease." Pediatr Nephrol **23**(9): 1395-1398.

Zeisberg, M., C. Yang, M. Martino, M. B. Duncan, F. Rieder, H. Tanjore and R. Kalluri (2007). "Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition." J Biol Chem **282**(32): 23337-23347.

Zekri, A. R., M. M. Hafez, A. A. Bahnassy, Z. K. Hassan, T. Mansour, M. M. Kamal and H. M. Khaled (2008). "Genetic profile of Egyptian hepatocellular-carcinoma associated with hepatitis C virus Genotype 4 by 15 K cDNA microarray: preliminary study." BMC Res Notes **1**: 106.

Zhang, Y. W. and G. F. Vande Woude (2003). "HGF/SF-met signaling in the control of branching morphogenesis and invasion." J Cell Biochem **88**(2): 408-417.

Zhang, Y. W., L. M. Wang, R. Jove and G. F. Vande Woude (2002). "Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis." Oncogene **21**(2): 217-226.

Zhao, W. M., Y. L. Qin, Z. P. Niu, C. F. Chang, J. Yang, M. H. Li, Y. Zhou and C. S. Xu (2016). "Branches of the NF-kappaB signaling pathway regulate proliferation of oval cells in rat liver regeneration." Genet Mol Res **15**(1).

Zhao, Y., T. Song, W. Wang, J. Wang, J. He, N. Wu, M. Tang, B. He and J. Luo (2012). "P38 and ERK1/2 MAPKs act in opposition to regulate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells." PLoS One **7**(8): e43383.

Zhou, W. C., Q. B. Zhang and L. Qiao (2014). "Pathogenesis of liver cirrhosis." World J Gastroenterol **20**(23): 7312-7324.

ABSTRACT

ROLE OF BMP9 IN CHRONIC LIVER DISEASE: IN VIVO AND IN VITRO STUDIES

INTRODUCTION

The liver performs many complex functions involved in maintaining the homeostasis of the organism. A very unique aspect is its high regenerative capacity after liver damage, which is carried out by parenchymal cells. However, if the regenerative capacity of these cells is compromised, such as in chronic injury states or in presence of substances inhibiting adult hepatocyte proliferation (Falkowski *et al.* 2003; Marshall *et al.* 2005), the hepatic progenitor cells (HPCs) are activated to support or take over the regenerative process.

HPCs (better known as oval cells in rodents) constitute a bipotential cell population from adult liver. Under chronic liver disease (CLD), they become activated, expand into liver parenchyma and differentiate into cholangiocytes and/or hepatocytes to compensate for the cellular loss and to help maintain liver homeostasis; therefore contributing to sustain liver regeneration during several hepatic disorders (Riehle *et al.* 2011). However, recent evidence supports a pro-fibrogenic role for these cells (Kuramitsu *et al.* 2013); which together with the fact that they can also be target of malignant conversion and become tumor-initiating cells (Lee *et al.* 2009), adds to the confusion of which is their precise role during liver injury. Certainly, an appropriate regulation of these cells in the context of CLD could be a major determinant of the response to liver injury and its subsequent outcome. This encourages studying the molecular mechanisms involved in the pro-regenerative or pro-tumorigenic potential of the hepatic progenitor cells, still poorly understood.

Hepatocyte growth factor (HGF) and its tyrosine kinase receptor, c-Met, has been demonstrated to be critical for a successful regenerative response after acute and chronic liver damage regulating both hepatocytes and progenitors population (Borowiak *et al.* 2004; Huh *et al.* 2004; Ishikawa *et al.* 2012). This pro-regenerative role is the consequence of its powerful anti-inflammatory, anti-fibrotic and anti-apoptotic activities. Interestingly, absence of c-Met has profound effects in oval cells, affecting multiple cellular processes required for regeneration, including proliferation, survival, differentiation and migration (Ishikawa *et al.* 2012).

Besides the role of TGF-β as a master cytokine in CLD participating in multiple aspects of the fibrogenic process, recent evidence indicates that the liver is also an important target of Bone Morphogenetic Proteins (BMPs), a subfamily within TGF-β superfamily. More particularly, an implication of BMPs in CLD has been suggested, although their physiopathological role and importance remains to be fully explored. Among the members of BMPs, BMP9 has emerged as a critical regulator of liver pathology. Results from our group and others have evidenced a pro-tumorigenic action of BMP9 in HCC cells (Herrera *et al.* 2013; Li *et al.* 2013); moreover, data obtained in collaboration with Dr. Breitkopf-Heilen show that BMP9 not only interferes with the liver regenerative response but it is directly involved in liver fibrosis, therefore acting as a pro-fibrogenic factor. To date nothing is known about the possible role of BMP9 on HPCs and its contribution to the HPC-mediated regenerative process.

AIMS

1. To analyze the effect of BMP9 deletion in the expansion of oval cells *in vivo* after induction of liver damage and the overall effect on the outcome of the regenerative response.

2. To characterize the signaling pathways and biological activities triggered by BMP9 in oval cells and the potential crosstalk with the HGF/c-Met pathway using an *in vitro* model of oval cell lines.

2.1 To analyze the effect of BMP9 on oval cell proliferation, survival and migration/invasion.

2.2 To investigate the potential functional crosstalk between BMP-9 and HGF/c-Met signaling pathways and to try to identify the mechanisms involved in this interaction.

2.3 To analyze the effect of chronic exposure to BMP9 in oval cells phenotype and properties.

RESULTS

1. BMP9 REGULATES OVAL CELL EXPANSION *IN VIVO*

Our results show that BMP9 plays as a negative regulatory function in the oval cell expansion since BMP9-KO presented a greater expansion of the oval cell population than WT in response to the DDC-induced cholestatic liver injury. In addition, we found that BMP9 and its major signaling components are downregulated during the ductular reaction in the DDC model, suggesting that BMP9 signaling downregulation might be critical to allow *in vivo* expansion of oval cells

Furthermore, the increased expansion of oval cells observed in BMP9-KO mice *in vivo* is accompanied by: (i) a significant increase in the activated levels of both AKT and ERK1/2-MAPKs along the DDC treatment; (ii) an increased activation of the HGF/c-MET pathway; (iii) an enhanced hepatic inflammatory response and (vi) a decreased expression of some fibrogenic markers. All this results in a reduced liver damage in BMP9-KO mice, confirmed by measuring liver function parameters, in particular alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin total levels, in serum of DDC fed mice. Altogether, these results suggest that the absence of BMP9 results in a more efficient liver regeneration in response to DDC-induced damage. Hence, BMP9 may act as a negative regulator of the oval cell mediated liver regeneration.

2. RELEVANCE OF BMP9-MEDIATED SIGNALING IN OVAL CELL FUNCTION. CROSSTALK WITH THE HGF/c-Met PATHWAY

2.1 ALK2 mediates BMP9-triggered signaling and suppressor effects in oval cells

First, we showed that oval cells respond to BMP9 with an activation of the canonical SMAD1,5,8, pathway at different doses and time points. BMP9 inhibits oval cell proliferation measured by [^{3}H] thymidine incorporation assay and induces a pro-apoptotic effect, measured by caspase-3 activity and apoptotic index.

BMP9 also positively regulates oval cell migration and invasion, analyzed by transwells migration assays, through a mechanism that remains to be further characterized.

In an effort to further elucidate BMP9 signaling pathway in oval cells, we next studied the receptors involved. As other members of the BMP family, BMP9 binds to a heterotetrameric transmembrane receptor complex comprised by type I and type II serine threonine kinase receptors. ALK1 is the high affinity receptor for BMP9, but ALK2 has been described to mediate BMP9 signaling in some cell types (Herrera *et al.* 2009; Luo *et al.* 2010). Our results indicate that ALK2 is the main type I receptor for BMP9 in oval cells: it mediates BMP9-triggered signaling and suppressor effect in oval cells, since shALK2 cells failed to phosphorylate SMAD1,5,8 in response to BMP9 and do not show evidences of BMP9 suppressor effect .

2.2 BMP9 and HGF/c-Met signaling crosstalk in oval cells.

Our *in vivo* data have revealed an over-activation of the HGF/c-Met pathway in BMP9-KO livers as compared to WT upon DDC providing the first evidence of a potential functional crosstalk between BMP9 and HGF/c-Met pathways in the regulation of oval cells. Based on this, we then aimed to deepen in the analysis of this potential crosstalk.

Our data indicated that HGF is able to prevent the BMP9 inhibitory effects, both in terms of cell loss and apoptotic effect. Moreover, oval cells that express a mutated inactive form of c-Met receptor lacking tyrosine kinase activity (Met$^{-/-}$) show a reduction in the BMP9-induced phosphorylation of SMAD1,5,8 compared to Met$^{flx/flx}$ oval cells. Intriguingly, the counteracting effects of HGF on BMP9 inhibitory actions in Met$^{flx/flx}$ cells are concomitant with a potentiation of BMP9-triggered SMAD1,5,8 signaling and we provide evidence that ALK1/SMAD1 signaling is actually required for HGF-mediated potentiation of BMP9 signaling as well as for HGF protective effects on BMP9-suppressor action in oval cells.

Apart from counteracting the suppressor activity of BMP9, HGF/c-Met signaling significantly increases the pro-invasive activity of BMP9 in oval cells, which is associated to an up-regulation of Mmp10, 13, Lama3 and CTGF expression, suggesting a role for these molecules in the regulation of cell invasion by HGF and BMP9.

3. EFFECT OF BMP9 CHRONIC TREATMENT ON OVAL CELL PHENOTYPE AND FUNCTION

The role of BMP9 in liver fibrosis is currently poorly understood, although there are some indirect evidences that have linked BMP9 with this process (Li and Liu 2007; Bi and Ge 2014; Munoz-Felix *et al.* 2016). Unpublished work performed in collaboration with Dr. Breitkopf-Heinlein present the first direct evidence for the pro-fibrogenic role of BMP9 in liver (Breitkopf-Heinlein et al., Gut, under revision). Consistent with this, we found that BMP9 and Id1 mRNA levels are increased in the CCl_4-induced liver fibrosis experimental model. Considering the association between chronic liver disease, fibrosis and oval cell expansion, we aimed to explore the effect of a chronic exposure of oval cells to BMP9, as an *in vitro* approach to resemble the *in vivo* liver fibrosis context.

Based on a detailed phenotypic characterization of these cells, we have found that oval cells chronically treated with BMP9 (named as B9T-OC) suffer a switch towards a more epithelial and mature hepatic phenotype. Consistent with this, these cells showed an increased capacity for urea synthesis suggesting that these cells acquire hepatocyte-like specific functions.

B9T-OC behavior differs in different aspects from their normal counterparts. Thus, B9T-OCs acquire growth advantage in response to the mitogenic signals present in the serum and have shown an intrinsic greater capacity to proliferate in the absence of serum. Additionally, B9T-OCs have higher invasive capacity, and are more resistant to TGF-β cytostatic and pro-apoptotic effects than the parental cells. Importantly, we have also found that BMP9 effects on oval cell viability and apoptosis are practically abolished in B9T-OCs.

Further experiments are required to better elucidate the importance of these changes in oval cells properties in an *in vivo* pathophysiological context.

DISCUSSION

In the first part of this Thesis project, we uncover a new role for BMP9 as a negative regulator of oval cell expansion and oval cell-mediated liver regeneration. First of all, we demonstrate that the absence of BMP9 in the liver results in an amplified oval cell expansion from the periportal regions and an improved liver regeneration in DDC-treated mice, which could be related to the strong activation of PI3K/AKT and ERK1/2 signalling after DDC diet in BMP9-KO mice. We also found an up-regulation of Hgf levels and an increase in the levels of active phosphorylated c-Met in DDC-treated BMP9-KO mice that make us speculate that HGF/c-Met signaling could be responsible for the strong activation of PI3K/AKT and ERKs. The down-regulation of the expression of BMP9 and its receptors upon cholestatic injury further support a role for BMP9 as a negative regulator of oval cell expansion. Our data also reveal that BMP9-KO showed an enhanced inflammatory response concomitant with an ameliorated liver damage, as evidenced by decreased expression of fibrosis markers and decreased serum levels of biochemical markers of liver damage. These data suggest a positive role of the inflammatory response in oval cell-mediated liver regeneration as previously proposed by other authors (Evarts *et al.* 1992; Knight *et al.* 2005; Xiang *et al.* 2012).

In the second part of our study, we confirm the suppressor action of BMP9 in oval cells *in vitro* as BMP9 treatment resulted in a decrease in oval cell number and an increase in apoptosis. Moreover, we have been able to demonstrate that ALK2 mediates BMP9-triggered signaling and suppressor effects in oval cells. Although oval cells express the BMP9-high affinity receptor ALK1, its levels are much lower than those of ALK2. Thus, our results are consistent with other data in the literature showing that ALK2 can be critical, either alone or together with ALK1, to mediate BMP9 effects in different cell types (Herrera *et al.* 2009; Olsen *et al.* 2014). Interestingly, the suppressor actions of BMP9 in oval cells are not mediated by Smad1. Certainly, BMPs effects (including BMP9 effects) may rely not only on the Smad canonical pathway, but also on non-canonical signaling pathways (Garcia-Alvaro *et al.* 2015; Ohta *et al.* 2016), so further work is needed to clarify the intracellular mechanism driving BMP9-suppresor effect in oval cells. On the contrary, BMP9 might be promoting oval cell invasion through Smad1 activation being this effect in accordance with data in literature (Katsuno *et al.* 2008). Importantly, our data also indicate that in oval cells there is a functional crosstalk between the BMP9 and the HGF/c-Met signaling pathways, as previously reported for other BMPs (Imai *et al.* 2005; Ye *et al.* 2008; Shibasaki *et al.* 2015). We have found that HGF/c-Met signaling amplifies BMP9-triggered Smad activation and oval cell migration and invasion which evidence a positive signaling crosstalk, but at the same time HGF/c-Met signaling also blocks the inhibitory effects of BMP9 in oval cells, evidencing a negative functional crosstalk. Additionally, we demonstrated that ALK1 is required for counteracting BMP9 suppressor actions in oval cells and that Smad1 activation is required for both the HGF/c-Met mediated protective effects against BMP9 and the pro-migratory/invasive activity of BMP9 alone or with HGF. Many questions regarding the mechanisms involved in this novel BMP9-HGF/c-Met crosstalk operating in oval cells are still opened and further studies are needed to clarify them.

In the last part of this study we have analysed the effects of chronic treatment with BMP9 in oval cells. Our results indicate that oval cell chronically exposed to BMP9 have higher proliferative, migratory and invasive capacities and are more resistant to pro-apoptotic stimuli, such as serum withdrawal and treatment with BMP9 or TGF-β. These cells also move further in their differentiation process toward hepatocyte and acquire a more epithelial phenotype. These observations lead us to propose a role for BMP9 as a differentiation factor and promoter/stabilizer of epithelial phenotype in hepatic progenitors. While a pro-fibrotic role of BMP9 is becoming clear, its long term effect on oval cells could rather contribute to increase the regenerative potential of this cell population. Additional experimental approaches should be done to understand the in vivo implications of such effect.

MAIN CONCLUSION

This study constitutes the first evidence supporting a role for BMP9 as an important regulator of oval cell biology. Although in vivo and in vitro approaches demonstrate that BMP9 act as a suppressor signal in oval cells, the response to BMP9 in these cells is far more complex than expected. Together with the inhibitory action we also reveal a pro-invasive activity for BMP9/Smad1 signaling. Furthermore, its effects are modulated by signalling crosstalk with the HGF/c-Met pathway and depend on the time of exposure to BMP9. Chronic treatment with BMP9 confers advantages to the cells that reverse the suppressor response and promote differentiation into hepatocytes. Altogether, our results suggest that BMP9 could significantly contribute to modulate the liver regenerative capacity by regulating oval cells. Full characterization of the mechanisms mediating these responses is needed to prove this hypothesis and may lead to novel therapeutic approaches in chronic liver disease.

REFERENCE LIST

Bi, J. and S. Ge (2014). "Potential roles of BMP9 in liver fibrosis." Int J Mol Sci 15(11): 20656-20667.

Borowiak, M., A. N. Garratt, T. Wustefeld, M. Strehle, C. Trautwein and C. Birchmeier (2004). "Met provides essential signals for liver regeneration." Proc Natl Acad Sci U S A 101(29): 10608-10613.

Evarts, R. P., H. Nakatsukasa, E. R. Marsden, Z. Hu and S. S. Thorgeirsson (1992). "Expression of transforming growth factor-alpha in regenerating liver and during hepatic differentiation." Mol Carcinog 5(1): 25-31.

Falkowski, O., H. J. An, I. A. Ianus, L. Chiriboga, H. Yee, A. B. West and N. D. Theise (2003). "Regeneration of hepatocyte 'buds' in cirrhosis from intrabiliary stem cells." J Hepatol 39(3): 357-364.

Garcia-Alvaro, M., A. Addante, C. Roncero, M. Fernandez, I. Fabregat, A. Sanchez and B. Herrera (2015). "BMP9-Induced Survival Effect in Liver Tumor Cells Requires p38MAPK Activation." Int J Mol Sci 16(9): 20431-20448.

Herrera, B., M. Garcia-Alvaro, S. Cruz, P. Walsh, M. Fernandez, C. Roncero, I. Fabregat, A. Sanchez and G. J. Inman (2013). "BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells." PLoS One 8(7): e69535.

Herrera, B., M. van Dinther, P. Ten Dijke and G. J. Inman (2009). "Autocrine bone morphogenetic protein-9 signals through activin receptor-like kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation." Cancer Res 69(24): 9254-9262.

Huh, C. G., V. M. Factor, A. Sanchez, K. Uchida, E. A. Conner and S. S. Thorgeirsson (2004). "Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair." Proc Natl Acad Sci U S A 101(13): 4477-4482.

Imai, Y., H. Terai, C. Nomura-Furuwatari, S. Mizuno, K. Matsumoto, T. Nakamura and K. Takaoka (2005). "Hepatocyte growth factor contributes to fracture repair by upregulating the expression of BMP receptors." J Bone Miner Res 20(10): 1723-1730.

Ishikawa, T., V. M. Factor, J. U. Marquardt, C. Raggi, D. Seo, M. Kitade, E. A. Conner and S. S. Thorgeirsson (2012). "Hepatocyte growth factor/c-met signaling is required for stem-cell-mediated liver regeneration in mice." Hepatology 55(4): 1215-1226.

Katsuno, Y., A. Hanyu, H. Kanda, Y. Ishikawa, F. Akiyama, T. Iwase, E. Ogata, S. Ehata, K. Miyazono and T. Imamura (2008). "Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway." Oncogene 27(49): 6322-6333.

Knight, B., V. B. Matthews, B. Akhurst, E. J. Croager, E. Klinken, L. J. Abraham, J. K. Olynyk and G. Yeoh (2005). "Liver inflammation and cytokine production, but not acute phase protein synthesis, accompany the adult liver progenitor (oval) cell response to chronic liver injury." Immunol Cell Biol 83(4): 364-374.

Kuramitsu, K., D. Y. Sverdlov, S. B. Liu, E. Csizmadia, L. Burkly, D. Schuppan, D. W. Hanto, L. E. Otterbein and Y. Popov (2013). "Failure of fibrotic liver regeneration in mice is linked to a severe fibrogenic response driven by hepatic progenitor cell activation." Am J Pathol 183(1): 182-194.

Lee, T. K., A. Castilho, S. Ma and I. O. Ng (2009). "Liver cancer stem cells: implications for a new therapeutic target." Liver Int 29(7): 955-965.

Li, M. X. and B. C. Liu (2007). "Epithelial to mesenchymal transition in the progression of tubulointerstitial fibrosis." Chin Med J (Engl) 120(21): 1925-1930.

Li, Q., X. Gu, H. Weng, S. Ghafoory, Y. Liu, T. Feng, J. Dzieran, L. Li, I. Ilkavets, M. Kruithof-de Julio, S. Munker, A. Marx, A. Piiper, E. A. Alonso, N. Gretz, C. Gao, S. Wolfl, S. Dooley and K. Breitkopf-Heinlein (2013). "Bone morphogenetic protein-9 (BMP-9) induces epithelial to mesenchymal transition (EMT) in hepatocellular carcinoma cells." Cancer Sci 104(3): 398-408.

Luo, J., M. Tang, J. Huang, B. C. He, J. L. Gao, L. Chen, G. W. Zuo, W. Zhang, Q. Luo, Q. Shi, B. Q. Zhang, Y. Bi, X. Luo, W. Jiang, Y. Su, J. Shen, S. H. Kim, E. Huang, Y. Gao, J. Z. Zhou, K. Yang, H. H. Luu, X. Pan, R. C. Haydon, Z. L. Deng and T. C. He (2010). "TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells." J Biol Chem 285(38): 29588-29598.

Marshall, A., S. Rushbrook, S. E. Davies, L. S. Morris, I. S. Scott, S. L. Vowler, N. Coleman and G. Alexander (2005). "Relation between hepatocyte G1 arrest, impaired hepatic regeneration, and fibrosis in chronic hepatitis C virus infection." Gastroenterology 128(1): 33-42.

Munoz-Felix, J. M., C. Cuesta, N. Perretta-Tejedor, M. Subileau, F. J. Lopez-Hernandez, J. M. Lopez-Novoa and C. Martinez-Salgado (2016). "Identification of bone morphogenetic protein 9 (BMP9) as a novel profibrotic factor in vitro." Cell Signal 28(9): 1252-1261.

Ohta, S., B. Wang, S. L. Mansour and G. C. Schoenwolf (2016). "BMP regulates regional gene expression in the dorsal otocyst through canonical and non-canonical intracellular pathways." Development 143(12): 2228-2237.

Olsen, O. E., K. F. Wader, K. Misund, T. K. Vatsveen, T. B. Ro, A. K. Mylin, I. Turesson, B. F. Stordal, S. H. Moen, T. Standal, A. Waage, A. Sundan and T. Holien (2014). "Bone morphogenetic protein-9 suppresses growth of myeloma cells by signaling through ALK2 but is inhibited by endoglin." Blood Cancer J 4: e196.

Riehle, K. J., Y. Y. Dan, J. S. Campbell and N. Fausto (2011). "New concepts in liver regeneration." J Gastroenterol Hepatol 26 Suppl 1: 203-212.

Shibasaki, S., S. Kitano, M. Karasaki, S. Tsunemi, H. Sano and T. Iwasaki (2015). "Blocking c-Met signaling enhances bone morphogenetic protein-2-induced osteoblast differentiation." FEBS Open Bio 5: 341-347.

Xiang, S., H. H. Dong, H. F. Liang, S. Q. He, W. Zhang, C. H. Li, B. X. Zhang, B. H. Zhang, K. Jing, S. Tomlinson, N. van Rooijen, L. Jiang, K. Cianflone and X. P. Chen (2012). "Oval cell response is attenuated by depletion of liver resident macrophages in the 2-AAF/partial hepatectomy rat." PLoS One 7(4): e35180.

Ye, L., J. M. Lewis-Russell, A. J. Sanders, H. Kynaston and W. G. Jiang (2008). "HGF/SF up-regulates the expression of bone morphogenetic protein 7 in prostate cancer cells." Urol Oncol 26(2): 190-197.

RESUMEN

PAPEL DE BMP9 EN LA PATOLÓGIA HEPÁTICA CRÓNICA: ESTUDIOS IN VIVO E IN VITRO

INTRODUCCIÓN

El hígado realiza numerosas funciones complejas implicadas en el mantenimiento de la homeostasis del organismo. Un aspecto muy característico es su elevada capacidad de regeneración después del daño hepático, que se lleva a cabo por las células parenquimáticas. Sin embargo, si se ve comprometida la capacidad de regeneración de estas células, como en una lesión crónica o en presencia de sustancias que inhiben la proliferación de los de hepatocitos (Falkowski *et al.* 2003; Marshall *et al.* 2005), las células progenitoras hepáticas (HPC) son activada para apoyar o asumir el proceso regenerativo.

Las HPCs (que son más conocidas en ratones como células ovales) constituyen una población de células bipotenciales del hígado adulto. Durante la patología hepática crónica, se activan, se expanden en el parénquima hepático y se diferencian a colangiocitos y/o hepatocitos para compensar la pérdida celular y ayudar a mantener la homeostasis de hígado, contribuyendo así a la regeneración del hígado en diferentes enfermedades hepáticas (Riehle *et al.* 2011). Sin embargo, algunas evidencias recientes apoyen el papel pro-fibrogénico de estas células (Kuramitsu *et al.* 2013); lo que junto al hecho de que estas células pueden ser objeto de una conversión maligna y transformarse en células iniciadoras de tumores (Lee *et al.* 2009) contribuye a alimentar la polémica sobre cual es el papel real que juegan durante el daño hepático. Ciertamente, una adecuada regulación de estas células en el contexto de la patología hepática crónica podría ser un determinante importante en la respuesta a la lesión hepática y su posterior desenlace. Todo esto anima a estudiar los mecanismos moleculares implicados en el potencial pro-regenerativo o pro-tumorigénico de las HPC, aún poco conocidos.

El factor de crecimiento hepático (HGF) y su receptor tirosina quinasa, c-Met son esenciales para promover una eficaz respuesta regenerativa después del daño hepático agudo y crónico, actuando en los hepatocitos así como en las HPC (Borowiak *et al.* 2004; Huh *et al.* 2004; Ishikawa *et al.* 2012). Este papel pro-regenerativo es la consecuencia de sus actividades anti-inflamatorias, anti-fibróticas y anti-apoptóticas. Es interesante destacar que la ausencia de c-Met tiene profundos efectos en las células ovales, afectando múltiples procesos celulares necesarios para la regeneración, incluyendo la proliferación, supervivencia, diferenciación y migración (Ishikawa *et al.* 2012).

Junto al papel conocido del TGF-β como citoquina clave en la patología hepática crónica por su participación en múltiples aspectos del proceso fibrogénico, evidencias recientes indican que el hígado es también una diana de la acción de las proteínas morfogenéticas de hueso (BMP, “Bone morphogenetic proteins”), que pertenecen a la superfamilia del TGF-β. Particularmente, se ha sugerido una posible implicación de las BMPs en la enfermedad hepática crónica, aunque su papel concreto y su importancia fisiopatológica aún no se han explorado completamente. Entre las BMPs, BMP9 ha emergido recientemente como un regulador crítico de la patología hepática. Específicamente, los resultados de nuestro grupo y otros han puesto de manifiesto una acción pro-tumorigénica de BMP9 en células HCC (Herrera *et al.* 2013; Li *et al.* 2013);

además, los datos obtenidos en colaboración con la Dra Breitkopf-Heinlein muestran que BMP9 no sólo interfiere con la respuesta regenerativa del hígado, sino que está directamente implicado en la fibrosis hepática, actuando por lo tanto como un factor pro-fibrogénico. Actualmente, no se sabe nada sobre el posible papel de BMP9 en las células HPC y su contribución al proceso regenerativo mediado por esta células.

OBJETIVOS

1. Analizar el efecto de la ausencia de BMP9 en la expansión de las células ovales *in vivo* después de la inducción de un daño hepático y su efecto global sobre el desenlace de la respuesta regenerativa.

2. Caracterizar las vías de señalización y las actividades biológicas desencadenadas por BMP9 en células ovales y su potencial interacción con la vía HGF/c-Met, utilizando modelos *in vitro* de líneas de células ovales.

- **2.1** Analizar el efecto de BMP9 sobre la proliferación, supervivencia, migración e invasión de células ovales.
- **2.2** Investigar la posible interacción funcional entre las vías de señalización de BMP9 y HGF/c-Met e intentar identificar los mecanismos implicados en esta interacción.
- **2.3** Analizar el efecto de la exposición crónica de las células ovales a BMP9 sobre sus propiedades fenotípicas y funcionales.

RESULTADOS

1. BMP9 REGULA LA EXPANSION DE LAS CÉLULAS OVALES *IN VIVO*

Nuestros resultados muestran que BMP9 actúa como un regulador negativo en la expansión de células ovales ya que los ratones BMP9-KO presentaron una mayor expansión de la población de células ovales respecto a los WT en respuesta a la lesión hepática colestásica inducida por el DDC. Además, se encontró que la expresión de BMP9 y otros componentes de su vía de señalización están disminuidas durante la reacción ductular en el modelo DDC, lo que sugiere que la regulación negativa de la señalización de BMP9 podría ser crítica para permitir la expansión de células ovales *in vivo*.

Además, la mayor expansión de células ovales observada en ratones BMP9-KO *in vivo* está acompañada por: (i) un aumento significativo en los niveles de AKT y ERK1/2-MAPKs fosforiladas/activadas a lo largo del tratamiento DDC; (ii) una activación incrementada de la vía de HGF/c-MET; (iii) una respuesta inflamatoria hepática más acentuada y (vi) una expresión aminorada de algunos marcadores fibrogénicos. Todo esto da como resultado una reducción del daño hepático en los ratones BMP9-KO, confirmada mediante la medida de los parámetros séricos de función hepática, en concreto, la alanina aminotransferasa (ALT), la aspartato aminotransferasa (AST) y los niveles totales de bilirrubina, en el suero de ratones sometidos a una dieta suplementada con DDC. Todo esto indica que la ausencia de BMP9 resulta en una regeneración hepática más eficiente en respuesta al daño inducido por DDC. Por lo tanto, nuestros datos sugieren que BMP9 puede actuar como un regulador negativo de la regeneración hepática mediada por células ovales.

2. RELEVANCIA DE LA SEÑALIZACIÓN MEDIADA POR BMP9 EN LA FUNCIÓN DE LA CÉLULA OVAL. INTERACCIÓN CON LA VÍA HGF/c-MET.

2.1 ALK2 media la señalización de BMP9 y sus efectos supresores en las células ovales

En primer lugar, hemos demostrado que las células ovales responden a BMP9 con una activación de la vía canónica SMAD1,5,8 a diferentes dosis y tiempos. BMP9 inhibe la proliferación de células ovales medida por el ensayo de incorporación de [^{3}H]-timidina e induce un efecto proapoptótico, medido mediante análisis de la actividad de la caspasa-3 y el índice apoptótico.

BMP9 también regula positivamente la migración de células ovales y la invasión, análisis realizado mediante ensayos de migración/invasión en transwells, siendo este efecto mediado por mecanismos que no están del todo esclarecidos. En un esfuerzo por elucidar la vía de señalización de BMP9 en células ovales, estudiamos los receptores involucrados. Como otros miembros de la familia de BMP, BMP9 se une a un complejo de receptor transmembrana heterotetramérico compuesto por los receptores de serina treonina quinasa de tipo I y tipo II. ALK1 es el receptor de alta afinidad para BMP9, pero también se ha descrito que ALK2 media la señalización de BMP9 en algunos tipos de células (Herrera *et al.* 2009; Luo *et al.* 2010). Nuestros resultados indican que ALK2 es el principal receptor de tipo I para BMP9 en las células ovales: ALK2 media la señalización desencadenada por BMP9 y su efecto supresor en las células ovales, ya que en las células shALK2 no se observa ni la fosforilación de SMAD1,5,8 ni el efecto supresor en respuesta a BMP9.

2.2 Interacción entre la señalización de BMP9 y HGF/c-Met en las células ovales.

Nuestros datos *in vivo* han revelado una mayor activación de la vía de HGF/c-Met en el hígado de ratones BMP9-KO en comparación con los WT después del tratamiento con DDC, sugiriendo la existencia de una interacción funcional entre BMP9 y la vía de HGF/c-Met en la regulación de las células ovales. En base a esto, a continuación, quisimos profundizar en esta interacción.

Nuestros datos indican que el HGF es capaz de prevenir los efectos inhibidores de BMP9, tanto en términos de pérdida de células como de su efecto apoptótico. Por otra parte, células ovales que expresan una forma inactiva y mutada de c-Met (que carece de actividad de tirosina quinasa), células Met$^{-/-}$, presentan una reducción en la fosforilación de SMAD1,5,8 inducida por BMP9 en comparación con las células ovales Metf$^{lx/flx}$. Curiosamente, los efectos neutralizantes del HGF sobre las acciones inhibitorias de BMP9 en las células Met$^{flx/flx}$ son concomitantes con una potenciación de la señalización SMAD1,5,8 disparada por BMP9, y proporcionamos evidencias de que la señalización vía ALK1/Smad1 es necesaria para la potenciación de la señalización de BMP9 mediada por HGF, así como para los efectos protectores de HGF sobre la acción supresora de BMP9 en las células ovales.

Aparte de contrarrestar la actividad supresora de BMP9, la señalización HGF/c-Met incrementa significativamente la actividad pro-invasiva de BMP9 en las células ovales, efecto que está asociado a una inducción de la expresión de MMP10, 13, Lama3 y CTGF, lo que sugiere que estas moléculas podrían estar implicadas en la regulación de la invasión por HGF y BMP9.

3. EFFECTO DEL TRATAMIENTO CRONICO DE BMP9 SOBRE EL FENOTIPO Y LA FUNCION DE LAS CELULAS OVALES.

El papel de BMP9 en la fibrosis hepática es actualmente poco conocido, aunque hay algunas evidencias indirectas de que BMP9 estaría relacionado con este proceso (Li and Liu 2007; Bi and Ge 2014; Munoz-Felix *et al.* 2016). El trabajo todavía no publicado llevado a cabo en colaboración con la Dra Breitkopf-Heinlein representa la primera evidencia directa del papel profibrótico de BMP9 en hígado (Breitkopf-Heinlein et al., Gut, en revisión). En consonancia con esto, encontramos que los niveles de mRNA de BMP9 e Id1 están incrementados en el modelo experimental de fibrosis hepática inducida por CCl_4, lo que sugiere una correlación entre BMP9 y la fibrosis hepática. Teniendo en cuenta la asociación que existe entre la enfermedad crónica del hígado, la fibrosis y la expansión de células ovales, quisimos explorar el efecto de una exposición crónica de células ovales a BMP9, como un abordaje *in vitro* que mimetizase el contexto *in vivo* de la fibrosis hepática.

Después de una caracterización fenotípica detallada de estas células, se ha encontrado que las células crónicamente tratadas con BMP9 (denominadas B9T-OC) sufren un cambio fenotípico hacia un fenotipo hepático más maduro y más epitelial. En consonancia con esto, estas células mostraron un aumento de la capacidad de síntesis de urea, lo que sugiere que estas células están adquiriendo funciones específicas de los hepatocitos.

El comportamiento de las células B9T-OC difiere de las células ovales normales en diferentes aspectos. De hecho, las células B9T-OC adquieren una ventaja de crecimiento en respuesta a las señales mitogénicas presentes en el suero, así como una mayor capacidad intrínseca para proliferar en ausencia de suero. Además, estas células poseen una mayor capacidad migratoria e invasiva y son más resistentes a los efectos citostáticos y pro-apoptóticos del TGF-β. Asimismo, hemos encontrado que los efectos de BMP9 sobre la viabilidad celular y la apoptosis se pierden casi por completo en las B9T-OC.

Se necesitan más experimentos para aclarar la importancia de estos cambios en las propiedades de las células ovales en un contexto fisiopatológico *in vivo*.

DISCUSIÓN

En la primera parte de este proyecto de tesis, hemos descubierto un nuevo papel para BMP9 como regulador negativo de la expansión de células ovales y de la regeneración hepática mediada por estas células. En primer lugar, se demuestra que la ausencia de BMP9 en el hígado da lugar a una expansión amplificada de las células ovales a partir de las regiones periportales y una mejor regeneración del hígado en los ratones tratados con DDC, lo que podría estar relacionado con la mayor activación de la señalización PI3K/Akt y ERK1/2 en respuesta a la dieta DDC en ratones BMP9-KO. Por otra parte, también encontramos un incremento en los niveles de expresión de

HGF y un aumento en los niveles de fosforilación de c-Met en los ratones BMP9-KO después del tratamiento, lo que nos hace especular que la señalización HGF/c-Met podría ser responsable de la mayor activación de PI3K/Akt y ERKs. La disminución de la expresión de BMP9 y sus receptores después de la lesión colestásica apoya aún más el papel de BMP9 como un regulador negativo de la expansión de la célula oval. Nuestros datos revelan también que los ratones BMP9-KO muestran una respuesta inflamatoria incrementada y un menor daño hepático, como se demuestra por la disminución de la expresión de marcadores de fibrosis y la disminución en los niveles séricos de marcadores bioquímicos de daño hepático. Estos datos sugieren un papel positivo para la respuesta inflamatoria en la regeneración hepática mediada por células ovales, de acuerdo con lo que ha sido previamente demostrado por otros autores (Evarts *et al.* 1992; Knight *et al.* 2005; Xiang *et al.* 2012).

En la segunda parte de nuestro estudio, hemos confirmado la acción supresora de BMP9 en las células ovales *in vitro*, ya que el tratamiento con BMP9 resulta en una disminución del número de células y en un aumento de la apoptosis. Además, se ha demostrado que ALK2 media los efectos supresores desencadenados por la señalización de BMP9 en las células ovales. Aunque las células ovales expresan el receptor de alta afinidad ALK1, sus niveles son mucho más bajos que los de ALK2. Estos resultados son consistentes con otros datos encontrados en la literatura que demuestran que ALK2, ya sea solo o junto con ALK1, puede ser crítico para mediar los efectos de BMP9 en diferentes tipos celulares (Herrera *et al.* 2009; Olsen *et al.* 2014). Resulta interesante destacar que estas acciones supresoras de BMP9 en las células ovales no están mediadas por Smad1. Se sabe que los efectos de las BMPs (incluyendo los efectos de BMP9) pueden depender no sólo de la vía Smad canónica, sino también de las vías de señalización no canónica (Garcia-Alvaro *et al.* 2015; Ohta *et al.* 2016), por tanto, es necesario seguir trabajando para dilucidar el mecanismo intracelular del que dependen los efectos supresores de BMP9 en células ovales. Contrariamente a lo observado para las acciones supresoras, BMP9 parece promover la invasión de células ovales a través de la activación Smad1, de acuerdo con datos recogidos ya en la literatura (Katsuno *et al.* 2008). Nuestro trabajo también indica que en las células ovales opera una interacción funcional entre BMP9 y la ruta de señalización de HGF/c-Met, como se ha descrito anteriormente para otras BMPs (Imai *et al.* 2005; Ye *et al.* 2008; Shibasaki *et al.* 2015). Hemos encontrado que la señalización de HGF/c-Met amplifica la activación de Smad disparada por el BMP9, y también incrementa el efecto migratorio /invasivo en las células ovales, evidenciando una señalización positiva; pero al mismo tiempo la señalización de HGF/c-Met también bloquea los efectos inhibidores de BMP9 en células ovales, lo que evidencia una interacción funcional negativa. Adicionalmente, hemos demostrado que se requiere ALK1 para contrarrestar las acciones supresoras de BMP9 en células ovales y que se requiere la activación de Smad1 tanto para los efectos protectores de HGF/c-Met frente a BMP9, como para la actividad promigratoria/invasiva de BMP9 solo o con HGF. Es evidente que quedan muchas preguntas por responder sobre los mecanismos implicados en esta nueva interacción cruzada BMP9-HGF/c-Met en células ovales, para lo cual son necesarios estudios adicionales.

En la última parte de este estudio hemos analizado los efectos del tratamiento crónico con BMP9 en células ovales. Nuestros resultados indican que las células ovales crónicamente tratadas con BMP9 presentan una mayor capacidad proliferativa, migratoria e invasiva y son más resistentes a estímulos pro-apoptóticos, como la retirada de suero y el tratamiento con BMP9 o TGF-β. Estas células están más adelantadas en su proceso de diferenciación hacia el hepatocito y además adquieren un fenotipo más epitelial. Estas observaciones nos llevan a proponer un papel para BMP9 como un factor de diferenciación y promotor/estabilizador del fenotipo epitelial en las HPC. Al mismo tiempo que el papel profibrótico de BMP9 se va consolidando, nuestros resultados apuntan a que el efecto de la exposición crónica de las células ovales a BMP9 podría por el contrario contribuir a aumentar la capacidad regenerativa de esta población celular. Experimentos adicionales serían necesarios para entender las implicaciones que estos efectos puedan tener *in vivo*.

CONCLUSIÓN FINAL

Este estudio constituye la primera evidencia que señala a BMP9 como un importante regulador de la biología de la célula oval. Aunque estudios in vivo e in vitro demuestran que BMP9 actúa como una señal supresora en las células ovales, la respuesta de estas células a BMP9 es mucho más compleja de lo esperado. Junto con su acción inhibidora, hemos encontrado que la señalización de BMP9/Smad1 dispara una respuesta pro-invasiva. Además, sus efectos son modulados por una interacción con la vía de HGF/c-Met y además dependen del tiempo de exposición a BMP9. El tratamiento crónico con BMP9 confiere a las células ciertas ventajas, como la reversión de la respuesta supresora y además promueve su diferenciación hacia hepatocitos. Conjuntamente, nuestros resultados sugieren que BMP9 podría contribuir de manera significativa a la regulación de la capacidad regenerativa del hígado, actuando sobre las células ovales. La caracterización completa de los mecanismos que median estas respuestas es necesaria para probar de manera inequívoca esta hipótesis y podría conducir al desarrollo de nuevas estrategias terapeúticas en la enfermedad hepática crónica.

BIBLIOGRAFIA

Bi, J. and S. Ge (2014). "Potential roles of BMP9 in liver fibrosis." Int J Mol Sci 15(11): 20656-20667.

Borowiak, M., A. N. Garratt, T. Wustefeld, M. Strehle, C. Trautwein and C. Birchmeier (2004). "Met provides essential signals for liver regeneration." Proc Natl Acad Sci U S A 101(29): 10608-10613.

Evarts, R. P., H. Nakatsukasa, E. R. Marsden, Z. Hu and S. S. Thorgeirsson (1992). "Expression of transforming growth factor-alpha in regenerating liver and during hepatic differentiation." Mol Carcinog 5(1): 25-31.

Falkowski, O., H. J. An, I. A. Ianus, L. Chiriboga, H. Yee, A. B. West and N. D. Theise (2003). "Regeneration of hepatocyte 'buds' in cirrhosis from intrabiliary stem cells." J Hepatol 39(3): 357-364.

Garcia-Alvaro, M., A. Addante, C. Roncero, M. Fernandez, I. Fabregat, A. Sanchez and B. Herrera (2015). "BMP9-Induced Survival Effect in Liver Tumor Cells Requires p38MAPK Activation." Int J Mol Sci 16(9): 20431-20448.

Herrera, B., M. Garcia-Alvaro, S. Cruz, P. Walsh, M. Fernandez, C. Roncero, I. Fabregat, A. Sanchez and G. J. Inman (2013). "BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells." PLoS One 8(7): e69535.

Herrera, B., M. van Dinther, P. Ten Dijke and G. J. Inman (2009). "Autocrine bone morphogenetic protein-9 signals through activin receptor-like kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation." Cancer Res 69(24): 9254-9262.

Huh, C. G., V. M. Factor, A. Sanchez, K. Uchida, E. A. Conner and S. S. Thorgeirsson (2004). "Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair." Proc Natl Acad Sci U S A 101(13): 4477-4482.

Imai, Y., H. Terai, C. Nomura-Furuwatari, S. Mizuno, K. Matsumoto, T. Nakamura and K. Takaoka (2005). "Hepatocyte growth factor contributes to fracture repair by upregulating the expression of BMP receptors." J Bone Miner Res 20(10): 1723-1730.

Ishikawa, T., V. M. Factor, J. U. Marquardt, C. Raggi, D. Seo, M. Kitade, E. A. Conner and S. S. Thorgeirsson (2012). "Hepatocyte growth factor/c-met signaling is required for stem-cell-mediated liver regeneration in mice." Hepatology 55(4): 1215-1226.

Katsuno, Y., A. Hanyu, H. Kanda, Y. Ishikawa, F. Akiyama, T. Iwase, E. Ogata, S. Ehata, K. Miyazono and T. Imamura (2008). "Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway." Oncogene 27(49): 6322-6333.

Knight, B., V. B. Matthews, B. Akhurst, E. J. Croager, E. Klinken, L. J. Abraham, J. K. Olynyk and G. Yeoh (2005). "Liver inflammation and cytokine production, but not acute phase protein synthesis, accompany the adult liver progenitor (oval) cell response to chronic liver injury." Immunol Cell Biol 83(4): 364-374.

Kuramitsu, K., D. Y. Sverdlov, S. B. Liu, E. Csizmadia, L. Burkly, D. Schuppan, D. W. Hanto, L. E. Otterbein and Y. Popov (2013). "Failure of fibrotic liver regeneration in mice is linked to a severe fibrogenic response driven by hepatic progenitor cell activation." Am J Pathol 183(1): 182-194.

Lee, T. K., A. Castilho, S. Ma and I. O. Ng (2009). "Liver cancer stem cells: implications for a new therapeutic target." Liver Int 29(7): 955-965.

Li, M. X. and B. C. Liu (2007). "Epithelial to mesenchymal transition in the progression of tubulointerstitial fibrosis." Chin Med J (Engl) 120(21): 1925-1930.

Li, Q., X. Gu, H. Weng, S. Ghafoory, Y. Liu, T. Feng, J. Dzieran, L. Li, I. Ilkavets, M. Kruithof-de Julio, S. Munker, A. Marx, A. Piiper, E. A. Alonso, N. Gretz, C. Gao, S. Wolfl, S. Dooley and K. Breitkopf-Heinlein (2013). "Bone morphogenetic protein-9 (BMP-9) induces epithelial to mesenchymal transition (EMT) in hepatocellular carcinoma cells." Cancer Sci 104(3): 398-408.

Luo, J., M. Tang, J. Huang, B. C. He, J. L. Gao, L. Chen, G. W. Zuo, W. Zhang, Q. Luo, Q. Shi, B. Q. Zhang, Y. Bi, X. Luo, W. Jiang, Y. Su, J. Shen, S. H. Kim, E. Huang, Y.

Gao, J. Z. Zhou, K. Yang, H. H. Luu, X. Pan, R. C. Haydon, Z. L. Deng and T. C. He (2010). "TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells." J Biol Chem 285(38): 29588-29598.

Marshall, A., S. Rushbrook, S. E. Davies, L. S. Morris, I. S. Scott, S. L. Vowler, N. Coleman and G. Alexander (2005). "Relation between hepatocyte G1 arrest, impaired hepatic regeneration, and fibrosis in chronic hepatitis C virus infection." Gastroenterology 128(1): 33-42.

Munoz-Felix, J. M., C. Cuesta, N. Perretta-Tejedor, M. Subileau, F. J. Lopez-Hernandez, J. M. Lopez-Novoa and C. Martinez-Salgado (2016). "Identification of bone morphogenetic protein 9 (BMP9) as a novel profibrotic factor in vitro." Cell Signal 28(9): 1252-1261.

Ohta, S., B. Wang, S. L. Mansour and G. C. Schoenwolf (2016). "BMP regulates regional gene expression in the dorsal otocyst through canonical and non-canonical intracellular pathways." Development 143(12): 2228-2237.

Olsen, O. E., K. F. Wader, K. Misund, T. K. Vatsveen, T. B. Ro, A. K. Mylin, I. Turesson, B. F. Stordal, S. H. Moen, T. Standal, A. Waage, A. Sundan and T. Holien (2014). "Bone morphogenetic protein-9 suppresses growth of myeloma cells by signaling through ALK2 but is inhibited by endoglin." Blood Cancer J 4: e196.

Riehle, K. J., Y. Y. Dan, J. S. Campbell and N. Fausto (2011). "New concepts in liver regeneration." J Gastroenterol Hepatol 26 Suppl 1: 203-212.

Shibasaki, S., S. Kitano, M. Karasaki, S. Tsunemi, H. Sano and T. Iwasaki (2015). "Blocking c-Met signaling enhances bone morphogenetic protein-2-induced osteoblast differentiation." FEBS Open Bio 5: 341-347.

Xiang, S., H. H. Dong, H. F. Liang, S. Q. He, W. Zhang, C. H. Li, B. X. Zhang, B. H. Zhang, K. Jing, S. Tomlinson, N. van Rooijen, L. Jiang, K. Cianflone and X. P. Chen (2012). "Oval cell response is attenuated by depletion of liver resident macrophages in the 2-AAF/partial hepatectomy rat." PLoS One 7(4): e35180.

Ye, L., J. M. Lewis-Russell, A. J. Sanders, H. Kynaston and W. G. Jiang (2008). "HGF/SF up-regulates the expression of bone morphogenetic protein 7 in prostate cancer cells." Urol Oncol 26(2): 190-197.

ANNEXES

ANNEX I

References of products used in this thesis:

Acetic Acid glacial (PanReac AppliChem, 141008.1611)

Acrylamide/Bisacrylamide 30%, 37.5:1 (BIO-RAD, 1610158)

Agarose low EEO (Agarose Standard) (PanReac AppliChem, A2114)

Amphotericin B (Gibco, 15290-026)

Aprotinin (from bovine lung) (Sigma, A3428)

APS (Ammonium Persulfate) (Fisher Scientific, BP179)

β-mercaptoethanol (2-Mercaptoethanol) (Sigma, M6250)

BMP9 (Recombinant Human BMP-9) (R&D SYSTEMS, 3209-BP)

Bradford reagent (BIO-RAD PROTEIN ASSAY) (BIO-RAD, 500-0006)

Bromophenol blue (BIO-RAD, 161-0404)

BS3 (Pierce™ Premium Grade BS3) (Thermo Scientific, PG82083)

BSA (Albumin Fraction V) (PanReac AppliChem, A1391)

Caspase-3 Ac-DEVD-AMC (BD Pharmingen, 556449)

CCl_4 (Carbon tetrachloride) (Sigma, 289116)

Collagen type IV Mouse (CORNING, 354233)

Crystal Violet (Sigma Ref C-0775)

DDC (3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-collidine) (Cymit Quimica, 3B-D0558)

DMEM (BioWhittaker, BE12-604F)

DMSO (Dimethyl sulfoxide) (Sigma, D8418)

DNA Ladder 1Kb (NIPPON Genetics EUROPE GmbH, MWD01)

DNase (Quiagen, 79254)

DSS (Disuccinimidyl suberate) (Thermo Scientific, 21655)

DTT (DL-Dithiothreitol) (Sigma, D5545)

dNTP MIX 10Mm each (BIOTOOLS, 20.031-4179)

Dulbecco's Phosphate Buffered Saline 10X (w/ Magnesium and w/ Calcium) (Dominique Dutscher, X0520)

ECL (Pierce ECL Western Blotting Substrate) (Thermo Scientific, 32106)

EDTA ((Ethylenedinitrilo)tetraacetic acid) (Sigma, 1233508)

EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid) (Sigma, 34596)

Ethanol (absolute for analysis) (PanReac AppliChem, A1613,0500PE)

Fast Start Universal SYBR Green Master (Rox) (Roche, 04913850001)

FBS (Life Techologies, 10270106)

Formalin solution, neutral buffered, 10% (Sigma, HT501128)

GelRed TM Nucleic Acid Gel Stain (Biotium, BTIU41003)

Glycerol (PanReac AppliChem, 131339.1211)

Glycine for molecular biology (PanReac AppliChem, A1067)

[^{3}H]-thymidine (GE Healthcare Life Sciences, TRK300)

Hepes (Sigma, H3375)

HGF (Recombinant Mouse HGF) (R&D SYSTEMS, 2207-HG)

Leupeptin (Sigma, L5793)

Luciferase Assay System (Promega, E1500)

Methanol (Panreac, 141091.1211)

Mineral Oil (Sigma, M5904)

NaCl (Panreac, 131689.1214)

Na_2CO_3 (Sodium carbonate) (Sigma, S7795)

NaF (Sodium fluoride) (Sigma, S7920)

NaOH (Sodium hydroxide) (Panreac, 141687.1211)

NaVO3 (Sodium orthovanadate) (Sigma, S6508)

NP40 (Calbiochem, 492016)

PageRuler Plus Prestained Protein Ladder (Thermo Scientific, 26619)

Penicillin (Sigma, P3032)

PFA (Paraformaldehyde) (Sigma, P6148)

PMSF (Phenylmethanesulfonyl fluoride) (Sigma, P7626)

Ponceau S solution (Sigma, P7170)

Primer p(dT) for cDNA synthesis (ROCHE, 10814270001)

Propidium iodide (PI) (Sigma, P4170)

Protein A Sepharose (GE Healthcare Life Sciences, 17-0780-01)

Proteinase K (from Tritirachium album) (Sigma, P6556)

Puromycin dihydrochloride (from Streptomyces alboniger) (Sigma, P8833)

Ribonuclease A (from bovine pancreas) (Sigma, R5503)

RNasin Ribonuclease Inhibitor (Promega, N2111)

SDS (Sodium dodecyl sulfate) (Sigma, L4509)

Sodium deoxycholate (Sigma, D6750)

SPINREACT kit Urea 37 (SPINREACT, UREA -37)

Streptomycin (Sigma, S9137)

Super Script III RT kit, is supplied with 5X first-strand buffer, and a vial of DTT (Invitrogen, 18080-044)

Taq Polymerase (BIOTAQ DNA Pol.) (BIOLINE, BIO-21040)

TEMED (N,N,N',N'-Tetrametiletilendiamina) (PanReac AppliChem, A1148)

TGFβ1 (Human, Recombinant, CHO Cell Line) (Merck Millipore, 616455)

Thymidine (powder, BioReagent, suitable for cell culture) (Sigma, T1895)

Tissue-Tek (OCT COMPOUND) (Sakura® Finetek | VWR, 25608-930)

Trichloroacetic Acid solution 10 % (PanReac AppliChem, A3844,1000)

Trizma base (Sigma, T1503)

Triton X-100 (Sigma, X100)

Trypan Blue solution (Sigma, T8154)

Tween-20 BioChemica (PanReac AppliChem, A1389)