Establishment and characterization of several liver cell lines as tools for the study of physio-pathological cellular interplay

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Abstract

Establishment and characterization of cell lines as tools for the study of physio-pathological liver cellular interplay

The liver is the largest internal organ of the body, constituting approximately 2% to 5% of body weight in the adult and 5% in the neonate. This organ plays a central role in metabolic homeostasis and it is responsible for the synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. The liver has a peculiar and fascinating ability: it is able to regenerate itself after loss of parenchyma for surgical resection or injury caused by drugs, toxins or acute viral disease.

Considering the variety of liver functions, it is not surprising that a large number of cell types and cell–cell interactions are required for its functionality. Most of the liver functions are carried out by the hepatocytes (about 70-75% of hepatic cells); these, together with cholangiocytes (10-5 %), both of endodermal derivation, constitute the hepatic parenchyma. The other 20% made up of non-parenchymal cells, includes: 1) Kupffer cells, essential for the phagocytosis of foreign particles as well as for the cytokines production, 2) stellate cells, that store vitamin A and produce extra-cellular matrix (ECM) components, 3) sinusoidal endothelial cells, that line the hepatic sinusoids providing a large surface for nutrients absorption and 4) lymphocytes, that mediate adaptive immune responses.

A unique architectural arrangement of hepatic parenchymal and non-parenchymal cells governs liver functionality.

The need to regenerate functional liver tissue in vitro is well established in three areas of application: model tissue for drug testing, bio artificial liver supports, and potentially engineered organs for implantation into patients. The ability of the mammalian liver to regenerate in vivo indicates that within correct stimulatory environment it should be possible to grow large quantities of liver tissue in vitro.

Our interest is always been to understand the minimal environmental signals that are required to generate liver tissue that is able to perform a physiological cellular interplay with parenchymal and non-parenchymal cells. For these reasons, we performed different cellular models.

We previously described the identification and characterization of an immortalized bipotential precursor cell within the MMH lines. MMHs (from Met murine hepatocyte) are

immortalized and untransformed cell lines derived from explants of liver derived from transgenic mice expressing a constitutively active truncated human Met receptor (cyto-Met) under control of the human α1-antitrypsin transcription unit. MMH lines present an epithelial cell polarity and the expression of hepatic functions. We used the MMHs model for the study: Hepatocyte transformation; Liver differentiation and transdifferentiation (EMT); Cholesterol metabolism; Retinol binding protein regulated secretions; Hemopoiesis and Hepatic influence of HSC.

In addition, we previously isolated characterized a number of stable liver stem cell lines named RLSCs (from resident liver stem cells) that spontaneously acquire an epithelial morphology and differentiate into hepatocyte named RLSCdH (from RLSC derived Hepatocytes). Thanks to this model we understood the hepatocyte post-differentiative patterning define "zonation": their spontaneous differentiation, in fact, generates periportal hepatocytes that may be induced to switch into perivenular hepatocytes by means of the convergence of Wnt signalling on the HNF4α-driven transcription. Recently, we demonstrate that RLSCs are able to differentiate in vivo (orthotopic transplants and heterotopic transplants) in epithelial and mesenchymal derivatives. Our data suggesting, for the first time, the existence of an adult stem/precursor cell capable of providing both parenchymal and non-parenchymal components to a complex epithelial organ.

For the study of environment signals necessary to liver regeneration in vitro, is necessary the presence of sinusoidal endothelial cellular model. In fact, liver endothelium is a prime example of organ-specific microvascular differentiation and functions.

The liver sinusoidal endothelial (LSECs) cells are a morphologically and functionally unique sub-population of liver endothelial cells that form the lining of the hepatic sinusoids. They possess fenestrations that are approximately 50–150 nm in diameter and most are aggregated into groups of 10–100, so-called liver sieve plates. The diameter and number of fenestrations are altered by various liver diseases, diabetes mellitus and old age and are influenced by cytokines and hormones . Alteration in the size and number of fenestrations influences the hepatic trafficking of lipoproteins , clearance of pharmaceutical agents , liver regeneration and interactions between lymphocytes and hepatocytes. Decapillarization is a dedifferentiation process that occurs in vitro over time with SEC in culture. Determinants of endothelial cell phenotype include heterotypic contact with pericytes or smooth muscle cells, paracrine effects of epithelial cells, shear stress, and the underlying substratum. Little

is known about the determinants of the normal SEC phenotype. Proximity to liver tissue can induce the SEC phenotype, but the pathways that regulate this have not been established.
This is because endothelial primary cells are generally difficult and time–consuming to isolate, limited in number, invariably contain impurities with other cell types, and may lack the features of pathologic vasculature. For these reasons, many authors performed different immortalization strategies on freshly isolated SECs (immortalization with SV40 Middle T or Large T antigens) to obtain a cellular model for the study endothelial-specific functions. Nevertheless these approaches can induce activation of endothelial- specific pathways in aberrant way.

Object

The current study is aimed to obtain an sinusoidal endothelial cell line as in vitro system for the identification of critical microenvironment factors involved in liver cellular interplay during physiology or pathology conditions. We hypothesis to improve protocols of liver tissue engineering using the our cellular models.

Results

The current study to go beyond the limits for maintaining LSECs in vitro, thanks the effects of soluble factors released by MMH lines ("Met Murine Hepatocytes" cells are immortalised and untransformed hepatocyte) on the sinusoidal endothelial cells phenotype in vitro and in vivo. Thanks hapatocytes soluble factors, we are able to obtain a "spontaneously immortalized murine liver sinusoidal endothelial cells line". In fact, Hepatocyte Conditioned medium was able to promote a substantial expansion of sinusoidal endothelial cells and their differentiation phenotype.
In Orthotopic transplants MLECs show an intrinsic capability to organize in physiological way and are able to maintain a differentiation state. Also, MLECs are able to promote cellular interplay between parenchymal and non-parenchymal cells, as shown by a sub-endothelial localization of pericyte, in proximity of sinusoids performed by MLECs.
In vitro studies showed that MLECs cultured with hepatocyte soluble factors, maintained the greatest degree of differentiation, as showed by high levels of endothelial cells markers expression, such as CD105, CD144, MECA32, VEGFR2. Also, MLECs are able to organize in tube-like structures on matrigel-coat as in vivo. It's induced that the maintenance of cell phenotype is dependent on micro environmental signals such as paracrine interactions (cell–cell), physical-chemical factors (oxygen tension, metabolites), mechanical stimuli, and cell–extracellular matrix (ECM) interaction.

Highlights these data suggest that MMH, RLSCs and MLECs are models that permit:
-the study of molecular bases of cellular interplay in physiology and pathology;
-to improve protocols of liver tissue engineering.

Abbreviations:

SECs/LSECs: sinusoidal/Liver sinusoidal endothelial cells; HSC: hepatic stellate cells; MMH: Met murine hepatocyte; RLSCs: resident liver stem cells; MH-CM: hepatocyte conditional medium; EGM 2:endothelial instructive medium.

Introductions

Chapter 1: Liver Functions and Regeneration

1.1 Liver Anatomy and Physiology (Liver functional Unit-Acinus)

The liver is a versatile organ which plays an important role in a variety of critical functions, including the efficient uptake of amino acids, carboydrates, bile acids, cholesterol, proteins, lipids and vitamins for storage and metabolism subsequent to release into bile and /or blood. The classical *structural* unit of the liver is the hepatic lobule (Figure 1).
The boundaries of the classical lobules are defined by connective tissue septa from the capsule. When viewed in cross section, the lobule has the shape of a polygon, usually a hexagon .The angles of the hexagon are called portal areas (or portal canals, or portal tracts). The liver cells are arranged in stacks of anastomosing plates, one or two cells thick, radiating from a central vein at the centre of the lobule towards the periphery to define the basic *functional* unit of the liver, known as the acinus which also serves as a microcosm of the major hepatic microenvironments, containing the essential cellular and physiological features that define the unique architecture of the liver tissue.

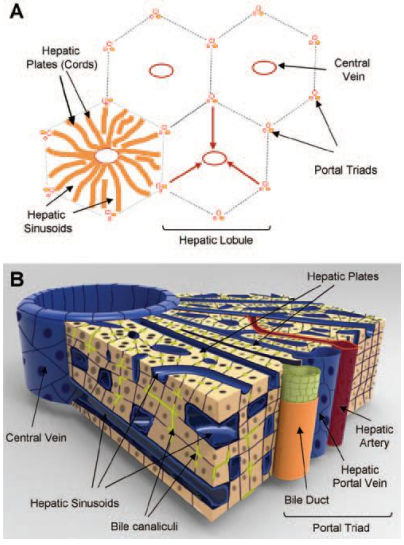

Figure 1. ***Representation of histotypic liver microstructure***. (A) Diagram of the basic hepatic lobule and acinus substructure showing the relative direction of blood flow from portal triads towards the central veins (red arrows). (B) Diagram illustrating the three-dimensional architecture of the liver between a portal triad and the central vein. The networks of bile canaliculi (yellow-green) run parallel and counter to the blood flow through the sinusoids.

Figure by LeCluyse E.L et al.,Critical review in tossicology,2012.

Hepatic plates or cords are generally one hepatocyte

thick and are separated from one another by the hepatic sinusoids (the “capillaries” of the liver) which are lined by sinusoidal endothelium.

The liver microanatomy is based on the concept of the liver lobule, which is polyhedral (hexagonal) prism of liver tissue, with a terminal hepatic venule at the center and rimmed by 6 portal tracts at the angles of a hexagon.

The lobule unit reference is used to describe the zonation of hepatocyte functions precise location of injury and other processes. The liver acinus is demarcated into three discrete zones: zone 1 is the *periportal region*; zone 2 is the *midlobular region*; and zone 3 is the *pericentral region*. Periportal hepatocytes cells are the first to regenerate. Midzonal (half-way between the portal tracts and central vein) hepatocytes receive blood that has less oxygen, toxins,etc. than periportal hepatocytes. Centrilobular hepatocytes, or hepatocytes surrounding the central veins, are most susceptible to ischemic insults and contain drug metabolizing. Blood enters the liver from the portal veins and hepatic arteries at the portal triads, flows through the sinusoidal microvasculature surrounded by the plates of parenchymal cells, and exits from the central vein. Due to the particular configuration of cells along the microvasculature and the directionality of flow through the lobular units, various chemical gradients and microenvironments are present. (Figure 2). Hepatocytes are exposed to gradients of nutrients and waste products leading to zonal metabolic specialization and zonal variation in susceptibility to ischemia and drug toxicity.

Different zones have different metabolic responsibilities, such as endogenous substrate utilization, oxygen tension, gene expression and xenobiotic clearance mechanisms.

Specific enzymatic/metabolic activities, i.e. carbohydrate metabolism, ammonia detoxification, bile formation/transport/secretion and drug biotransformation, are confined to the perivenular (PV, i.e. near the centrolobular vein) or periportal (PP, i.e. near the portal vein) zones of the hepatic lobule (41).

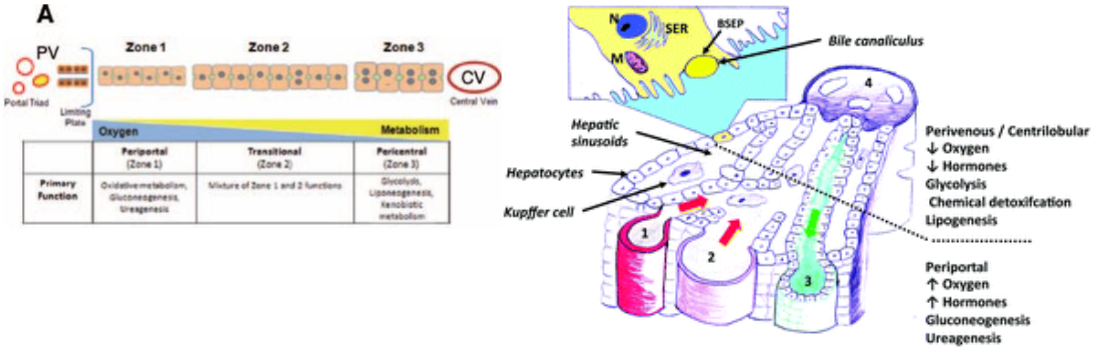

Figure 2.Structural and functional zonation of the liver. (A) Discrete zones of the liver between the portal vein (PV) and central vein (CV) illustrating the differences in cell size, phenotype and gradients in oxygen tension and metabolism.
In figure to right, is described hepatocytes metabolic zonation
Figure 2A by LeCluyse E.L et al.,Critical review in tossicology, 2012, and Figure 2B by Williams P.D. et al., 2013.

The liver is comprised of cells that are broadly into two categories: parenchymal cells and non-parenchymal cells (NPC). Hepatocytes are the most numerous and comprise 60% of the total cells and 80% of the volume of the liver. Sinusoidal endothelial cells (SECs), Kupffer cells, hepatic stellate cells (HSC), and biliary epithelium make up a significant number (3-20% each) of the remaining biologically important cells. Hepatocytes are arranged in plates or laminae of cords 1 cell thick (called muralium) that branch and anastomose in a continuous labrynth with limiting plates being at the capsule and portal regions. The 6 or more surfaces of the hapatocyte either abut adjacent parenchymal cells, border bile canaliculi, or are exposed to the perisinusoidal space (this surface being covered by microvilli). Being the workhorses of the liver, hepatocytes contain the machinery necessary to carry out the thousands of vital functions (44).
Biliary epithelium primarily acts as a lining of the conduit for bile flow but it also modifies canalicular bile and concentrates bile in the gall bladder. Biliary epithelia are “effective communicators” with neighboring cells in producing mediators that are involved in cell growth and response to injury.
The sinusoidal endothelial cells (SECs) are the primary barrier between blood and hepatocytes and they act to filter fluids, solutes, and particles between the blood and space

of Disse and represent up to 20% of the liver cells. SECs are unique type of endothelial cells in that they have fenestrae, lack a basal lamina, and can transfer molecules and particles by endocytosis. Blood cells pass through sinusoids that gently "massage" fluids though fenestrae and, being dynamically active, it is not surprising that SECs contain extensive cytoskeletal support. For these particular functions, SECs are involved in different liver disease.
Kupffer cells represent 15% of the liver cells (30% of sinusoidal cells) and are derived from circulating monocytes. They can proliferate locally, are phagocytic, are the, major producers of cytokines as mediators of inflammation and provide "cross-talk" with other cells. Hepatic stellate cells (HSC) comprise about 5% of liver cells and were previously called Ito cells or fat-storing cells. HSC normally produce extracellular matrix, control microvascular tone, store and metabolize vitamin A and lipid, and when actived transform to myofibroblasts. In the activated myofibroblast form, they typically express desmin and smooth muscle actin filaments.
The extracellular matrix (ECM) is important in the regulation and modulation of hepatic function. Five to 10% of the liver is collagen. The ECM has numerous components including matrix metalloproteinases; the glycoproteins, laminin, fibronectin, vitronectin, undulin, nidogen (entactin); and proteoglicans such heparin sulphate.

1.2 Liver regeneration

The liver has a peculiar and fascinating ability: it is able to regenerate itself after loss of parenchyma for surgical resection or injury caused by drugs, toxins or acute viral disease. The ancient myth of Prometheus highlighted this capability: the Titan Prometheus was bound for ever to a rock as punishment by Zeus for his theft of the fire; each day great eagle ate his liver and each night the liver was regenerated, only to be eaten again the next day.
The liver compensatory regeneration is a rapid and tightly orchestrated phenomenon efficiently ensuring the reacquisition of the original tissue mass and its functionality. Primarily, it involves the re-entry into cell-cycle of parenchymal hepatocytes which are able to completely recover the original liver mass (27). The liver anatomical and functional units reconstitution also requires non parenchymal cells (sinusoidal endothelial cells, cholangiocyte, Kuppfer cells, stellate cells). It is yet not clear if each cell histotype is

involved in the proliferative process or if the regeneration requires the activity of a cell with multiple differentiations potential. Recently, the bipotentiality of hepatocytes, able to divide giving rise to both hepatocytes and cholangiocytes, has been suggested. Furthermore, when injury is severe or the hepatocytes can no longer proliferate a progenitor cell population, normally a quiescent compartment is activated. A population of a small portal cells named "oval cells" is indicated a heterogeneous population of bipotent transient ampliflying cells, originating from Canale of Hering (16). These cells are normally quiescent but, after injury, rapidly and extensively proliferate and differentiate in hepatocytes and cholangyocites .
The observation that oval cells are mixed precursor population suggests their differentiation from liver stem cells (67). Since the hepatocytes are able to regenerate themselves to compensate liver mass loss, the existence of a liver stem cells, able to drive regeneration in conditions of extreme toxicity affecting the same hepatocytes, has long been debated. Today, there is growing evidence that the liver stem cell exists and its isolation from the organ, its numerical expansion in vitro and its characterization are joint effectors in many laboratories. The interest of the scientific community in the identifications, isolation and manipulation of the hepatic stem cell also depends on the fact that the great hopes placed in the use of mature hepatocytes in cell transplantation protocols for the treatment of liver disease have been disappointed. The basis of these unsatisfactory therapeutic approaches lie in the paradox, not yet resolved, of the inability of hepatocytes, which show in vivo a virtually unlimited proliferative potential, to grow in vitro to quantitatively and qualitatively amount suitable for cell transplantation in adults

1.3 Hepatocytes

The parenchymal cells or hepatocytes are highly differentiated epithelial cells that comprise the cell plates of the liver lobule (Figure 4). They perform a majority of the physiological functions commonly associated with the liver, including xenobiotic biotransformation and elimination. Hepatocytes are involved in protein, steroid, and fat metabolism as well as vitamin, iron, and sugar storage and display marked morphologic, biochemical and functional heterogeneity based on their zonal location. Under healthy non adaptive conditions, parenchymal cell size increases from Zone 1 to Zone 3, accompanied by

distinctive zonal variations in morphological features of the cells, such as mitochondria, endoplasmic reticulum, lipid vesicles and glycogen granules (Figure 2A).

Much of the functional diversity of hepatocytes is also revealed in their cytological features. Hepatocytes are cuboidal in shape and possess one or more nuclei with prominent nucleoli (Figure 4). The fraction of hepatocytes that are polyploid (4N and 8N), which results from mitotic division of the nucleus without accompanying cytokinesis, increases across the liver lobule from Zone 1 to Zone 3. Generally, hepatocytes possess abundant mitochondria with Golgi complexes localized mainly adjacent to the bile canaliculi. The cytoplasm is rich in both rough endoplasmic reticulum (RER), which is indicative of the hepatocyte's secretory nature, and smooth endoplasmic reticulum (SER), with many of the enzymes involved in phase 1 and 2 biotransformation of drugs and other xenobiotics. Lysosomes are scattered throughout the cytoplasm and play a central role in the degradation of extracellular and intracellular macromolecules including organelles and proteins (autophagy) that results from environmental stress, such as nutrient or serum deprivation.

Hepatocytes are also highly polarized cells with distinct sinusoidal and canalicular junctional complexes. These membrane domains exhibit ultrastructural, compositional, and functional differences and are essential for the hepatocyte's role in the uptake, metabolism, and biliary elimination of both endogenous and exogenous substrates(45).

In the intact liver, hepatocytes exhibit efficient transport of a wide variety of endogenous and exogenous substances from blood into bile. Physiologically, biliary transport is concerned primarily with the production and secretion of bile components which are necessary for fat absorption in the gut but is also an important step in the detoxication of both endogenous and exogenous compounds. The production of bile requires the coordinated participation of transport mechanisms selectively localized to the sinusoidal and canalicular membranes of the hepatocytes. Perturbation of these transport mechanisms by drugs and other xenobiotics is one cause of intrahepatic cholestasis that can lead to accumulation of substrates to toxic levels in both the liver and plasma.

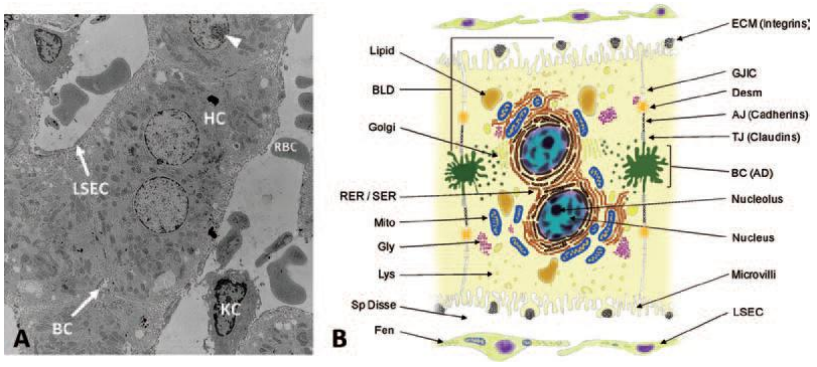

Figure 4. Histological and architectural structure of the liver parenchyma and endothelium. (A) Transmission electron micrograph of whole liver showing histotypic configuration and cytoarchitecture of hepatocytes (HC), including bile canaliculi (BC) and nucleoli (arrowhead). Sinusoids contain red blood cells (RBC) and resident macrophages (Kupffer cells, KC), and are lined with sinusoidal endothelial cells (LSEC).(B) Diagram illustrating the diverse morphological features of the mature hepatocyte including bile canaliculi, junctional complexes, and various subcellular organelles. Hepatocytes exhibit cellular polarity of subcellular organelles, cytoskeletal elements, and biochemical composition of membrane domains. BLD, basolateral domain; AD, apical domain; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; Mito, mitochondria; Gly, glycogen granules; Lys, lysosomes; Sp Disse, space of Disse; Fen, fenestrations; ECM, extracellular matrix; GJIC, gap junction intercellular communication; Desm, desmosome; AJ, adherence junction; TJ, tight junction; BC, bile canaliculi; LSEC, liver sinusoidal endothelial cell.
Figure by LeCluyse E.L et al.,Critical review in tossicology,2012.

The functional and structural specialization of the hepatocyte is related to selective activation and the sustained expression of a distinct set of gene programs encoding specific categories of proteins. The expression of hepatocyte-specific genes is primarily regulated at the transcriptional level and depends on signals from both inside and outside the cell.
Extracellular soluble (e.g. growth factors, cytokines, other hormones) and insoluble (e.g. extracellular matrix composition) signals play a major role in determining which combination of genes is expressed.

1.3.1 Hepatocytes and regeneration

Regeneration of the original liver mass after damage has been extensively studied in rodents after two-thirds partial hepatectomy (PH) (9). Regeneration of the liver depends on both hyperplasia and hypertrophy of the hepatocytes, cells that in a normal adult liver exhibit a quiescent phenotype. Hypertrophy begins within hours after PH then hyperplasia follows (65). This occurs first in the periportal region of the liver lobule then spreads toward the pericentral region.

The restoration of liver volume depends on three steps involving the hepatocytes: *i) initiation, ii) proliferation and iii) termination phases.*

The initiation steps depends on the "priming" of parenchymal cells, mainly via the signaling pathways triggered by cytokines IL-6 and TNF-α secreted by Kuppfer cells, rendering the hepatocytes sensitive to growth factors and competent to replication.

After the G0/G1 transition in the initiation phase, the hepatocytes will enter into the cell cycle (Figure 3) (65). Growth factors, primarily HGF, epidermal growth factors (EGF) and TGF- α, are responsible of this second steps of regeneration in which the hepatocytes both proliferate and grown in cell size, activating the IL6/STAT 3 and PI3/PDX1/Akt pathways respectively. The fist signaling cascade regulates the cyclin D1/p21 and also protects against cell death, for example by up-regulation FLIP, Bcl 2 and Bcl-XL. The latter pathway regulates cell size via mammalian target of rapamycin (mTOR) (28;64;58;31). Numerous growth factors (for example HGF, TGF- α, EGF, glucagon, insulin and cytokines like TNF, IL-1, IL-6 and somatostatin (SOM) are implicated in the regeneration process.

The **HGF** is a potent growth factors mainly acting on hepatocytes in a paracrine manner binding to its specific trans-membrane receptor tyrosine kinase c-met. HGF is secreted as an inactive precursor and stored in the extracellular matrix (ECM), then activated by the fibrinolitc system (38). The HGF/met signaling is transduced to its downstream mediators, i.e. the Ras-Raf- MEK, ERK 1/ 2 (7), PI3K/PDX1/Akt (55) m TOR/S6 kinase pathway, resulting in cell cycle progression.

TGF-α, is another growth factor relevant in liver regeneration (70). It belongs to the EGF family, of which all members (EGF, heparin binding EGF-like factor and amphiregulin) transduce trough the common receptor EGF receptor (EGFR) and exert overlapping functions (26). This factor acts in autocrine and paracrine fashions and its production and secretion are induced by HGF. IL-6 induces mitotic signals in hepatocytes through the

activation of STAT-3 (18) The IL-6/STAT-3 signaling involves several proteins: the IL-6 receptor, gp130, receptor-associated Janus kinase (Jak) and STAT-3. The IL-6 receptor is in a complex with gp130, which, after recognition by IL-6, transmits the signal. Jak is responsible of gp130 and STAT-3 activation after IL-6 binding. The STAT-3 form released by gp130 dimerizes and translocates to the nucleus to activate the transcription. STAT3 controls cell cycle progression from G1 to S phase regulating the expression of cyclin D1. In fact, in the liverspecific STAT3-KO model mice, mitotic activity of hepatocytes after PH is reduced significantly (43).

The PIK/PDK1/Akt signaling pathways are activated by receptor tyrosine kinases or receptors coupled with G proteins by IL-6, TGF- α, HGF, EGF, TGF- α, and others (39). An important downstream molecule of Akt for cell growth is mTOR. The activation of this pathway coexists with STAT-3 signaling. In STAT-3-KO mice no significant differences were observed macroscopically in liver regeneration in comparison to control animals, reaching the liver of these mice after PH an equal size. This observation may be explained considering the increase in size of the hepatocytes. Increase in cell size corresponds to marked phosphorylation of Akt and its downstream molecules p70 S6K, mTOR and GSK3beta (33).

The third phase in liver regeneration is the termination step. A stop signal is necessary to avoid an inappropriate liver functional size but the molecular pathways involved in this phenomenon are not yet clear. A key role is exerted by the cytokine TGF-α, secreted by hepatocytes and platelets, that inhibits DNA synthesis (50). In fact, within 2-6 hours after PH, the insulin growth factor (IGF) binding protein-1 (IGFBP-1) is produced to counteract its inhibitor effects.

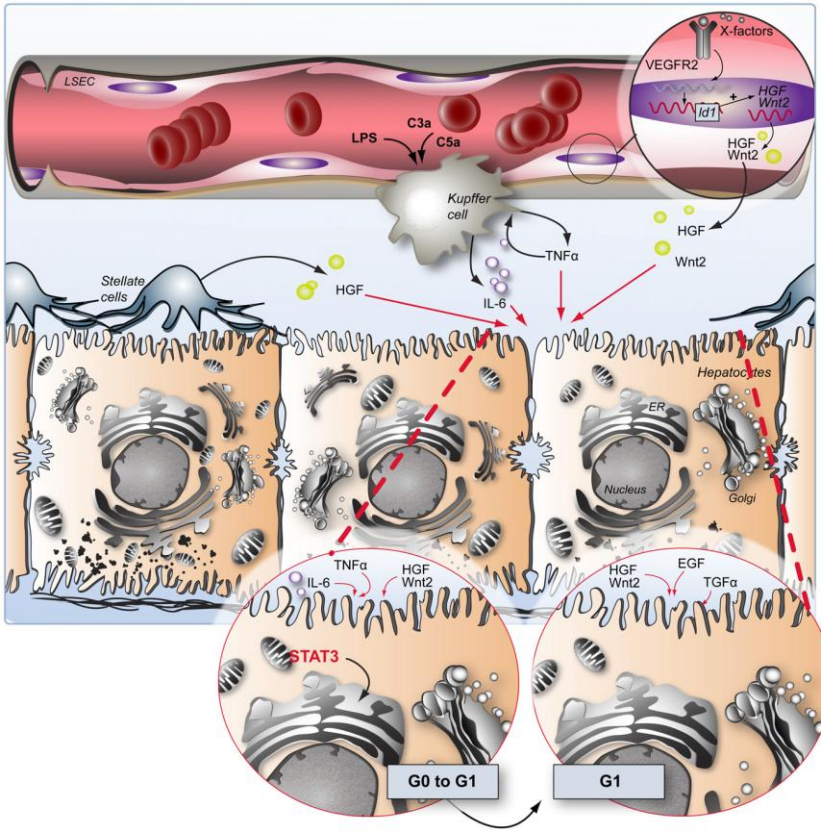

Figure 3. Schematic representation of the priming events leading to liver reconstruction after partial hepatectomy.
Hepatocytes priming is induced by the cumulative action of TNFa and IL6 secreted by activated Kupffer cells, HGF secreted by activated stellate cells, and HGF and Wnt2 secreted by LSECs.
Figure by Moniaux N. et al., 2011.

1.4 Liver sinusoidal endothelial cells (LSECs)

THE LIVER SINUSOIDAL ENDOTHELIAL CELLS (LSECs) are a morphologically and functionally unique sub-population of liver endothelial cells that form the lining of the hepatic sinusoids. These cells comprise the vast majority of endothelial cells within the liver, but differ dramatically from endothelia of other organs. They have a unique phenotype that is well integrated into the special needs of the liver. They possess fenestration and are lack of membrane basal.

Fenestrations are approximately 50–150 nm in diameter and most are aggregated into groups of 10–100, so-called liver sieve plates (19). The diameter and number of fenestrations are altered by various liver diseases, diabetes mellitus and old age and are influenced by cytokines and hormones . Alteration in the size and number of fenestrations influences the hepatic trafficking of lipoproteins, clearance of pharmaceutical agents , **liver regeneration** and interactions between lymphocytes and hepatocytes (20).This freer access to blood permits greater oxygenation of hepatocytes and more efficient clearance of drugs and perhaps also of chylomicron remnants. Cross-talk between SEC and hepatocytes may also be critical for recovery of hepatocytes from toxic injury. Capillarization is a change in the phenotype of the SEC to avascular phenotype with loss of fenestration and formation of an organized basement membrane. Capillarization is a dedifferentiation process that can be reproduced with experimental interventions in vivo and in vitro in SEC (22) but that also occurs in vitro over time with SEC in culture. Capillarization precedes the onset of alcoholic liver disease in humans and mice and has been seen in various rat models of fibrosis and cirrhosis (22). Aging is accompanied by a change in SEC phenotype with loss of fenestration but with less extensive changes in the basement membrane. Given the limited changes in the basement membrane, this change in SEC phenotype has been referred to as pseudocapillarization (21). Determinants of endothelial cell phenotype include heterotypic contact with pericytes or smooth muscle cells, paracrine effects of epithelial cells, shear stress, and the underlying substratum. Little is known about the determinants of the normal SEC phenotype. Proximity to liver tissue can induce the SEC phenotype (31), but the pathways that regulate this have not been established. One of the limitations to performing studies of SEC phenotype has been the lack of a practical marker for the normal, differentiated phenotype. While the above concepts represent significant advances in our understanding of the physiology and pathology of the unique endothelia within in liver, many aspects of the biology of these cells remain poorly understood, due in part to the

relative paucity of appropriate in vitro models. The development of several methods to isolate liver endothelial cells from experimental animals, while a significant and critically important advancement, still leaves certain limitations in term of rapid, high-troughtput, and reproducible hypothesis testing. This is because primary cells are generally difficult and time–consuming to isolate, limited in number, invariably contain impurities with other cell types, and may lack the features of pathologic vasculature. Further, the isolation procedures themselves may affect cell viability and phenotypic homogeneity(46).

A defining feature of sinusoidal endothelial cells is the presence and type of adhesion molecules. **CD31**, or platelet endothelial cell adhesion molecule, is an adhesion molecule that is present on the cell-cell junctions of most endothelial cells and that facilitates leukocyte transmigration. Immunohistochemical studies have demonstrated that differentiated SEC in situ do not express CD31 but that CD31 becomes detectable on SEC in cirrhosis and in focal nodular hyperplasia (19), presumably in areas with capillarization. In contrast, more recent studies (19) have detected CD31 on normal SEC in situ. A recent paper was able to culture human SEC over time and maintain some degree of fenestration and an absence of basement membrane. In these fenestrated SEC, CD31 could only be demonstrated by flow cytometry after permeabilization, suggesting an intracellular localization (35). If this is indeed the case, then the discrepancy in previous studies as to whether CD31 is expressed in normal SEC might be related to differences in permeabilization techniques. SEC are small, flat cells, and the localization of CD31 would not be appreciated on light microscopy of SEC in situ. More studies used also other markers as sinusoidal endothelial markers, such as: CD105 (endoglin), CD144 (VE-Cadherin), KDR (VEGF receptor 2), vWF (von-Willembrand-factor), MECA 32 (pan endothelial cell antigen), Lyve 1 (receptor scavenger 1) and caveolin.

The peculiarity of LSEC is presence of fenestrations, but no markers have been reported that specifically label fenestrations and the mechanisms for the regulation of their formation and size remain unclear. The most consistent findings of biological relevance are that fenestrations are increased by actin-disrupting agents and by the angiogenic cytokine, vascular endothelial growth factor (VEGF) (65). The mechanisms that regulate fenestrations need to be clarified in order to develop strategies to improve lipoprotein metabolism in old age and liver disease (46) and to enhance liver regeneration. Fenestrations are smaller than

the limit of resolution of light microscopy and most studies have relied upon electron microscopy with inherent problems related to fixation of tissue. Recently three dimensional structured illumination fluorescence light microscopy (3D-SIM) was applied to LSECs and their fenestrations (35). The morphology of the fenestrations and sieve plates was very effectively resolved by 3D-SIM, providing for the first time a detailed three-dimensional map of their structure.

1.4.1 Sinusoidal endothelial cells and regeneration

The precise molecular and cellular mechanism driving the regenerative capacity of the liver are still not fully comprehended. Liver regeneration after partial hepatectomy is characterized by a compensatory hyperplastic response of the mature differentiated remnant liver cells, which lasts until the original liver mass is restored.
The groundbreaking work of Ding et al.(23) recently published reveals that the liver sinusoidal endothelial cells (LSECs), which harbour a specific VEGFR2+ VEGFR3+ CD34- VE-cadherin+ factor VIII+ CD45- phenotype play a crucial role in the triggering of hepatocyte proliferation. These authors report a biphasic proliferative wave of hepatocytes during the first 3 days after partial hepatectomy, and then of LSEC from 4 day to day 8. Using knockout mice models, they showed that partial hepatectomy induced VEGFR2 activation at cell surface of LSECs, initiating Id1 up-regulation and secretion of HGF and Wnt2 angiocrine factors. The production of HGF and Wnt2, as well as the direct contact between LSECs and hepatocytes were clearly necessary conditions for the first wave of hepatocytes proliferation to occur. Subsequently, the VEGFR2-Id1 pathway promoted neoangiogenesis to ensure blood supply of the growing liver. Ding et al. raise the unsolved question of how LSECs sense partial hepatectomy and suggest that they respond to some imbalance of the inhibitory factors, which maintain the mass of the liver. These conclusions may be compared to those of recent work by Ninomiya et al.(49) showing that a decrease in liver regenerative speed caused by ERK/MEK inhibitors reduced the small-for-size syndrome in 70% or 90% partial hepatectomy in rats. The latter authors conclude that the abrupt regenerative response of hepatocytes to resection stifles the sinusoids, resulting in hypoxia of the hepatocytes and liver disfuction. A *syncronized* replication of hepatocytes and SECs is thus a crucial requirement for proper liver regeneration (Figure 5)(45).

In conclusion, Ding et al. have a cast light on a several crucial aspects of the regulation of the initial steps of liver regeneration and DNA synthesis in hepatocytes. Using VEGFR2 and Id1 knockout mice enabled them to show that HGF production by non-endothelial cells, such as stellate cells, was not a sufficient condition for hepatocytes proliferation. Secretion of HGF and Wnt2 by LSECs was also necessary. The identification and characterization of the soluble factors that promote activation of LSECs and HGF/Wnt2 secretion will provide valuable tools of future therapeutic developments. In agreement, the close contact between LSECs and hepatocytes was necessary for triggering liver regeneration. Hence, recent research findings point toward the importance of SECs in the hepatocyte response to partial hepatectomy and should impact the design of future regenerative medicine based on hepatocyte or stem cell transplantation for the treatment of end-stage liver disease.

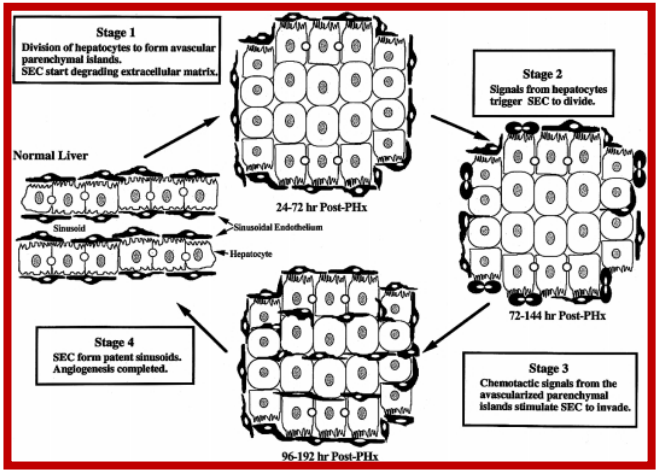

Figure 3. Schematic representation of the role of SECs during the priming events leading to liver reconstruction after partial hepatectomy.
Hepatocytes priming is induced by the cumulative action of TNFα and IL6 secreted by activated Kupffer cells, HGF secreted by activated stellate cells, and HGF and Wnt2 secreted by LSECs. Hepatocytes induces SECs proliferations at 7sh after PH. Activated SECs produce angiogenic factors and were induced by avasculated parenchymal cells to invade the liver mass. After 96-120h, SECs forms sinusoids and complet the angiogenic processes
Figure by Ross M.A. et al., 2001

1.5 Hepatic Stellate cells (HCSs)

HSC, also called perisinusoidal cells, Ito cells or fat-storing cells, reside in the space of Disse – the perisinusoidal space between the basolateral surface of hepatocytes and the anti-luminal side of sinusoidal endothelial cells (4). Under normal physiological conditions in the adult liver, HSC are morphologically characterized by extensive dendrite-like extensions that wrap around the sinusoids, essentially "embracing" the endothelial cells (29). This close contact between HSC and their neighboring cell types facilitates intercellular communication by means of soluble mediators and cytokines. HSC store vitamin A, control turnover and production of ECM, and are involved in regulation of sinusoid contractility. HSC can be identified by the expression of desmin, a typical intermediate filament protein within contractile cells. Mature HSC produce both network and fibrillar collagens (large amounts of type I collagen and lower levels of type III, IV and V collagen), large amounts of elastin and both heparan sulfate proteoglycans (HS-PG) and chondroitin sulfate proteoglycans (CS-PG). HSC also produce important cytokines and growth factors for intercellular communication in normal and injured liver. These include hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), three potent growth factors for hepatocyte proliferation during liver regeneration (4;29). TGF-α and EGF also stimulate mitosis in stellate cells themselves, creating an autocrine loop for cellular activation. Insulin-like growth factor (IGF-I and II) and platelet-derived growth factor (PDGF), among the most potent HSC mitogens, are also secreted by stellate cells.

Collectively, these factors allow HSC to influence their own gene expression and phenotype as well as that of other cells of the liver. Following liver injury, HSC become activated to a myofibroblastic (MF) phenotype characterized by a loss of vitamin A and expression of α-smooth muscle actin (α-SMA) (29). In this activated state, MF-HSC produce growth factors and cytokines, such as transforming growth factor β (TGF-β), which play a key role in the regulation of hepatocyte growth and the development of inflammatory fibrotic response of the liver (42). Connective tissue growth factor (CTGF) is also expressed by HSC and promotes fibrogenesis. HSC participate significantly in the inflammatory response of the liver through secretion of cytokines, such as macrophage colony-stimulating factor (M-CSF), which regulates macrophage accumulation and growth, interleukins-8 and -6 (IL-8, IL-6), monocyte chemotactic peptide (MCP)-1, CCL21, RANTES, CCR5, and the anti-inflammatory IL-10. Activated HSC express toll-like receptors (TLRs) allowing them to recognize bacterial endotoxin lipopolysaccharide (LPS) and function as APC. HSC also

amplify the inflammatory response by inducing infiltration of leukocytes. HSC are involved in the onset and progression of cirrhosis, which is typically associated with highly activated cells leading to a fibrotic response, a progressive increase in deposition of ECM proteins and scar tissue formation throughout the liver. A major contributing factor includes the production of the potent vasoconstrictor ET-1. ET-1 has a prominent contractile effect on HSC and MF-HSC, which may contribute to portal hypertension in the cirrhotic liver. Activated HSC also produce elevated levels of extracellular matrix proteins (e.g. collagen types I, III, IV, V) and various basal adhesion molecules (fibronectin, and laminin α1 and γ1 chains) that contribute to scar tissue formation throughout the liver (42).

1.5.1 Hepatic stellate cells (HSC) and regeneration

Hepatic stellate cells (HSCs) can be found within the progenitor cell niche in normal and regenerating liver, which is situated near the Canals of Hering (63). The functional importance of these stellate cells is supported by evidence that mouse fetal liver derived-Thy+ cells, which express classical features of hepatic stellate cells (a-SMA, desmin and vimentin), promote maturation of hepatic progenitors through cell-cell contact in culture. Similarly, another stellate cell-derived morphogen, pleiotrophin, is also secreted by stellate cells and may contribute to hepatocyte regeneration. Stellate cells may also be vital to the development of intrahepatic bile ducts during development.

In addition to their emerging role in hepatic development, there is growing evidence that stellate cells are also vital to the hepatic regenerative response in adult liver, but further investigation is urgently needed. An important study has identified neurotrophin signaling as a paracrine pathway in stellate cells that contributes to hepatocellular growth after injury, in part trough stimulation of HGF secretion by stellate cells. In a similarly approach, mice heterozygous for the FPXf1 fork head transcription factors display defective stellate cells activation after CCl4 administration, as assessed by a-SMA expression. At the same time, these animals have increased liver cell injury and apoptosis, but reduced fibrosis. Also, the animals have defective epithelium regeneration, although it is difficult to tease out potential mechanism based on these data alone (29).

The role of HSCs in hepatic regeneration would be ideally addressed if methods are developed to determine the impact of ablating or inactivating these cells on regeneration of

normal liver following partial hepatectomy. A genetic model of cell-specific deletion has been quite informative in understanding the role of hepatic macrophages in liver injury and repair, for example.
A number of potential hepatocytes mytogens are secreted by stellate cells, including HGF, epidermal growth factors, epimorphism, and pleiotrophin. As yet, however, their relative contribution and modes of regulation during hepatic regeneration have not been clarified.
Even more interesting, a subset of hepatic stellate cells express CD133 which have a pleuripotent potential in developing or adult liver. This very intriguing finding merits further exploration, as two recent studies have identified CD133 as a marker of stemlike cells in several tissue, including colon cancer.
HSCs respond to a proliferative stimulus of partial hepatectomy similarly to other liver cells, by a semi-synchronous wave of proliferation that comes to a halt when the liver has achieved its original mass. The factors initiating and terminating this proliferative surge are emerging.
Rat HSCs produce IL-6 and have receptors for IL-6, suggesting an autocrine role for IL-6 in HSC proliferation. Stimulation of rat HSCs with human recombinant IL-6 augmented HSC proliferation. In contrast, transgenic mice overexpressing the IL-6 receptor showed inhibition of hepatocyte proliferation, correlated with an increase in p21, a cell cycle inhibitor protein, suggesting that hyperstimulation with IL-6 can also inhibit liver regeneration, at least in this transgenic model.
Today, is needed to understand the role of HSCs during liver regeneration or the possible role of Stellate Progenitor Cells. this is a progenitor cells not yet identified and for which community scientific try an answer.

1.6 Liver progenitor cells

HPC are bi-potential stem cells residing in human and animal livers that are able to differentiate towards the hepatocytic and the cholangiocytic lineages. The HPC reside in a compartment contained within the canals of Hering. These canals represent the smallest and most peripheral branches of the biliary tree connecting the bile canalicular system with the interlobular ducts. In normal adult liver, HPC are small, quiescent cells with elongated or vesicular nuclei, small nucleoli and scant cytoplasm. Under normal circumstances they have

a relatively low proliferation rate and represent a reserve compartment that is activated only when the mature epithelial cells of the liver are continuously damaged or inhibited in their replication, or in cases of severe cell loss. Under these conditions, resident HPC are activated and expand from the periportal to the pericentral zone giving rise to mature hepatocytes and/or cholangiocytes). In rat liver, the HPC are activated and induced to proliferate by various hepatocarcinogens and other noxious stimuli whereupon their nuclei acquire an oval shape, thus the name ‘oval cell’ in the early literature

The HPC niche is defined as the cellular and extracellular microenvironment which supports the stem cell populations and contributes to sustain self-renewal and is composed of numerous cells, such as LSEC, HC, cholangiocytes, KC, pit cells and other inflammatory cells (1). All of these cells in combination with numerous hormones and growth factors interact and cross-talk with progenitor cells influencing their proliferative and differentiative processes. The unique microenvironment and interaction with the specific cell types is thought to be a key mechanism in regulating the maintenance of self-renewal and maturation capacities by stem cells. Nevertheless, a number of different types of signaling and adhesion molecules within the niche influence stem cell quiescence, self-renewal and cell fate decisions. In fact, this niche environment has been associated with regulating key stem cell functions, such as maintaining stem cell quiescence and providing proliferation- or maturation-inducing signals when numerous progenitor cells are required to generate mature cell lineages.

1.6.1 Liver progenitor cells and regeneration

When liver parenchyma damage is particularly serious and hepatocytes are no longer able to proliferate, liver regeneration can occur through the intervention of bipotent progenitor cells that can proliferate and differentiate into hepatocytes and bile duct cells.

It was 1950 when Wilson and Leduc, studying the regeneration of rat liver after severe nutritional damage, observed for the first time these particular cells, located within or immediately adjacent to the Canal of Hering, and their differentiation into two histological types of liver epithelial cells (77). In 1956 Faber called these cells, which are found in the liver of mice treated with carcinogens (25), "oval cells" for their morphology. The first characterization of oval cells has shown the simultaneous expression of bile ducts (CK-7,

CK-19 and OV-6) and hepatocytes (alpha-fetoprotein and albumin) markers (41). Subsequent studies have shown the activation, during oval cell compartment proliferation, of stem cell genes such as c-kit (30), CD34 (55) and LIF (56) .

Stable lines of oval cells, useful for in vitro and in vivo studies of differentiation and of liver colonization, were obtained from normal rat liver F-334, or from rats fed with DL-ethionine (63) or treated with ally alcohol (79). In addition, these precursors were stabilized starting from liver explants of animal models of Wilson disease (78) of transgenic mice expressing Ras of p53 knockout mice fed with choline-free diet and finally of human liver (24).

The oval cell is currently the best characterized liver progenitor cell although several studies have demonstrated the presence of precursors/stem cells either residing in the liver or coming from blood. Regardless of the species in which were observed and the name that was given to them, the progenitor cells of the liver have common characteristics:

- they are very few and hardly recognizable in the healthy liver, but clearly evident as a result of chronic liver injury near the terminal trait of biliary duct;
- they express cholangiocyte and hepatocyte markers;
- they are basophilic, with a high ratio of nucleus/cytoplasm and are smaller than mature hepatocytes (10 μM in diameter compared to 50 of hepatocytes);
- they are immature and have a great proliferative capacity.

Further than oval cells, other bipotential precursor cells able to differentiate and colonize diseased liver in animal models have been isolated from rodent and human livers, allowing the study of molecular mechanisms triggering their differentiation.

The development of an *oval cell reaction* in response to hepatocyte replicative senescence has also been demonstrated in a transgenic mouse model of fatty liver and DNA damage. In both humans and mice, the extent of this reaction is dependent on the severity of the damage. This so-called ‘oval cell’ or ‘ductular reaction’ amplifies a cholangiocyte derived (biliary) population before these cells differentiate into either hepatocytes or cholangiocytes.

The AAF/PH model in the rat will be used to illustrate both the cellular biology and growth factor/receptor systems involved in stem cell-energized liver regeneration. In this experimental system, a rapid and extensive proliferation of oval cells takes place after PH, first in the periportal area; later, these cells expand into the liver acinus and differentiate into small basophilic hepatocytes. (69).The powerful activation of the stem cell compartment seen in the AAF/PH model is a consequence of a close to complete mitoinhibitory effect of AAF on the adult rat hepatocytes that prevents the regeneration from the remaining liver

tissue. It has been established that proliferation of desmin-positive Ito cells is closely associated with the early stages of oval cell proliferation in the AAF/PH model. Early population of oval cells can be identified by the use of the monoclonal antibody OV-6 and distinguished from proliferating desmin-positive Ito cells. Results from a detailed time course study of activation of hepatic stem cells in the AAF/PH model, utilizing a combination of immunohistochemistry with OV-6 and desmin antibodies and autoradiography after [3H]thymidine administration shortly after the PH, indicate that the earliest population of proliferating OV-6 positive cells is located in the small bile ductules (69). In addition, these early populations of OV-6-positive cells express albumin and a-fetoprotein (AFP). These data clearly show that the majority of thymidine-labeled, OV-6-positive cells first observed after PH in the AAF/PH model reside in the bile ductules. Moreover, at the time when few of the OV-6-positive cells in the large bile ducts become labelled with thymidine, the ductular-derived OV-6-positive and thymidine labeled "oval" cells expressing both albumin and AFP have already started to infiltrate into the liver acinus (69). It therefore seems likely that the major source of oval cells, at least in the AAF/PH model, is derived from the lining cells of the biliary ductules and that these cells constitute the dormant/facultative hepatic stem cell compartment.

During normal hepatic regeneration as well as during renewal from the stem cell compartment, several growth factors appear to affect the proliferation and differentiation of hepatic cells. The question therefore arises as to whether the same growth factors known to be involved in normal hepatic regeneration are also involved in regeneration from the stem cell compartment.

There are three "primary" growth factors associated with normal liver regeneration: transforming growth factor alpha (TGF-α), hepatocyte growth factor (HGF), and acidic fibroblast growth factor (aFGF). Each of these growth factors is also capable of inducing replication of primary hepatocytes in vitro . In addition, transforming growth factor-beta 1 (TGF-β1) is also expressed during hepatic regeneration, and it has been proposed that TGF- β1 may provide at least part of the negative growth signals controlling liver size after the compensatory hyperplasia that occurs after loss of liver mass (69). The first cells entering DNA synthesis after PH in the AAF/PH model are the OV-6 and desmin-positive bile duetular and Ito cells, respectively, in the periportal area. Coincident with the appearance of these cells, an increase in the expression of TGF-α, HGF, and TGF- β1 is observed, whereas increased expression of aFGF is first seen 24 h later. All the growth

factors are then expressed at high levels throughout the period of expansion and differentiation of the oval cells and return to levels seen in normal liver at the end of the regeneration process. The cellular distribution of the growth factor transcripts differs: TGF-α and aFGF transcripts are found both in Ito cells and oval cells, whereas the HGF transcripts are only found in Ito cells. The TGF-β1 transcripts are located mainly in Ito cells, but the early population of oval cells also contain the TGF-β1 transcripts. The cellular distribution of the transcripts for all the receptors corresponding to the growth factors has revealed that all are located on oval cells. These data suggest that the same primary growth factors involved in liver regeneration from existing differentiated parenchyma are also involved in regeneration from the stem cell compartment.

Another mechanism is the ligand/receptor system, the stem cell factor (SCF)/c-kit system, which may be uniquely involved in the earliest stages of hepatic stem cell activation, was discovered. In the AAF/PH model, the expression of both SCF and c-kit is seen before the expression of AFP, and the levels of both the SCF and the c-kit transcripts decline before those of TGF-α, aFGF, HGF, and TGF-β1. It has also been shown that in contrast to TGF-a, HGF, aFGF, and TGF-β1, the SCF/c-kit system is only slightly and transiently activated in regeneration after PH in normal liver. The SCF/c-kit signal transduction system is believed to play a fundamental role in the survival, proliferation, and migration of stem cells in hematopoiesis, melanogenesis, and gametogenesis. It appears that in all cases, SCF and c-kit are involved in the early stages of stem cell activation. Whether the SCF/c-kit system in the early hepatic stem cell population interacts with other hepatic growth factors so as to influence the frequency of lineage commitment of progenitor cells is not known at present. However, the hepatic expression pattern and cellular location of the SCF/c-kit system indicate that this signal transduction system is required only during the early activation and transitional phase of the oval cell differentiation. Once the oval cells have differentiated into the small basophilic hepatocytes, the expression of both SCF and c-kit is abolished. After the hepatocyte population is reduced by, for example, PH, the residual hepatocytes proliferate promptly, continue to cycle until the deficit is repaired, and continue to function while proliferating. Under these conditions, no apparent contribution to the regeneration process is provided by the stem cell compartment. Activation of oval cell proliferation and differentiation by injury, which is more severe and/or qualitatively different from the simple loss that triggers only hepatocyte proliferation, results in transient

reestablishment of a hepatocytic lineage that has all the characteristics of a potential or facultative stem cell system.

Cells in the normally quiescent stem cell compartment are activated to produce poorly differentiated oval cell progeny. Oval cells proliferate extensively to yield a large population of cells that migrate throughout the parenchyma, some of which differentiate as they migrate. Hepatic progeny of oval cells merge into the functional compartment of mature hepatocytes and help restore the parenchyma. Similar to the generation of new hepatocytes after simple loss, the production of hepatocytes via the stem cell (oval cell) mechanism is also episodic and transient. These two distinct mechanisms of hepatocyte formation are both subjected to several points of stringent control. Controls are required to regulate the reinitiation of hepatocyte formation from the normally quiescent hepatocytes, as well as to regulate the activation of potential stem cells that energizes cell flow through the entire lineage. Although the controls may differ between the two mechanisms of hepatocyte formation, it is probable that both pathways are simultaneously activated after loss of liver mass, including that after simple PH.

One of the earliest phenotypic indications of liver stem cell activation is the expression of AFP. A transient expression of AFP is also seen after simple PH and similar to that seen in stem cell activation in the AAF/PH model, the AFP transcripts are located in the bile ductules. Expression of both SCF and c-kit is also transiently elevated after standard PH. However, there is no evidence indicating that the stem cell-derived hepatocytes significantly contribute to regeneration of liver mass after simple PH. These observations suggest that the activation, and in particular, the expansion of liver stem cells, are stringently controlled during hepatocyte-driven liver regeneration.

In a healthy liver, the reparative renewal of the hepatocyte and biliary epithelial cell populations is accomplished in most instances by proliferation of residual differentiated cells of each types , resulting in only a transient activation of the stem cells. However, under conditions in which the hepatocytes are unable to respond to the regenerative stimuli and/or are functionally compromised, a sustained activation of the stem cells and their progeny ensues, generating the differentiated cell lineages needed for the liver regeneration. An essential requirement for the stem cell-driven liver regeneration is a sustained expression of a set of growth factors, including those known to be involved in liver regeneration after simple PH.

1.7 Liver Tissue engineering

Tissue engineering (TE) is a multidisciplinary science aimed at developing "biological substitutes to restore maintain or augment tissue function", and holds premise for the development of innovative alternatives to hepatic disease. The ideal biological liver substitute should perform most or all of the liver-specific detoxification, synthetic and biotrasformation functions. As most of these functions are still unknown, mature liver cells (e.g. primary or immortalized) or cells that may differentiate into hepatocytes (e.g. stem or progenitor oval cells) have been used in these substitutes to perform liver-specific functions. Typically, liver constructs are engineering *in vitro* by culturing liver (or like-liver) cells in/on synthetic scaffolds which provide the template for cell adhesion, re-arrangement, proliferation and development (6).

The development of constructs with metabolic functions equivalent to those of the liver poses technical challenges well beyond that of developing new culture techniques (e.g. 2D monolayer vs.3D culture) for the complexity of liver cell physical-chemical requirements and the scale of the constructs. In fact, the liver is a highly structured organ with many distinct cell sub-population. Liver cells are spatially organized to optimize communication and transport. Cells communicate directly through cellular and gap junctions, and via chemical signals dissolved and blood-borne or present in the macromolecules forming the ECM that surrounds them. The signals that cells exchange promote differentiation, proliferation and functions. Furthermore, metabolic (e.g. carbohydrate metabolism) and detoxificant (e.g. CYP450 enzymes) activities of the hepatocytes change spatially along the length of the sinusoid, apparently regulated by gradients of oxygen, hormones and ECM composition, a phenomenon termed "liver zonation". Information on the structure-function relationship for normal and pathological liver tissue is still lacking. Fostering the same cellular relationship existing in the normal liver also in the TE liver construct is considered fundamental for cells to function as in the natural liver (11).

Over the years, research has mainly focused on the procurement of large amounts of suitable liver cells and the development of in vitro culture techniques.

Cultured primary and immortalized hepatocytes have been used for decades to address a wide variety of pharmacological and toxicological research topic (6).One shortcoming of conventional 2-D monocultures of hepatocytes utilized traditionally for compound testing is the partial or complete loss of viability and phenotype over time in culture. When reflecting

on the various factors that dictate the expression of normal hepatic phenotype *in vivo*, it is easy to understand that much of the conditional loss of structure and function *in vitro* is due to the loss of physiological context under conventional culture conditions. In many respects, the loss of normal cell structure and function *in vitro* is in reality an adaptation to the preparation and cultivation process that causes a shift in the gene program expressed in the cells as requisite contextual signals are lost.
Hepatotoxicity *in vivo* is often dependent on specific anatomical, morphological and phenotypic properties of the individual cell types that comprise the liver microenvironments *in vivo*.
The three-dimensional relationships of the unique cell types within the microenvironments of the liver (e.g. periportal versus pericentral), the regional hemodynamic flow patterns, and other physiological factors, such as oxygen tension and cytokine profiles, all play important roles in determining the toxicokinetics and toxicity of particular compounds(45).
Current cell-based models that are routinely utilized to perform toxicity testing *in vitro* are generally simple culture platforms (typically standard microtiter plate formats) employed under static, nonphysiologic conditions. Due to their simplicity, these static, monoculture model systems often represent suboptimal models for drug and chemical safety testing that are not able to mimic or predict more complex MOA. One of the biggest challenges to the development of more organotypic *in vitro* models of the liver is the integration of the architectural and cellular complexities of the liver, while incorporating the important elements of the localized hemodynamics of the regional microenvironments (6).

Increasing research effort is being devoted to the development of biomaterials for liver cells scaffolding at whose surface topological, morphological and biochemical signals are present to attract cells and make them adhere and rearrange their cytoskeleton, and to the characterization of the dissolved biochemical and physical signals that control cell differentiation, proliferation and apoptosis.
Worldwide, there is a growing interest for such innovative cell-based therapies as an alternative for orthotopic liver transplantation. If numerous clinical reports have established the efficacy of cell infusion therapies for the correction of inborn metabolism disorders, their clinical potential for the treatment of acute liver failure remain uncertain, mostly because engraftment efficiency can be poor in a situation without proper liver architecture. In the light of the current results, improvement of these therapies may require to co-infusion

of hepatocytes-stem cells with SECs or the use of a mimetic scaffold. In addition, Uygun et al. demonstrated that hepatocytes engraftment into a decellularized liver scaffold allowing a perfect lining of hepatocytes along fully functional SECs, was much more efficient than direct transplantation within the liver (45).
Until now, exist different approach to construct a bioartifical liver.
Three dimensional (3D) implantable constructs are made of porous biomaterials that degrade and resorb at controlled rates to permit their replacement with the extracellular matrix (ECM) produced by the cells, and cell colonization of the construct. Once implanted *in vivo*, the graft has to fully integrate into the body of the host. Two-dimensional (2D) or 3D non-implantable constructs may be used *ex vivo* for the EC support of ALF patients till a tissue compatible organ is available on the patient's own liver heals(6).

1.8 Bioreactor devices

A bioreactor may possibly be defined as a volume (or vessels) in which one or more biochemical or biological processes take place. Indeed, bioreactors are extensively used at any step of the assembly of a TE liver construct. In fact, primary cells enzymatically isolated from autogenous or allogenic liver tissue (or the whole organ) are often cultured in Petri dishes or T-flasks prior to their seeding in the scaffold to let them recover from isolation and purification stress.
Immortalized and primary progenitor or stem liver cells are generally expanded in Petri dishes or T-flasks, often under conditions facilitating differentiation to mature hepatocytes of the first two cell types, of inhibiting differentiation to maximize proliferation of the third cell types.
Liver cells (often of different types) are harvested and seeded in/on synthetic scaffolds in bioreactors under conditions that should facilitate cell attachment to the scaffold surface and penetration into its pores. The cell-seeded scaffolds are then cultured in bioreactors under tightly controlled and closely monitored environmental conditions to provide cells with biochemical and physical cues that should promote cell reorganization into liver-like aggregates and differentiation to make the construct functionally equivalent to liver tissue(6).

The first step in the development of constructs for liver replacement is to seed a large mass of liver cells uniformly on, or throughout, a scaffold. Then, the adherent cells have to be provided with adequate amounts of oxygen and nutrients to survive and proliferate, and adequate biochemical/physical signals to re-organize and differentiate to yield cellular structures and metabolic zonation resembling that the liver. Attaining adequate cell seeding, nutrient and oxygen supply to cells, and the control of biochemical signals gradients and concentrations in large scaffolds is not easy and depends on the bioreactor configuration and operation, on how mass is transported outside and inside the scaffold, and on cell metabolism. Bioreactors used for extracorporeal liver support have also to be connected to the patient so as to guarantee the unhindered transport of soluble species from the patient's circulation to the cells in the bioreactor and vice versa.

Cell seeding is thought to play a crucial role in the development of in vitro engennered tissue. Seeding cells at high initial density may favour tissue formation. High seeding efficiency would also limit the amount of organ tissue from which primary cells are isolated and cell expansion. Uniform initial cell distribution on 2D or throughout 3D scaffold has been related to the uniformity of engineered cartilage and bone tissue. Uneven cell distribution in the scaffold might lead to spatial variations in nutrients, oxygen and metabolic concentrations that would condition the survival and metabolism of cells at different positions in the scaffold. Seeding cells efficiently and uniformly on/in a scaffold is challenging, in particular throughout 3D porous scaffolds. In fact, closed or tight pores inside the scaffold may limit cell access and the seeding efficiency and distribution. Cell distribution in accessible pores depends on the balance between the rate at which cells are physically transported from the medium bulk to the outer scaffold surface (i.e. external transport) and from there towards its innermost pores (internal transport), and the rate at which cell bind to the biomaterial pore surface or to other cells in the feed suspension and form clusters (57). In particular, the resistance to external transport and cell uniformity in the feed suspension may be varied by changing the mixing intensity in the bioreactor where seeding takes place. Resistance to internal transport depends on the cell-to- pore size ratio but also on the transport mechanism within the scaffold.

Another limits on the bioreactor's perform "nutrient and metabolite transport". Growing liver tissue *in vitro* for liver replacement or support is more difficult than other tissues. In fact, cells have to be cultured at the high density typical of the natural liver, much higher than in many other tissues. Liver cells have also important nutrient requirements and are

sensitive to waste metabolites. *In vivo*, the liver is efficiently provided with soluble nutrients by a high blood flow that reaches the innermost cells in the organ by means of a fine network of capillaries (i.e. the sinusoids). This keeps the diffusion distance between cells and the blood small (within a few hundreds micron). Providing ad analogous system to supply basic substrates (oxygen, glucose and amino acids) to or clear waste metabolites (CO2, ammonia, urea, lactate) from liver cells in large 3D constructs is a formidable challenge, and a pre-requisite to promote cell growth, differentiation and a long-term survival (11). In fact, nutrients are continuously consumed (and products formed) by the cells while they are transported from the source into the cell mass (or from the cells to the skin). This causes the concentration of soluble nutrients in the cell mass to be generally less than that near their source, and the formation of concentration gradients across the cell construct.

1.9 Cell sourcing

In response to the increasing incidence of liver disease and the relative shortage of donor organs, many investigators have developed cellular therapies using isolated hepatocytes. Such approaches must consider both the source of hepatocytes and crucial stabilization of liver-specific function. Cell-based therapies that are reviewed can be generally categorized as extracorporeal devices, cell transplantation, and tissue-engineered constructs.
The choice of cell type in any cellular therapy is of paramount importance. Unfortunately, the full complement of cellular functions required to replace the liver and positively affect clinical outcomes has not been determined. For example, the mediators of hepatic encephalopathy resulting from liver decompensation are not fully understood although many theories, such as accumulation of ammonia,17 benzodiazapine,18 or gut-derived neurotransmitters,19 have been proposed. Hence, functionality of cellular devices is determined by “surrogate” markers of each class of liver-specific functions including synthetic functions, metabolic functions, detoxification (phase I and II pathways), and biliary excretion. The implicit assumption is that hepatocytes capable of a wide array of known functions will also express those unmeasured (or unknown) functions that are central to their metabolic role. Tissue-engineering applications may now consider sources other than primary cells as new cell lines are developed and stem cell lineages are elucidated (45).

Primary hepatocytes are the most common cellular component in current engineered therapies. Most devices undergoing clinical evaluation use porcine hepatocytes, which are readily available but, compared with rodent models, are poorly characterized *in vitro*. Whereas some functions such as albumin secretion may be stable, others such as cytochrome *P*-450 decline under standard culture conditions.21,22 In general, primary hepatocytes require specific microenvironmental cues to maintain the hepatic phenotype *in vitro*, and continuing investigation of culture conditions is likely to improve the stability of primary porcine hepatocytes *in vitro* as has been the case for rodent hepatocytes. Primary human cells are a preferred source for cellular therapies, but like whole organs, they are in limited supply.

Further *in vitro* characterization of human hepatocytes will provide key information affecting the development of improved cell-based therapies. The development of highly functional hepatocyte cell lines for use in cellular therapies is an obvious strategy to overcome the growth limitations of primary cells. A common approach to immortalizing hepatocytes is retroviral transduction of the simian virus 40 tumor antigen gene (SV40 Tag) whose gene product binds to cell cycle regulator proteins Rb and p53. Cell lines have also resulted from spontaneous immortalization of hepatocytes in collagen gel sandwich cultures or co-cultures (37). A third type of hepatic cell line is derived from liver tumors, as in the case of HepG2.

All these cell lines are growth competent but must be evaluated on the basis of liver-specific function and safety. Immortalized hepatocytes typically underperform primary cells and may not respond to important physiologic cues. The primary safety concern with the use of cell lines is the transmission of oncogenic factors to the host, especially with implanted cells. Efforts to improve the safety of immortalized cells has resulted in the use of temperature-sensitive SV40 Tag,35 Cre–*loxP*-mediated oncogene excision, and integration of suicide genes such as herpes simples virus thymidine kinase (HSV-tk). In the case of tumor-derived or spontaneously immortalized lines, limiting patient exposure to cells and preventing tumorgenesis may prove more difficult. In addition to primary cells and cell lines, stem cells are being considered for use in cellular therapies for liver disease. Stem cells are self-renewing cells that have the potential to differentiate into specialized cell types. The study of liver stem cell biology is rapidly evolving. Potential stem cell sources for use in cell-based therapies are embryonic stem cells, adult liver progenitors, and transdifferentiated non hepatic cells. Although embryonic stem cells may ultimately provide

a cell source, differentiation along the early hepatocyte lineage *in vitro* has been reported only in murine embryonic stem cells. The oval cell is a "facultative," bipotential stem cell that emerges in the setting of hepatic injury coupled with the inability of the adult hepatocyte to undergo repair.42 However, despite the fact that oval cells can be propagated *in vitro*, some transplantation studies indicate that they have less repopulation potential than mature hepatocytes. "Progenitor" cells have also been isolated from adult and fetal tissues that have not been subject to an oval cell protocol. Certain progenitor cells have been characterized as multipotent hepatic stem cells with self-renewal capability *in vitro*. In addition, it appears that hematopoietic stem cells can generate hepatocytes directly as well as through an oval cell intermediate, depending on the mode of injury and the model system. This has been shown in rodent models and confirmed in humans by a retrospective study of recipients of bone marrow and liver transplantation. Although it is not clear which stem cell source would be optimal, stem cells that can proliferate yet retain the ability to differentiate into hepatocytes would provide an ideal source for engineered cellular therapies.

Each of the cell sources currently under evaluation, that is, primary cells, cell lines, and stem cells, has inherent advantages and limitations. Independent of the source, mature hepatocytes in cellular therapies will likely require long-term functional stability to prove effective.

The success of cellular therapies ultimately depends on the stability of the hepatocyte phenotype and its regulation by microenvironmental cues. For years, investigators have developed culture models based on features of liver architecture to recapitulate the complex hepatocyte microenvironment. These features include extracellular matrix as found in the space of Disse, physicochemical stimuli imposed by sinusoidal blood flow, and cell–cell interactions present in the hepatic cord.

Cell–cell interactions, both homotypic (hepatocyte–hepatocyte) and heterotypic (hepatocyte–non parenchymal cell), have been shown to improve viability and function. Restoration of hepatocyte interactions as in spheroidal aggregates promotes formation of bile canaliculi, gap junctions, tight junctions, and E-cadherins and stabilizes function. The heterotypic interactions in hepatocyte–non parenchymal co-cultures are thought to present a highly conserved signal that greatly augments liver-specific functions. Cell patterning methods have been used to study the "co-culture" effect by tightly controlling the amount of cell–cell interaction to identify specific signaling pathways. Whatever the nature of the hepatocyte therapy, the issue of phenotypic stability must be addressed. Elucidation of

specific molecular mechanisms that stabilize hepatocyte function would have broad impact in this field.

Chapter 2: Aims of work and Cellular Models

2.1 Aims of the work

The need to regenerate functional liver tissue in vitro concerns several areas of bio-engineering application e.g. model tissue for drug testing, bio artificial liver supports and, finally, potentially engineered organs for implantation into patients.

Liver showed a complex and peculiar architecture, to study the single events that induce liver organization, functions and disease, is necessary to obtain a single cellular models for understand a single role in physiology and pathologic conditions.

We dispose of peculiar tools to in vitro study of the physiological cellular interplay in the liver:

i) the differentiated murine hepatocytes cell lines MMH, a wide range proteomic analysis highlighted as confluent MMHs also retain "in vivo features" in terms of cell-cell contact influences on proliferation and differentiation.

ii) in addition, we recently reported the isolation, characterization and reproducible establishment in line of resident liver stem cells (RLSCs) with immunophenotype (Sca1+, CD34-, CD45-, Alpha-fetoprotein+, Albumin) that locate them in a pre-hepatoblast/liver precursor cells hierarchical position and differentiative potentiality spanning from endodermal to mesenchymal and ectodermal derivatives. (Conigliaro et al., 2008).

To perform in vitro approaches of liver bioengineering, it is also necessary to dispose of sinusoidal endothelial cells, being liver endothelium a prime example of organ-specific microvascular differentiation and functions.

The liver endothelial cells, that form the lining of the hepatic sinusoids, represent a morphologically and functionally unique sub-population named liver sinusoidal endothelial cells (LSECs). Considering that endothelial primary cells are difficult and time–consuming to isolate, limited in number, invariably contain impurities with other cell types, and may lack the features of pathologic vasculature, the in vitro study of endothelial-specific functions has been hampered up today. Many authors tried to obtain an in vitro cellular model by immortalization strategies on freshly isolated SECs (immortalization with SV40

Middle T or Large T antigens), nevertheless these approaches failed inducing aberrant activation of endothelial-specific pathways.

Our efforts have been focused to the isolation and establishment in line of a liver sinusoidal endothelial cell and to the characterizations of resident liver stem cells line
i) to dispose of an in vitro system for the identification of critical microenvironment factors involved in liver cellular interplay during physiology or pathology conditions and
ii) to possibly improve protocols of liver tissue engineering.

2.2 Met Murine Hepatocytes: MMH soluble factors are able to maintain sinusoidal endothelial cells in differentiate state.

Met Murine Hepatocytes (MMHs) are immortalized cell lines derived from explants of embryonic, fetal, and new-born liver derived from transgenic mice expressing a constitutively active truncated human Met receptor (cyto-Met) under control of the human α 1-antitrypsin transcription unit (2). Although the immortalization of transgenic liver cells was reproducible, the event was rare: a small number of epithelial islands emerged and grew from a large number of cells in the primary cultures (Amicone L. et al., 1997). Making use of the MMH hepatocytes cell model we demonstrated the influence of soluble factors in conditioned medium released by murine hepatocyte conditioned medium (MH-CM) on human CB CD34+ progenitors (7); in long- term MH-CM culture we obtained growth of: (i) a bulk CD34+ population differentiating toward the endothelial lineage and (ii) single CD34+ cells expressing both haematopoietic (CD45) and endothelial (CD144) markers. In recent our studies, we explored the potential of human CB CD34+ HPCs to differentiate into haemogenic endothelium. In long-term culture, the addition of MH-CM stimulates the initial CD34+45+144− HPCs to generate adherent CD45−144+ endothelial precursors capable of self-renewal/proliferation and to differentiate in endothelial cells *in vivo*. These cells, instructed by haematopoietic growth factors (HGFs), rapidly differentiate into CD45+144+ cells that in turn generate either (i) CD45+ haematopoietic cells (mainly of erythroid and megakaryocytic type) when grown in haematopoietic medium, or (ii) CD144+ ECs if cultured in endothelial medium (59).

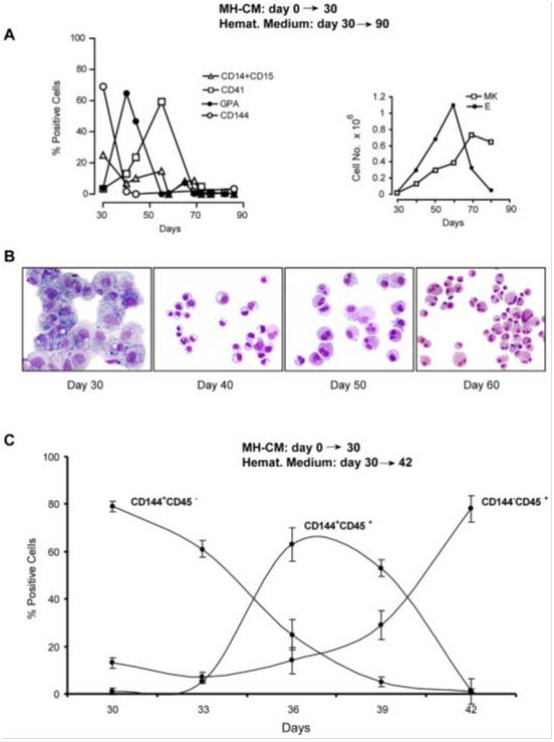

Figure 5. Immunophenotypic and morphological analysis of adherent ECs progeny following transfer into haematopoietic culture.
A- Left: Time course FACS analysis of adherent cells generated by CD34+ cells cultured in MH-CM and then transferred, at day 30, to haematopoieticmedium and characterized for lineage -specific antigen expression. A representative experiment out of 5 is shown. - Right: Growth curve of erythroid (E) and megakaryocytic (Mk) cells generated from the culture of day- 30 adherent cells in haematopoietic medium. A representative experiment out
of 5 is shown. B- Morphological analysis of adherent cells generated by CD34+ cells cultured in MH-CM for 30 days, then transferred to haematopoietic medium and grown for additional days (day 30 = day 0 in haematopoietic medium, day 40 = day 10 in haematopoietic medium, day50 = day 20 in haematopoietic medium, day 60 = day 30 in haematopoietic medium). At day 40, the large majority of cells had a morphology typical of the erythroid lineage elements at various stages of maturation. At later days of culture (day 50 and 60), erythroid cells were replaced by a cell population with a morphology compatible with the cord blood derived megakaryocytes (i.e. showing limited capacity of polyploidization). Pictures of a representative experiment out of 3 are shown. C- CD144 and CD45 expression analysis of adherent cells generated by CD34+ cells cultured in MHCM for 30 days, then transferred to haematopoietic medium (i.e. day 33 = day 3 in haematopoietic medium, day 36 = day 6 in haematopoieticmedium and so on). The percentage of CD144+452, CD144+45+ and CD144245+ cells from 8 independent experiments is reported (mean values 6SEM).

2.3 Murine Resident Liver Stem Cells (RLSCs)

In this study we also made use of an immortalized liver progenitor cell line isolated in our laboratory among the MMH lines (Spagnoli, F.M., et al., 1998). The MMH lines are composed of two distinct cell types: the expected epithelial cells as well as a cell type of spreading fibroblast-like morphology that we designate **palmate**. The epithelial cells express LETFs (Liver Enriched Transcription Factors). In addition, many express hepatic functions, or are competent to do so upon induction. In contrast, the palmate cells express neither LETFs nor hepatic functions. We demonstrated that palmate cells show properties of resident liver stem cells, or **RLSC** (14). The palmate RLSCs are **bipotential progenitors (**$Sca1^+$, $CD34^-$, $CD45^-$, α-$fetoprotein^+$, $Albumin^-$) that give rise to epithelial cells of both hepatocyte and cholangiocyte lineages, spontaneously upon continuous culture, or precociously under the appropriate environmental conditions. Epithelial progeny of palmate cells express LETFs and are competent to express hepatic functions. In addition, in three-dimensional cultures, palmate cells form hollow tubules lined with microvilli, reminiscent of bile ducts. Unequivocal demonstration that palmate cells can give rise to epithelial-hepatocytes is provided by cloning of individually fished cells and characterization of their progeny. All of these findings demonstrate that palmate cells are the precursors of hepatocytes in MMH cell lines.

Interestingly, RLSCs display a peculiar transcriptome profile, with coexistence of epithelial and mesenchymal markers (32). Such **metastable phenotype**, peculiar to stem cells, is perpetuated over cell generations in absence of any instructive signal and in basal culture conditions.

Recently, we demonstrate that RLSCs are able to differentiate in vitro and in vivo (orthotopic transplants and heterotopic transplants) in epithelial and mesenchymal derivatives, thus suggesting for the first time the existence of an adult stem/precursor cell capable of providing both parenchymal and non-parenchymal components to a complex epithelial organ (Conigliaro et al., submitted).

Chapter 3: MATERIALS AND METHODS

3.1 In vitro studies

Primary sinusoidal endothelial cell culture

Primary culture were performed in accord to previously described protocol (DELEVE) [91] with some modifications. Briefly, LSECs were isolated from CD1 mice by enzymatic digestion (Collagenase IV SIGMA Cat.C5138, Pronase SIGMA Cat.P6911 , DNAsi IV SIGMA Cat.D5025, Ialuronidase IV SIGMA Cat.H6254), percol discontinues density gradient centrifugation and centrifugal elutriation. The centrifugation method permit the separation of non parenchymal and parenchymal cells fractions. Sinusoidal endothelial cells was comprise in non parenchymal cells fraction. This fraction was maintained in different culture conditions: plated at high density on and not collagen I (Transduction Laboratories, Lexington, UK) coated dishes (Falcon-BD, Franklin Lakes, NJ) on in hepatocyte conditional medium (MH-CM), RPMI-1640, supplemented with 10% Foetal Bovin Serum (FBS) (both Gibco, Carlsbad, CA), 50 ng/ml Epidermal Growth Factor, 30 ng/ml Insulin like Growth Factor II (PeproTech Inc, Rocky Hill, NJ), 10 μg/ml insulin (Roche, Mannheim, Germany), 2 mmol/L L-glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin (Gibco); Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (basal medium); IMDM containing 10% FBS. After 12-24 hours the cultures were washed to remove all unattached cells and medium replaced. The cultures were maintained without transfer for several weeks with medium replacement twice a week. Within 4 weeks of culture, only cells plated on in coated dishes in MH-CM were survived and colonies with distinct cell morphology became visible; these cells are named "mix populations"
After sub cloning, we found a single sinusoidal endothelial cell clone that is maintained only on in MH-CM in collagen I coated dishes.

Immortalization strategies

Mix populations were immortalized by two different strategies: spontaneously (END S) and retroviral transduction (END M). For the first strategy the cells were plated in coated dishes on in MH-CM with medium replacement twice a week.

For the second strategy, cells were immortalized using a pantropic lentivirus to overexpress the SV 40 middle T-antigen. Briefly, viral supernatant containing high-titer SV40 virus was diluted 1:2 in culture media and added to mix populations 24h after plating. Cells were incubated for 48h, and then washed and cultured in MH-CM for 24h.
Within 4 weeks of culture, cells were characterized by FACS for CD105, MECA32, CD144, LYVE1.

Adenoviral transduction of Cell Culture

Primary mix populations were cultured in the presence of adenoviral vectors carrying green fluorescent protein (EGFP). Cultures were analyzed at 48h by FACS analysis.

Single cell cloning

In order to obtain clonal cell lines we performed limiting dilution seeding cells in microtitration plate at a concentration of 0,1 cell/well. Passages are calculated from initial thawing.
The medium replacement twice a week. Within 40 days of culture, we obtained 6 clones from END M and 4 clones from END S. After, these clones were expanded in cultures on in MH-CM and EGM-2 (endothelial instructive medium). Both cells were analysed for sinusoidal endothelial cells markers by FACS and Real Time PCR.

3.2 In vivo Studies:

Animals care and treatment

Animal management and experiments were performed according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' of the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23 revised 1985).

Orthotropic transplantation

CD1 newborn mice (2 days after birth) were transplanted, by transcutaneous injection into the liver (i.h.), with $1x10^6$ EGFP/LSECs or EGFP/RLSCs in 15ml PBS using a 30-gauge needle (Hamilton Bonaduz AG,Bonaduz, Switzerland).

Mice were sacrificed by cervical dislocation after 60 days and the livers removed, formalin fixed, mounted onto OCT and cooled by liquid nitrogen.

Heterotopic transplantation in GF-reduced Matrigel

Mouse sinusoidal endothelial cells (LSECs) cells or resident liver stem cells (RLSCs) (1 $x10^6$) were suspended in 1ml of Matrigel Growth factor-reduced (BD Biosciences Labware, San Diego, CA) with an equal quantity of mouse resident liver stem cells in differentiate state (RLSCdH) or Huvec for RLSCs transplants. Aliquots of about 0,7 ml of suspension were injected subcutaneously in the back of skid beige mice (Charles River Laboratories, Raleigh, NC, U.S.A.), carefully positioning the needle between the epidermis and the muscle layer. Mice were sacrificed by cervical dislocation after 21 days and transplants were immediately fixed for histology.

3.3 Cell and tissue analysis

RNA extraction, Reverse Transcription and Real Time quantitative PCR (qRT-PCR)

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, San Diego) according to the manufacturer's instructions. For RT-PCR analyses, single-stranded cDNA was obtained by reverse transcription of 1µg of total RNA using MMLV-reverse transcriptase (Promega, MI, Italia). cDNA was amplified by PCR using GoTaq enzyme (Promega, MI, Italia).

qRT-PCRs were performed using Rotor gene 6000 series. The Light Cycler Fast Start DNA Master SYBR Green I (Roche) was used to produce fluorescent-labeled PCR products during repetitive cycling of the amplification reaction. 40 ng of cDNA was used as template and cycling parameters were 95°C for 3 min, followed by 45 cycles of 95°C for 30s, 60°C for 1 min, 72°C for 30s, 60°C +0,5°C for 10 min. Fluorescence intensities were analyzed using the manufacturer's software and relative amounts were obtained using the $2^{-\Delta\Delta Ct}$ method and normalized for the β-actin. For a list of specific primers see following table (table 1).

Table 1. Mouse oligonucleotides used for quantitative real-time RT-PCR

Mouse β-ACTIN	5'- ACCACACCTTCTACAATGAG-3' 5'- AGGTCTCAAACATGATCTGG -3'
Mouse IL 6	5'-CACCAAGAACGATAGTCA A-3' 5'-TTTCCACGATTTCCCAGA-5'
Mouse KDR (VEGFR2)	5'-GCTGAAGATAATGACTCACC-3' 5'-CTGTGATGATGTAAATGGGAC-3'
Mouse CD144	5'-TAGCAAGAGTGCGCTGGAGATTCA-3' 5'-ACACATCATAGCTGGTGGTGTCCA-3'
Mouse vWF	5'-TGGCAAGAGAATGAGCCTGTCTGT-3' 5'-TAGGGCATGGAGATGCTTTGGTCA-3'
Mouse CD31	5'-ACCTGTAGCCAACTTCACCATCCA-3' 5'-TCTCCTCGGCGATCTTGCTGAAAT-3'
Mouse ANGPTL3	5'-AACAAGATGACCTTCCTGCCGACT-3' 5'-TGGACTGCCTGATTGGGTATCACA-3'
Mouse STABILIN2	5'-AAACTCCAGTGCAAATGCCTTCCC-3' 5'-ACTGGCAGACACACTTGACCATCT-3'

Immunofluorescence

Formalin-fixed frozen liver tissues were sliced (7μM) and stained with: anti-GFP Ab 1/200 (Santa Cruz Biotechnology sc-8334), anti-EGFP aB 1/600 (Abnova MAB1765), anti-HNF4α Ab 1/100 (Abcam ab41898), anti-Albumin Ab 1/200 (Novus Biologicals ab19196), anti-PanCytokeratin Ab 1/500 (Dako z0622), anti-Cytokeratin 7 Ab 1/200 (Abcam ab9021), anti-Desmin Ab 1/200 (Thermo Scientific RB-9014), anti-GFAP Ab 1/200 (Millipore MAB3402), anti CD31 Ab 1/200 (BD Biosciences 55027), anti- PDGFR2 ab1/300 (LS bio-LS-C106587) anti-EpCAM Ab 1/200 (BD Biosciences 552370), anti αFetoProtein Ab 1/200 (Santa Cruz Biotechnology sc-8108), anti E-Cadherin Ab 1/50 (BD Biosciences 610181), anti αSMA Ab 1/200 (Sigma-Aldrich A5228).

Secondary antibodies (Alexa-Fluor 488 and Alexa-Fluor 594 diluted 1/500) were from Molecular Probes, Eugene, OR, USA. The nuclei were co-stained with TOPRO3 (Invitrogen). Preparations were examined with a Leica TCS2 confocal microscope.

From heterotopic transplant sections, 5μm thick, embedded in paraffin were stained with hematoxylin and eosin. Immunolocalization was performed using standard

immunoperoxidase (DAB reaction) and counterstained with hematoxylin.
For immunocytochemistry, cells were grown on collagen I-coated dishes, fixed and treated as described previously.

Flow cytometry

Cells were trypsinited and pellets were suspended following two protocols, one for membrane proteins and one for the cytosol proteins. For the membrane proteins: pellets were suspended with antibody diluted 1:50 in PBS1, BSA, sodium azide, and incubated for 20 minutes at 4°C darkness. After cells were suspended with 100µl of buffer and centrifugated; final cell pellet were suspended in FACS flow buffer or in its addition with 100µl of PAF1%.
For the cytosol proteins: cells pellet were suspended with antibody diluted 1:50 in PBS 1x, Saponine 0,1%, BSA, sodium azide for 20 minutes at 4°C darkness. After pellet suspended in PBS, BSA, 0,1% saponine, sodium azide and centrifuged. Final pellet were suspended in FACS flow buffer or in its addition with 100µl of PAF1%. Cell fluorescence was analyzed with the FACSCalibur (Becton Dickinson).
For a list of specific antibodies see following table (Table 2).

Table 2. Mouse antibodies used for FACS analysis

PE anti mouse CD 105	eBioscience-cat. 12-1051-81
APC anti mouse CD144	eBioscience-cat. 17-1441
Biotin Rat Anti-Mouse Panendothelial Cell Antigen	BD Bioscience-cat. 558773
Anti-Mouse Lyve-1 Alexa Fluor® 488	eBioscience-cat. 53-0443-80
FITC Anti mouse CD31	eBioscience- cat.11-0311-82
PE anti mouse ALCAM (CD166)	eBioscience- cat.12-1661-82
FITC Rat anti mouse Ly-6A/E	BD Bioscience- cat.557405
Anti mouse-VIMENTIN FITC	eBioscience- cat.11-9897-80
Anti mouse VEGFR 1	ReD system-cat. MAB4711
PERCP-e Fluor 710 anti mouse CD324 (E-CADHERIN)	eBioscience- cat.46-3249-80

Tube formation assay

Vascular tube formation assay were performed on growth factor-reduced Matrigel (BD Bioscience, Bedford, MA ; USA). Briefly, MLECs were trypsinized and seeded onto the Matrigel (10.000cells/well) in the presence of MH-CM or EGM-2. Random fields were photographed at 2h-4h-6h after seeding. Vascular tube formation was assessed with optic microscopy.

SEM analysis

Scanning electron microscopy was performed to studies the presence of fenestrate on c. Cells were fixed in 2.5 gluteraldehyde for 1h, post-fixed in 1%osmium tetroxide on ice 30min, dehydrated,critical point dried, sputter or carbon coat, and image at 3kV using S-4700 scanning electron microscope (Hitachi).

Chapter 4: Resident liver stem cells (RLSC) result's

4.1 RLSC coexpress epithelial and mesenchymal markers and undergo into mutually exclusive differentiations

Two RLSC lines, WTE14/1and MetE14/3, initially isolated from explants of embryonic livers at 14 days post coitum and previously characterized for their broad differentiation capacity 15, have been seeded at clonal limiting dilution (0,2 cell/well) and expanded. A single clonal progeny from each of the two lines (WTE14/1-1 and MetE14/3-1) was randomly selected and used as starting populations for these studies; the results obtained in cell culture and in vivo experiments and described below were equivalent for the two clones that, from now on, will be referred to simply as RLSCs.

FACS analysis of the starting undifferentiated RLSC clones revealed as the cells homogenously express the stem marker SCA1 and, notably, co-express epithelial and mesenchymal markers such as E-Cadherin, PanCK and Vimentin (Figure 1A). An immunocytochemical analysis confirmed the co-expression of these proteins (Figure 1B) unveiling for E-Cadherin a cytoplasm distribution. A broader characterization by FACS analysis showed that cells were negative for CD144, CD45, and Thy1 (data not shown) so excluding an hematopoietic origin.

Surprisingly, low serum culture condition gave rise, within the same culture, to cells with either epithelial or mesenchymal morphology (Figure 1C). The unexpected appearance of two morphologically discrete subpopulations within a clonal cell line rendered mandatory to gather observations at single cell level. To this end we marked RLSCs with a retrovirus expressing enhanced green fluorescent protein (EGFP) and followed single cells proliferation/differentiation by live imaging. This analysis allowed to re-conduct the dimorphism to single mother cells: as shown in Figure 1D and in the supplementary video, single EGFP-RLSC followed for 5 days gave rise to cells either co-expressing E-Cadherin and Vimentin or expressing one of these two markers in a mutually exclusive fashion.

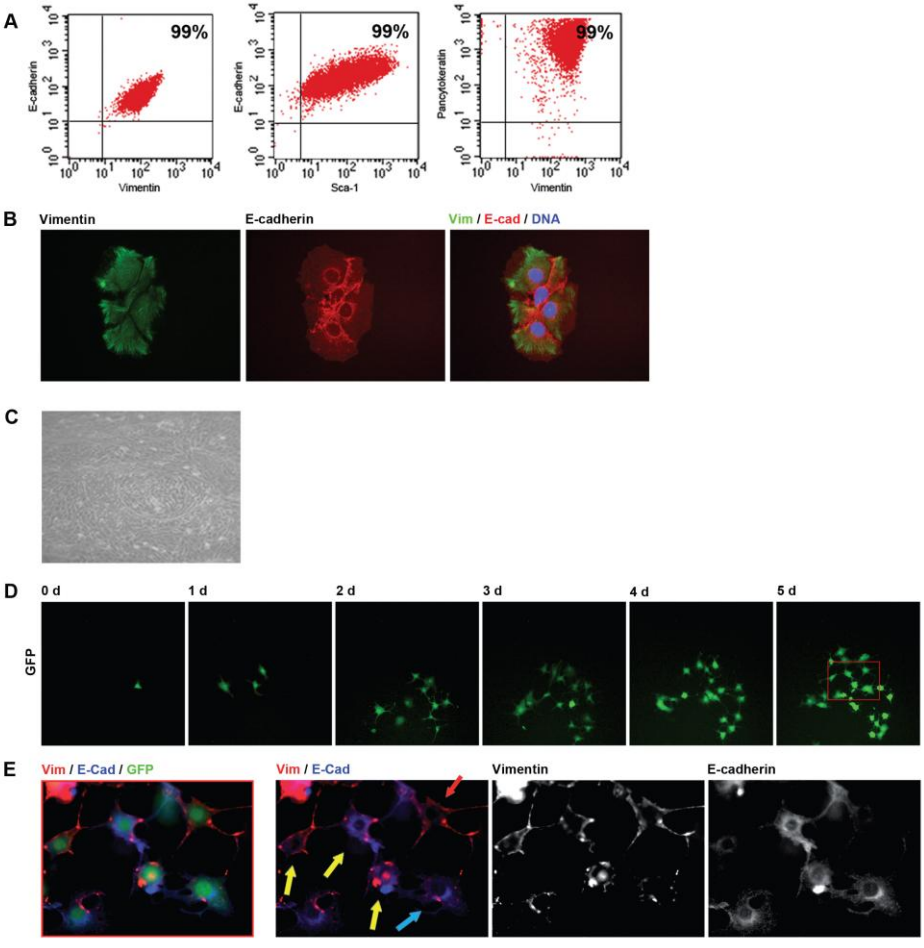
A
99%
E-cadherin
Vimentin
99%
E-cadherin
Sca-1
99%
Pancytokeratin
Vimentin
B
Vimentin
E-cadherin
Vim / E-cad / DNA
C
D
0 d
1 d
2 d
3 d
4 d
5 d
GFP
E
Vim / E-Cad / GFP
Vim / E-Cad
Vimentin
E-cadherin

Figure 1. RLSC characterization and in vitro differentiation.
A) FACS analysis for the indicated antigens highlighted as the RLSC starting population homogeneously expresses the stemness marker SCA1, the epithelial markers Pan-CK and E-cad and the mesenchymal marker Vimentin.
B) Immunocytochemical analysis for the indicated markers highlighted as the RLSC starting population co-expresses epithelial and mesenchymal markers.
C) Within 10 days RLSCs, in low serum culture condition, gave rise to derivatives that acquired distinct epithelial and mesenchymal phenotypes as observed by phase contrast (original magnification 20X).
D-E) Time lapse analysis (see also video1) indicates as, within five days, a single EGFP-RLSC cell, cultured in low serum, gave rise to clonal progeny composed by cells co-expressing Vimentin and E-cadherin (yellow arrows), cells expressing E-cadherin (blue arrow) and cells expressing Vimentin (red arrow).

To further characterize the segregation of epithelial and mesenchymal markers to different cell populations, a dimorphic cell culture, obtained at confluence after 10 days of starvation, was analyzed by FACS and immunofluorescence. FACS analysis highlighted that cells undergoing to epithelial differentiation down-regulate both stem and mesenchymal markers, while cells undergoing to mesenchymal differentiation down-regulate both stem and epithelial markers (Figure 2A). Immunofluorescence analysis for Vimentin/E-Cadherin and for a-SMA/HNF4 highlighted as cells express these markers in a mutually exclusive fashion and in coherence with the corresponding cellular habit. The epithelial-shaped progeny was further characterized as expressing the HNF4 while the mesenchymal-shaped one as expressing the myofibroblast-associate marker α- smooth muscle actin (αSMA) (Figure 2B). Quantitative analysis of markers segregation is shown in figure 2C.
These results indicate an intrinsic dual differentiation potentiality of RLSCs.

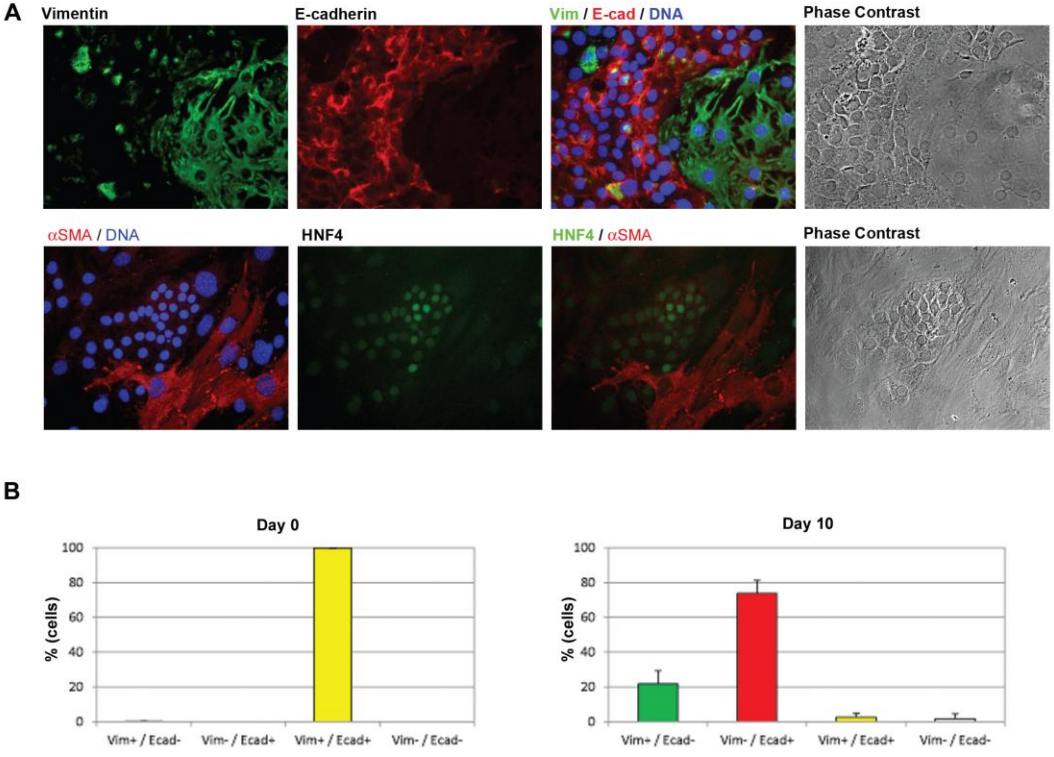

Figure 2. Segregation of epithelial and mesenchymal markers into distinct RLSC derivatives.
A) Immunocytochemical analysis for the indicated markers highlighted as RLSCs, cultured in low serum for 10 days, undergo into either epithelial or mesenchymal differentiation. The morphology of the two different cellular subpopulations is coherent with the expression of specific markers.
B) Cells were scored in four categories based on their Vimentin and E-Cadherin expression. Data show percentage of cells for each category. Average of 3 experiment. N=500 cells were counted for each experiment. Data are shown as mean ± SEM.

4.2 RLSC derivatives display liver-specific epithelial and mesenchymal phenotypes in orthotopic transplant

To challenge *in vivo* the intrinsic dual differentiation potentiality of RLSC, EGFP+ cells were injected directly into the liver of untreated immuno-tollerant (CD1) mice at the first day after birth, through a percutaneous trans-abdominal puncture, according to Bissig *et al.* 17. Animals were sacrificed at various times and the peak of colonization was found around two months after cell transplantation. RLSC progeny, only capitalizing on the differentiating and proliferative stimuli of the growing organ and in competition with endogenous cells, was found to reproducibly colonize the recipient livers in 40/50 injected animals. In 30 animals, EGFP+ cells expressing hepatocyte markers (i.e. the transcription factor HNF4α and Albumin) engrafted in the hepatocytic muralium with an efficiency until 0.01% of total hepatocytes (Figure 3 A).

Notably, in each of the 40 engrafted mice, EGFP+ cells with a mesenchymal shape and expressing Glial Fibrillary Acidic Protein (GFAP) and Desmin were also observed (Figure 3 B). The sub-endothelial localization of these cells (in peri-sinusoidal spaces, Figure 3C), together with their immunophenotype, corresponds to those of Hepatic Stellate Cells.

Taken together, these data indicated that clonal RLSCs could contribute to both epithelial and mesenchymal specific compartments of the liver.

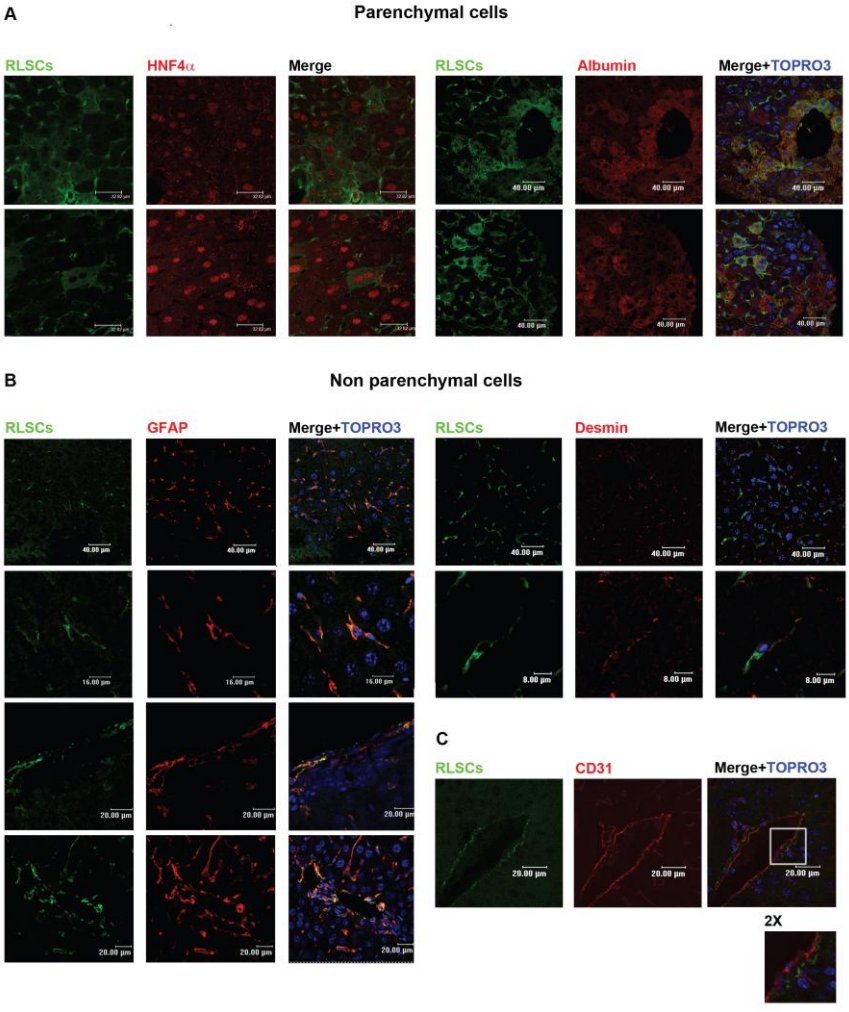
A
Parenchymal cells
RLSCs
HNF4α
Merge
RLSCs
Albumin
Merge+TOPRO3
B
Non parenchymal cells
RLSCs
GFAP
Merge+TOPRO3
RLSCs
Desmin
Merge+TOPRO3
C
RLSCs
CD31
Merge+TOPRO3
2X

Figure 3.Characterization of RLSC derivatives in orthotopic transplants.
Immunohistochemical analysis of RLSCs engrafted livers. The RLSC's EGFP progeny was detected in untreated CD1 null mice injected at 2 days after birth and sacrificed after two months.

A- EGFP parenchymal cells were found either integrated into the epithelial cords expressing hepatocyte markers HNF4 and Albumin.

B- EGFP non- parenchymal cells expressing GFAP and Desmin were found both scattered in the parenchyma and C) in sub-endothelial positions. The endothelium was decorated with CD31antibody.

4.3 RLSC give rise to liver-specific epithelial and mesenchymal derivatives in heterotopic transplants

In order to determine to what extent the differentiation of transplanted progenitors into hepatic lineages was induced by liver-specific local cues, EGFP+ RLSCs were transplanted subcutaneously into the back of SCID beige mice, as described in Methods. The co-transplants with Huvec cells was performed in order to guarantee an homogenous vascularization inside the implant.

Histological and immuno-histochemical analysis of implants showed RLSC derivatives with both epithelial and mesenchymal phenotypes (Figure 4A); these cells were found to express a number of liver histotype-restricted markers including EpCAM, α-fetoprotein, E-Caderin, CK7, Albumin, detected in cells with epithelial morphology, and GFAP and α-SMA, observed in those displaying mesenchymal shape. (Figure 4B-C). The expression of EpCAM and α-fetoprotein (endodermal precursor markers), in particular, suggests that in an extra-hepatic context, RLSC derivatives proliferate retaining precursor features; moreover the low expression of Albumin and CK7 indicates also the RLSC aptitude to differentiate toward hepatocytes/cholangiocytes. With respect to mesenchymal differentiation, the expression of α-SMA and GFAP suggested that the ability of RLSCs to generate a HSC-like phenotype was also inherent and independent of exogenous, liver restricted cues. Overall, heterotopic transplantations highlighted that RLSCs retain a binary, mesenchymal-epithelial potential, independent of soluble factors, cell-to-cell and cell-to-ECM organ-specific instructive signals. Taken together these results indicate an intrinsic dual potential of RLSCs to differentiate into either epithelial or mesenchymal derivatives. This dual potential, per se cell-autonomous as reproduced in cell culture, may harness organ-specific cues *in vivo*, critical for proper maturation toward liver-specific cell types.

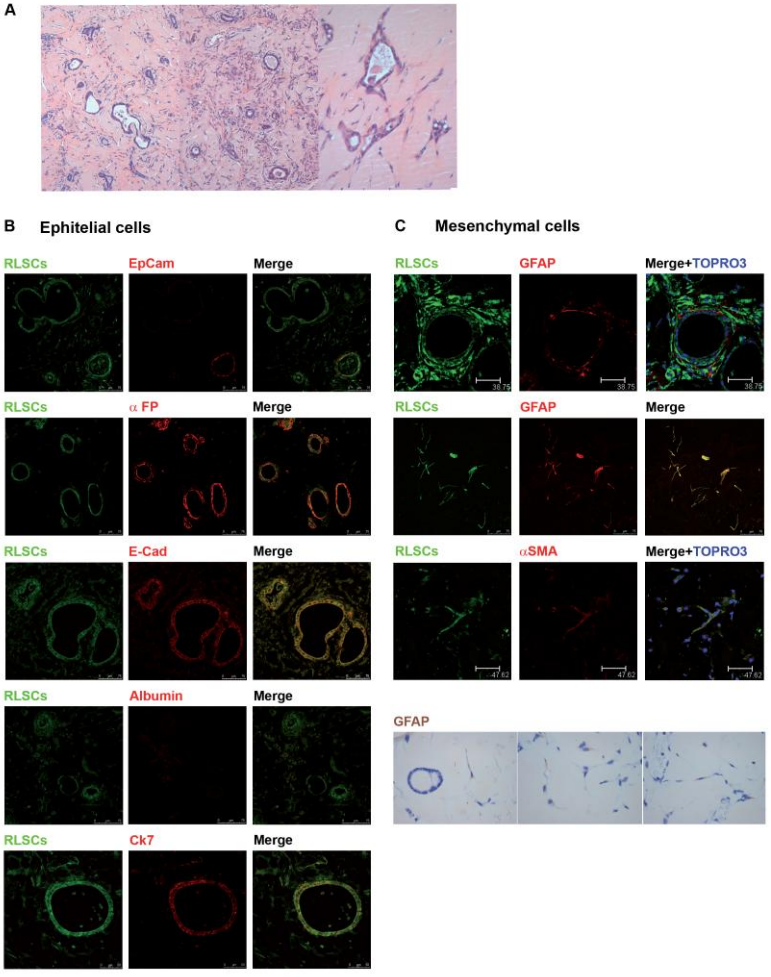

Figure 4. Characterization of RLSC derivatives in heterotopic transplants.
H&E Immunohistochemical and Immunofluorescence analysis of RLSCs engrafted in SCID adult null mice. RLSCs were injected in Matrigel in the epifascial region and animals were analyzed one month after injection.
A) H&E revealed epithelial-like RLSC derivatives found either arranged in compact islands or delimiting empty spaces. Mesenchymal-like RLSC derivatives were found scattered in the matrigel scaffold.
B) The EGFP epithelial cells were found to express the hepatic progenitor/hepatocyte markers, EpCAM, αFP, E-Cad, Albumin and CK7.
C) The EGFP mesenchymal cells were found to express GFAP and αSMA by IF and IHC (bottom panel).

Chapter 5: Sinusoidal Endothelial Cells result's

5.1 Characterization of elutriated non parenchymal cells (NPC)

Primary cultures were prepared from livers of mice CD1 14 days post coitum. LSECs were isolated by enzymatic digestion of the liver with Collagenase IV, Ialuronidase IV, DNAsi IV and Pronase, and Percol discontinues density gradient centrifugation. Cell elutriation procedure was performed to separate parenchymal from non parenchymal cells, including sinusoidal endothelial cells. Non parenchymal cell fraction was cultured in hepatocytes conditional medium (MH-CM) (Figure 1A) and after 15 days analysed by FACS for expression of endothelial markers.
FACS analysis showed that 15% of cells express both the endothelial markers CD105 and MECA 32. Less of than 7% of cells express the mesenchymal progenitor markers, ALCAM (Figure 1B), this indicating the presence of progenitor cells in non parenchymal fraction.
Notably, after 2 weeks of culture the cells have been shown to be capable of forming tubular structures spontaneously (i.e. without instructive stimuli), as wells as the well-differentiated endothelial cells. (Figure 1C-C').

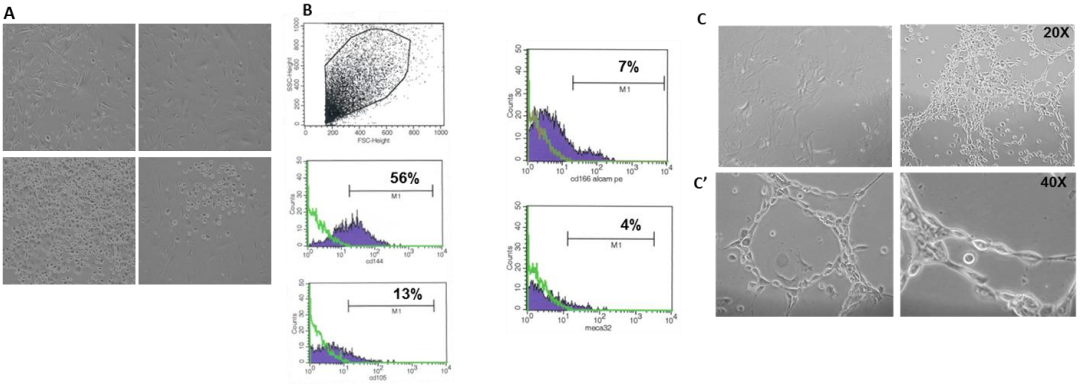

Figure 1. Immuno-phenotypic and morphological analysis of non parenchymal cells fractions (NPC).

A- Left: Morphological analysis of non parenchymal cells maintained on in MH-CM in collagen I coated dished.
B- FACS analysis of non parenchymal cells. We analysed sinusoidal endothelial marker, MECA 32; endothelial markers: CD105 and CD144; mesodermal progenitors marker ALCAM.
C- Right: Morphological analysis of non parenchymal cells. Cells are able to form spontaneously tubules in collagen coated dished. Imagines acquired to original magnifications 20X (the first line of images)and 40X (the second line of images).

5.2 Establishment and characterization of non parenchymal cell lines

The mix populations of non parenchymal cells were immortalized by two different strategies:

1) cells were left for 14 days in MH-CM, until immortalized elements emerged from senescent cultures (spontaneously immortalized endothelium, END S);
2) cells were infected with a pantropic lentivirus carrying the SV 40 middle T-antigen (Middle T immortalized endothelium, END M).

- Immortalized cells were **immunophenotypic characterized** by FACS.

The characterization showed that END S and END M express the typical endothelial marker CD105 together with the markers of sinusoidal fenestrae MECA 32, LYVE1, (Figure 2A).
Notably, both populations do not express typical endothelial marker CD144; this is in line with many studies suggesting that this protein is not essential to function of liver sinusoidal endothelial cells, in which its expression is strictly related to the age.

- Both immortalized cell lines required collagen as substrate.
- END M cell line, as direct consequence of the transforming middle T antigen expression, has a doubling time shorter than END S (data no shown).

- To explore the **tumorigenicity** of both cell lines, END M and END S marked with a retrovirus expressing EGP, were transplanted subcutaneously in SCID mice. No tumours development was observed for both cell lines after 4 weeks from the transplantation.
  (Figure 2B).

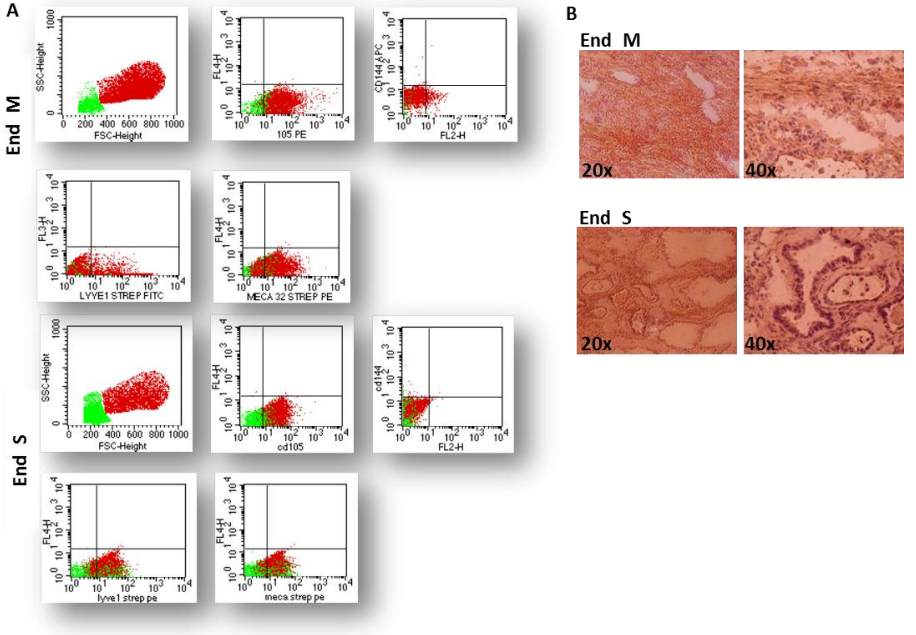

Figure 2. Immunophenotypic and morphological analysis of END M and END S.

A- Left: FACS analysis of mother populations END M (immortalized by SV40 Middle T antigen) and END S (spontaneously immortalized endothelium). Cells characterized for endothelial markers: CD105, CD144; and sinusoidal endothelial markers MECA 32 and LYVE 1.

B- Right: Histological analysis of Heterotopic transplants. END M and END S were transplanted subcutaneously into the SCID mice. Mice were sacrificed by cervical dislocation after 21 days and transplants were immediately fixed for histology. Both mother populations not shown a tumorigenic capability.

5.3 Establishment and characterization of clonal endothelial cell lines.

In order to obtain single sinusoidal endothelial clones expressing SECs features, we performed a cloning assay by limiting dilution of END M and END S cells.
After 40 days of culture we obtained 6 clones from END M cells (named M1,M2,M4,M6) and 4 clones from END S (named S1,S2,S3,S4) (Figure 3A).
Characterization of two immortalized clonal cells END M and END S were performed comparing two different culture conditions, MH-CM and EGM 2. This is an endothelial instructive medium, normally used for the maintenance of endothelial cells culture; it is added with angiogenic growth factors, as VEGF, FGFb; ascorbic acid and EGF.

- **Immuno-phenotypic characterization** by FACS analysis revealed as all clones express markers even if at different levels: in both cultured conditions, all clones showed high levels of CD105, MECA 32, LYVE1 and CD144. Interestingly, only when clones were cultured in MH-CM showed approximately 96% positive cells for all sinusoidal endothelial markers (Figure 3B).

- To investigate on the **endothelial functions** we performed a **tubulogenesis assay** in the same culture conditions. Clones cultured in MH-CM are able to form tubules in only 4 hours respect to clones cultured in EGM2, in which we observed tubules after 6h from the seeding (Figure 4). In particular, between all clones, the S2 clone show a better capability to form tubules in vitro, in terms of structure and quantity, in both experimental conditions.

A

	M1	M2	M4	M6	S1	S2	S3	S4
CD105	97%	23%	96%	88%	97%	100%	90%	97%
Lyve1	80%	18%	92%	87%	91%	84%	35%	40%
Meca 32	57%	24%	86%	81%	65%	73%	5%	35%
CD144	51%	31%	68%	17%	98%	100%	19%	33%

B

	M4 MH-CM	M4 EGM-2	M6 MH-CM	M6 EGM-2	S1 MH-CM	S1 EGM-2	S2 MH-CM	S2 EGM-2
CD105	96%	80%	88%	84%	97%	99%	98%	92%
Lyve1	92%	53%	93%	87%	91%	99%	94%	91%
Meca 32	86%	26%	74%	50%	79%	69%	93%	83%
CD144	68%	69%	37%	17%	98%	82%	100%	93%

Figure 3. Immuno-phenotypic of clones derived from END S and END M populations

A- FACS analysis of clones generated by END M cells: M1-M2-M3-M4, and clones generated by END S cells: S1,S2,S3,S4. Cells cultured in MH-CM and characterized for lineage -specific antigen expression. Clones showed, in particular, a variable expression of specific sinusoidal endothelial markers, Lyve 1 and Meca32

B- FACS analysis of clones cultured in different medium as MH-CM and endothelial instructive medium EGM-2 for 15 days. Cells showed a high expression of sinusoidal endothelial markers in presence of hepatocytes soluble factors. In particular, S2 clone had a typical sinusoidal endothelial phenotype.

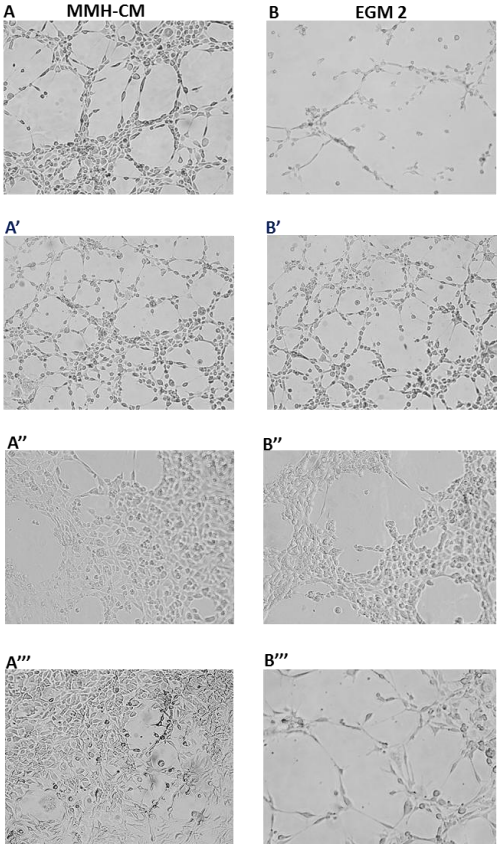

Figure 4. Tube formation assay. Inverse microscopic images illustrating clones maintained in MH-CM (A-A'-A''-A''') and clones maintained in EGM 2 (B-B'-B''-B''') and put in matrigel for different duration of cultivation. Imagines acquired when cells showed better tubes formation. Original magnification 20x.

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- Furthermore, by the use of **Scanning Electron Microscopy** we evaluated the number and dimensions of cytoplasmatic fenestrae, typical of sinusoidal endothelial structure. We observed fenestrae with a mean diameter of 60-100nm only in clones cultivated in MH-CM (Figure 5).

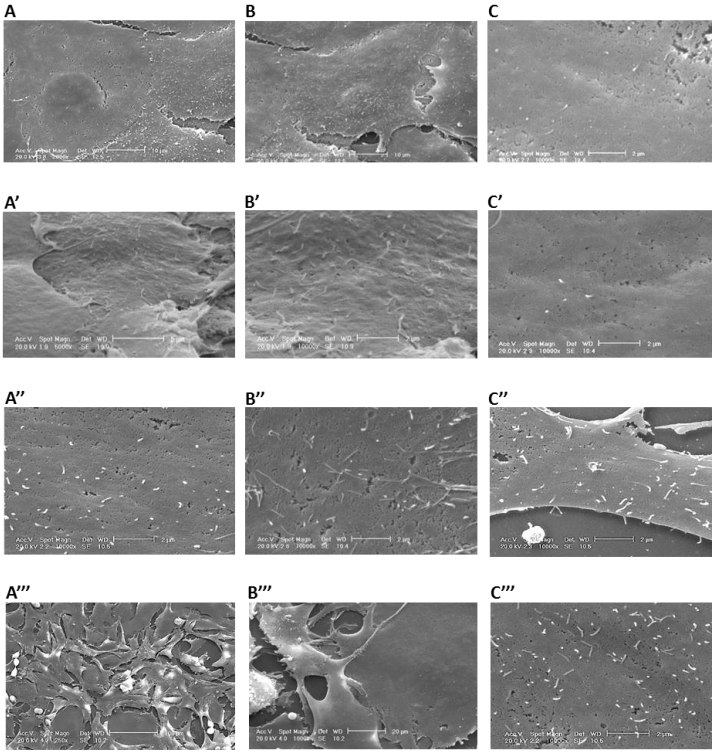

Figure 5.SEM analysis. We evaluated cell morphologic by SEM ,of clones S1 (A-B-C) , S2 (A'-B'-B'), M4 (A''-B''-C''), M6 (A'''-B'''-C''') maintained in MH-CM. We found the fenestrature with following diameters:
S1(A-B-C) : 60-100nm
S2 (A'-B'-B'): 50-80nm
M4 (A''-B''-C''): 50-100nm
M6 (A'''-B'''-C'''): 50-100nm

- In order to explore the **angiogenesis capability in vivo** we performed heterotopic transplants. The clones were co-transplanted with hepatocytes subcutaneously into SCID mice.

Firstly, we observed that clones derived from END M (M4, M6) acquired tumorigenicity capability (Figure 6B), while those derived from spontaneous immortalization events are not transformed and organize themselves in tubular structures driving correctly the architectural organization of co-transplanted hepatocytes. In particular, END S clones are able to forms vessels, as shown by CD31 expression, and to induce an organization of hepatocytes in cords highly reminiscent of the epithelial muralium in the liver (Figure 6A).
In the tumoral tissue formed starting from END M clones, we observed a disorganized vessel system unable to drive a correct hepatocyte organization.

Over all, the endothelial cell lines characterization showed as hepatocyte soluble factors play a pivotal role in the maintenance of sinusoidal endothelial cells phenotype and functions.

All further studies were performed with **S2 clone**, because it showed a *better phenotype and endothelial features in vitro and in vivo*

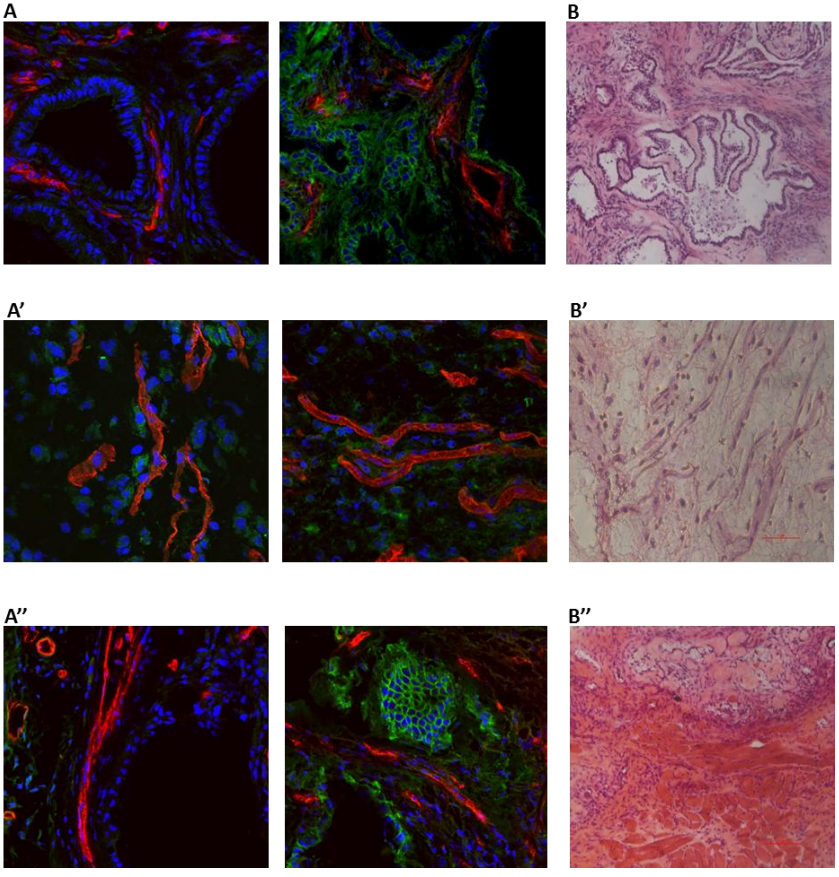

Figure 6. Heterotopic transplants. H&E Immunohistochemical and Immunofluorescence analysis of sinusoidal endothelial clones co-injected with hepatocytes in SCID adult null mice. Cells were injected in Matrigel in the epifascial region and animals were analyzed one month after injection.

A- Clones cells were found to express the endothelial marker CD31,as shown in red spot. (A'-A''-A''') .Hepatocytes were identified by the E-cadherin expression (as shown in green spots) near endothelial clones CD31 positive, as shown by images.

B- Sinusoidal Endothelial clones derived from END S showed a non-tumorigenic aptitude, while cell clones derived by END M showed a strong tumorigenic capability.

5.4 Liver endothelial cells are able to maintain the differentiate state in vitro

S2 clone is now named **MLECs** from **M**ouse **L**iver **E**ndothelial **C**ells.
A further immunophenotypic characterization of MLECs cultured in MH-CM (M-MLECs) and in EGM2 (E-MLECs) were performed.

FACS analysis showed as cells cultured in hepatocyte conditional medium were approximately 100% positive for the sinusoidal endothelial markers.
In particular cells maintained in MH-CM were: 96% positive for MECA 32 and 2% positive for Lyve 1, typical markers of fenetrature, respect same cells in EGM 2 in which they were: 90% positive for MECA32 and 5% positive for LYVE.1.
Notably, we showed in both cells (M-MLECs and E-MLEC), different levels of expression of typical endothelial markers; in fact in presence of MH-CM cells were 96% double-positive to CD144/CD31 and 97% positive to CD105, respect in EGM2 in which cells were 76% double-positive CD144/CD31 and 75% positive to CD105.

These data were confirmed by *RT-qPCR analysis*, in which observed high levels of mRNA expression of CD144 and CD31 in presence of hepatocyte soluble factors.
Interestingly, mRNA expression of *liver endothelial and endothelial specific genes* were increased in presence of MH-CM respect in the EGM2, such as KDR, vWF for endothelial markers and Stabilin2, Angptl3 and IL6 for sinusoidal endothelial markers.

These data suggests that hepatocytes soluble factors are able to promote and to maintain differentiation state of SECs .

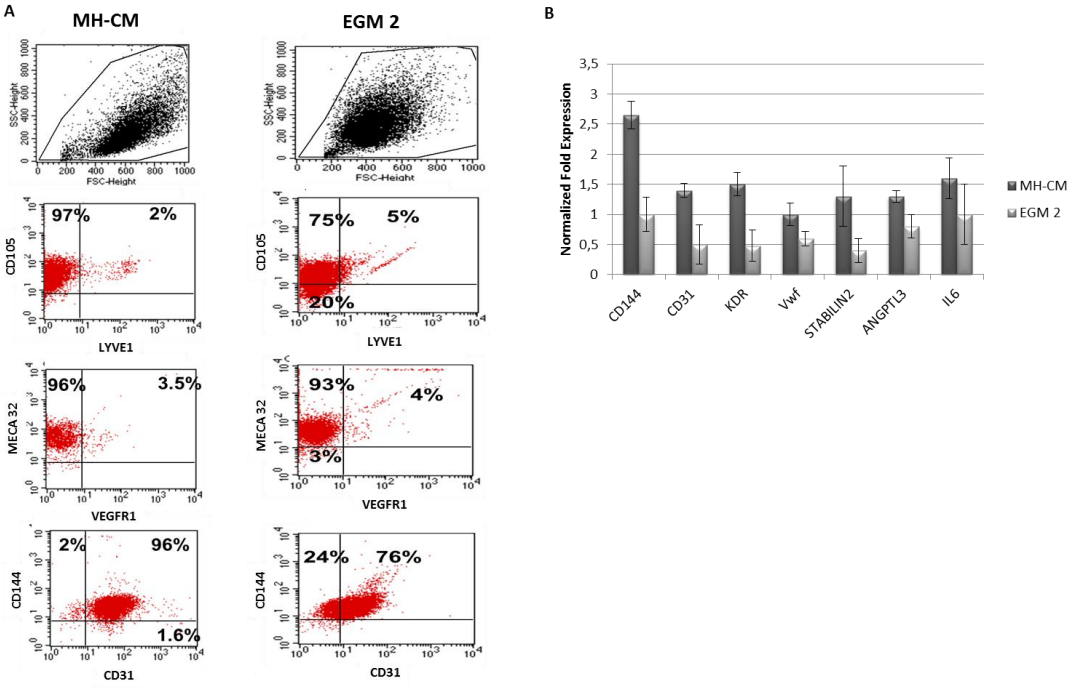

Figure 7. Phenotypic and transcriptional analysis of MLECs

A- Left: FACS analysis of MLECs maintained in MH-CM (A)and in endothelial instructive medium (A''). We analysed a specific sinusoidal endothelial markers, such as LYVE1, MECA3; and endothelial markers, such as CD144, CD105 and CD31. In dot plot is reported the percentages of positive cells to each markers. Data suggesting as MLECs show sinusoidal endothelial features although are cultured for a long time in particular in presence of hepatocytes markers.

B- Right: Transcriptional analysis of endothelial and sinusoidal endothelial markers on MLECs maintained in hepatocytes soluble factors or in endothelial soluble factors. The real-time PCR results were calculated by ΔΔCT Method. mRNA levels are relative to endogenous β-actin.

5.5 MLECs forms sinusoids in Orthotopic Transplants

To value in vivo the intrinsic capability to organization and differentiation of SEC, MLECs EGFP+ cells were injected directly into the liver of untreated immuno-tollerant (CD1) mice at the first day after birth, through a percutaneous trans-abdominal puncture. Animals were sacrificed at various times and the peak of colonization was found around two month after cell transplantation. MLECs capitalizing on the differentiating and proliferative stimuli of the growing organ and in competition with endogenous cells suggesting a capability of MLECs to play a physiological role.
MLECs EGFP+ were identified by EGFP and CD31 co-expression. EGFP+ cells are able to form sinusoids, but also, a large vessels, as shown by Figure 9.

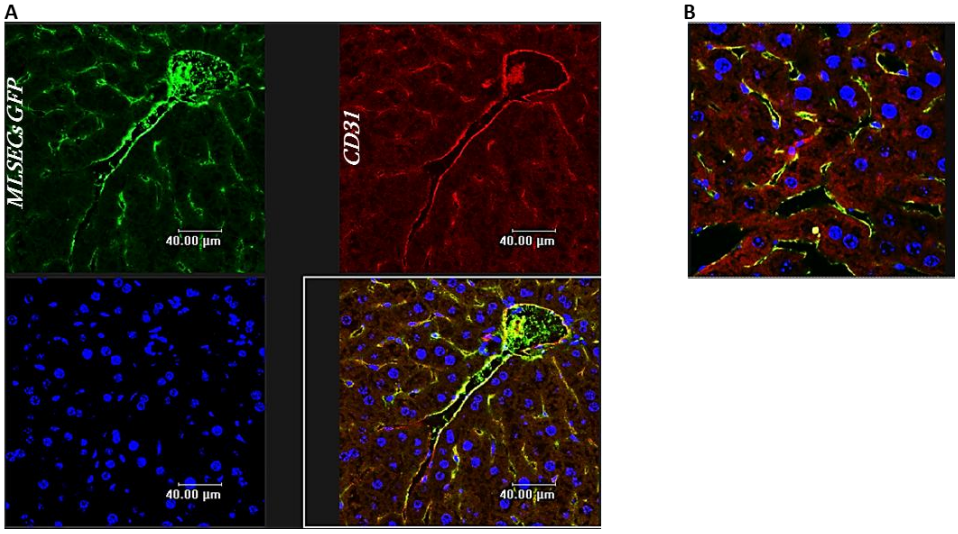

Figure 9. Characterization of MLECs in orthotopic transplants.
MLECs, previously genetically marked with a retroviral vector expressing GFP, were injected intrahepatically in CD1 newborn mice; two months after injection the livers were collected and immuno-stained.
A- IF analysis shown that MLECS EGFP+ co-express sinusoidal endothelial markers, CD31 (red), and promote the interaction with endogenous sinusoidal endothelial cells.
B- A particular of sinusoidal endothelial cells organization's in vivo.

MLECs EGFP+ displays a physiological interaction with endogenous sinusoidal endothelial cells during liver growth.

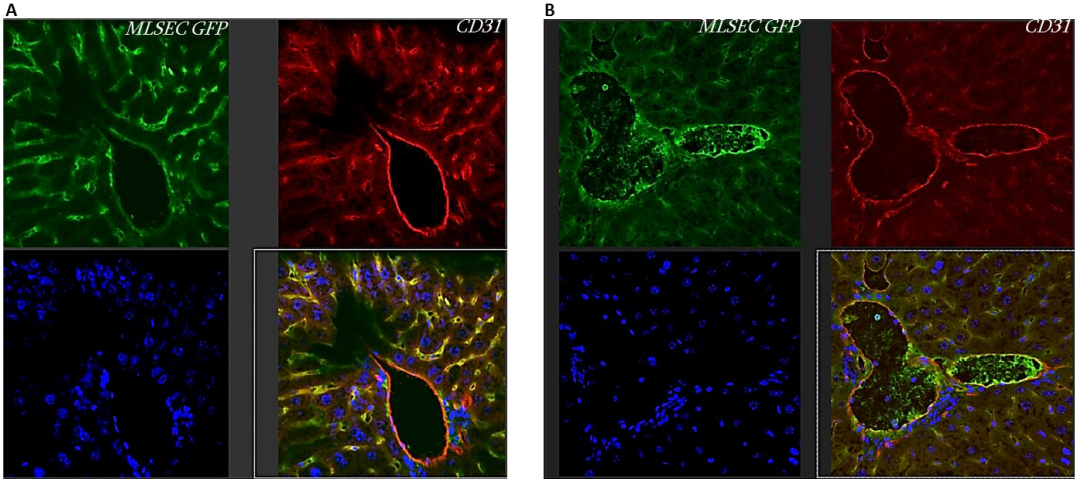

Figure 10. Orthotopic transplants.
IF analysis shown that MLECs display physiological interactions with endogenous cells, during the formation of sinusoids and large vessels.
In figure A is noted a correct cellular organization between endogenous and exogenous SECs; it is observable the co-expression of CD31 and EGFP in sinusoids near to a large vessels that express only CD31.
In figure B is showed in large vessels a cell contact between MLECs and SECs, while sinusoids have co-expression of CD31 and EGFP.

In particular, MLECs EGFP+ displays a physiological interaction with endogenous cells, as shown by pericyte localization near exogenous SECs (Figure 11).
Taken together, these date indicated that SECs are able to promote cellular interplay between parenchymal and non-parenchymal cells also in vivo.

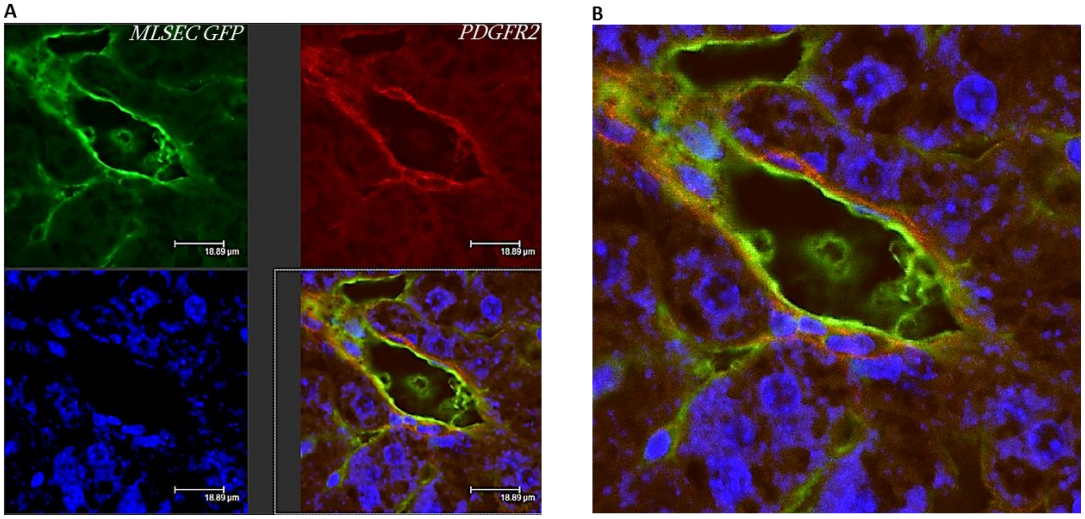

Figure 11. Orthotopic transplants.

IF analysis shown that MLECs display physiological interactions with endogenous cells, as shown by pericyte localization near exogenous MLECs.

In figure A is noted a correct cellular organization between endogenous and exogenous SECs; it is observable the co-expression of CD31 and EGFP in sinusoids near to a large vessels that express only CD31.

In figure B is showed in large vessels a cell contact between MLECs and SECs, while sinusoids have co-expression of CD31 and EGFP.

Chapter 6: Discussion

Liver is a prime example of the pivotal role played by cellular interplay in the organ functions.
For years, investigators have developed culture models based on features of liver architecture to recapitulate the complex hepatocyte microenvironment. These features include extracellular matrix as found in the space of Disse, physicochemical stimuli imposed by sinusoidal blood flow, and cell–cell interactions present in the hepatic cord.
Cell–cell interactions, both homotypic (hepatocyte–hepatocyte) and heterotypic (hepatocyte–non parenchymal cell), have been shown to improve viability and function. Restoration of hepatocyte interactions as in spheroidal aggregates promotes formation of bile canaliculi, gap junctions, tight junctions, and E-cadherins and stabilizes function. The heterotypic interactions in hepatocyte–non parenchymal co-cultures are thought to present a highly conserved signal that greatly augments liver-specific functions. Cell patterning methods have been used to study the "co-culture" effect by tightly controlling the amount of cell–cell interaction to identify specific signaling pathways.

This study sought a first analysis of the cellular interactions underlying micro-environmental controlled differentiation and functions of LSEC, HSCs and liver stem cells in physiologic and pathologic liver conditions. Here we present, in fact, a clonal cell line of resident liver stem cell (RLSC) (14) and clonal cell lines of sinusoidal endothelium as tools useful to improve knowledge of cell-cell dialogue in the liver and to approach liver tissue engineering.

Regarding RLSCs, derived for murine liver explants and established in lines, we described the unexpected finding of their differentiation bi-potentiality that allows to obtain, from the same progenitor cell, epithelial and mesenchymal derivatives.
With respect to the in vitro differentiation experiments, RLSC, previously shown in mitogenic media to differentiate coherently into hepatocytes, were found here, when cultured in low serum, to be also able of accomplishing an alternative mesenchymal differentiation path. The tracking of the initial stages of differentiation highlighted as RLSCs spontaneously endeavor a mutually exclusive path that correlate with the loss of stem signature (i.e. SCA1).

With respect to the in vivo differentiation experiments, RLSC, orthotopically transplantated in neonatal healthy growing liver, contribute to the formation of the adult organ giving derivatives matching anatomical, morphological and molecular features of both epithelial and mesenchymal cells. In the mesenchymal progeny characterization, both immunophenotype and localization of RLSC derivatives are highly indicative of Hepatic Stellate Cells. The interesting evidences that HSCs in adult liver may (also) arise from a precursor compartment should be considered as a self-standing advance since, as yet, HSC precursors in adult animals have to be identified.
In heterotopic transplants again both RLSC mesenchymal and epithelial derivatives were found: mesenchymal cells expressed markers shared by HSCs (i.e. GFAP, alphaSMA); epithelial derivatives were found mainly aggregated in tubular structures and expressing markers of endodermal precursors (i.e. EpCam, αFP), and only few cells weakly positive for markers of hepatocytes (i.e. Albumin). This indicates that in vivo only liver-specific cues allow driving the full liver-specific differentiation.
Overall, RLSCs can conceivably be proposed as cells retaining features of the "meso-endodermal" liver progenitor. To the best of our knowledge, this is the first time that a liver progenitor cell is described in vivo to differentiate toward a lineage with characteristics attributable to HSCs. In the frame of this last conclusion, further dedicated studies in disease-prone animals will clarify whether RLSC may contribute to the onset of liver fibrosis and whether the precursor compartment should be considered an additional therapeutic target.

Regarding sinusoidal endothelium cells, here we present the isolation, the establishment in clonal cell lines and the characterization of murine endothelial cells, MLECs.
The major advancement coming from this part of my work is the set-up of culture conditions, based on the use of a hepatocyte conditioned medium, useful for the maintenance of liver specific sinusoidal endothelium. In fact, although several endothelial cell lines from non-liver sources exist, a cellular model displaying the unique structures and functions of hepatic sinusoids, as well as the pathological changes they undergo during chronic liver disease, was lacking.
We found that hepatocytes soluble factors support the liver endothelial cells proliferation, permits the retaining of the unique characteristics of SECs, primarily the trans-cytoplasmic fenestrations together with the expression of specific protein.

By the use of these particular culture conditions we obtained stable endothelial cell lines able to form, heterotopically (when transplanted together with hepatocytes) and orthotopically, functional sinusoidal vessels. When transplanted in neonatal livers, in particular, MLECs forms sinusoids and large vessels, establish correct interactions with endogenous SECs, drive the architectural spatial organization of the hepatocyte into epithelial cord, interact with pericytes.

In conclusion, we established in cell lines several liver histotypes, so realizing unique and high innovative cellular tools useful for the study of cellular interplay in physiology and pathology; the improvement of protocols of liver tissue engineering.

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